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Heterogeneity in habitat and nutrient availability facilitate the co-occurrence of N_2 fixation and denitrification across wetland–stream–lake ecotones of Lakes Superior and Huron

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Abstract Great Lakes coastlines are mosaics of wetland, stream, and lake habitats, characterized by a high degree of spatial heterogeneity that may facilitate the co-occurrence of seemingly incompatible biogeochemical processes due to variation in environmental factors that favor each process. We measured nutrient limitation and rates of N_2 fixation and denitrification along transects in 5 wetland–stream–lake ecotones with different nutrient loading in Lakes Superior and Huron. We hypothesized that rates of both processes would be related to nutrient limitation status, habitat type, and environmental characteristics including temperature, nutrient concentrations, and organic matter quality. We found that median denitrification rates (914 µg N m^{-2} h^{-1}) were 166×higher than N_2 fixation rates (5.5 µg N m^{-2} h^{-1}), but the processes co-occurred in 48% of 83 points measured across all 5 transects and habitat types. N_2 fixation occurred on sediment and macrophyte substrate, while denitrification occurred mostly in sediment. Nutrient-diffusing substrate experiments indicated that biofilm chlorophyll-*a* was limited by N and/or P at 55% and biofilm AFDM was limited at 26% of sample points. N_2 fixation and denitrification rates did not differ significantly with differing nutrient limitation. Predictive models for N_2 fixation and denitrification rates both included variables related to the composition of dissolved organic matter, while the model for N_2 fixation also included P concentrations. These results demonstrate the potential for heterogeneity in habitat characteristics, nutrient availability, and organic matter composition to lead to biogeochemical complexity at the local scale, despite overall N removal at broader scales.

Keywords N_2 fixation · Denitrification · Dissolved organic matter · Wetland · Stream · Lake

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Introduction

Wetland–stream–lake ecotones are critical systems regulating complex biogeochemical cycling (Hedin 1998, Sierzen et al. 2012, Flint and McDowell 2015). Wetlands and lakes are known to store nutrients in sediments, and wetlands can decrease outflowing concentrations of some nutrients through retention

and removal, whereas streams can transport and transform material as it moves downstream in addition to storage in sediments (Knuth and Kelly 2011, Sierzen et al. 2012, Flint and McDowell 2015). These three aquatic habitats, though diverse, are spatially connected through cross-ecotone processes that alter material form and export magnitude (Kling et al. 2000; Jones 2010; Baker et al. 2016). For example, streams and wetlands are important sources of nutrients and organic matter to lakes, where the nutrients and organic matter are used to support primary and secondary production in the lakes (Biddanda and Cotner 2002; Dila and Biddanda 2015). Stream inflows deliver organic matter and invertebrates to lakes, creating hotspots of productivity and biodiversity (Richardson et al. 2021). Lakes in watersheds control the hydrology, water temperature, and flux of nutrients to outflow streams and wetlands, which can affect metabolic and biogeochemical processes within downstream environments (Baker et al. 2016). Upstream wetlands can supply dissolved organic carbon to streams, and the presence of embedded lakes in wetland-stream networks can influence the flux of dissolved nutrients (Lottig et al. 2013). Therefore, differences in environmental variables created by spatial heterogeneity within and across wetland–stream–lake ecotones can have consequences for biogeochemical processes.

Nutrient limitation could facilitate spatial heterogeneity of biogeochemical processes across wetland–stream–lake ecotones. There is abundant evidence that primary producers in the water column of the Great Lakes are primarily limited by phosphorus (P) (Schelske et al. 1987). However, both nitrogen (N) and/or P may limit primary producers in tributary streams and coastal wetlands of the Great Lakes, where nutrient diffusing substrate experiments have shown that increased N concentrations can increase biofilm standing crops and microbial activity (Allen and Hershey 1996; Wold and Hershey 1999; Cooper et al. 2016). Moreover, these experiments have also shown that in wetlands that are degraded by high N inputs, biofilms can become P limited (Cooper et al. 2016), which could create conditions more suitable for different microorganisms to perform processes that were not favorable when N was limiting, like denitrification. Therefore, spatial gradients in nutrient limitation across wetland–stream–lake ecotones may promote the co-occurrence of different biogeochemical processes—particularly N_2 fixation and denitrification, which have long been thought to be mutually exclusive in freshwater ecosystems (Marcarelli et al. 2008; Eberhard et al. 2018). N_2 fixation is the conversion of N_2 gas into biologically available N, while denitrification is the metabolic conversion of nitrate (NO_3^-) into N_2 gas, both of which are microbially-mediated in aquatic ecosystems (Schlesinger and Bernhardt 2013). Together these two processes control net N_2 flux (Fulweiler and Heiss 2014), however both processes are rarely studied together in aquatic ecosystems because different environmental factors favor each process (Marcarelli et al. 2008). Traditionally it has been assumed that a difference in N concentrations was the major factor driving the occurrence of these processes (Marcarelli et al. 2008). Under low NO_3^- concentrations, N_2 fixation should be favored because the process has significant energy costs to the organism, while denitrification requires higher concentrations of NO_3^- to use as an oxidant (Grimm and Petrone 1997; Arango et al. 2007). However, the occurrence of these processes cannot be consistently predicted by N concentrations alone, and their rates are also controlled by other environmental variables, like P and carbon (C) availability, across ecosystems (Marcarelli et al. 2008; Eberhard et al. 2018).

Beyond nutrient limitation, the co-occurrence of N_2 fixation and denitrification may be driven spatially by other environmental variables across wetland–stream–lake ecotones. N and C concentrations and composition can exhibit spatial patterning with the presence of plants, water depth, organic matter, and soil moisture in wetland and floodplain ecosystems (Bellinger et al. 2014; Orr et al. 2014; Wang et al. 2016). In stream ecosystems, a positive relationship between denitrification rates and the amount and quality of organic matter has long been recognized (Holmes et al. 1996; Groffman et al. 2005; Barnes et al. 2012; Eberhard et al. 2018). However, these types of relationships have not been widely studied spatially across wetland–stream–lake ecotones (Larson et al. 2013, 2016). Flow paths through terrestrial alder stands can result in hot spots of N inputs into streams and streamside wetlands (Callahan et al. 2017). Oxbow wetlands can receive stream and storm flow that result in the wetlands being significant sinks of N through loss via denitrification (Harrison et al. 2014). Examining the spatial heterogeneity of environmental variables across wetland–stream–lake

ecotones may better explain N cycling at the local scale within ecotones.

The rates and net contributions of denitrification and N_2 fixation have been relatively understudied in the Great Lakes region where P limitation of water column primary producers is common (Schelske and Roth 1973). The lakes themselves are oligotrophic with dissolved N concentrations that are high and/or rising (McDonald et al. 2010). N_2 fixation may occur at low to negligible rates in the water column of the Great Lakes since primary producers are primarily P-limited (Mague and Burris 1973), whereas denitrification may occur in lake sediment if there is sufficient NO_3^- availability (Small et al. 2014b). However, some studies in the Great Lakes have shown that N_2 fixation is important to offsetting biological N deficits in phytoplankton communities despite differing levels of N concentrations, and in periods of N limitation in eutrophic waters of Lake Erie, N_2 fixation rates can exceed NO_3^- and NH_4^+ uptake (Salk et al. 2018; Natwora and Sheik 2021). In coastal regions of the Great Lakes, denitrification can occur in stream sediments when there is sufficient NO_3^- availability and organic matter content (Bellinger et al. 2014). In a tributary stream of Lake Erie, denitrification has been shown to lead to N limitation in downstream wetlands when the outlet stream was blocked by a sand barrier that forms periodically on the lake shoreline (McCarthy et al. 2007). Wetlands in this region are typically thought of as sinks of N and P via retention and removal (Small et al. 2014a). Outside of the Great Lakes region in constructed wetlands, the ratio of N:P can decrease downstream as N is permanently removed through denitrification, creating ideal conditions for N_2 fixing organisms downstream (Scott et al. 2005, 2008). Since wetlands are shallow, they may have warmer temperatures than surrounding streams and lakes that could be more conducive to organisms performing N_2 fixation, as higher temperatures have been shown to stimulate N_2 fixation activity (Marcarelli and Wurtsbaugh 2006; Welter et al. 2015). N_2 fixing cyanobacteria and diatoms have been observed in Great Lakes coastal wetlands where abundance of the algae was higher in N-limited wetlands and negatively correlated with dissolved N concentrations (Cooper et al. 2016). Wetlands and lakes also have the potential for N_2 fixation through attached epiphytes on macrophytes (Finke and Seely 1978, Doyle and Fisher 1994; Scott et al. 2005; Marcarelli and Wurtsbaugh 2009). Quantifying these processes along the full spatial continuum of wetland–stream–lake ecotones could change our understanding of the importance of these two processes to the local N cycle of Great Lakes coastal ecotones.

