High Daily and Year-Round Variability in Denitrification and Nitrogen Fixation in a Northern Temperate River

Kevin C. Nevorski and Amy M. Marcarelli*

Department of Biological Sciences, Michigan Technological University, Houghton, MI, United States

Rates of nitrogen (N) cycling processes like denitrification and dinitrogen (N₂) fixation, which together are the primary contributors to N₂ flux rates from surface waters, may change at different time scales from seasons to weeks to days. Yet, we know little about the magnitude, mechanisms or drivers of these temporal changes, especially at shorter daily and weekly timescales. Quantifying variation in rates and drivers across temporal scales is essential to understand how nutrient cycling processes operate in aquatic ecosystems and predict how they may respond to shifting seasonal dynamics caused by global change (i.e., earlier snowmelt and extreme weather events). This study quantified denitrification and N₂ fixation rates seasonally and daily in a northern temperate river, and explored how environmental conditions such as discharge, light, and N and phosphorus (P) concentrations were related to that variation at different time scales. We measured denitrification and N₂ fixation rates on biweekly and daily intervals at a single 20-m long sampling reach in the Pilgrim River in Michigan’s Upper Peninsula from May 2017 through May 2019. We found high rates of daily change (difference in rate from one day to the next) for both processes in all seasons (maximum daily change 5,690 µg N/m²/h for denitrification and 38 µg N/m²/h for N₂ fixation). No detectable differences in rates among seasons were detected using Multiple Response Permutation Procedure (MRPP). Day-to-day variation did not change before and after elevated discharge events, including a 1,000-year flood that occurred in June 2018. Partial least squares (PLS) regression identified total dissolved N, dissolved organic N, and ammonium as important predictors of denitrification and N₂ fixation, but explained only 15–28% of the variation in all measured rates. The unexpectedly high daily variation and lack of seasonal difference in rates found in this study demonstrate the need to use caution when studying these processes and/or extrapolating rates across time scales, as discrete and infrequent measurements may be misleading.

**Keywords:** nitrogen fixation, denitrification, river, nitrogen cycle, temporal variability

**INTRODUCTION**

Denitrification and N₂ fixation are N transformation processes which can both control and be controlled by environmental factors (Vitousek et al., 2002; Seitzinger et al., 2006). Together with anammox, N₂ fixation and denitrification are the primary biological processes that control N₂ flux from ecosystems (Fox et al., 2014; Nifong et al., 2020). Denitrification is a form of anaerobic
respiration in which microbes break down organic carbon (C) for energy and use nitrate (NO$_3^-$) in the place of oxygen (O$_2$) as an electron acceptor, so supply of organic C and NO$_3^-$ are important limitations on denitrification rates (Wall et al., 2005). Along with the required reactant availability, environmental factors including oxygen concentration and temperature can alter denitrification rates. Biological N$_2$ fixation is the microbially-mediated conversion of N$_2$ gas into ammonium (NH$_4^+$); it is an energetically expensive process so high availability of NH$_4^+$ and/or other forms of dissolved inorganic N (DIN) may cause microbes to reduce the process rate to conserve energy (Burris and Roberts, 1993; Bandyopadhyay et al., 2013). Light may be especially important for controlling N$_2$ fixation rates in shallow aquatic ecosystems like streams, where it is often carried out by cyanobacteria who obtain their energy via photosynthesis (Burris and Roberts, 1993; Scott and Marcarelli, 2012). Light intensity along with these other factors vary through time, and therefore they may lead to temporal variation in rates of both of these N transformation processes in aquatic ecosystems. Yet, the mechanisms by which environmental factors affect rates of denitrification and N$_2$ fixation may differ depending on the time scale.

In streams and rivers in temperate climate zones, seasonal changes in denitrification and N$_2$ fixation rates in response to changes in environmental conditions may be gradual over the course of days to weeks, caused by the abundance and/or activity of microbes increasing or decreasing, or the identity of the microbes within the community changing. Environmental characteristics that show strong seasonal changes in temperate streams and rivers include nutrient availability, temperature, and light availability (Coble et al., 2019). Low biotic uptake rates in winter can lead to increased inorganic N concentrations in temperate forested streams (Stottlemeyer and Toczydlowski, 1999a,b) which could provide reactants for denitrification and reduce rates of N$_2$ fixation. Temperate forests have large inputs of allochthonous organic matter during the fall and high dissolved organic C (DOC) flux during snowmelt (Stottlemeyer and Toczydlowski, 1999a), either of which could provide a C source for denitrifying bacteria. Discharge varies seasonally corresponding to rainy seasons or snowmelt, which leads to predictable patterns of disturbance that can move, scour and mobilize riverbed substrates, disturbing microbial communities, and the microenvironments of these communities, which control nutrient cycling processes (O’Connor et al., 2012). Seasonal changes in discharge can also alter nutrient concentrations both by altering patterns of nutrient delivery and via dilution (Meyer and Likens, 1979; Horner et al., 1990; Stottlemeyer and Toczydlowski, 1999a). Denitrification and N$_2$ fixing microbial communities can both be sensitive to seasonal temperature changes, with N$_2$-fixing microbes often preferring warmer temperatures (Scott and Marcarelli, 2012), and denitrification activity decreasing during periods with colder temperatures such as in the winter (Christensen et al., 1990; Kim et al., 2006). Light also varies seasonally, especially in forested streams where canopy cover will shade streams differently depending on the season and vegetation type (Roberts and Mulholland, 2007; Bowes et al., 2012). 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 (Roberts and Mulholland, 2007).

