Biofilm ecoenzymatic activity, organic carbon and nitrogen in Lake Superior tributary streams

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Abstract

I compared the ecoenzymatic activity of naturally occurring epilithic biofilm to stream water carbon and nitrogen concentrations in Lake Superior tributary streams along the south and north shores of the western end of Lake Superior. My goal was to determine if the ecoenzymatic activity of biofilm would reflect water chemistry. The streams drain catchments ranging in size from 14-172 km^2 of primarily deciduous forest, coniferous forest and woody wetlands. Measurements and samples were collected during base stream flows (June-September). The streams represented a broad range of organic carbon, nitrogen and phosphorous concentrations, which correlated to physical habitat parameters and land usage. I used light absorbance in the ultraviolet and visible light (UV-VIS) spectrum to show differences in dissolved organic matter (DOM) between sites on the north and south shore. The UV-VIS proxies of E2:E3 and $SUVA_{254}$, which indicate molecular size and degree of aromaticity, were correlated with the percentage of wetlands in the catchment, as well as the specific conductance, pH and the C, N and P concentrations in the water. Eleven different ecoenzymes involved in the breakdown of organic matter were measured. The activities of these enzymes were often positively correlated to each other and correlated to the measured water chemistry. The slopes from Type II linear regression of β-N-acetylglucosaminidase to phosphatase (0.77), β-d-glucosidase to phosphatase (0.68), and β-d-glucosidase to β-N-acetylglucosaminidase (0.88) are presented here as metabolic stoichiometric ratios. The molar C:N ratio in epilithic biofilm (11:1) was positively correlated to the ratio of dissolved organic carbon

to total dissolved nitrogen (DOC:TDN) in filtered (<0.45 µm) stream water and negatively correlated with biofilm peptidase activity. The expression of the peptidase activity by both L-alanine aminopeptidase and L-leucine aminopeptidase increased in response to dissolved organic nitrogen (DON). Increasing stream-water inorganic nitrogen (DIN) concentrations reduced or inhibited L-alanine aminopeptidase expression and had no apparent influence on L-leucine aminopeptidase. These results along with other studies of biofilm show that the ecoenzymatic activity of biofilm and the C:N ratio in biofilm reflects water chemistry, providing further evidence linking the stoichiometric theory of ecology to metabolic theories of ecology. The ecoenzymatic activity is a measurement of metabolic requirements mediating the stoichiometry of incorporation of nutrients by the biofilm. This study was the first to compare natural epilithic biofilm ecoenzyme activity in the Lake Superior region to water chemistry, land use and habitat characteristics.

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Introduction

Epilithic biofilm is one component of aquatic ecosystems that is responsive to nutrient additions, often demonstrating changes in community structure and biomass (Tank and Dodds, 2003; Carr, Morin and Chambers, 2005; Olapade and Leff, 2006; Artigas *et al.*, 2009). Biofilm in freshwater streams can serve as an indicator and integrator of ecosystem processes and environmental variables. Biofilm also acts as a trophic link, trapping and bringing dissolved nutrients to higher ecosystem levels (Freeman *et al.*, 1995; Battin *et al.*, 2003b; Hill *et al.*, 2010b). The quantity and quality of nitrogen and carbon exports from streams is a significant aspect of global biogeochemical N and C cycling, and biofilm represents a functional hot spot at the terrestrial-aquatic interface in these cycles (McClain *et al.*, 2003; Battin *et al.*, 2007). Creating a better understanding of biofilm response to stream chemistry may allow biofilm to be a sensitive indicator of changes in ecosystem processes, such as nutrient availability.

Nitrogen export from catchments to downstream receiving waters has doubled globally and is continuing to increase as a result of atmospheric deposition and non-point source runoff (Vitousek *et al.*, 1997). Lake Superior, the sensitive ultra-oligotrophic downstream receiving water in this study, has a long-term trend of increasing levels of NO_x attributed to high levels of reduced nitrogen (e.g. organic nitrogen) inputs and low levels of denitrification (Finlay, Sterner and Kumar, 2007; Sterner *et al.*, 2007). Carbon exports from catchments to lakes have also been

increasing, and have been linked to global climate change, as the role of lakes in carbon cycling is increasingly investigated (Evans, Monteith and Cooper, 2005; Tranvik *et al.*, 2009). Lake Superior has been shown to be net heterotrophic and the C:N ratio of its dissolved organic matter (DOM) is indicative of terrestrial inputs (e.g. riverine) representing a major source of nutrients to microbially mediated respiration (Urban *et al.*, 2005). Changes in nutrient concentrations and molar ratios (C:N) in freshwater streams should be reflected in biofilm structure and function. Enhancing our understanding of how elevated water nutrient concentrations and C:N stoichiometry affect stream ecosystem processes is an important aspect of managing these anthropogenic impacts and understanding downstream export of nutrients (Sterner and Elser, 2002; Dodds *et al.*, 2004).

Recently, it was shown that the mean ratio of certain ecoenzymatic activities in soils and sediments are constant across many orders of magnitude and that, when scaled with measures of production, both bacterioplankton and biofilm demonstrate a similar mean relationship between certain types of ecoenzymatic substrate turnover (Sinsabaugh, Hill and Follstad Shah, 2009; Sinsabaugh *et al.*, 2010). Sinsabaugh, Hill and Follstad Shah (2009) suggested that these ecoenzymatic activities provide a link between the theory of ecological stoichiometry and metabolic theory of ecology (MTE), discussed by Allen and Gilooly (2009) and also investigated by Hill et al. (2010b). The metabolic theory of ecology (MTE) posits that the flux of nutrients into an organism, and the transformation and allocation within the organism, is governed by a metabolic rate

(Brown *et al.*, 2004). This metabolic rate is a function of body size, temperature, and life history. This relationship is based on the threshold elemental ratio (TER), the point where growth limitation switches from one element to another (Frost *et al.*, 2006). There is substantial evidence for the significant role of microbial assemblage control and response to the biogeochemical cycling of nutrients in streams (Brookshire *et al.*, 2005). Ecoenzyme activity of natural epilithic biofilm has not been evaluated across wide ranges in water chemistry and I suggest that ecoenzymatic activity of naturally occurring epilithic biofilm can serve as an indicator of nutrient ratios in streams, as was shown by Hill et al (2010a, 2010b) for stream sediments.

Epilithic biofilm consists of microorganisms (algae, bacteria, protozoa and fungi) organized in a matrix composed of exopolysaccharides and attached to a rock surface (Lock *et al.*, 1984). The exopolysaccharide matrix facilitates retention of dissolved and particulate nutrients and creates a microenvironment that enhances the growth and metabolism of the microorganisms and favors enzyme induction and secretion (Fiebig and Marxsen, 1992; Jass, Roberts and Lappin-Scott, 2002). This microbial assemblage in stream biofilm plays an important role in the degradation and re-mineralization of organic matter, facilitating the transfer of nutrients within this organic matter to higher trophic levels (Dodds *et al.*, 2000; Peterson *et al.*, 2001 and others). Nutrients from low molecular weight (LMW) organic molecules (i.e. amino acids and other monomers) and inorganic forms of nitrogen and phosphorous cycle within the biofilm and are preferentially taken up by bacteria from water, leading to higher growth efficiencies

(Paul, Duthie and Taylor, 1991, Kirchman, 1994). Stream biofilm has been shown to be responsible for a large portion of in-stream metabolism of dissolved organic matter (DOM), with the high molecular weight (HMW) portion of DOM often eliciting greater respiration by bacteria versus lower molecular-weight DOM or samples replete with inorganic nutrients (Wiegner and Seitzinger, 2001; Amon and Benner, 1996; Olapade and Leff, 2006). DOM consists of a combination of elements (e.g., C, H, O, N, P, S) combined into many structures, including higher molecular weight compounds such as polysaccharides, lignins, tannins, and proteins. It is often tracked and quantified by focusing on carbon (as DOC) or nitrogen (as DON). The breakdown and uptake of DOM to acquire nutrients by microbial and fungal assemblages of the biofilm is facilitated by extracellular, non-membrane bound enzymes which are retained in the exopolysaccharide matrix of the biofilm (ecoenzymes, sensu Sinsabaugh, Hill and Follstad Shah, 2009). The ecoenzymes catalyze reactions that breakdown structures within DOM into molecules more readily available for uptake across cell membranes. Sinsabaugh and Foreman (2001) suggested that, in spite of the large variability in the concentration of DOM on a seasonal and daily basis, microbial community structure and ecoenzyme activity should reflect the DOM composition integrated over longer periods of time.