The goal of this study was to evaluate how the spatial heterogeneity of environmental characteristics across wetland–stream–lake ecotones control the net N_2 flux in these ecosystems. We first hypothesized that the spatial heterogeneity of wetland–stream–lake ecotones would lead to spatial variability in nutrient limitation of biofilms. Secondly, we hypothesized that the spatial variability in nutrient limitation of biofilms would be related to the co-occurrence of N_2 fixation and denitrification across wetland–stream–lake ecotones, where sites with N or N+P limitation of biofilm chlorophyll-*a* and biomass would have higher rates of N_2 fixation, and sites with P limitation of biofilms would have higher rates of denitrification than sites with N or no biofilm nutrient limitation. Finally, we hypothesized that spatial patterns of physical and chemical conditions would predict rates of these processes. Particularly, denitrification rates would be highest where there is high quality organic C available (relatively low aromaticity, with indices correlated with degradation) and anoxic conditions (e.g., wetland, lake, and stream sediments), while N_2 fixation would occur where there are warm temperatures and low nitrate (e.g., stream microhabitats, shallow water in wetlands, epiphytes on macrophytes in wetlands and lakes).

Methods

Study area

This study was conducted in 5 wetland–stream–lake ecotones in Lakes Superior and Huron, selected to span a gradient of nutrient loading and human impact conditions (Fig. 1, Tables S1 and S2 in Supplemental Information). The Sioux and Mackinac ecotones were selected as sites where we expected low levels of human impact, while Nara was selected as a site with moderate levels of impact, and the Saganing and Wildfowl ecotones were selected as sites with high levels of impact. The Nara ecotone was in the Nara Nature Area in Houghton, MI that encompasses part of the Pilgrim River, a tributary to the Keweenaw Waterway, which flows into Lake Superior. Nara is managed and owned by the city of

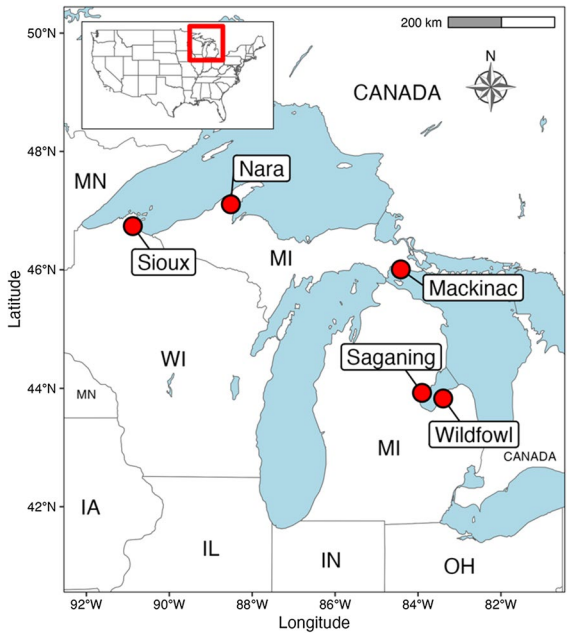

Fig. 1 Map of the states of Michigan and Wisconsin and the 5 wetland–stream–lake ecotones sampled in this study marked by a red dot. The 5 sites were Sioux, Nara, Mackinac, Saganing, and Wildfowl

Houghton, MI. The Sioux ecotone was along the Sioux River in Washburn, WI which is a tributary to Lake Superior and managed by WI Department of Natural Resources. The Mackinac Bay ecotone was located near the Les Cheneaux Islands in northern Lake Huron and managed by the Little Traverse Conservancy. Both the Saganing and Wildfowl Bay ecotones drain into Saginaw Bay of Lake Huron. Saganing is managed by the Saginaw Bay Land Conservancy and Wildfowl is managed by the Michigan Department of Natural Resources. All ecotones were categorized as shrub swamp and emergent marsh cover types in the Great Lakes Coastal Wetland Mapping tool (Bourgeau-Chavez et al. 2015). The Wildfowl and Saganing transects were the only transects noted for the presence of the invasive plant *Phragmites australis* at the time of mapping in 2015.

Study design

Transect setup

The Nara, Sioux, and Mackinac ecotones were sampled in summers 2018 and 2019, while the Saganing and Wildfowl ecotones were only sampled in summer 2020 (Table 1, see also Figs. S1-5 in Supplemental Information). Although we had planned to sample all 5 sites in summer 2020, this was prevented by limitations to field work caused by the COVID-19 pandemic. Each interface was sampled across 1–3 sampling days in each year due to the number of transect points and the duration of incubations. Sampling days in each ecotone were typically sequential, but in some cases, there was a day (and in one case 3 days) between sampling due to inclement weather (Table 1). On the first day at each ecotone, a transect of 8–15 points was established that encompassed the wetland–stream–lake ecotone. Based on visual classifications of the 83 total transect points among all transects, 37 were wetland, 18 were transition zones from wetland to stream, 15 were stream, 2 were stream to lake transition zones and 11 were lake sites (Table 1). The number of wetland sites per transect ranged from 3 to 7, the number of stream sites ranged from 0 to 3, and the number of lake sites ranged from 0 to 4. Wetland to stream transition zones ranged from 1 to 4 sites per transect and the number of stream to lake transition zones were either 0 or 1 in each transect. In the Saganing and Wildfowl transects, there were no sites sampled that could be strictly classified as stream or lake because the stream bed itself and nearshore lake areas were too deep to safely deploy chamber incubations given our sampling equipment.

Nutrient limitation

To test the first hypothesis that the spatial heterogeneity of environmental characteristics across the wetland–stream–lake ecotones would lead to spatial variability of nutrient limitation for biofilms, we deployed nutrient diffusing substrates (NDS, Tank et al. 2017) in summers 2019 and 2020 at all transect points except those that were too deep, had high wave action, or had no standing water (see Tables S3 and S4 in Supplemental Information). NDS were constructed using 45 mL plastic containers filled with a 2% (by weight) agar solution amended with 0.8 M N added as $NaNO_3$ (N treatment), 0.05 M P added as NaH_2PO_4 (P treatment), both (N + P treatment), or neither as a control treatment. A 25 mm porous porcelain disc (Leco Corporation, St. Joseph, MI) was placed on top of each hardened NDS for biofilms to grow on. At transect points, a total of 16 NDS were deployed

Table 1 The sampling design of each interface is explained including years each transect was sampled, total transect points, total number of sites classified as wetland (standing and no standing water), number of sites classified as wetland to stream transition zones, number of sites classified as stream, number of sites classified as stream to lake transition zones, number of sites classified as lake, dates sampled, and GPS coordinates in °N and °W

Transect	Year sampled	# of Transect points	# of Wetland (no standing water)	# of Wetland (standing water)	# of Wetland-stream	# of Stream	# of Stream-Lake	# of Lake	Sample dates	GPS Coordinates (°N, °W)
Nara	2018	15	4	2	4	3	0	2	18-Jul, 19-Jul	47.10612, − 88.51456
Sioux	2018	9	0	4	1	3	0	1	14-Jul	46.73722, − 90.87925
Mackinac	2018	12	3	1	1	2	1	4	22-Aug, 23-Aug	46.00581, − 84.41543
Nara	2019	12	1	2	4	3	0	2	26-Aug, 30-Aug, 2-Sep	47.10612, − 88.51456
Sioux	2019	9	0	4	1	3	0	1	24-Jul, 25-Jul	46.73722, − 90.87925
Mackinac	2019	8	1	3	1	1	1	1	17-Jul, 18-Jul	46.00581, − 84.41543
Saganing	2020	9	0	7	2	0	0	0	7-Sep, 9-Sep	43.924037, − 83.904138
Wildfowl	2020	9	0	5	4	0	0	0	10-Sep, 11-Sep	43.828629, − 83.392688

Wetland sites with no standing water were saturated soils near sites in the wetland with standing water. Not all transects were sampled every year and some transects varied in the total number of transect points from year to year as well as the classification type of the transect points

with 4 control, 4 N, 4 P, and 4 N+P replicates. NDS were deployed at transect points two weeks prior to the first sampling day at a site. After these two weeks, the discs were collected, wrapped in aluminum foil and frozen for later analysis of biofilm biomass using chlorophyll-*a* and ash free dry mass (AFDM), which provides an estimate of the total organic material present in a sample. Laboratory analysis of chlorophyll-*a* followed standard methods using a Thermo Scientific 10 s UV–Vis spectrophotometer and ethanol extraction (APHA 2005). All discs were also analyzed for AFDM as the difference between the mass of the ashed samples and the initial dry samples. AFDM samples were dried at 50 °C, weighed for dry mass and then ashed in a muffle furnace at 550 °C, rewetted, and dried before a final weighing.