Environmental factors in temperate streams can also shift much more rapidly over hours to days, which could instead affect rates through enzymatic regulation (Grimm, 1987; Marcarelli and Wurtsbaugh, 2006; Marcarelli et al., 2008). N cycling rates can both increase and decrease over the course of hours with factors such as temperature or substrate availability affecting enzyme activity (Grimm, 1987; Marcarelli and Wurtsbaugh, 2006). Temperature variation can occur on an hourly scale, and the enzymes and microbes facilitating N cycling processes may have reduced activity at cold temperatures (Yvon-Durocher et al., 2010; Boulètreau et al., 2012). Also, changes in light can alter N$_2$ fixation rates by cyanobacteria in some ecosystems on a diel cycle because it is tightly coupled with photosynthesis (Howarth et al., 1988; Grimm and Petrone, 1997). Nutrient concentrations can rapidly change due to storm runoff, which could also alter N process rates. Because N$_2$ fixation is so energy intensive, enzymes can be rapidly deregulated to save energy following an influx of DIN (Howarth et al., 1988; Grimm and Fisher, 1989; Grimm and Petrone, 1997). But, the distinction between community or enzymatic shifts can be complicated because some environmental factors such as temperature and light vary at multiple time scales. For example, hydrological disturbances such as flooding caused by storms are overlain on seasonal patterns created by snowmelt and rainfall, and may therefore alter biogeochemical process rates through a mix of shorter-term enzymatic changes and longer-term community shifts (Grimm and Fisher, 1989). Storm-driven surface runoff can carry nutrients (Meyer and Likens, 1979; Horner et al., 1990; Stottlemeyer and Toczydlowski, 1999a), change water temperature, and create short-term increases in water velocity and benthic disturbance (O’Connor et al., 2012), all of which may act independently and in concert to create variation in process rates.

Understanding relationships between N cycling rates and different environmental drivers that vary across time scales may provide insight into how climate change will affect these processes in the future. Climate change can lead to temperature, stream flow, and growing season changes (Barnett et al., 2005; Backlund et al., 2008; Christiansen et al., 2011), all environmental characteristics which may be influential to denitrification and N$_2$ fixation rates. Storms are becoming separated by longer periods of drought, but overall higher rainfall can occur driven by more intense storm events (Mathboul et al., 2018). It is also not well-understood how resistant and resilient rates of N transformations are to hydrologic disturbances, which is important in the face of ongoing climate change. Resistance and recovery (or resilience) are concepts used throughout ecological studies, including studies on the impact of hydrological disturbances, anthropogenic disturbances, and climate change (Holling, 1973; Bahadur et al., 2013; Pope et al., 2014; Reisinger et al., 2017). Resistance describes how much the structure and processes of an ecosystem change in response to disturbances of different magnitudes, while recovery describes how quickly the ecosystem returns to the same structure and/or function after a disturbance event (Hershkovitz and Gäsith, 2013;
Reisinger et al., 2017). 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. Understanding how large hydrological disturbances affect denitrification and N\textsubscript{2} fixation can provide insight into how they will be affected by changing climactic conditions.

The complexity of seasonal vs. 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 N cycle processes vary. The objectives of this study were to characterize how denitrification and N\textsubscript{2} fixation rates vary over seasonal, weekly, and daily time 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 N\textsubscript{2} fixation rates vary seasonally? We hypothesized that rates would differ among seasons due to changes in the environmental conditions that directly and indirectly control process rates. (2) How do denitrification and N\textsubscript{2} fixation rates vary daily and in their response to hydrological disturbances? We hypothesized low day to day variation in process rates; however, after large hydrological events (i.e., flooding caused by storms or snowmelt), rates would decline due to disturbance 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 N\textsubscript{2} fixation rates? We hypothesized seasonal drivers including light and temperature would also be drivers of these rates as we expected large differences in rates between seasons. We addressed these questions by quantifying these processes in relation to changes in environmental conditions at daily and seasonal time intervals over 2 years.

**MATERIALS AND METHODS**

**Study Site and Sampling Design**

Sampling occurred in a single 20 m-long reach on the main branch of the Pilgrim River (47.10138° north, 88.51750° west; [Figure 1]). The Pilgrim River and its 4 tributaries stretch 34.9 km, draining a 52 km\textsuperscript{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\textsuperscript{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

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**FIGURE 1** | The 20-m long study reach (location indicated by triangle) was located on the Pilgrim River on the Upper Peninsula of Michigan, USA. The inset shows the location of the study area within the state of Michigan. Map of Pilgrim River derived from Hydrography Lines_	extsubscript{v}17a\textsuperscript{a} shapefile from State of Michigan GIS Open data (https://gis-michigan.opendata.arcgis.com/).
river from December to 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 ~2/3 sand cover and 1/3 patches of large cobbles.