The determination of biofilm response to nutrient additions and other environmental conditions in an aquatic environment is not well understood, due to rapid assemblage changes, phenotypic responses, localized conditions and the

variability in the quantity and chemical nature of nitrogen-containing and carbon-containing compounds in streams. Additionally, the mixture of biotic factors and environmental conditions that control ecoenzyme production is not well understood, partially due to the large number of enzymes necessary to catalyze the breakdown of the complex mixture of nitrogen and carbon compounds in natural aquatic environments (Findlay *et al.*, 2003).

Multiple studies have focused on enzyme activities associated with sediment, soil, or epilithic biofilm in mesocosms or on specific substrates with amendments, which show that ecoenzymatic activity reflects water chemistry through shifts in allocation and activity as a function of nitrogen and carbon availability (Romani *et al.*, 2004; Olapade and Leff, 2005; Wilczek, Fischer and Pusch, 2005; Findlay and Sinsabaugh, 2006). Biofilms have also been used as qualitative indicators of nutrient enrichment or limitation in streams based on changes in gross primary production, biomass, and structure (Johnson, Tank and Dodds, 2009; Porter-Goff, Boylen and Nierzwicki-Bauer, 2010). The ecoenzyme activity of epilithic biofilm has also been linked to the ratio of carbon to nitrogen (C:N) in the water column, demonstrating a negative correlation between peptidase activity and water C:N ratios (Olapade and Leff, 2005; Artigas, Romani and Sabater, 2008). Sediment ecoenzyme activity ratios of classes of enzymes (glycosidases, peptidases and phosphatases) reflect uptake length, land usage and nutrient limitations in wetlands and rivers (Hill *et al.*, 2006; Hill *et al.*, 2010a).

Biofilm microbial growth and certain ecoenzymatic activity are positively correlated with dissolved inorganic nitrogen (DIN) concentrations and biofilm is a source and a sink for DIN, as ammonia (NH_3) and nitrate/nitrite (NO_x) (Sala *et al.*, 2001; Findlay and Sinsabaugh, 2006; Olapade and Leff, 2006). Stream uptake and demand for DON increases in response to both increases and decreases in DIN (Stepanauskas, Leonardson and Tranvik, 1999; Johnson, Tank and Arango, 2009). Uptake of DON exceeds DIN and DOC from DOM in headwater streams which can lead to increases in stream water C:N (Brookshire *et al.*, 2005). Thus the differing biological availability of DIN and DON versus the demand for N, needs to be addressed in order to understand nitrogen cycling in streams. Few studies have looked at broad ranges of ambient DON concentrations and recent research indicates that more DON is utilized than previously measured in aquatic environments (Berman and Bronk, 2003; Brookshire *et al.*, 2005; Wiegner *et al.*, 2006). DON availability ranging from 15-71% measured with incubations of water from forested mountain streams over a two year period and microbial uptake of DON declines logarithmically with DIN addition (Kaushal and Lewis, 2005). The bioavailability of DON in boreal streams ranged from 19-28% during base flow, with demonstrated spikes in availability (45%-55%) during spring runoff events as measured with saline bacteria inoculums to better understand stream export to oceans (Stepanauskas, Laudon and Jorgensen, 2000). The DON fraction of total nitrogen (TN) can vary dramatically in relative abundance and bioavailability both temporally and spatially, i.e.,

between regions and catchments with different %wetlands and storm discharge flow regimes (Pellerin *et al.*, 2004; McNamara *et al.*, 2008; Stanley and Maxted, 2008).

The utilization of DOC by biofilm has been examined in a number of ways to gain a better understanding of carbon distributions and cycling in biofilm, where ecoenzymatic activity is correlated to DOC (Sobczak and Findlay, 2002; Romani *et al.*, 2004). Generally, carbon is not considered a limiting nutrient in an aquatic environment, though there are a number of citations of carbon limitation on microbial productivity (Hill *et al.*, 2006; Ardon and Pringle, 2007; Hill *et al.*, 2010b; Sinsabaugh and Shah, 2011). The relative recalcitrance of the carbon compounds available will affect uptake by biofilm and ecoenzymatic activity (Findlay, Hickey and Quinn, 1997). The affect of carbon enrichment has been measured by adding DOM isolated from natural systems or specific carbon compounds, where measurements of isotopically-labeled reduced-carbon substrates measured with primary production have shown that the outside supply of carbon is more important in lower nutrient streams (Olapade and Leff, 2005). DOM molecular size and composition play significant roles in bioavailability. In open-water, marine/estuarine systems, high molecular weight DOM molecules have been shown support higher bacterial growth and respiration rates than smaller DOM molecules, even when C concentrations were comparable. However higher molecular weight DOM has a higher C:N, which tends to increase inorganic N uptake versus the inorganic N generation observed when using low molecular weight DOM as a food source (Amon and Benner, 1996). The use of ultraviolet and visible absorbance (UV-Vis)

and spectral slope ratios on river water samples has been used in a number of cases to quickly evaluate the relative molecular weight of organic carbon compounds and to provide a qualitative measure of DOM composition, e.g., the aromatic character (Hood, Gooseff and Johnson, 2006; Helms *et al.*, 2008; Minor and Stephens, 2008).

In this study, I investigated a gradient of organic and inorganic nitrogen concentrations in stream water and the ecoenzymatic activity of co-occurring epilithic biofilm from the naturally occurring rocks in these streams. I also measured the C:N ratio of the biofilm, collected physical habitat parameters and used scanning UV-Vis as a quick qualitative method for looking at DOC chemical composition and compared these measurements with ecoenzymatic activity. My primary objective was to evaluate possible stoichiometric relationships between biofilm, water chemistry and ecoenzymatic activity in biofilm. I hypothesized that the ratio of DIN to DON concentrations in streams significantly affects biofilm composition and metabolism, and that changes in this ratio would be indicated by ecoenzyme activity and C:N ratios in the biofilm. I further hypothesized that the ecoenzymes used to cleave various carbon bonds (glycosidases and oxidases) would reflect differences in organic carbon abundance (measured by DOC analyzer) or quality (as determined by UV-VIS proxies for relative size and aromaticity).

Methods

Study Sites

I chose 8 sites on 7 2nd and 3rd order Lake Superior tributary streams near Duluth, Minnesota, based on differences in DIN/TN ratio determined in samples collected during base flow periods in 1998-1999 (Detenbeck *et al.*, 2003; Detenbeck *et al.*, 2004) (Fig.1). This same study identified a number of physical differences in the streams and their catchments (e.g. soils, geology, forested land cover and water storage capacity) that could be linked to differences in water chemistry. The catchments of the sample sites were delineated using 10 meter (1/3 arc-second) resolution elevation data from the National Elevation Dataset (Gesch, 2007) and the 24K National Hydrologic Dataset (U. S. Geological Survey and U.S. Environmental Protection Agency, Fig. 1). The ArcHydro (v.1.3, ESRI, Redlands, CA, USA) tool set was used to delineate the catchments using the NHD to ensure proper drainage direction and patterns (Maidment, 2002). Land cover and usage were estimated from the 2006 National Land Cover Dataset (NLCD) (Xian, Homer and Fry, 2009). The south shore streams were the Flag, Amnicon, and Middle Rivers. Based on the EPA level IV Ecoregions map, the Flag is located in the Lake Superior Clay Plain and the St. Croix Pine Barrens, while the Middle and Amnicon sites are located in the Minnesota/Wisconsin Upland Till Plain (Omernik, 1987). The Flag and the Amnicon Rivers have catchments of 80.1 km^2 and 172 km^2 respectively. Two sites were located on the Middle River because they showed significant differences in DON:DIN ratios based on the previous study. The Middle 3 site is located downstream of the Middle 2 site and is fed by two additional tributary streams. Catchment area for Middle 2 is 85.2 km^2 while catchment area of Middle 3 is 95.2 km^2. The north shore

streams included Amity Creek, and the French, Talmadge, and Sucker Rivers, located within the North Shore Highlands and Toimi Drumlins EPA Level IV ecoregions (Omernik, 1987). The north shore catchments ranged in size from 14.0 km^2 (Talmadge) to 95.5 km^2 (Sucker). Land cover for both the north and south shore catchments is primarily deciduous and coniferous forests, with some woody wetlands. The north shore streams tend to be steeper, flashier and rockier with a smaller percentage of wetlands located near the headwaters. The south shore streams have lower elevation gradients and a greater percentage of wetlands in their catchments, and flow paths that meander through clay and sand substrates, leading to higher concentrations of sediment and organic material (Brazner *et al.*, 2005).