N cycling rate measurements

Chamber incubations were used to measure rates of N_2 fixation and denitrification in all transect points. The chambers used during these incubations varied by substrate type. 2-L polycarbonate food storage containers were used for larger macrophyte substrate (Gettel et al. 2007; Eberhard et al. 2018). The chamber lids were sealed airtight with a Viton o-ring, and were fit with a 13 × 20 mm septum for sample collection. For sediment and smaller macrophyte substrate, chambers were made from pint size glass mason jars and lids were similarly fit with an airtight sampling septum. Macrophytes were collected using chamber lids to approximate surface area of macrophyte to sample, then pulling from the root and placing in chambers. Sediment substrate was collected using a 7 cm diameter suction corer to collect ~ 200–400 mL of sediment that was then placed into chambers. For each transect point, there were 1–4 sample chambers and 1–4 blank chambers, with each sample chamber having a paired blank chamber. The blank chambers were filled with stream water and incubated alongside sample chambers to simulate an environment with minimal N_2 fixation or denitrification to control for chamber effects. Blank chambers were confirmed to have minimal to no N_2 fixation or denitrification activity.

N_2 fixation rates were measured using acetylene reduction (Capone 1993; Dodds et al. 2017). An acetylene-filled balloon was added to each chamber. Chambers were filled with stream water and sealed

underwater, then balloons were popped with a needle through the sampling septum to introduce a 20% acetylene headspace. Chambers were shaken for approximately 20 s to equilibrate the gas dissolved in the water with that in the headspace, and initial gas samples were collected within 5 min of sealing the chambers. Chambers were placed in the water for a 2-h incubation to maintain ambient water temperatures, then shaken again to equilibrate and final samples were collected. All gas samples were placed into evacuated 9-mL serum vials and kept in the dark until analyzed. Ethylene concentrations were measured using a SRI 8610C gas chromatograph equipped with a Hayesep T column, He carrier gas, and a flame ionization detector. The column oven was set to 40 °C. To obtain N_2 fixation rates, ethylene concentrations in the chambers were compared to 100 ppm ethylene standards (Matheson Tri Gas). N_2 fixation rates were calculated following Capone (1993) and Dodds et al. (2017), then converted to µg of N assuming a ratio of 3 mol of ethylene produced for every 1 mol of N_2 gas potentially fixed (Capone 1993).

Denitrification rates were measured using the acetylene block method (Groffman et al. 2006). We chose this method because most previous stream studies have used this method and we wanted to be able to compare estimates to these studies, and because this method is quick and easy to run with a large number of replicates to estimate rate variability. However, the acetylene block method inhibits nitrification, so measuring without amendments of nitrate can underestimate denitrification rates (Dodds et al. 2017). Therefore, we amended each chamber with 0.62 g L^{-1} Glucose as a C source and 0.62 g L^{-1} $NaNO_3$ as an N source, and also added chloramphenicol (2 g L^{-1}) to suppress additional protein synthesis during the incubation in all chambers. Chambers were not sparged with nitrogen or helium to create anoxic conditions. After the amendment, acetylene was introduced, chambers were incubated, and initial and final gas samples were collected as described previously for N_2 fixation. Nitrous oxide (N_2O) concentrations were measured using a SRI 8610C gas chromatograph equipped with a Hayesep D column, He carrier gas, and an electron capture detector. The column oven was set to 40 °C. N_2O concentrations in chambers were compared to standard concentrations of 1000 ppm N_2O (Matheson Tri Gas). Denitrification rates were calculated following Dodds et al. (2017).

To scale process rates by substrate area, all substrate material was collected and analyzed after incubations. Sediment and macrophyte material were analyzed for AFDM as described in the above *Nutrient Limitation* section. Surface area and volume of all substrates were also measured for use in scaling process rates for surface area. Sediment surface area was calculated as the diameter of the corer. Macrophyte surface area was calculated as the diameter of the chamber lid. Sediment volume was determined by multiplying the surface area by average sediment core depth in the jar and macrophyte volume was measured by displacement in a graduated cylinder.

Environmental characteristics

To test the third hypothesis that variation in physical and chemical conditions would predict the occurrence of N_2 fixation and denitrification process rates, we measured depth at each transect point and measured canopy cover (%) using a spherical densiometer (Lemmon 1956). We also collected ~ 40 mL water samples from each transect point. The water was filtered using Millipore 0.45 µm nitrocellulose membrane filters into 60 mL bottles. Samples were frozen until laboratory analysis for NO_3^-−N, ammonium (NH_4^+-N), soluble reactive phosphorus (SRP), total dissolved phosphorus (TDP), dissolved organic carbon (DOC), and total dissolved nitrogen (TDN). NH_4^+−N was analyzed using a fluorometric method (Holmes et al. 1999; Taylor et al. 2007) on a Turner Aquafluor (Turner Designs, Palo Alto California). NO_3^-+NO_2 samples were analyzed on a SEAL AQ_2 discrete water analyzer using the AQ_2 method EPA-127-A Rev. 9. DIN concentration was then calculated by adding concentrations of NH_4^+−N and NO_3^-+NO_2. SRP samples were analyzed on a SEAL AQ_2 discrete water analyzer using the AQ_2 method EPA-155-A Rev. 0. TDP samples were analyzed on a Thermo Scientific 10 s UV–Vis spectrophotometer using the ascorbic acid method and molybdenum antimony colorimetric determination methods (APHA 2005). For TDP samples, an ammonium persulfate digestion was used prior to this analysis. DOC and TDN samples were run on a Shimadzu TOC-L_{CPH} analyzer with TNM-L module in the AQUA lab at Michigan Tech.

Water was also filtered using Millipore 0.45 µm nitrocellulose membrane filters into 60 mL amber

bottles with no headspace for DOM analysis. Samples were kept refrigerated until analysis for absorption and fluorescence spectra using a Horbia Aqualog fluorometer (Horiba–Jobin–Yvon Aqualog C; Horiba Co., Edison, New Jersey) in 1 cm quartz cells (Starna Cells, Inc) to determine fluorescence excitation-emission matrices (EEMs). Absorption spectra were run from 240 to 600 nm at 3 nm resolution. Fluorescence spectra were collected at intervals of 1 nm excitation wavelengths from 240 to 800 nm and emission was recorded at 3 nm resolution from 240 to 640 nm. Fluorescence spectra were corrected for inner filter effects and converted to Raman units (R.U.) as described in detail in Meingast et al. 2020. Samples with absorbance greater than 0.6 at $\lambda = 254$ were diluted to satisfy the assumption of detector linearity required by modeling (Horiba Corp. 2012). Absorption and fluorescence spectra were then used to calculate different parameters indicative of the structure and/or composition of DOM (Chen et al. 2003; Miller et al. 2006). Two specific spectral slopes and their spectral ratio (SR) were derived for $S_{275–295}$ and $S_{350–400}$ from the absorption spectra as indicators of average DOM molecular weight (Helms et al. 2008). The absorbance index E2:E3 ($\lambda = 254:365$ nm)—an inverse index of both molecular size (De Haan and De Boer 1987; Helms et al. 2008; Zhang and He 2015) and electron acceptor capacity (Sharpless et al. 2014) was also measured. We also calculated the biological index (BIX), which is used to differentiate between terrestrial reference standards of DOM and phytoplankton derived DOM, and the humification index (HIX), which is used as an index of soil humification (Ohno 2002; Osburn et al. 2019; Meingast et al. 2020). To further distinguish the source of DOM, we also calculated the fluorescence index (FI) as the ratio of the emission intensity at 450–550 nm acquired with an excitation of 370 nm from corrected sample EEMs (McKnight et al. 2001). We also calculated a "redox index", which indicates the proportional amount of reduced components (fluorophores) associated with quinone-like biomolecules (Miller et al. 2006). $SUVA_{254}$, a metric used to quantify the amount of chromophoric DOM in a sample, was calculated following Weishaar et al. 2003, where the specific absorbance at 254 nm was normalized by the DOC concentration of the sample.

Statistical analysis

To evaluate our first hypothesis that spatial heterogeneity of wetland–stream–lake ecotones would lead to spatial variability in nutrient limitation for biofilms, we used a two-way analysis of variance (ANOVA) with N and P as factors to test whether chlorophyll-*a* and AFDM concentrations were significantly different (p-value ≤ 0.05) among NDS treatments at each transect point (Tank and Dodds 2003; Tank et al. 2017). Single nutrient limitation was indicated if just one of the individual treatments (N or P) indicated a positive response, but the interaction term of the ANOVA was not significant. Co-limitation by N and P was determined when either both individual treatments indicated a positive response, the interaction term of the ANOVA was significant, or if both the interaction term of the ANOVA was significant and one of the individual treatments indicated a positive response. No significant terms (p-value ≥ 0.05) indicated no nutrient limitation. ANOVAs were performed in RStudio (R version 4.1.2).

To evaluate the second hypothesis that spatial variability in nutrient limitation would facilitate the co-occurrence of N_2 fixation and denitrification across wetland–stream–lake ecotones, we performed Kruskal–Wallis tests in RStudio (R version 4.1.2), as the data were not evenly distributed across different nutrient limitation categories. We ran four separate tests with N_2 fixation or denitrification rates as the response variable and nutrient limitation status with chlorophyll-*a* or AFDM as the predictor variable. Similarly, we also used Kruskal–Wallis tests to evaluate if rates of N_2 fixation and denitrification varied significantly among the different habitat types of the ecotones (wetland, wetland to stream transition zone, stream, stream to lake transition zone, and lake).