Sampling occurred every 2–6 weeks year-round between May 2017 and May 2019 except for N\textsubscript{2} fixation, which was not sampled from November 2017 to April 2018. Additionally, once per season sampling occurred every day over a 1 to 2-week period. This nested design allowed us to measure both seasonal and daily variation in rates, as well as calculate recovery when disturbances occurred. During certain time periods, some sampling dates were skipped (Table 1) due to unsafe conditions such as extreme cold, storms, and high discharge. 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 (Coble et al., 2019; Meingast et al., 2020). 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 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 season start or end dates could vary among the years of our study based on conditions in that year.

### Sampling Methods

Denitrification and N\textsubscript{2} fixation rates were measured in sediment and on rock substrates using acetylene block and acetylene reduction assay techniques as described by Dodds et al. (2017) and Eberhard et al. (2018). Sediment and rock were measured because these were the predominant substrates found in the river. To measure denitrification and N\textsubscript{2} fixation on each date, 200 mL sediment cores or enough rocks to cover ~0.0045 m\textsuperscript{2} surface area were collected from <1 m deep water and placed in chambers (pint glass mason jars with lids drilled to fit 13 × 20 mm septa, but 2-L polycarbonate food storage containers 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 stream water. This allowed us to account for chamber effects such as gas leakage or processes occurring in the water column. Blank chambers also confirmed that there was minimal denitrification or nitrogen fixation activity in the water column of this river, which justifies the focus on benthic substrate as the main locations for these processes.

Acetylene reduction assays were used to quantify rates of N\textsubscript{2} fixation by introducing acetylene gas to a chamber. Nitrogenase, the enzyme that fixes N\textsubscript{2}, will also convert acetylene to ethylene if it is present. We measured the change in ethylene concentration to estimate the amount of N that could have been fixed (Capone,
We estimated the amount of 

| TABLE 1 | Sampling frequency and study site characteristics (mean ± standard deviation) for all variables measured during each season throughout the sampling period. |
|----------|-----------------|-----------------|-----------------|-----------------|
|          | Fall            | Spring          | Summer          | Winter          |
| # Sample days N\textsubscript{2} fixation—rock | 11 ± 0.1158 | 0.1239 ± 0.1506 | 0.0360 ± 0.0363 | 0.0366 ± 0.0042 |
| # Sample days N\textsubscript{2} fixation—sediment | 1.9 ± 0.9 | 2.5 ± 1.4 | 1.5 ± 0.6 | 1.6 ± 0.8 |
| # Sample days denitrification—rock | 14 ± 0.0211 | 0.0327 ± 0.0313 | 0.0117 ± 0.0120 | 0.0406 ± 0.0374 |
| # Sample days denitrification—sediment | 0.0048 ± 0.0116 | 0.0070 ± 0.0103 | 0.0116 ± 0.0116 | 0.0116 ± 0.0116 |
| Rock AFDM (g) | 0.0568 ± 0.0048 | 0.0285 ± 0.0344 | 0.03891 ± 0.01126 | 0.3569 ± 0.1108 |
| Sediment AFDM (g) | 6.2357 ± 0.0032 | 0.0024 ± 0.0032 | 0.0017 ± 0.0067 | 0.0049 ± 0.0043 |
| Chlorophyll a (mg/m\textsuperscript{2}) | 0.0044 ± 0.00021 | 0.0063 ± 0.0032 | 0.0117 ± 0.0067 | 0.0049 ± 0.0043 |
| Temperature (°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 |
| PAR (W/m\textsuperscript{2}) | 0.0067 ± 0.00031 | 0.0056 ± 0.0024 | 0.0080 ± 0.00070 | 0.0103 ± 0.00105 |
| Discharge (L/s) | 327 ± 208 | 1,177 ± 662 | 978 ± 444 | 353 ± 246 |
| DOC (mg C/L) | 1.161 ± 0.549 | 2.184 ± 2.548 | 973 ± 601 | Ice |
| TDO (mg N/L) | 6.2357 ± 2.3783 | 6.2737 ± 0.8997 | 7.5097 ± 2.7486 | 5.7153 ± 1.9237 |
| NO\textsubscript{2} + NO\textsubscript{3} (mg N/L) | 0.1323 ± 0.00351 | 0.0916 ± 0.0387 | 0.1267 ± 0.0611 | 0.1856 ± 0.0798 |
| NH\textsubscript{4} (mg N/L) | 0.0044 ± 0.00021 | 0.0063 ± 0.0032 | 0.0117 ± 0.0067 | 0.0049 ± 0.0043 |
| DON (mg N/L) | 0.2091 ± 0.0780 | 0.1902 ± 0.0285 | 0.2682 ± 0.1021 | 0.1756 ± 0.0895 |
| SRP (mg P/L) | 0.0067 ± 0.00031 | 0.0056 ± 0.0024 | 0.0080 ± 0.00070 | 0.0103 ± 0.00105 |
| TDP (mg P/L) | 0.0084 ± 0.00044 | 0.0104 ± 0.0048 | 0.0130 ± 0.0048 | 0.0111 ± 0.0116 |
| N\textsubscript{2} fixation rate—rock (µmol N/m\textsuperscript{2}/h) | 2.30 ± 3.49 | 5.17 ± 11.24 | 2.47 ± 4.39 | 1.83 ± 1.25 |
| N\textsubscript{2} fixation rate—sediment (µmol N/m\textsuperscript{2}/h) | 1.03 ± 1.76 | 1.86 ± 3.00 | 3.60 ± 7.05 | 2.33 ± 4.16 |
| Denitrification rate—rock (µmol N/m\textsuperscript{2}/h) | 1264.8 ± 2515.3 | 48.7 ± 95.9 | 355.4 ± 967.5 | 228.1 ± 361.5 |
| Denitrification rate—sediment (µmol N/m\textsuperscript{2}/h) | 815.7 ± 1322.1 | 1049.3 ± 1917.2 | 1351.3 ± 1840.2 | 286.3 ± 410.9 |