Physical Habitat Characterization

Physical habitat data were collected from 11 transects perpendicular to the thalweg and spaced equidistantly throughout the 150 m to 240 m reach length. The reach lengths were previously established using 35x the mean stream width (MSW). Habitat parameters included canopy cover, streambed substrate characterization, and stream channel dimensions (Lazorchak et al., 2000). Canopy density was estimated (8 measurements per transect) after full canopy development (mid-June) as percent cover using a convex spherical densitometer (model-A, Robert E. Lemmon, Forest Densiometers, Bartlesville, OK, USA). Dominant streambed substrate was determined (using a modified Wolman pebble count) measured at 5 sample locations on each of the 11 cross-sections and an additional 10 sub-stations, located halfway between each

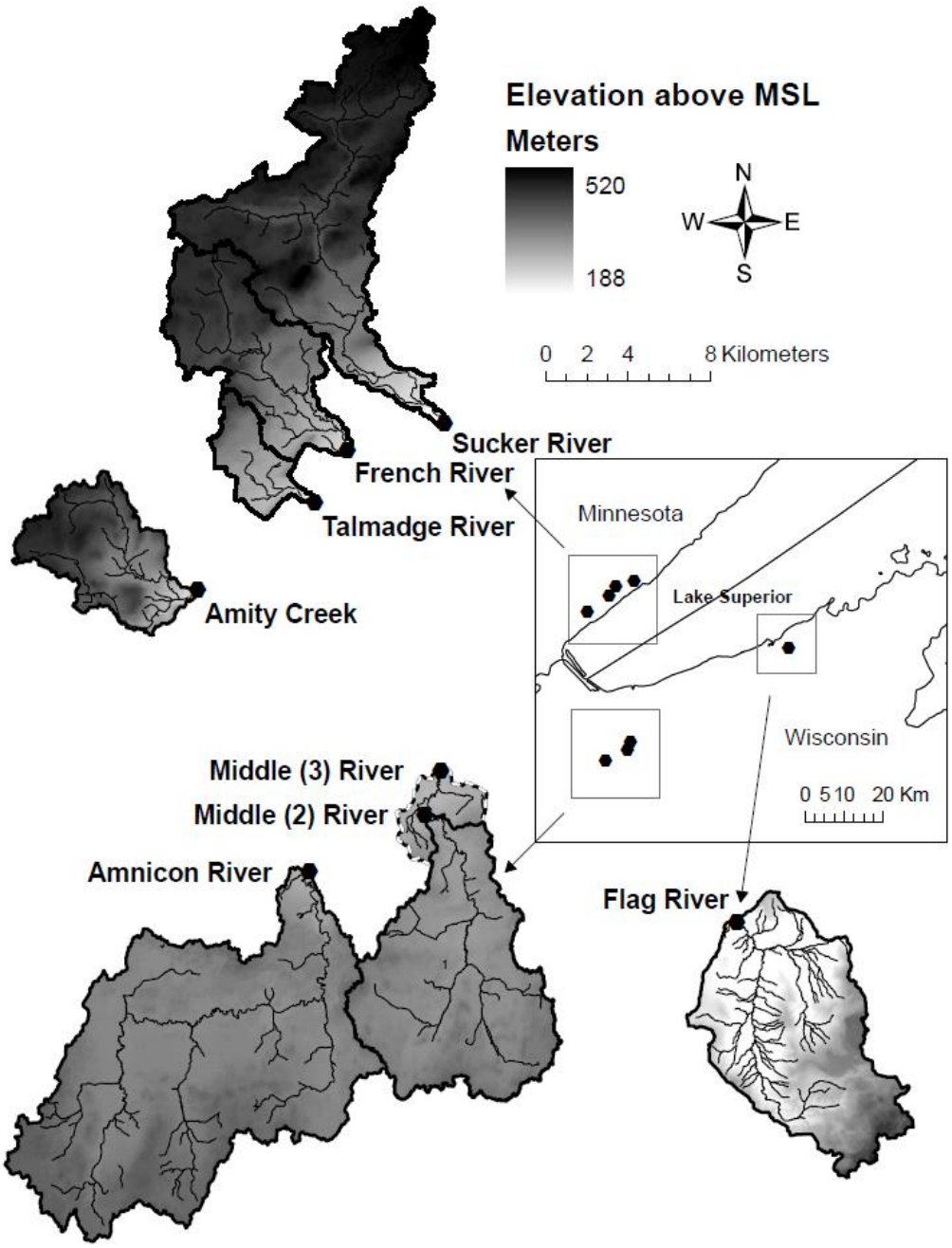

Figure 1. Locations of sampled streams, delineated catchments, and elevation above mean sea level and national hydrologic dataset (NHD) stream lines (24K). Elevation is visually exaggerated using the hillshade function (z=2) to make relief more apparent. Sample sites are located at the base of the delineated catchments. The projected coordinate system is NAD 1983 UTM Zone 15 N.

transect. The median particle size was calculated as the sum of the geometric mean particle size for each classification multiplied by the proportion of that class identified (Kaufmann *et al.*, 1999). Cross-sectional area, the product of the wetted channel width and depth, was measured at the 11 transects and wetted width at the 10 sub-stations (Lazorchak et al., 2000). On each sampling date, dissolved oxygen (DO, mg/L), specific conductivity (µS/cm), temperature (°C), turbidity (NTU) and pH were measured using a Hydrolab sonde equipped with a luminescent DO sensor (Model 6120, Hach Hydromet, Loveland, CO, USA).

Water samples were collected in well mixed areas at the base of the reach. Sampling took place from June-September 2009, with biweekly collections on all streams, except the Flag River, which was not sampled as often due to its distance from the laboratory. Sample containers were acid-washed (10% HCl), rinsed (deionized water, DI), high density polyethylene containers (HDPE), which were rinsed at least 3 times with stream water prior to sample collection. Duplicate water samples were immediately placed on ice in a cooler until they could be taken back to the lab, where they were homogenized and then split into aliquots for whole water and filtered nutrient analyses. The filtered-nutrients aliquot was vacuum filtered through a 0.45 µm nylon membrane. Samples were immediately frozen for later analysis, or placed in 40-mL amber vials and acidified to pH 2 (using reagent-grade HCl) for subsequent organic carbon and nitrogen measurement. The acidified aliquots for total and dissolved

organic carbon (TOC and DOC) and total and dissolved N (TN and TDN) were stored (4 C, <30d) prior to being run on a high temperature combustion TOC/TN analyzer (Apollo 9000, Teledyne Tekmar, Mason, OH, USA). Samples were mixed using hydrocarbon-free-air (zero-grade) in the sample vials for 15 seconds to re-suspend larger particles. Using established protocols for this instrument, non-purgeable organic carbon was measured as the average of 3 injections in which the organic carbon was converted to CO_2 and quantified, using a high resolution, non-dispersive infrared sensor. The instrument was calibrated for carbon using potassium hydrogen phthalate (KHP) with additional KHP check standards, blanks and duplicates included to monitor instrument performance. Nitrogen was measured (as NO) using chemoluminesence, and was calibrated with a complex nutrient standard (Wastewater, Kjedahl Nitrogen, Environmental Resource Associates, Arvada, CO, USA) with additional check standards of nitrate and urea. All samples were corrected using DI water blanks and filter blanks where appropriate. Filter blanks measured generally less than 0.3 mg C/L and less than 0.1 µg N/L.

Total phosphorus (TP), NO_x (NO_3+NO_2) and ammonia (NH_3) were measured using standard methods (APHA, 1998) on an automated flow injection analyzer (Model 8000, Lachat Instruments, Milwaukee, WI, USA). Filtered water samples were measured for dissolved inorganic nitrogen (DIN) as NO_x plus NH_3, with DON calculated as TDN-DIN. NH_3 was measured on frozen water samples immediately following a quick defrost to minimize volatilization, using an alkaline phenol and hypochlorite method (1 cm cell,

absorbance at 630 nm; APHA, 1998). NO_x was measured using a copper-cadmium reduction column with sulfanilamide and N-(1-napthyl)-ethylenediamine dihydrochloride method (1 cm cell, absorbance at 520 nm; APHA, 1998). Whole water samples for TP and standards were digested using a persulfate method prior to reacting phosphate with ammonium molybdate and potassium antimonyl tartrate to form a complex which when reduced using ascorbic acid, creates a colored reaction, measured at 880 nm with a 2 cm detection cell (APHA, 1998). Method detection limits for the dissolved nutrients NH_3, NO_x and TP were determined to be 1.3 µg/L, 0.7 µg/L, and 2 µg/L respectively, using 3 times the standard deviation of 5 replicates of the lowest standard for each of the methods.