Since the dataset for this study was nonlinear and spatially autocorrelated, we chose to use predictive modeling to evaluate the third hypothesis that spatial patterns of nutrients, light availability, and quality of organic matter would predict rates of these processes. Predictive modeling is a mathematical process that uses known results to create and validate a model that generates predictions accurately, although there is a trade-off between accurate predictability and direct interpretability of models (Kuhn and Johnson 2013). Separate models were generated with N_2 fixation rates and denitrification rates as individual response

variables. For all models, the predictor variables were substrate type, canopy cover (%), depth (cm), temperature (°C), AFDM (g/m^2), $S_{275–295}$, $S_{350–400}$, E2_E3, SR, BIX, HIX, FI, EEM tryptophan index, EEM tyrosine index, EEM redox index, $SUVA_{254}$, SRP (mg/L), TDP (mg/L), dissolved inorganic nitrogen (DIN, mg/L), DOC (mg/L), and TDN (mg/L) concentrations. All predictor variables included in the models were based on a priori hypotheses and general knowledge of biogeochemistry. Nutrient limitation of biofilms from NDS was not used as a predictor variable because this data was only collected in 2019 and 2020 and the models used process rates from 2018, 2019, and 2020 as response variables. Nine total transect points were removed from the data matrix because they were categorized as terrestrial or with no standing water, so they did not have the full suite of predictors collected at those sites. All data were pre-processed by centering, scaling, removing near-zero variables, and imputing missing variables using 5-nearest neighbors. Variables were tested for high correlation at a cutoff value of 85%, but no variables had to be removed (Table S5 in Supplemental Information). Each dataset was split into training and testing sets using stratified random sampling based on transect name and sampling year, so that each set would have an even distribution of the transects and years sampled. 80% of the data was placed into a training set to build and tune the models and 20% of the data was placed into a testing set to estimate the models' predictive performance. Replacement was used due to the small size of each dataset (105 observations, 21 variables) and we used Monte Carlo and bootstrapping resampling methods with 10 resamples for each test model. Data were placed into different sets to find the most realistic predictive model performance without overfitting. We then trained a variety of regression-based models including: partial least squares, ridge regression, elastic net/lasso, neural networks, support vector machines, MARS/FDA, K-nearest neighbors, single trees, model trees/rules, bagged trees, random forest, boosted trees, and cubist (summarized in Kuhn and Johnson 2013). For each model the seed was set to 100 and test set performance was evaluated. Best fit models were selected based on lowest root mean square error (RMSE) and a high (> 10%) R^2 value. For each best fit model, we then looked at the predictor variables of most importance to evaluate our hypotheses. All predictive modelling was done in RStudio (R version 4.1.2) using the caret package (Kuhn 2019).

Results

Nutrient limitation

We observed spatial variability in nutrient limitation of biofilms determined using NDS across 4 of the 5 ecotones (Fig. 2). Of all 31 transect points measured, chlorophyll-*a* limitation by N was indicated at 32%, co-limitation by N and P was indicated at 23%, and no nutrient limitation at was indicated at 45% of the points. At the Nara, Wildfowl, and Saganing transects, we observed a range of chlorophyll-*a* limitation responses, with N limitation, P limitation and co-limitation of N and P at different points along the transects. In contrast to these 3 ecotones, at the Sioux transect, only N limitation of chlorophyll-*a* was observed at 4 sites, while 4 sites showed no nutrient limitation. Of the 27 transect points with AFDM data, there were 7% of sites with P limitation, 19% with co-limitation of N and P, and 74% where no nutrient limitation was indicated. As with chlorophyll-*a*, we observed co-limitation of AFDM by N and P at different points along the transects at the Nara, Wildfowl, and Saganing transects. No NDS data were available from the Mackinac transect because most were lost due to high-water levels and storms.

Process co-occurrence and nutrient limitation

N_2 fixation and denitrification co-occurred across all wetland–stream–lake ecotones. Rates of N_2 fixation ranged from 0 to 1950 μg N m^{-2} h^{-1} with a median of 5.49 μg N m^{-2} h^{-1}, while denitrification rates ranged from 0 to 16,536 μg N m^{-2} h^{-1} with a median of 914 μg N m^{-2} h^{-1}. When comparing across all transects denitrification rates were overall much higher than N_2 fixation rates indicating that these systems are overall sinks of N (Fig. 3). However, there were some transect points where N_2 fixation rates were high and denitrification rates were near zero, indicating that within transects there can be locations that are sources of N (Fig. 3). Contrary to our first hypothesis,there were no significant differences in rates of N_2 fixation or denitrification across habitat types of the ecotones ($\chi^2 = 2.57$, df = 4,

		Chl-a			AFDM		
Transect	Type	N effect	P effect	Interaction NxP	N effect	P effect	Interaction NxP
Nara 2019	Wetland	p-value < 0.01		p-value = 0.03	p-value = 0.01		p-value = 0.02
	Wetland	p-value < 0.01					
	Wetland-Stream	p-value < 0.01					p-value = 0.03
	Wetland-Stream	p-value = 0.01	p-value = 0.02				
	Wetland-Stream	p-value < 0.01	p-value = 0.05	p-value = 0.03			
	Stream						
	Lake	p-value = 0.02	p-value = 0.04	p-value = 0.03			
Sioux 2019	Wetland	p-value = 0.01					
	Wetland						
	Wetland	p-value = 0.04					
	Wetland						
	Wetland-Stream						
	Stream	p-value = 0.03					
	Stream	p-value < 0.01					
	Stream					p-value < 0.01	
Saganing 2020	Wetland			p-value = 0.02	p-value = 0.02	p-value = 0.02	
	Wetland-Stream						
	Wetland					p-value < 0.01	
	Wetland						
	Wetland						
	Wetland						
	Wetland						
	Wetland-Stream	p-value < 0.01	p-value = 0.02				
	Wetland-Stream						
Wildfowl 2020	Wetland						
	Wetland	p-value = 0.02					
	Wetland	p-value < 0.01				p-value = 0.04	p-value < 0.01
	Wetland	p-value = 0.03					
	Wetland	p-value < 0.01	p-value < 0.01	p-value = 0.01			
	Wetland-Stream						
	Wetland-Stream	p-value < 0.01			p-value = 0.04	p-value = 0.01	

Fig. 2 Nutrient limitation data based on chlorophyll-*a* (Chl-a) and ash free dry mass (AFDM) concentrations (g m^{-2}) collected from nutrient diffusing substrates (NDS) for 4 of the 5 transects. Transect points with N effect are colored blue, P effect yellow, and N:P effect green. No nutrient limitation is colored gray; black indicates samples were lost due to a lab error. N = nitrogen and P = phosphorus. P-values are denoted where significant (p-value ≤ 0.05)

p-value = 0.63 and $\chi^2 = 4.74$, df = 4, p-value = 0.31 respectively, Fig. 4). The highest rates of N_2 fixation occurred in wetlands and wetland to stream transition zones, while the highest denitrification rate occurred in a stream site, but high rates of denitrification were observed across all habitat types except the stream to lake transition zones (Fig. 4).

Across habitat types, N_2 fixation occurred on both sediment and macrophytes (Figs. 5, 6). Of the 114 chamber measurements across all transects, 82 were on sediment substrate and 32 were on macrophyte substrate. Mackinac 2018 and Saganing had the highest number of overall macrophyte samples (n = 7, n = 8, respectively), while all other transects had 2–4 macrophyte samples. Of the 27 transect points with rates higher than the 75th percentile of all N_2 fixation rates (> 45.1 µg N m^{-2} h^{-1}), 14 of those rates occurred on sediment substrate and 13 occurred on macrophyte substrate (Figs. 5, 6). Denitrification occurred mostly in sediments, but occasionally on macrophytes. For denitrification, of the 29 transect points with rates higher than the 75th percentile (> 3129 µg N m^{-2} h^{-1}), 26 of those rates occurred