Discharge was not measured in winter due to ice coverage over the river. AFDM, ash free dry mass; PAR, photosynthetically active radiation; DOC, dissolved organic carbon; TDO, total dissolved nitrogen; NO\textsubscript{2} + NO\textsubscript{3}, nitrate and nitrite; NH\textsubscript{4}, ammonium; DON, dissolved organic nitrogen; SRP, soluble reactive phosphorous; TDP, total dissolved phosphorous.

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 an SRI 8610C Gas Chromatograph with Haysep T column with hydrogen carrier gas, a flame ionization detector, and column oven set at 40°C ramping to 110°C after 2.5 min. 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 using water volume, the solubility constant for ethylene, and equations from Dodds et al. (2017). We estimated the amount of N fixed by assuming 3 mol of acetylene converted to ethylene is equal to 1 mol of N\textsubscript{2} being fixed (Capone, 1993; Kim et al., 2006).

Ethylene block assays were used to quantify rates of denitrification. Acetylene prevents the complete transformation of NO\textsubscript{3} to atmospheric N, causing N\textsubscript{2}O to accumulate, which can be measured to estimate denitrification rate (Smith and Tiedje, 1979). C, N, and chloramphenicol (to prevent bottle effects) were added to each chamber to a final concentration of 34 mg/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 N\textsubscript{2} fixation assays. The gas samples were analyzed for N\textsubscript{2}O using the SRI 8610C Gas Chromatograph with Haysep D column and helium or ultra-high purity N\textsubscript{2} (for samples analyzed after February 2019) carrier gas, electron capture detector, and column oven set at 80°C ramping to 180°C after 5 min. A 1,000 ppm N\textsubscript{2}O standard was used to convert peak height to N\textsubscript{2}O 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 and Getty, 2006).

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 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 (Nusch, 1980; APHA, 2005). 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 (AFDM). AFDM and
chlorophyll concentrations 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 P (SRP), total dissolved P (TDP), NO\textsubscript{3}− + nitrite (NO\textsubscript{2}− + NO\textsubscript{3}−), NH\textsubscript{4}+, total dissolved N (TDN) and DOC. NO\textsubscript{3}− + NO\textsubscript{2}− and SRP were analyzed using a SEAL AQ2 discrete water analyzer. NO\textsubscript{3}− + NO\textsubscript{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, Maryland) for DOC and TDN analysis. Dissolved Organic N (DON) was calculated by subtracting NO\textsubscript{3}− + NO\textsubscript{2}− and NH\textsubscript{4}+ concentrations from TDN concentrations. TDP concentration was analyzed using the molybdenum—antimony method following an ammonium persulfate digestion (APHA, 2005).

Discharge was determined using a USGS installed gauge (USGS 04043016 Pilgrim River at Paradise Road Near Dodgeville, MI: May 2017–June 2018) and by measuring directly using a Marsh Mc Birney Flo-mate (May 2017–May 2019). The Flo-mate was attached to a wading rod to measure velocity (m s\textsuperscript{-1}) at 0.6 * stream depth (m) at 10 equidistant points on a transect of the stream perpendicular to shore. The area of each segment was determined by multiplying segment width by segment depth. Discharge in each segment was determined by multiplying velocity by segment area, then added together across all segments to get total discharge (m\textsuperscript{3}/s). Canopy cover was measured using a spherical densitometer (Lemmon, 1956). Photosynthetically active radiation (PAR) was retrieved from the Upper Great Lakes Observing System station located at Michigan Tech’s Great Lakes Research Center (station name GLRCMET; http://uglos.mtu.edu/), which is ≈3 km from the study site. A MiniDO\textsubscript{2}T logger from PME was deployed to continuously measure O\textsubscript{2} and temperature at the site, and open water metabolism, which includes gross primary production (GPP) and ecosystem metabolism (ER), was modeled using the Stream Metabolizer software package (Appling et al., 2018; github.com/USGS-R/streamMetabolizer).