Scanning UV-VIS Spectrometry (UV-VIS)

Whole water and filtered samples were analyzed for colored dissolved organic matter on a scanning spectrophotometer (Genesys 6, Thermo Fisher Scientific, Waltham, MA, USA) measuring absorbance from 800 to 250 nm using a 1 cm quartz cell. Milli-Q water blanks and sample duplicates were run every five samples. Samples were corrected with the Milli-Q blanks and also corrected for backscatter and machine error by subtracting the mean absorbance from 700-800 nm, based on the assumption that absorbance at these wavelengths is zero (Green and Blough, 1994). The absorbance at each wavelength was converted to an absorption coefficient using the Beer-Lambert law:

$$\text{absorption coefficient a } \lambda = 2.303 * \frac{\text{absorbance A } \lambda}{\text{path length m}} \quad (1)$$

From the UV-VIS absorption data, two proxies for organic matter quality (E2:E3 and $SUVA_{254}$) were calculated. The ratio of the absorbance coefficients at 250 nm and 360 nm (E2:E3) has been shown to be inversely correlated to molecular size (Dehaan and Deboer, 1987; Peuravuori and Pihlaja, 1997). The absorption coefficient at 254 nm normalized to the DOC concentration in mg C L^{-1} (Weishaar *et al.*, 2003) is a measure of the relative degree of aromaticity of the sample.

Biofilm Sampling and C:N

Biofilm samples were collected from the exposed portion of a shallow (<0.5 m depth) streambed substrate 2-5 times during the sampling period, using the U.S. Environmental Protection Agency (EPA) Environmental Monitoring and Assessment Program (EMAP) periphyton methods (Lazorchak et al., 2000). Biofilm was removed from a 12 cm^2 area of the rock surface by brushing the surface of the rock with a stiff bristled toothbrush for no more than 30 seconds. The toothbrush was cleaned between transects with stream water and rinsed in 10% HCL and DI water between streams. The loosened biofilm was washed into a 1 liter HDPE sample container using a funnel and approximately 30 ml of stream water. This was done at each of the 11 transects then combined into one sample which was stored on ice and frozen for later analysis. The compositing of the 11 transects minimizes differences in UV exposure and hydrologic patterns and is more representative of the stream reach. Biofilm samples were

homogenized and mixed prior to aliquots being taken. Duplicate aliquots of biofilm samples (approx. 20 g) were put into aluminum trays, weighed and dried for dry weight (DW, 24 hours at 110°C) calculation. Aliquots were placed into tin capsules for analysis of carbon and nitrogen on a CHN elemental analyzer (Flash EA 1112 NC Soil Analyzer, Thermo Fisher Scientific, Waltham, MA, USA). Each sample was blank subtracted and compared to a calibration curve based on acetanilide. The remaining sample was combusted at 450°C for four hours, to determine the ash free dried mass (AFDM) and % organic matter (%OM) (Stevenson, 1996).

Epilithic Biofilm Ecoenzymatic Activity

Ecoenzymatic activity was measured on previously frozen biofilm samples that were thawed and re-homogenized with a vortex mixer. The use of frozen disrupted biofilm has been used in other studies and was chosen to facilitate comparison. This method ensures uniformity in substrate and minimal instrument variation by assaying samples from different dates at the same time. Assays for hydrolytic ecoenzymes used fluorescently coupled methylcoumarin (MCM) or methylumbelliferyl (MUB) substrates (Sinsabaugh *et al.*, 1997; Sinsabaugh and Foreman, 2001). Two aminopeptidases (L-alanine [ALA] and L-leucine aminopeptidase [LAP]) and six glycosidases (β-d-glucosidase [BGLU], α-d-galactosidase [AGAL], β-d-galactosidase [BGAL], and β-N-acetylglucosaminidase [NAG], β-d-xylosidase [XYL] and cellobiohydrolase [CELL]) were measured on the samples. NAG is also presented here as a peptidase (N-acquiring) enzyme. Phosphatase [AP] activity was assayed as a measure of microbial phosphorous

acquisition. All buffers, samples and substrates were prepared in sterilized, deionized water. Enzymes were incubated in the dark at 20°C for 15 minutes to 4 hours as in previous studies (Hill et al., 2006; 2010). Enzyme activities were measured in quadruplicate on a 96 well microplate at an excitation wavelength of 365 nm and an emission wavelength of 450 nm on a fluorometer (FLX 800T, BioTek Instruments Winooski, VT, USA). Fluorescence quenching, which is the decrease in emissions caused by other chemical species, was controlled for by measuring the decrease in emissions for a standard combined with the biofilm sample. Biofilm phenol oxidase (POX) and peroxidase (PO) activities were measured using L-3,4-dihydroxyphenylalanine (L-Dopa) and hydrogen peroxide (3%) as substrates respectively. These activities were measured on a spectrophotometer (Synergy 4, BioTek Instruments Winooski, VT, USA) as absorbance at 460 nm (Sinsabaugh, Osgood and Findlay, 1994). Ecoenzymatic activity is presented here as nmol substrate turnover g^{-1} C h^{-1}). The two peptidases (LAP and ALA) were summed for a total peptidase activity and total glycosidase was calculated as the sum of AGAL, BGAL, NAG, CELL and BGLU.

Statistical methods

Each biofilm sampling date was treated as a discrete event regardless of sample location or date and compared to the water chemistry on that date. All chemistry ratios are presented based on molar concentrations. Ecoenzyme activity and chemistry data were transformed (*ln*) to ensure normality and mitigate scaling and magnitude issues. Correlations were tested using a Spearman rank correlation, with p-values <0.05

deemed significant and r<0.30 are not shown. A one-way analysis of variance (ANOVA) was used to compare differences between sample dates at the sites, and between the north and south shore sites. We used a paired t-test to compare UV-Vis results for filtered vs. unfiltered water samples. Type II linear regression (Deming regression) was used to compare between the enzymes as independent variables. Statistical tests and figures were completed using Sigma Plot 12 (Systat Software Inc. San Jose, CA, USA) and STATISTICA (StatSoft Inc. Tulsa, OK, USA).

Results

Habitat Parameters

Most of the habitat parameters were measured once during the course of this study; stream widths (MSW) and mean stream depths (MSD), however, were measured at the base of the reach on each sampling date. The width and depth data are reflective of the differences between streams on their respective sampling dates and showed little variability. The reach lengths used were 35 to 40x the MSW, with the exception of the Sucker River (30x), which was widened in a flood event since the previous study. Land use is primarily mixed forests and wetlands, with the lowest percentage of forest and the highest percentage of wetlands (55.0% and 33.6%, respectively) in the Amnicon River catchment (Table 1). The Sucker River catchment had highest percentage of forest (89.8%) and the Amity Creek catchment had the lowest percentage of wetlands (0.9%). The %forests and %wetlands were negatively correlated ($r=-0.83$, $p<0.001$). The Amnicon and Flag River sites were the deepest, and the shallowest sites were the

Middle 3 and Amity locations. Median particle size (MPS) was higher at the north shore sites and ranged from 0.20 cm (Flag) to 188 cm (Amity), averaging 46.0 ± 22.0 cm (mean ± SE). Canopy coverage varied from 50-80% for all streams with a mean of 61.25 ± 3.5%.

Background water chemistry

All of the in situ water parameters were averaged based on site and found to be normally distributed with the exception of turbidity and mean substrate size. Water temperature was variable across the sampling dates, but averages for the sites tended to be similar (16.2 ± 0.6 °C; Table 1). The coldest temperatures were at the Flag River (13.1 °C, n=2) and the warmest were at Amity (18.4 ± 1.18 °C n=7). Dissolved oxygen levels were near saturation relative to water-saturated air, with means ranging from 8.61 to 10.2 mg/L. Turbidity was generally low, ranging from 2.4 to 10.1 NTU, excluding the Flag River, which had a mean of 31.1 NTU. Water pH was negatively correlated to the %wetlands ($r=-0.73$, $p=0.01$; Table 3) and higher at the north shore versus the south shore locations with pH ranging from 7.32 to 8.20 and not significantly different between sampling dates at the sites. Specific conductance ranged from 95.6 to 310 µS/cm and decreased as catchment area, mean stream width and depth increased (Table 3).