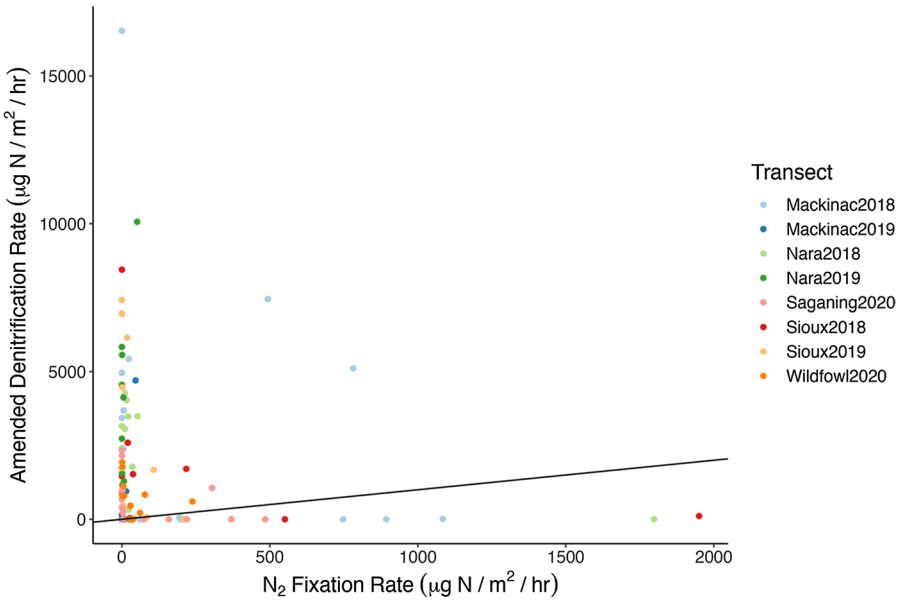

Fig. 3 Scatter plot comparing the rates of denitrification and N_2 fixation ($\mu g\ N\ m^{-2}\ h^{-1}$) at each transect point with a 1:1 line. Different colors represent a different year and transect combination. Note the Y-axis for denitrification is 6 × higher than for N_2 fixation

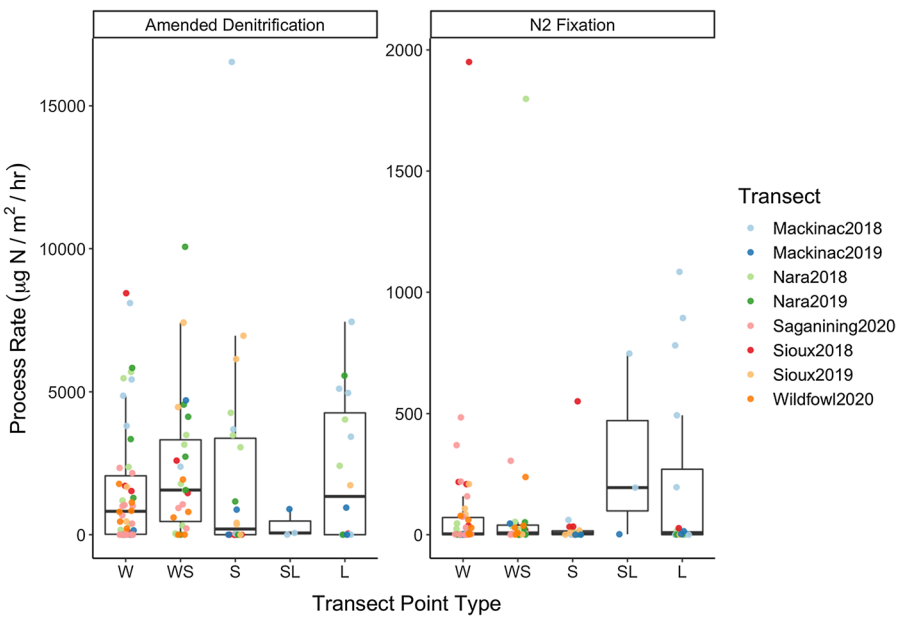

Fig. 4 Rates of denitrification and N_2 fixation ($\mu g\ N\ m^{-2}\ h^{-1}$) among all transects compared to transect point classification. The classifications were wetland (W, chamber n = 46), wetland to stream transition (WS, n = 27), stream (S, n = 22), stream to lake transition (SL, n = 3), and lake (L, n = 16). Note the Y-axis for denitrification is 6 × higher than for N_2 fixation

on sediment substrate and 3 occurred on macrophyte substrate (Figs. 5, 6).

To test our second hypothesis that spatial variability in nutrient limitation would facilitate co-occurrence of N_2 fixation and denitrification, we found no significant relationship between either N_2 fixation or denitrification rates and biofilm chlorophyll-*a* nutrient limitation status ($\chi^2 = 5.45$, df = 2, *p*-value = 0.07 and $\chi^2 = 2.04$, df = 2, *p*-value = 0.36 respectively, Fig. 7). Also, no significant relationship was found between biofilm AFDM nutrient limitation status and rates of either N_2 fixation or denitrification ($\chi^2 = 0.38$, df = 2, p-value = 0.83 and $\chi^2 = 4.62$, df = 2, *p*-value = 0.10 respectively, Fig. 7). Observationally, the highest rates of N_2 fixation were in sites with no biofilm chlorophyll-*a* and AFDM nutrient limitation followed by AFDM P limitation, whereas for denitrification the highest

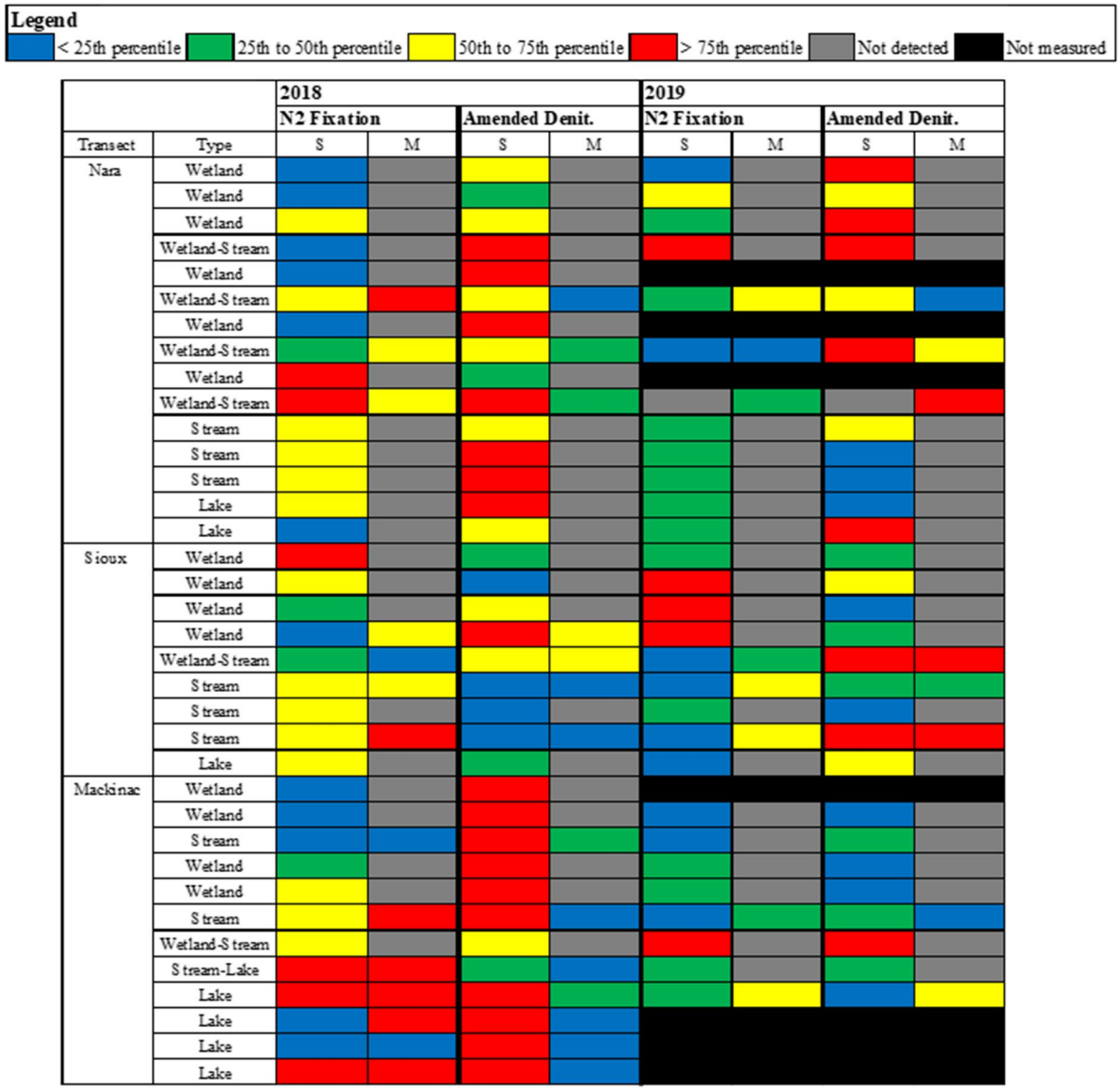

Fig. 5 A heatmap of N_2 fixation and amended denitrification (amended with N and C as described in methods) rates (μg N m^{-2} h^{-1}) across the Nara, Sioux, and Mackinac transects in 2018 and 2019 and substrate type (M = macrophyte or S = sediment). Transects began from the wetland and moved to stream to lake. Rates of both processes are color coded based on quartiles across all 5 transects. Grey indicates no rate was detected for the transect point and substrate combination and black indicates no measurements were taken. For N_2 fixation Q1 = 0.02, Q2 = 5.49, and Q3 = 45.1 μg N m^{-2} h^{-1}, and for denitrification Q1 = 0, Q2 = 913.8, and Q3 = 3129.1 μg N m^{-2} h^{-1}

rate was in a site with biofilm chlorophyll-*a* N limitation and AFDM N + P limitation, followed by high rates in sites with no biofilm chlorophyll-*a* nutrient limitation and sites with AFDM P limitation (Fig. 7).