**Calculations and Statistical Analyses**

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, MRPP determined if the distance of points among groups was different from the distance of points within groups, which is reported as the parameter A (significance determined as \( p < 0.05 \)). First, to ensure the selected seasonal ranges were distinct we used a MRPP to determine if there was grouping in sampling dates by season 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 PAR, water temperature, DOC, TDN, NO\textsubscript{3}− + NO\textsubscript{2}− , SRP, and TDP. Principle components analysis (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, N\textsubscript{2} fixation rates on rocks, and N\textsubscript{2} fixation rates in sediment to see if they also grouped by season.

To address question 2 about how denitrification and N\textsubscript{2} 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:

\[
daily\ change = abs(X^i - X^{i+1})
\]

Where abs is the absolute value, \( X^i \) is the rate at day \( i \) and \( X^{i+1} \) is the rate one day later. MRPP was performed for daily change in denitrification rates on rock, denitrification rates in sediment, N\textsubscript{2} fixation rates on rock and N\textsubscript{2} fixation rates in sediment to determine if daily change differed between spring, summer, and fall. Due to less frequent sampling dates in winter (Table 1), daily change was not calculated for this season.

Resistance, or the measure of how much a process rate changes in response to a disturbance, and recovery, or the measure of how quickly rates return to what they were pre-disturbance, 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, we predicted that N\textsubscript{2} fixation and denitrification processes would be resistant if the rates did not change before and after a hydrological disturbance. Resistance was calculated as:

\[
R = 1 - \frac{X_{\text{before}} - X_{\text{after}}}{X_{\text{before}}}
\]

Where \( R \) is resistance, \( X_{\text{before}} \) is the rate before a disturbance, and \( X_{\text{after}} \) is the rate after a disturbance. Values closer to 1 indicate high resistance, while those closer to 0 indicate low resistance (Hershkovitz and Gasith, 2013). Recovery or resilience is how quickly the rates post-disturbance return to pre-disturbance levels if they decreased because of the disturbance. Recovery for each rate was calculated as the linear slope of the relationship between rate and day in the time period directly following the disturbance (Reisinger et al., 2017).

Environmental factors related to N\textsubscript{2} fixation and denitrification rates on rock and in sediment were explored using partial least squares (PLS) regression with 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).

RESULTS

Throughout the year, both rates and environmental variables ranged greatly (Table 1, Figures 3, 4). Denitrification in sediment averaged 984.65 ± 1032.46 µg N/m²/h (mean ± standard deviation; Figure 3) for the entire sampling period, while N₂ fixation on rocks averaged 3.29 ± 7.23 µg N/m²/h. Discharge measured during sampling averaged 1.18 ± 1.04 m³/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 of the study reach occurred on 19 Jun 2018, during a 1,000-year flood event with an estimated maximum discharge of 208 m³/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.

Seasonal Patterns in N₂ 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 PAR, water temperature, DOC, TDN, NO$_3^- +$ NO$_2^-$, SRP, TDP (Figure 5). The 1st principal component explained 35% of the
variation (Supplementary Table S1) and was primarily driven by PAR (0.27), DOC (−0.26), TDN (−0.55), NO₃⁻ + NO₂⁻ (−0.33), SRP (−0.44), and TDP (−0.51). Principal component 2 explained an additional 27% of the variation and was driven by temperature (−0.62). However, MRPP showed no seasonal separation for any of the rates: N₂ fixation on rock (A = −0.004098, p = 0.54, spring n = 24, summer n = 30, fall n = 11, winter n = 4); N₂ 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 N₂ Fixation and Denitrification Rates and Their Response to Hydrological Disturbances**

Day-to-day differences in denitrification and N₂ fixation could be both large or small for both rocks and sediment in spring.
summer, and fall. For the entire study period the highest daily change (difference in rates between one day and the next) in denitrification rates on rock was 5,690 µg N/m²/h, denitrification in sediment was 5,202 µg N/m²/h, N₂ fixation on rocks was 38 µg N/m²/h, and N₂ fixation in sediment was 27 µg N/m²/h. The lowest daily changes were 0 µg N/m²/h for both rates on both substrates (Figure 6). 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 $n = 5$), N₂ fixation on rocks ($A = 0.01658$, $p = 0.23$, spring $n = 19$, summer $n = 20$, fall $n = 4$), or N₂ fixation in sediment ($A = 0.01067$, $p = 0.274$, spring $n = 20$, summer $n = 19$, fall $n = 4$).

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 7). 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/m²/h mean ± SE) and N₂ fixation on rocks (1.5 ± 1.9 µg N/m²/h mean ± SE) were frequently high, with rates appearing similar to before the storm (Figure 7). For denitrification in sediment, resistance was calculated to be 0, with a rate of 415 µg N/m²/h measured before the storm and 0 µg N/m²/h measured after the storm. The before-storm rate was one order of magnitude lower than the highest denitrification rate measured over the 3-week recovery period (3,525 µg N/m²/h; average = 697 µg N/m²/h). Recovery was 185 N/m²/h/day, with rates increasing from 0 to 970 µg N/m²/h in 3 days. For N₂ 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).