Ammonia levels were fairly low across all of the sites with a mean of 8.6 ± 0.9 µg/L, with the highest levels detected at Middle 2 (18.1 ± 2.32 µg/L) and the lowest at Sucker (2.4 ± 0.5 µg; Table 2). NO_x concentrations were variable across sites (2.3 ± 0.3 to 172 ± 35.4 µg/L) with a mean of 47.1 ± 9.92 µg/L. NO_x represented ≈84% of the total

dissolved inorganic nitrogen (DIN) for the sites, and was correlated with NH_3 (r=0.60, p<.001, n=55; Table 5). The DON was variable, with a mean of 587 ± 58.3 µg/L, and ranged from 39.3 µg/L ± 19.2 µg/L to 1125 ± 126 µg/L (Table 2). TN averaged 709 ± 60.4 µg/L and ranged from 131± 32.8 µg/L to 1382 ± 73.0 µg/L and was correlated to TDN (r=0.93, p< 0.01; Table 5). TDN (DON+DIN) represented ≈81% of the TN for all the sites and ranged from 48% (Flag) to 94% (Middle 2) of TN. At many of the sites, the TDN was greater than 99% of the TN, particularly during August and September samplings.

TN was correlated to TP (r=0.70, p<0.05). TP concentrations ranged from 1.70 to 40.5 µg/L and averaged 18.2 ± 2.12 µg/L, with the highest levels detected at the south shore sites (Table 2). TOC was variable with averages ranging from 0.96 mg/L in the Flag River to 24.5 ± 2.29 mg/L at the Middle 2 site and was positively correlated to TN (r=0.96, p<0.01). DOC was correlated to TOC (r=0.97, p<0.01) and TDN (r=0.92, p<0.01) at the study sites (Table 5). DOC means ranged from 5.7 ± 0.28 to 26.15 ± 2.23 mg/L (Table 2). The mean C:N ratio was calculated as the molar ratio of organic carbon to total nitrogen, with both whole water and filtered samples being fairly invariant and averaging approximately 16:1 and 18:1, respectively (Table 2). Water chemistry was correlated to specific habitat parameters, particularly land use and MSW (Table 3).

UV-Vis Spectrometry

The E2:E3 ratios (n=46) for the filtered samples ranged from 4.8 - 6.0 and the $SUVA_{254}$ (n=47) ranged from 8.4 - 12.5 (Table 2). The E2:E3 and $SUVA_{254}$ values were

Table 1. Summary of stream habitat and mean water quality parameters. The habitat parameters were measured once and the water quality parameters are reported as mean (standard error). No standard error is reported for the Flag, as it was only sampled twice.

	Amity	French	Sucker	Talmadge	Amnicon	Middle 2	Middle 3	Flag
% Forests	81	85	90	77	55	57	59	85
% Wetlands	1	8	6	14	34	31	29	3
Mean stream width (m)	5.1	5.0	7.7	3.7	11.7	9.0	10.0	5.0
Mean stream depth (m)	0.16	0.19	0.17	0.05	0.30	0.26	0.14	0.30
Median particle size (cm)	188	31.0	16.5	111	5.60	12.4	5.40	0.20
% canopy	61	57	50	80	51	54	65	72
Water Samples (n)	7	7	8	7	5	6	8	2
Temp (^{o}C)	18.4 (1.18)	16.1 (1.98)	17.2 (1.72)	15.90 (2.27)	17.0 (1.97)	16.4 (1.63)	16.1 (1.34)	13.1
DO (% Saturation)	101.4 (1.38)	99.8 (1.69)	99.7 (2.72)	89.0 (3.19)	98.0 (2.03)	91.2 (3.91)	95.9 (3.49)	100
DO (mg/L)	9.48 (0.18)	9.47 (0.54)	9.27 (0.43)	8.61 (0.50)	9.21 (0.26)	8.64 (0.85)	9.14 (0.41)	10.25
Turbidity (NTU)	4.7 (1.0)	2.4 (0.3)	2.4 (0.2)	4.8 (1.1)	4.0 (0.8)	6.7 (2.2)	10.2 (4.3)	31.1
pH	8.20 (0.10)	8.14 (0.12)	8.13 (0.09)	7.54 (0.09)	7.32 (0.13)	7.45 (0.08)	7.61 (0.11)	7.75
Specific conductance (µS/cm)	310 (8.70)	209 (8.40)	203 (11.5)	255 (10.7)	95.6 (12.3)	175.5 (12.9)	178.6 (9.86)	159.25

Table 2. Mean (standard error) of water chemistry and UV-VIS proxies for all of the sites. Sample size (n) varied from 5 to 8 with the exception of the Flag (n=2 to 3). Measurements performed on filtered (<0.45µm) samples are denoted with a *. The C:N values are molar ratios. $SUVA_{254}$ values for Flag are not included because of questionable accuracy.

	Amity	French	Sucker	Talmadge	Amnicon	Middle (2)	Middle (3)	Flag
NH_3 (µg/L) *	7.9 (1.9)	4.1 (0.2)	2.4 (0.5)	6.3 (1.0)	8.7 (1.8)	18.1 (2.3)	13.4 (2.6)	8.1 (1.5)
NO_x (µg/L) *	13.1 (4.79)	5.5 (1.1)	2.3 (0.3)	58.8 (21.1)	16.7 (8.85)	172 (35.4)	42.6 (11.9)	17.5 (5.45)
DON (µg/L) *	265 (43.4)	263 (24.3)	345 (31.3)	503 (84.2)	692 (127)	1125 (126)	825 (102)	39.3 (19.2)
TN (µg/L)	372 (57.5)	292 (21.2)	403 (23.6)	630 (64.4)	851 (111)	1382 (73.1)	1118 (107)	131 (33.1)
TP (µg/L)	7.1 (1.7)	1.7 (0.2)	3.5 (0.9)	6.9 (4.2)	14.8 (2.6)	40.5 (3.3)	25.5 (4.5)	22 (8.5)
TOC (mg/L)	6.51 (0.98)	6.08 (0.32)	8.08 (0.28)	9.96 (1.94)	18.8 (2.74)	24.5 (2.29)	21.88 (3.39)	0.96
E2:E3	5.6 (0.1)	5.5 (0.1)	5.7 (0.1)	5.6 (0.2)	4.6 (0.0)	5.1 (0.1)	5.1 (0.1)	2.6
$SUVA_{254}$	7.9 (0.3)	9.4 (1.2)	8.7 (0.1)	8.7 (0.3)	11.8 (0.4)	10.8 (0.1)	10.8 (0.5)	---
DOC (mg/L)*	6.40 (0.86)	5.70 (0.28)	7.68 (0.20)	9.55 (1.48)	17.6 (2.44)	26.2 (2.23)	20.9 (2.40)	0.86
E2:E3*	6.0 (0.1)	5.7 (0.2)	5.9 (0.1)	5.9 (0.2)	4.9 (0.1)	5.3 (0.1)	5.3 (0.1)	4.8
$SUVA_{254}$ *	7.6 (0.3)	8.5 (0.2)	8.9 (0.4)	8.4 (0.2)	11.2 (0.4)	10.6 (0.1)	10.3 (0.4)	---
C:N	15.3 (1.2)	18.3 (1.6)	18.1 (0.5)	14.2 (1.6)	20.1 (0.8)	15.2 (1.0)	16.7 (0.6)	5.3 (0.9)
C: N*	20.9 (3.2)	17.1 (1.2)	20.1 (1.8)	14.6 (1.5)	22.8 (2.1)	17.4 (2.2)	21.3 (2.1)	11.3 (0.8)