Environmental variation

We observed variation in environmental characteristics across all the transects. Canopy cover, temperature, AFDM, NH_4^+−N, NO_3^-+NO_2, DIN, SRP, TDP, DIN:TDP, DOC, and TDN concentrations

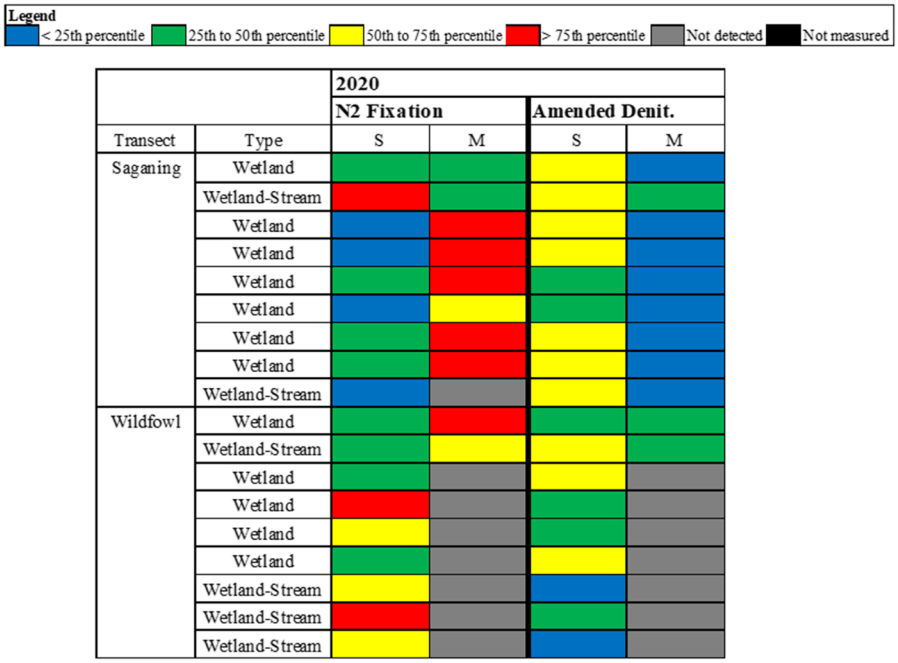

Fig. 6 A heatmap of N_2 fixation and amended denitrification (amended with N and C as described in methods) rates (μg N m^{-2} h^{-1}) across the Wildfowl and Saganing transects in 2020 and substrate type (M = macrophyte or S = sediment). Transects began from the wetland and moved to stream to lake. Rates of both processes are color coded based on quartiles across all 5 transects. Grey indicates no rate was detected for the transect point and substrate combination and black indicates no measurements were taken. For N_2 fixation Q1 = 0.02, Q2 = 5.49, and Q3 = 45.1 μg N m^{-2} h^{-1}, and for denitrification Q1 = 0, Q2 = 913.8, and Q3 = 3129.1 μg N m^{-2} h^{-1}

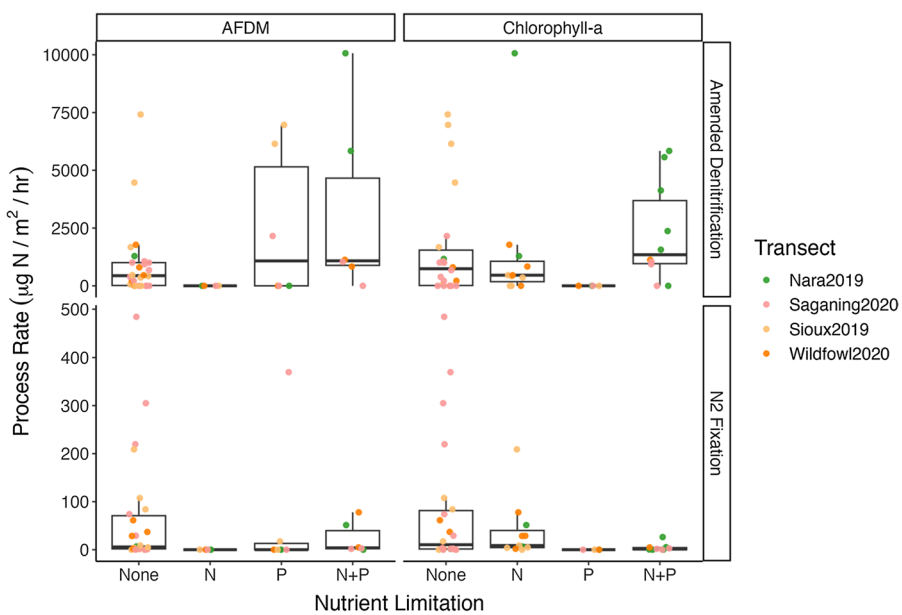

Fig. 7 Rates of N_2 fixation and denitrification (μg N m^{-2} h^{-1}) in comparison to nutrient limitation status of biofilms based off chlorphyll-*a* and ash free dry mass (AFDM) concentrations (g m^{-2}). Note the Y-axis for denitrification is 20 × higher than for N_2 fixation. N = nitrogen, P = phosphorus, and N + P = co-limitation of nitrogen and phosphorus

were more variable among transects than within, except for a few cases in which individual transects had higher variability within. (Supplemental Information Tables S1 and S2, Fig. S6). For DOM metrics, BIX, fluorescence tryptophan, tyrosine, and redox indices, FI, HIX, and SR were the most variable among transects, except for a few cases in which individual transects had higher variability within. E2_E3 was the most variable in Mackinac 2020. Both $S_{275\text{-}295}$ and $S_{350\text{-}400}$ showed similar variation among transects as within. $SUVA_{254}$ was more variable within transects than among transects (Supplemental Information Table S5, Fig. S7).

Environmental factors as predictors of process rates

Predictive modeling for denitrification did support our hypothesis that variables related to available and high quality organic C would facilitate higher rates of denitrification. The best fit model for denitrification rates was a MARS (multivariate adaptive regression splines) model using Monte Carlo resampling with a RMSE of 1.53 and a R^2 of 15% (Table 2). MARS models are nonlinear regression-based models that use surrogate features to create a piecewise linear model where each new feature models a portion of the original dataset. The surrogate features are created by breaking the predictor into two groups based on a cut point and modeling the linear relationship between the predictor and the outcome of that group (Kuhn and Johnson 2013). The top three variables of importance to the model were substrate (macrophyte or sediment), and two variables related to DOM quality and source: $SUVA_{254}$ and SR (Fig. 8). $SUVA_{254}$ is a measurement for the aromaticity of DOM and SR is the slope ratio used to determine the molecular

Table 2 Predictive modeling results for the response variables N_2 fixation rates and denitrification rates

Response variable	Model type	Monte Carlo		Bootstrapping	
		RMSE	R^2	RMSE	R^2
N fixation rates	Partial least squares	1.48	< 0.01	1.48	< 0.01
	Ridge regression	1.64	0.02	1.62	< 0.01
	Elastic net/lasso	1.47	< 0.01	1.49	< 0.01
	Neural networks	1.52	0.02	1.52	0.01
	Support vector machines	1.63	0.02	**1.45**	**0.13**
	MARS/FDA	1.47	0.02	1.54	< 0.01
	K-nearest neighbor	1.62	< 0.01	1.59	< 0.01
	Single trees	1.54	< 0.01	1.54	< 0.01
	Model trees	1.47	0.06	1.42	0.06
	Bagged trees	1.49	< 0.01	1.49	< 0.01
	Random forest	1.48	< 0.01	1.47	< 0.01
	Boosted trees	1.45	0.03	1.43	0.04
	Cubist	1.63	< 0.01	1.53	< 0.01
Denitrification rates	Partial least squares	1.71	< 0.01	1.64	0.11
	Ridge regression	1.72	< 0.01	1.72	< 0.01
	Elastic net/lasso	1.64	0.17	1.65	0.21
	Neural networks	1.64	0.06	1.64	0.08
	Support vector machines	1.72	0.08	1.79	0.06
	MARS/FDA	**1.53**	**0.15**	1.67	0.04
	K-nearest neighbor	1.68	0.02	1.70	0.01
	Single trees	1.62	0.01	1.62	0.01
	Model trees	1.61	0.13	1.61	0.13
	Bagged trees	1.69	0.02	1.69	0.02
	Random forest	1.70	< 0.01	1.70	< 0.01
	Boosted trees	1.70	0.01	1.71	< 0.01
	Cubist	1.61	0.14	1.74	0.04

Items in bold represent the model of best fit based on the lowest RMSE and $R^2 > 0.10$. RMSE = root mean square error

weight of DOM and indicate if the source is richer in DOM.