### Environmental Factors Related to Variation in Denitrification and N₂ Fixation Rates

Inorganic N availability and DOC appeared to be the important driver for denitrification rates, and to a lesser degree for N₂ fixation rates. Partial least squares regression showed that 27.52% of the variation in rates of denitrification in sediment was explained by Component 1, ($n = 88$, Supplementary Table S2). Component 1 was driven by DOC, TDN, and DON with loadings $> 0.4$. Component 2 explained an additional 8.74% with ecosystem respiration and chlorophyll concentration having loadings $> 0.4$. For denitrification on rocks, 22.64% of the variation in rates was explained with the first component ($n = 71$, Supplementary Table S3) which was driven by DOC, TDN, and DON with loadings $> 0.4$. Component 2 explained an additional 29.56% and was driven by ER and DOC. For N₂ fixation on rocks, 25.87% of the variation in rates was explained with the first component ($n = 71$, Supplementary Table S4) which was driven by TDN, DON, DOC, and ER with loadings $> 0.4$. Component 2 explained an additional 10.36% and was driven by TDN and NH⁴⁺ with loadings $> 0.4$. For N₂ fixation in sediment, 15.09% of the variation in rates was explained with the first component ($n = 90$, Supplementary Table S5) which was driven by DOC, TDN, and NH⁴⁺ with loadings $> 0.4$. Component 2 explained an additional 20.54% and was driven by PAR.

### DISCUSSION

We found that N cycling processes in the Pilgrim River were temporally dynamic and resilient to hydrologic disturbances. Unexpectedly, rates were not significantly different among seasons; rather, within-season variation and daily changes in rates were as large as variation among seasons. Day-to-day variation was high throughout the year, with no discernible difference between seasons and in response to hydrologic events. Because rates were so dynamic, traditional methods for calculating resistance 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. The primary environmental drivers of this variation appeared to be dissolved inorganic and organic N concentrations and DOC, although they explained relatively small amounts of the overall variation in process rates.

### Seasonal Patterns in N₂ Fixation and Denitrification Rates

Contrary to the findings of other studies, rates and variation in N₂ 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 NO₃⁻ concentration, dissolved O₂, and organic C.
FIGURE 6 | Daily change in denitrification and N$_2$ fixation in different seasons. Daily change was significantly different between seasons for denitrification on rocks but not for the other rate/substrate combinations.

Studies of N$_2$ fixation in streams have detected seasonal rate shifts associated with light, temperature, and N flux. Perhaps the most complete understanding of N cycling in a single stream exists for Sycamore Creek in the Sonoran Desert, where seasonal variation in N$_2$ fixation was detected and correlated with light and temperature (Grimm and 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, it is located in a temperate forested ecoregion, in general does not support the growth of large cyanobacterial mats, has much lower rates of N$_2$ fixation, and has different temperature ranges and nutrient ratios than Sycamore Creek. Streams in the subalpine, coniferous forested Sawtooth Mountains of central Idaho exhibited N$_2$ fixation rates more comparable (10–610 µg N/m$^2$/h) to those measured in the Pilgrim River (0–50 µg N/m$^2$/h), and in those streams seasonal changes in N$_2$ fixation rates were related to seasonal changes in N flux (Marcarelli and Wurtsbaugh, 2009). Comparatively, the Pilgrim River had higher overall N concentrations than the Sawtooth Mountain...
streams, with no apparent seasonal variation. Additionally, for both denitrification and N\textsubscript{2} fixation, the sampling frequency in most seasonal studies is lower 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 N\textsubscript{2} fixation and different environmental conditions in the Pilgrim River, could explain why no seasonal variation in N\textsubscript{2} fixation or denitrification was observed in this study.

**Daily Variation in N\textsubscript{2} Fixation and Denitrification Rates and in Their Response to Hydrological Disturbances**

Resistance and recovery of lotic systems are difficult to characterize due to frequent hydrological shifts (Friberg, 2014; Jaiswal and Pandey, 2021). 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 > 1,000-year flood (T. Weaver, USGS, personal communication) denitrification in sediment did decrease, but returned to pre-flood rates in 3 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 at this location in this river. N\textsubscript{2} fixation showed similar trends to denitrification in which variation pre and post-flood were similar and recovery was difficult to decipher. Interestingly, immediately after the flood N\textsubscript{2} fixation rates increased, although we are unable to tell if that was due to the disturbance or due to normal daily variation.

As this study was only performed in a single location, we are unable to draw conclusions about the responses of these processes to disturbances in the river as a whole, nor how they may vary among different locations on the river. Assessing resistance and recovery becomes more complicated when looking at landscapes because one part of a landscape may be resistant to a disturbance while another part may not be. Yet, this broader spatial understanding is necessary for proper management, and methods are being developed to characterize the recovery of an entire landscape (Cushman and McGarigal, 2019). For purposes of this study, we do know from other studies on the Pilgrim River that biogeochemical processes vary both within and among stream reaches. Schipper (2021) measured gross primary production, ecosystem respiration and decomposition at 4 reaches along the Pilgrim River, finding the rates differed significantly among the different sites due to changes in water temperature and canopy cover. Eberhard (2022) measured denitrification and N\textsubscript{2} fixation rates in different substrate patches within a 657 m\textsuperscript{2} reach that included the location used in the current study and found that rates of N\textsubscript{2} fixation varied relatively little, but rates of denitrification varied 4 orders of magnitude among patches on a single day. These additional studies on the same stream show that the variation of rates for biogeochemical cycles are highly variable both temporally and spatially.