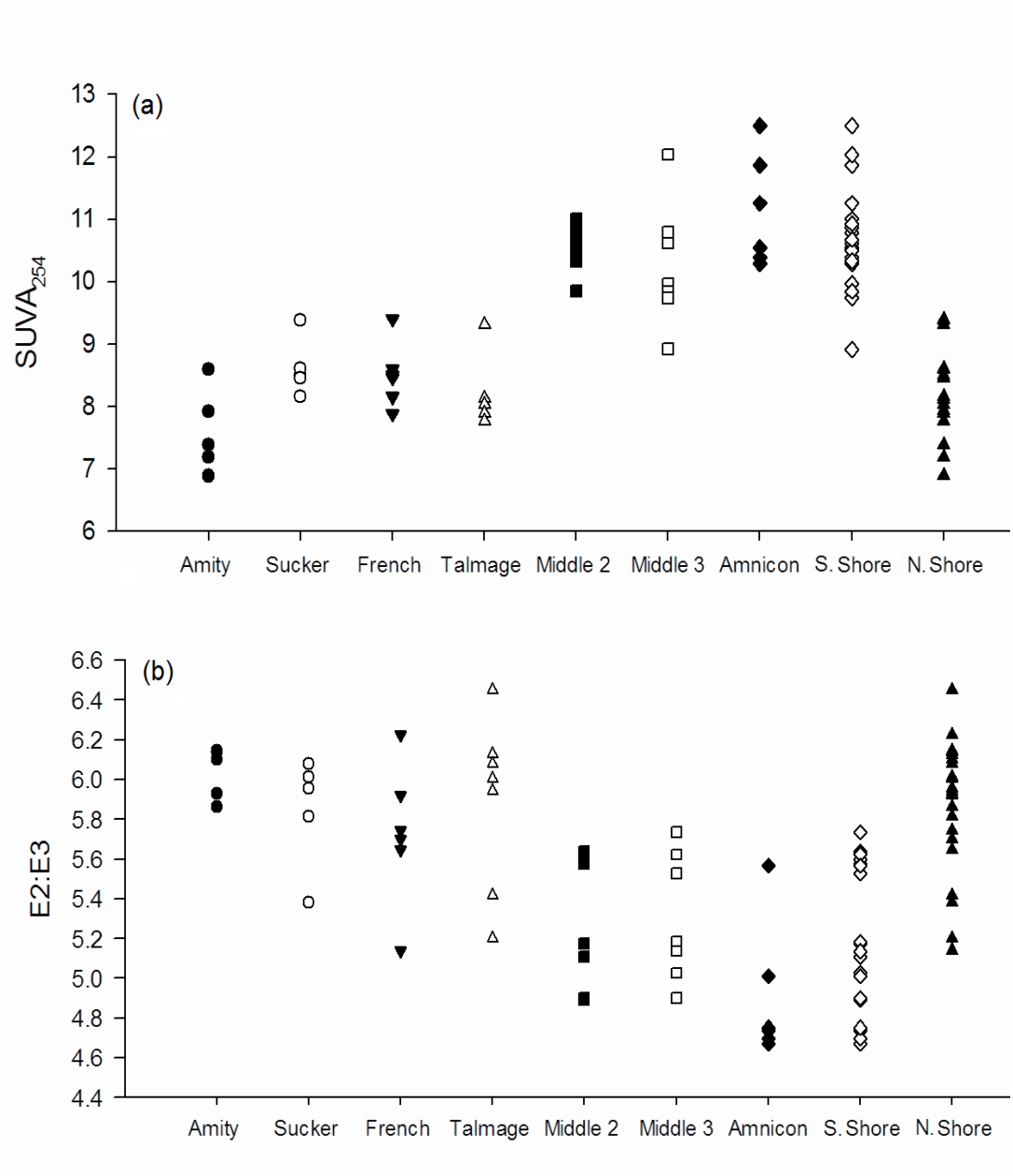

Figure 2. Filtered water sample aromaticity (a; $SUVA_{254}$) and molecular size (b; E2:E3) grouped by site and location. The unfiltered samples were not statistically significantly different from the filtered ones and no significant differences were found between sample dates within the sites. Amity, Sucker, French and Talmage are grouped in N. Shore. Middle 2, Middle 3 and Amnicon are grouped into S. Shore.

Table 3. Spearman correlation coefficients (r) for physical habitat parameters versus stream chemistry. P-values are indicated as <0.05 (*) and <0.01 (**). The (—) indicates that r < 0.30 or p-value >0.05

	Sp.Cond (µS/cm)	DO (mg/L)	Turbidity (NTU)	pH	Temp. (C)	Canopy (%)	MSD (m)	MSW (m)	MPS (m)	Wetlands (%)	Forests (%)	Area (km2)
DO (mg/L)	—											
Turbidity (NTU)	—	—										
pH	0.45 **	—	—									
Temp. (C)	—	-0.60 **	—	—								
Canopy (%)	—	—	—	—	—							
MSD (m)	-0.62 **	—	—	—	—	-0.60 **						
MSW (m)	-0.67 **	—	—	—	0.41 **	-0.56 **	0.41 **					
MPS (m)	0.76 **	—	-0.50 **	—	—	—	-0.47 **	-0.60 **				
Wetlands (%)	-0.64 **	—	—	-0.73 **	—	—	—	0.63 **	-0.44 **			
Forests (%)	0.52 **	—	—	0.72 **	—	—	—	-0.63 **		-0.89 **		
Area (km^2)	-0.65 **	—	—	—	—	-0.71 **	0.51 **	0.86 **	-0.68 **	0.48 **	—	
TP (µg/L)	-0.48 **	—	0.70 **	-0.50 **	—	—	—	—	-0.45 **	0.52 **	-0.58 **	—
TN (µg/L)	-0.40 *	—	0.40 *	-0.54 **	—	—	—	0.57 **		0.77 **	-0.76 **	—
DON (µg/L)	-0.54 **	-0.52 **	0.45 **	-0.61 **	0.57 **	—		0.55 **		0.75 **	-0.72 **	—
NOx (µg/L)	—	—	0.47 **	-0.44 **	—	—	—	—	—	0.47 **	-0.58 **	—
NH4 (µg/L)	—	—	0.45 **	—	—	—	—	—	—	0.43 **	-0.54 **	—
TOC (mg/L)	-0.47 **	-0.44**	—	-0.62 **	—	—	—	0.67 **	—	0.78 **	-0.74 **	0.47 **
DOC (mg/L)	-0.52 **	-0.40 **	—	-0.63 **	—	—	—	0.65 **	—	0.80 **	-0.77 **	0.43 **
C:N <.45	—	0.48 **	—	—	—	—	—	0.43 **	—	—	—	0.40 **
$SUVA_{254}$ (<.45)	-0.82 **	—	—	-0.49 **	—	—	0.52 **	0.67 **	-0.79 **	0.70 **	-0.62 **	0.57 **
E2:E3 (<.45)	0.82 **	—	—	0.41 *	—	—	-0.53 **	-0.56 **	0.71 **	-0.60 **	0.54 **	-0.48 **

negatively correlated (r=-0.78, p<0.01, Table 5) indicating that as aromaticity increased, the relative molecular size of the DOM also increased (E2:E3 is inversely related to size). Based on correlations, DOM molecular size and aromaticity increased as MSD, MSW, %wetlands, N, C , P, and catchment size increased (Table 3). Conversely molecular size and aromaticity decreased as measurements of specific conductance, pH, MPS, and %forests increased. For each sampling event, the UV-Vis data for filtered and unfiltered water samples were not significantly different. There was a clear geographic signature in absorbance with the north shore streams being similar to each other, but significantly different from the south shore streams. These results indicate that organic matter in the south shore streams had relatively larger molecular weight and higher relative aromaticity compared to the north shore streams (Fig. 3).

Biofilm C/N and ecoenzymatic activity

The highest relative rates of ecoenzyme activity were measured for AP, BGLU, NAG, PO, POX (Fig. 3). The lowest rates were measured for XYL and CELL and overall the lowest activity for all enzymes was measured from the Flag River. The Amnicon River often had the highest levels of activity for many of the ecoenzymatic assays, with the exception of peptidase, AGAL and BGAL activity, which were higher at the Middle 2 and Middle 3 sites (Table 4). The ecoenzyme activities are positively correlated to each other (data not shown) and correlated in many cases to the carbon and nitrogen steam chemistry on the particular day they were sampled (Table 5). AGAL and BGAL had the highest correlation to the TOC (0.62 and 0.63 respectively, p<0.01). ALA activity, a

measure of biofilm N acquisition, was positively related to the DON:DIN ratio (r=0.65, p<0.01, Table 5, Fig. 5a).