Predictive modeling for N_2 fixation did not support our hypothesis that temperature and NO_3^- concentrations would be important to predicting N_2 fixation rates. The best fit model for N_2 fixation was a support vector machine (SVM) with bootstrap resampling, with a RMSE of 1.45 and an R^2 of 13% (Table 2). SVMs are highly flexible nonlinear regression based models that use a kernel function to map complicated data patterns in a more simplistic way and minimize the effect of outliers on the regression equations (Kuhn and Johnson 2013). The nature of SVMs make them black box type models where they are not directly interpretable. The top three variables of importance to the model were TDP, AFDM, and the EEM redox index, which relates to a change in redox state (oxidized or reduced) of fluorophores (fluorescent chemical compounds) in DOM (Miller et al. 2006, Fig. 9).

Discussion

Our results demonstrate that N_2 fixation and denitrification do co-occur across habitats in wetland–stream–lake ecotones of Lakes Superior and Huron, and that the occurrence of these processes cannot simply be explained by differences in biofilm nutrient limitation or nutrient concentrations across the ecotones. When evaluating our first hypothesis that nutrient limitation would vary spatially across the ecotones, we found there was N, N+P, and/or no nutrient limitation of biofilm chlorophyll-*a* and AFDM at transect points across ecotones. When evaluating our second hypothesis that spatial variation in nutrient limitation would facilitate the co-occurrence of N_2 fixation and denitrification across the ecotones, there were no significant differences across nutrient limitation responses of biofilm chlorophyll-*a* or AFDM and rates of either N_2 fixation or denitrification. N_2 fixation and denitrification

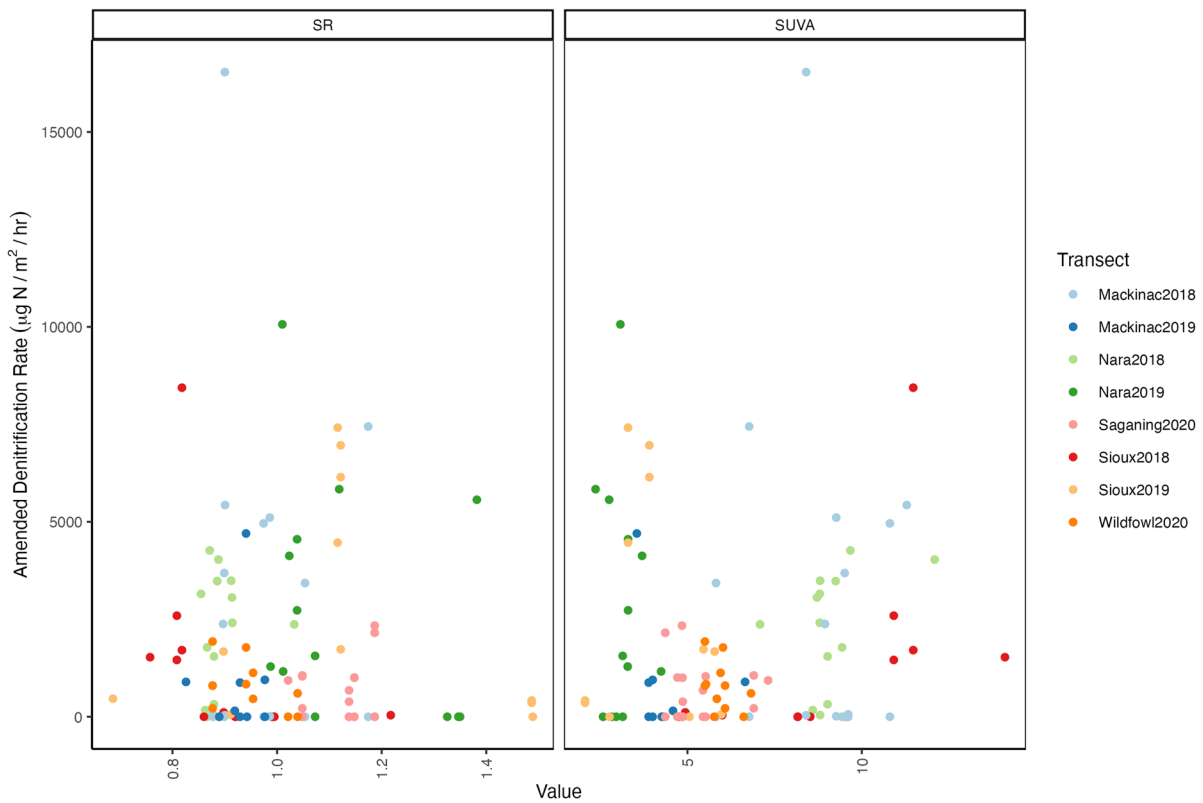

Fig. 8 Scatter plots of variables of importance to the MARS model predicting denitrification rates (µg N m^{-2} h^{-1}). SR stands for spectral slope and SUVA is the DOC-specific absorbance at 254 nm, or $SUVA_{254}$ metric. Dots are colored by transect name and year

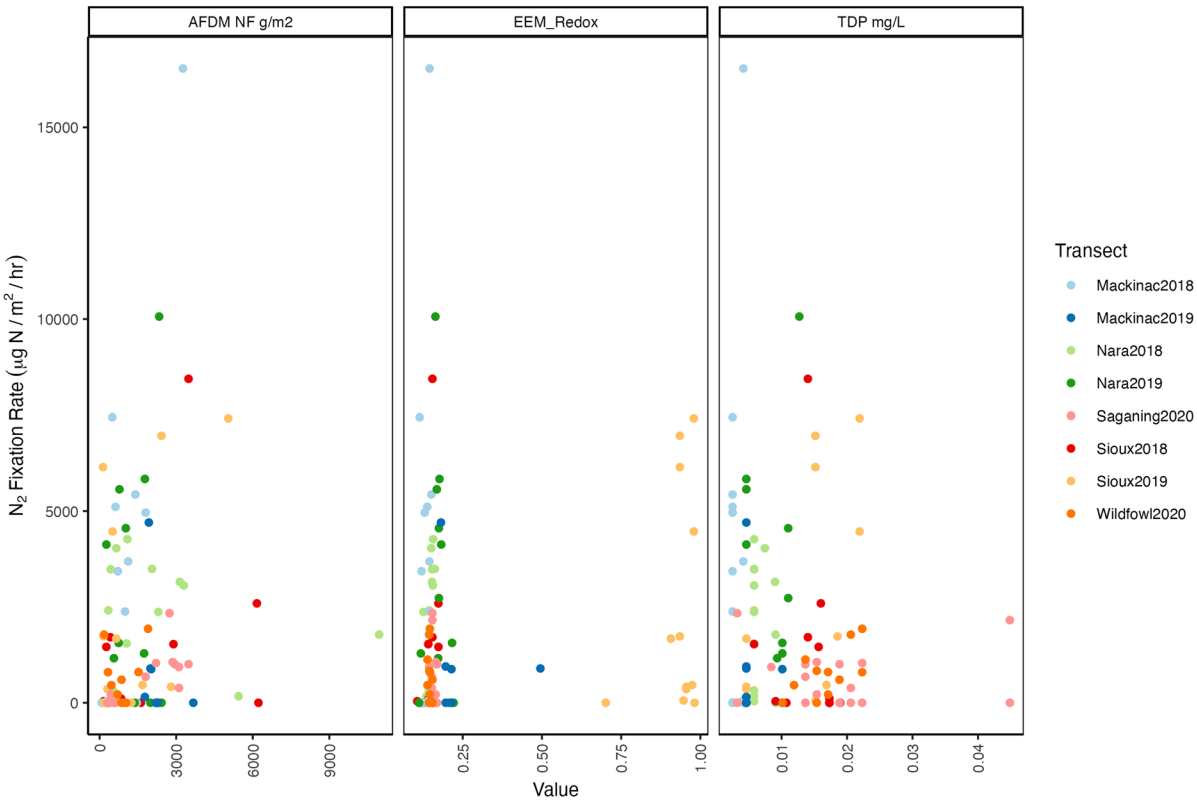

Fig. 9 Scatter plots of the top 3 variables of importance to the SVM model predicting N_2 fixation rates (µg N m^{-2} h^{-1}). AFDM is ash free dry mass in g m^{-2}, EEM_redox is the redox index for dissolved organic matter, and TDP is total dissolved phosphorus in concentrations of mg L^{-1}. Dots are colored by transect name and year

co-occurred across a wide range of N and P concentrations (DIN = 0.026 to 0.260 mg/L, TDP = 0.003 to 0.017 mg/L among sites). Rates of both processes varied among the different habitat types within transects, but rates were not significantly different among habitat types. Predictive modelling did support our hypothesis that high rates of denitrification would be related to availability and quality of organic C, but did not support our hypothesis that high rates of N_2 fixation would be related to NO_3^- concentrations or temperature. Instead, predictive modelling showed that variables related to DOM composition were among the most important predictors of both N_2 fixation and denitrification rates, while TDP was also important to N_2 fixation rates. However, the models only explained 13–15% of the variation in both rates. Together, our results show that N_2 fixation and denitrification co-occur in wetland–stream–lake ecotones and may be related to spatial variability in substrate, DOM composition, and TDP and as such, N cycling at the local scale is complex. Despite this fine scale heterogeneity in N cycling, wetland–stream–lake ecotones are collective sinks of N, which is common in the Great Lakes region and globally where many wetlands remove N (Small et al. 2014a; Jordan et al. 2011).