**Environmental Factors Are Related to Variations in Denitrification and N\textsubscript{2} Fixation Rates**

N concentrations and DOC were the most important explanatory factors of rate variation using PLS regression, although the variability explained by a single component was low (15–28%). As denitrification requires both organic C and N 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. Sediment AFDM was not an important variable implicated by PLS, which was surprising as denitrification requires C. However, we did not measure DOM, N or P concentrations in the sediment or in sediment pore water, which may have been more important indicators than the water column nutrient concentrations that we did measure. For N\textsubscript{2} fixation, both DON and TDN were implicated in variation of rates. It was interesting that N:P was not selected as a predictor of N\textsubscript{2} fixation, as substantial prior research suggests that N:P could be a more important predictor of N\textsubscript{2} fixation that N concentration alone (Smith, 1990; Paerl et al., 2016). However, the 1st component of the...
PLS had many variables with high loadings including TDP, suggesting that variability in N₂ fixation rate may coincide with many different environmental variables. Yet, it is important to note that this 1st component only explained 23% of the overall variation.

These results have important consequences for considering how N cycling processes in northern temperate rivers may respond to climate change. Two likely environmental shifts that are expected with climate change in this region are shifts in the timing and duration of seasons, especially spring and fall (Creed et al., 2015; Contosta et al., 2016), and increasing frequency and magnitude of summer storms (Hayden and Hayden, 2003; Marcarelli et al., 2019). Both of these were inconsequential for variation in denitrification and N₂ fixation rates in this river. However, climate change could affect these two processes by alterations to the environmental factors that were related to variation in process rates through time through direct and indirect pathways. For example, changing growing seasons could alter rates of primary production (Barnett et al., 2005; Backlund et al., 2008; Christiansen et al., 2011), which can in turn alter N availability (Roberts and Mulholland, 2007), which our analyses identified as an important explanatory variable for denitrification and N₂ fixation rate. DOC concentrations, which was also related to denitrification and N₂ fixation in our analyses, are increasing in northern temperate streams globally due to decreases in sulfur deposition, increased temperatures, and increased precipitation (Roulet and Moore, 2006; de Wit et al., 2016); however, long-term changes in DOC concentrations in streams in the Upper Peninsula of Michigan, where this study was located, are less clear (Marcarelli et al., 2019; Meingast et al., 2020). Yet, each of these environmental drivers explained a relatively small proportion of the variation in process rates, which poses a challenge for predicting the responses of N cycling processes that could result from these changes.

Our results suggest that caution is needed when studying biogeochemical processes in rivers because the processes may vary to a greater extent than previously recognized. Assumptions of predictable seasonal variation 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 N₂ fixation and denitrification rates observed in this study. Additionally, the environmental conditions commonly assumed to control denitrification and N₂ fixation rates such as nutrient concentration, although relevant, may not be sufficient for evaluating the occurrence or changes in processes within a single stream. It is likely these relationships are much more complicated, involving many predictor variables interacting in ways we do not yet understand.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The datasets generated for this study can be found in the Environmental Data Initiative data repository (https://doi.org/10.6073/pasta/62a51b36519e73e4ca42d0bb52f0ce3e3).

AUTHOR CONTRIBUTIONS

KN and AM contributed to the study conception and design and revisions and prepared the final manuscript. KN led data collection and analysis and wrote the first draft of the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTS MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frwa.2022.894554/full#supplementary-material

REFERENCES

APHA (2005). Standard Methods for the Examination of Water and Wastewater. Washington, DC: American Public Health Association (APHA).

Appling, A. P., Hall, R. O., Yackulic, C. B., and Arroita, M. (2018). Overcoming equifinality: Leveraging long time series for stream metabolism estimation. J. Geophys. Res.: Biogeosci. 123, 624–645. doi: 10.1002/2017JG004140.

Backlund, P., Schimel, D., Janetos, A., Hatfield, J., Ryan, M. G., Archer, S. R., et al. (2008). “Introduction. The effects of climate change on agriculture, land resources, water resources, and biodiversity in the United States”, in United States Climate Change Science Program Synthesis and Assessment Product, Vol. 4, 11–20.