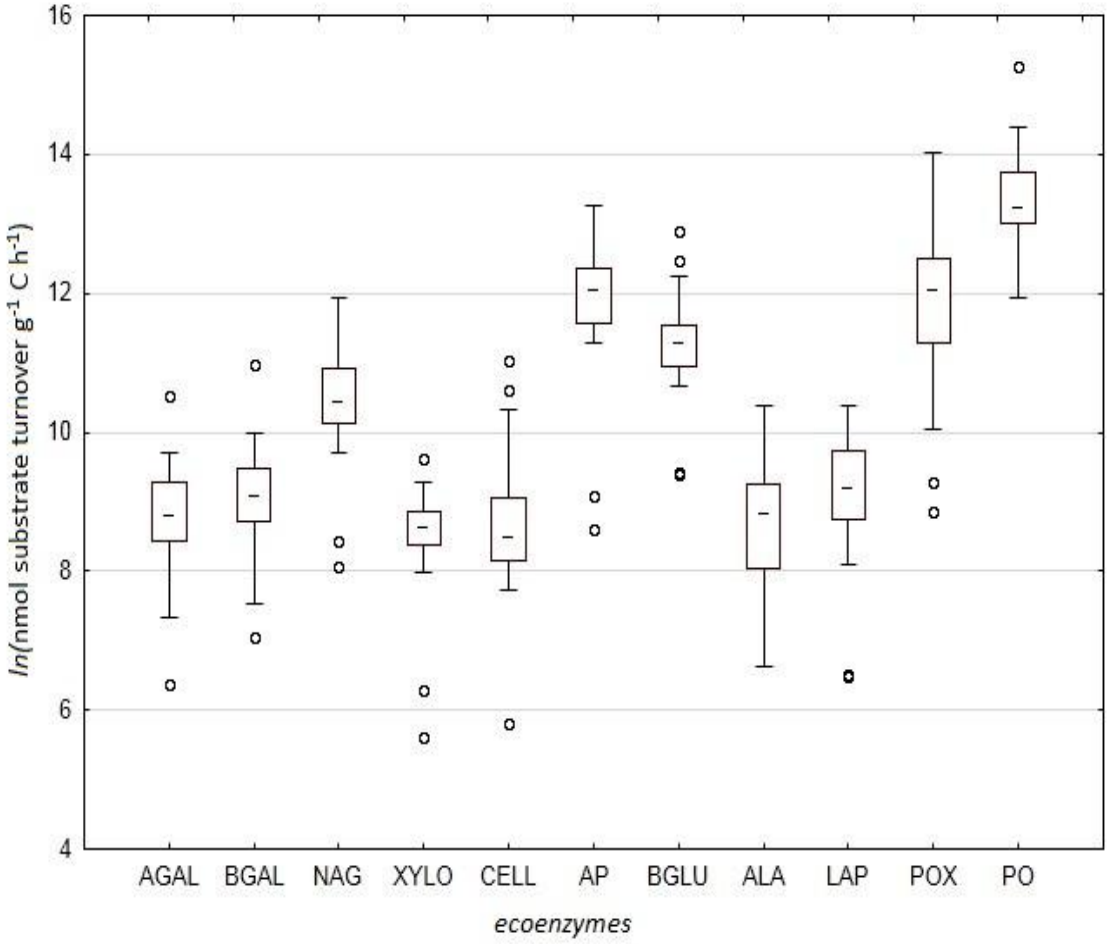

Figure 3. Box plots showing the mean, inter-quartile range, and 5th and 95th percentile for the ln of ecoenzymatic activity. n=27-28 with the exception of POX and PO (n=21). The open circles indicating extreme outliers.

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Table 4. Mean of biofilm C:N ratio and *ln* of ecoenzymatic activity (nm substrate g^{-1} C h^{-1}) at the various sites (n=2-5)

	Amity	Amnicon	Flag	French	Middle 2	Middle 3	Sucker	Talmadge
C:N	11.35	11.64	12.10	13.88	9.70	11.37	11.33	11.66
AGAL	8.68	9.13	6.86	8.72	9.78	8.81	9.05	8.71
BGAL	9.04	9.55	7.30	9.09	10.25	9.22	9.06	8.65
NAG	10.31	11.19	8.25	10.60	10.66	10.41	10.40	10.61
XYLO	8.58	9.05	5.95	8.47	8.65	8.60	8.87	8.55
CELL	8.46	9.87	6.79	8.26	8.92	8.59	8.98	8.57
AP	12.17	12.81	8.85	12.06	12.04	11.90	12.20	11.58
BGLU	11.18	11.90	9.41	11.28	11.44	11.14	11.45	11.55
ALA	8.22	9.05	7.16	8.28	9.00	9.50	9.24	7.98
LAP	8.97	9.30	6.49	9.30	10.09	9.67	9.39	8.45
POX	11.51	13.34	9.73	11.87	12.45	12.26	12.10	11.57
PO	12.96	14.69	12.49	12.88	14.07	13.85	13.60	14.33

Table 5. Spearman correlation coefficients relating averaged ecoenzymatic activity and water chemistry based on sample sites. All values used are natural log (*ln*) transformed and p-values are indicated as <0.05(*) and <0.01 (**). The (—) indicates p-values >0.05 or r<0.30

	Glycosidases	Peptidases	E2:E3	$SUVA_{254}$	TN	TOC	TP	C:N (<.45)	DOC	DON	DON/IN
C/N Biofilm	—	-0.49 **	—	—	—	-0.45 *	-0.47 *	0.48 *	—	-0.48*	—
AGAL		—	—	—	0.49 *	0.62 **	—	—	0.58 **	0.48 *	—
BGAL		—	—	—	0.54 **	0.63 **	0.56 **	—	0.59 **	0.58 **	—
NAG		—	—	—	0.40 *	0.46 *	—	—	—	—	—
XYLO	0.53 **	—	—	—	0.40 *	0.44 *	—	—	—	—	—
CELL		—	—	—	0.49 *	0.55 **	0.42 *	—	0.44 *	0.50*	—
AP	0.48 *	—	—	—	—	—	—	—	—	—	—
BG		—	—	—	—	—	—	—	—	—	—
ALA	—		—	—	—	0.46 *	—	—	0.38 *	—	0.65 **
LAP	—		—	—	0.55 **	0.58 **	0.55 **	—	0.44 *	0.56 **	—
POX	—	—	—	—	0.54**	0.66**	—	—	0.60**	0.54*	—
PO	—	0.56*	—	—	0.73**	0.76**	0.62 **	—	0.74**	0.68**	—
Glycosidases		—	—	—	0.43 *	0.48 **	—	—	0.40 *	—	—
Peptidases			—	—	0.54 **	0.62 --	0.48 *	—	0.46 *	0.55 **	0.42 *
E2:E3				-0.78 **	-0.43 **	-0.49 **	-0.40 **	0.43 *	-0.52 **.	-0.42 **	—
$SUVA_{254}$					0.63 **	0.69 **	0.53 **	-0.62 **	0.64 **	0.60 **	—
TN						0.96 **	0.70 **	-0.69 **	0.96 **	0.93 **	—
TOC							0.58 *	-0.57 **	0.97 **	0.92 **	—
TP								-0.77 **	0.62 **	0.69 **	0.40 **
C:N (<.45)									-0.62 **	-0.78 **	—
DOC										0.92 **	—
DON											—

LAP also was correlated to the concentration of DON ($r=0.55$ $p<0.01$). The PO activity correlated to the TP ($r=0.62$, $p<0.01$), TOC ($r=0.76$, $p<0.01$) and other water chemistry. Type II linear regression comparing BGLU, NAG, and AP, showed increases in ecoenzymatic activity that tended to increase at relatively constant ratios, based on the slope of the regression (Fig. 5a-c). The P acquiring AP activity increased relative to the nitrogen and carbon acquiring activities of NAG and BGLU. The average measured C and N composition of the dried biofilm mass was 5.76+2.38% and 0.45+0.23%, respectively, corresponding to a molar C:N ratio of 11:1 across all of the samples, with the highest mean values from the French River (13.9) and the lowest from the Middle 2 (9.70). This biofilm C:N ratio was negatively correlated to the peptidase activity and positively correlated with the C:N (DOC:TDN) ratio of the filtered water samples (Table 5, Fig. 5b).