The spatial variability of habitat type is important to the co-occurrence of N_2 fixation and denitrification in wetland–stream–lake ecotones. Our results show that N_2 fixation and denitrification do occur across all habitat types within wetland–stream–lake ecotones. Sediment substrate was important for denitrification as substrate type was one of the variables of importance identified using predictive modeling, and because we observed that denitrification occurred most frequently on sediment substrates across all the study transects (Figs. 5 and 6). Sediment substrate can be a source of organic matter and anoxic conditions that can promote denitrification in streams (Holmes et al. 1996; Groffman et al. 2005; Eberhard

et al. 2018). In contrast, high rates of N_2 fixation were observed evenly among sediment and macrophyte substrate. Macrophytes could host bound epiphytes that have the potential to fix N_2 (Scott et al. 2005), while sediment could be an important habitat for heterotrophic N_2 fixers or cyanobacteria in sediment-bound microphytobenthos (Scott et al. 2008; Newell et al. 2016). Therefore, the variability of sediment and macrophyte substrate promoted the spatial variability and co-occurrence of these two seemingly incompatible processes across the study transects. Furthermore, at some transect points we observed both N_2 fixation and denitrification occurring at the same time on the same substrate, despite that these processes have vastly different controls when studied on the organismal level. This suggests that our sampling techniques are probably obscuring finer-scale habitat heterogeneity that allows microorganisms to carry out these two seemingly incompatible processes at the same apparent location and time.

The spatial variability of biofilm nutrient limitation in these ecotones also highlights the importance of habitat complexity for nutrient dynamics. Overall, we observed that at most transect points there was no evidence of nutrient limitation of biofilm chlorophyll-*a* and AFDM using the NDS assay, and that the spatial variation in nutrient limitation measured by NDS had no significant relationship to rates of N_2 fixation or denitrification rates in our study. This may not be surprising as the NDS technique may not target the organisms carrying out these processes, like sediment microbes and macrophyte-associated epiphytes. However, these patterns of nutrient limitation may have influences on other biogeochemical processes, and the patterns of nutrient limitation within and among sites we observed are similar to other studies of nutrient limitation in the regions. Similar to NDS studies targeting primary producers in Great Lakes wetlands (Cooper et al. 2016), we did find more N limitation followed by co-limitation of N and P (7:4 based on chlorophyll-*a*) at sites classified as wetland. Previous studies in streams of Lake Superior have shown a predominant co-limitation of N and P of biofilms on NDS (Wold and Hershey 1999), but we observed sites where biofilms were not nutrient limited, were N limited, and were co-limited by N and P in our study streams. Due to sample recovery, we only had nutrient limitation data for benthic biofilms at one lake site, which were N and P co-limited. Primary producers in the water column of the Great Lakes are primarily limited by phosphorus (P) (Schelske et al. 1987), but studies in other lakes have shown that nutrient limitation can differ between species of periphyton (Fairchild et al. 1985) and between benthic and planktonic organisms (Bonilla et al. 2005; Steinman et al. 2016). In a eutrophic lake, benthic algae were found to be co-limited by N and P, while phytoplankton were P-limited (Steinman et al. 2016), which indicates that nutrient limitation of primary producers within lakes can be complex, and that we may not have captured the full suite of organismal nutrient limitation across our study sites with our biofilm-focused NDS.

Of all the environmental variables that we measured as potential predictors of N_2 fixation and denitrification rates, variables related to DOM composition, and in particular descriptors of the molecular weight, aromaticity, and redox state of DOM, were the most frequently selected variables of importance included in both predictive models. P and C concentrations in the forms of TDP and AFDM were also important to the model predicting N_2 fixation. P availability has been shown to limit N_2 fixation rates in aquatic ecosystems (Marcarelli and Wurtsbaugh 2007) and AFDM, a measure of organic matter content, may affect N_2 fixation rates as studies have shown higher availability of organic matter is often associated with cyanobacterial blooms, which are known fixers of N (Howarth et al. 1988). Interestingly, N concentrations were not important to the models for N_2 fixation or denitrification rates. This finding is similar to other studies suggesting the relationship between N_2 fixation rates and N concentrations are not always direct (Knapp et al. 2016; Eberhard et al. 2018; Tang et al. 2020). However, our models suggest that variability in DOM composition across wetland–stream–lake ecotones may play a role in facilitating the co-occurrence of N_2 fixation and denitrification in these ecosystems. We found that the redox state of DOM was important to the model predicting rates of N_2 fixation, which could be because the redox state is related to oxygen availability, another limiting factor for N_2 fixation activity. In peatlands, increased dissolved oxygen concentrations caused by aerenchymatous roots and lowering water tables can result in more oxidized DOM (Kane et al. 2019). Also, the redox state of DOM could be related to the availability of trace metals like molybdenum and iron through chelation that are used in the nitrogenase enzyme (Howarth

et al. 1988). For denitrification rates, the dissolved aromatic carbon content of DOM ($SUVA_{254}$) and the slope ratio (SR) characterizing molecular weight were important to predicting process rates. Lower $SUVA_{254}$ has been associated with more bioavailable DOC that can be used to promote higher denitrification capacity in seepage wetlands (Chibuike et al. 2020). Overall, the availability of nutrients and composition of organic matter across wetland–stream–lake ecotones were important to the co-occurrence of N_2 fixation and denitrification.

Our study did not directly assess how temporal variability may play a factor in the spatial variability of environmental characteristics and rates of N_2 fixation and denitrification across wetland–stream–lake ecotones. Recent literature has noted that a fundamental trait of spatial areas of high rates of a biogeochemical process, or hot spots, is that they are temporally dynamic and that these areas should be reconceptualized as "control points" that can be turned on or off depending on the timing and magnitude of delivery of limiting factors (Bernhardt et al. 2017). Rates of both N_2 fixation and denitrification have been found to vary day-to-day (maximum daily change of 4390 μg N m^{-2} h^{-1} for denitrification and 39 μg N m^{-2} h^{-1} for N_2 fixation) across seasons in the Pilgrim River (Nevorski and Marcarelli 2022), which was part of the Nara transect in the current study. There is also evidence of temporal dynamics in the patterns of process rates of N_2 fixation and denitrification in the same transect points that we sampled in 2018 and 2019, with high rates one year and low or no rates the next year. Due to rising water levels in the Great Lakes in summer 2019, we were not able to access all the transect points that were sampled in 2018 in Nara or Mackinac, and in some cases substrate that was prevalent at a transect point in one year was absent in the other. Rising water levels could affect temperature and light availability. Water level fluctuations have previously been shown to alter sediment and water nutrient exchange in Great Lakes coastal wetlands (Steinman et al. 2012, 2014). Therefore, temporal variability in environmental characteristics should play an important role in the biogeochemical complexity within wetland–stream–lake ecotones along with the spatial variability.

Spatial heterogeneity of habitat, nutrient availability, and the chemical composition of DOM in wetland–stream–lake ecotones facilitate the co-occurrence of N_2 fixation and denitrification in these ecosystems. This means that losses via denitrification must be considered relative to inputs from N_2 fixation to accurately understand the role that wetlands play in nutrient uptake and load mitigation. Additionally, the occurrence of both processes across wetland–stream–lake ecotones could affect N dynamics of the larger Great Lakes. For example, recent studies have shown that Lake Superior may be seeded with cyanobacteria through fluvial and/or wetland inputs (Reinl et al. 2020). Therefore, alterations to the stream and/or wetland N dynamics could have an effect on what is being transported to the larger bodies and their biogeochemical cycles. The spatial heterogeneity within wetland–stream–lake ecotones is key to maintaining complex nutrient cycling. Anything that may reduce physical habitat or biodiversity complexity, such as the invasive wetland plant *P. australis*, will alter the way that wetlands cycle, store, and transport nutrients (Duke et al. 2015; Judd and Francoeur 2019). Therefore, from a restoration and conservation perspective, it is important to maintain and restore spatial heterogeneity in these ecosystems to preserve their function in complex biogeochemical cycling.

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Author contributions Conception and design: EE and AM; data collection: EE; analysis and interpretation of results: EE, EK, and AM; manuscript preparation and revision: EE, EK, and AM. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during this study are publicly available through the

Environmental Data Initiative https://doi.org/10.6073/pasta/6707bb3c21ae0e63e593e7a82aab9146.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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