Bahadur, A. V., Ibrahim, M., and Tanner, T. (2013). Characterising resilience: unpacking the concept for tackling climate change and development. Clim. Dev. 5, 55–65. doi: 10.1080/17565529.2012.762334.
Meyer, J. L., and Likens, G. E. (1979). Transport and transformation of phosphorus in a forest stream ecosystem. *Ecography* 60, 1255–1269. doi: 10.2307/1936971

Nifuong, R. L., Taylor, J. M., Adams, G., Moore, M. T., and Farris, J. L. (2020). Recognizing both denitrification and nitrogen consumption improves performance of stream diel N2 flux models. *Limnol. Oceanogr.: Methods* 18, 169–182. doi: 10.1002/lom3.10361

Nusch, E. A. (1980). Comparison of different methods for chlorophyll and phaeopigment determination. *Arch. Hydrobiol. Beih.* 14, 14–36. doi: 10.1007/BF00027688

O’Connor, B. L., Harvey, J. W., and McPhillips, L. E. (2012). Thresholds of flow-induced bed disturbances and their effects on stream metabolism in an agricultural river. *Water Resour. Res.* 48:W08504. doi: 10.1029/2011WR011488

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). vegan: Community Ecology Package. R package version 2.5-6. Available online at: https://CRAN.R-project.org/package=vegan (accessed March 05, 2022).

Paerl, H. W., Scott, J. T., McCarthy, M. J., Newell, S. E., Gardner, W. S., Havens, K. E., et al. (2016). It takes two to tango: when and where dual nutrient (N and P) reductions are needed to protect lakes and downstream ecosystems. *Environ. Sci. Technol.* 50, 10805–10813. doi: 10.1021/acs.est.6b02575

Pope, K. L., Allen, C. R., and Angeler, D. G. (2014). Fishing for resilience. *Trans. Am. Fish. Soc.* 143, 467–478. doi: 10.1080/00028487.2014.880735

R Core Team (2020). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. Available online at: https://www.R-project.org/ (accessed March 05, 2022).

Reisinger, A. J., Rosi, E. J., Bechtold, H. A., Doody, T. R., Kaushal, S. S., and Groffman, P. M. (2017). Recovery and resilience of urban stream metabolism following Superstorm Sandy and other floods. *Ecosphere* 8, e01776. doi: 10.1002/ecs2.1776

Roberts, B. J., and Mulholland, P. J. (2007). In-stream biotic control on nutrient biogeochemistry in a forested stream, West Fork of Walker Branch. *J. Geophys. Res.: Biogeosci.* 112, 1–11. doi: 10.1029/2007JG000422

Roulet, N., and Moore, T. R. (2006). Environmental chemical: burning the waters. *Nature* 444, 283. doi: 10.1038/444283a

Schipper, R. (2021). Controls on Autotrophic Respiration, Heterotrophic Respiration, and Decomposition in Northern Forested Streams (Open Access Master’s Thesis). Michigan Technological University.

Scott, J. T., and Marcarelli, A. M. (2012). “Cyanobacteria in freshwater benthic environments,” in *Ecology of the Cyanobacteria II: Their Diversity in Time and Space*, 271–289. doi: 10.1007/978-94-007-3855-3_9

Seitzinger, S., Harrison, J., Bohlke, J., Bouwman, A., Lowrance, R., Peterson, B., et al. (2006). Denitrification across landscapes and waterscapes: a synthesis. *Ecol. Appl.* 16, 2064–2090. doi: 10.1890/1051-0761(2006)016[2064:DALAWA]2.0.CO;2

Smith, M. S., and Tiedje, J. M. (1979). Phases of denitrification following oxygen depletion in soil. *Soil Biol. Biochem.* 11, 261–267. doi: 10.1016/0038-0717(79)90071-3

Smith, V. H. (1990). Nitrogen, phosphorus, and nitrogen fixation in lacustrine and estuarine ecosystems. *Limnol. Oceanogr.* 35, 1852–1859. doi: 10.4319/lo.1990.35.8.1852

Stottlemeyer, R., and Toczydlowski, D. (1999a). Seasonal change in precipitation, snowpack, snowmelt, soil water and stream water chemistry, northern Michigan. *Hydrogeol. J.* 2231, 2215–2231. doi: 10.1002/SICi.1083(199910)13:14/15<2215::AID-HYPI882>3.0.CO;2-V

Stottlemeyer, R., and Toczydlowski, D. (1999b). Seasonal relationships between precipitation, forest floor, and stream water nitrogen, Isle Royale, Michigan. *Soil Sci. Soc. Am. J.* 63, 389–398. doi: 10.2136/sssaj1999.03615995006300020018x

Vitousek, P. M., Cassman, K., Cleveland, C., Crews, T., Field, C. B., Grimm, N. B., et al. (2002). Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* 57–58, 1–45. doi: 10.1007/978-94-017-3405-9_1

Wall, L. G., Tank, J. L., Royer, T. V., and Bernot, M. J. (2005). Spatial and temporal variability in sediment denitrification within an agriculturally influenced reservoir. *Biogeochemistry* 76, 85–111. doi: 10.1007/s10533-005-2199-6

Warton, D. I., Wright, T. W., and Wang, Y. (2012). Distance-based multivariate analyses confound location and dispersion effects. *Methods Ecol. Evol.* 3, 89–101. doi: 10.1111/j.2041-210X.2011.00127.x

Yvon-Durocher, G., Jones, J. I., Trimmer, M., Woodward, G., and Montoya, J. M. (2010). Warming alters the metabolic balance of ecosystems. *Philos. Trans. R. Soc. B: Biol. Sci.* 365, 2117–2126. doi: 10.1098/rstb.2010.0038

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