Discussion

The measured stream nitrogen, phosphorous and carbon concentrations were all within the ranges reported from the previous study and within the range of values reported by other studies of streams in this region (Wold and Hershey, 1999; Detenbeck *et al.*, 2003; Detenbeck *et al.*, 2004). The measured DON/DIN ratios in my study were similar to the values used to pick the sample sites and showed the broad range that we expected (Detenbeck *et al.*, 2003; Detenbeck *et al.*, 2004). The values of DON and DIN were similar to those reported by Stanley and Maxted (2008) in Wisconsin streams and

showed a similar pattern of DON representing a large percentage of TN at concentrations below 2 mg/L. My values for DIN are consistent with a previous study of north shores streams (Wold and Hershey, 1999). The measured carbon concentrations in the water column were similar for the north shore sites and higher at the Middle 2, Middle 3 and Amnicon sites compared to other studies of Lake Superior tributary streams (Urban *et al.*, 2005). My measured $SUVA_{254}$ values were within the ranges of values reported by other researchers in freshwater streams (Weishaar *et al.*, 2003; Hood, Gooseff and Johnson, 2006; Jaffe *et al.*, 2008). I had generally higher carbon concentrations than the streams included in Jaffe et al. (2008) and my E2:E3 and $SUVA_{254}$ values were positively correlated to the organic carbon in the study. Generally E2:E3 increases and $SUVA_{254}$ decreases in a downstream transect indicating a decrease in molecular size and aromaticity from a more terrestrial source to a more open water signature as DOM is increasingly re-mineralized (Stephens and Minor, 2010). We show this same pattern based on increasing %wetlands, which for our sites had a higher relative molecular size and greater aromaticity. Our measured %wetlands were slightly less than the calculated %storage in the Detenbeck et al. (2004) study, which included a more comprehensive field assessment and took place 10 years prior to our study. In their broader study, they showed that %storage and maturity of forests were the significant factors affecting water chemistry. My results agree with these findings, as water chemistry parameters were correlated with %wetlands (e.g. %storage), %forests and to the UV-Vis proxies. Our results show carbon and nitrogen concentrations

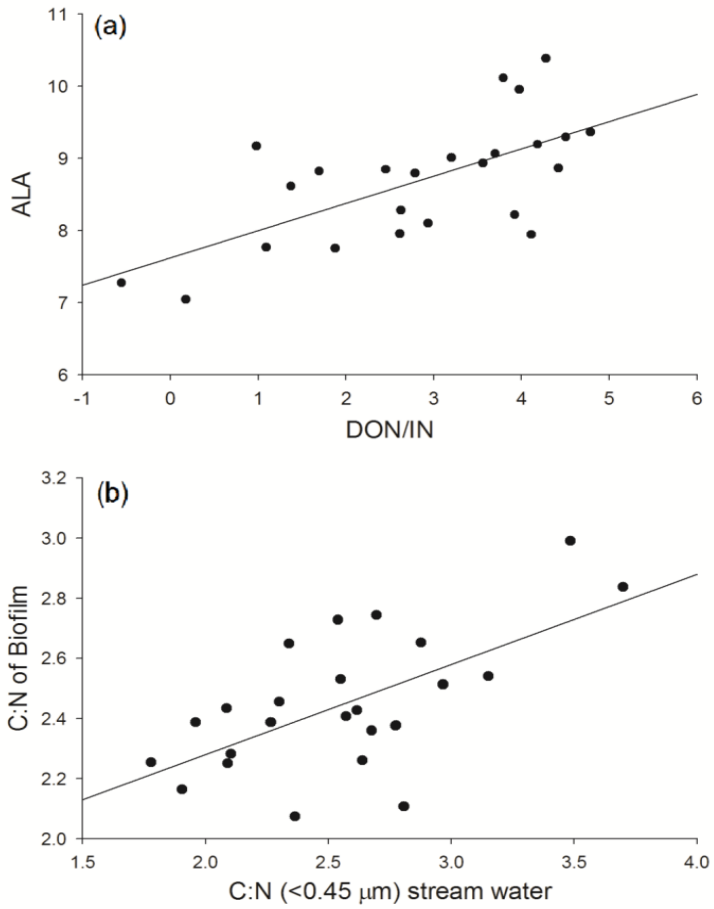

Figure 4. (a) Scatter plot with linear regression (r^2=0.43, slope=0.38, p<0.01 , n=24) for ALA activity as a function of the *ln*(DON/IN) ratio. (b) Scatter plot with linear regression (r^2 =0.39, slope=0.30, p<0.01, n=25) for *ln*(C:N) of biofilm as a function of the *ln*(C:N) of water (<0.45 μm). C:N is calculated as the molar ratio of DOC:TDN

increasing with increasing %wetlands as has been previously shown in Wisconsin streams and northeastern U.S. rivers and streams (Pellerin *et al.*, 2004; Stanley and Maxted, 2008).

The ecoenzyme activities tended to be slightly higher than values for attached biofilm in other studies (Findlay and Sinsabaugh, 2006; Hill *et al.*, 2010b). However, they were within the range reported by Sinsabaugh et al. (2009) for soils, wetlands and lotic sediment biofilm. The higher activity likely arises from differences in collection methods. I collected well established periphytic biofilm, in which Porter-Goff et al. (2010) found significantly higher organic matter and ecoenzymatic activity versus incubated substrates, using similar collection methods. My mean C:N for biofilm sample was 11:1, which was similar to other studies for biofilm and slightly higher than estimates of C:N in microbial soil (Romani *et al.*, 2004; Cleveland and Liptzin, 2007; Artigas, Romani and Sabater, 2008). Artigas et al. (2008) reported that C:N ratios for biofilm were negatively correlated to peptidase activity and tended to increase along with the C:N ratio of the dissolved component of the water chemistry. The same correlations were present in my study, although my work, unlike the Artigas et al (2008) study, used dissolved organic carbon rather than total dissolved carbon (organic and inorganic) in the calculation of stream chemistry ratios. Lower C:N ratios have been reported for freshwater stream periphyton than found in the biofilm of the streams studied here. A ratio of 7.5:1 for biofilm has been suggested to be the optimal growth

circumstance and ratios of greater than 10:1 imply nitrogen limitation (Hillebrand and Sommer, 1999; O'Brien and Wehr, 2010). AP activity did not correlate with TP concentrations in the streams which could be for a number of reasons, including: constitutive expression, a highly developed biofilm may be recycling P internally or changing pH within the biofilm to encourage the precipitation of phosphorous during daytime and dissolution during the evening (Steinman, Mulholland and Beauchamp, 1995). There were relatively higher concentrations of TP in the Middle 2, Middle 3, and Flag River sites compared to the north shore streams, suggesting the north shore streams may be more likely to be phosphorous limited which was also suggested by Detenbeck et al. (2003). Wold and Hershey (1999) found that both N and P, but often some other unknown factor was limiting periphyton growth in North Shore streams, as determined using nutrient releasing substrates. The previous paradigm of a specific nutrient causing limitation for stream microbial communities seems to be evolving into a complex model where co-limitation and the dynamic nature of the system need to be addressed.

In addition to nutrient availability, ecoenzymatic activity is influenced by other environmental variables, including pH, temperature and conductivity (Ainsworth and Goulder, 2000). The specific conductivity was negatively correlated to LAP and ALA activity, which contradicts Ainsworth and Goulder (2000) finding of a positive correlation, but specific conductivity also showed a similar negative correlation to

stream water C and N concentrations. The pH and temperature did not correlate to any measurements of ecoenzymatic activity.

The relative abundance and complexity of biofilms are also influenced by physical aspects such as flow and light patterns in streams (Battin *et al.*, 2003a; Romani *et al.*, 2004). There is evidence that the autotrophic and heterotrophic components in biofilms will show differing responses to nutrient addition. For example, biofilm grown in light is more likely to demonstrate nutrient limitation and more heterotrophic biofilm generally demonstrates lower ecoenzymatic activity (Romani and Sabater, 2000; Johnson, Tank and Dodds, 2009). In this study, ecoenzyme activity was negatively correlated with %canopy for most ecoenzymes ($p<0.05$; data not shown), with the exception of BGLU, and positively correlated with the mean stream width (e.g. less canopy coverage), which does support this hypothesis for our study streams.

UV-Vis proxies were not a useful predictor of ecoenzymatic activity. Lack of significant differences in the measured UV-Vis parameters between sampling dates and the strong correlations to our water quality parameters suggests that the chemical composition of TOC and DOC was fairly constant and the measured differences in UV-VIS proxies, may be a function of hydrologic discharge. However, despite the subtle changes in the UV-Vis data between sites and the correlation with the DOC, I did not see a relationship between glycosidases and E2:E3. This type of result may indicate that that controls on expression for these streams do not include relative molecular size or that the UV-Vis technique, which can only address those organic molecules absorbing

light in the measured wavelength range, cannot resolve the different triggers in expression. However, the UV-Vis proxies did show some strong relationships to both water chemistry and land usage, suggesting the possibility of applying this relatively quick method for qualitatively understanding stream chemistry patterns in this region.

The LAP and ALA showed some interesting differences, suggesting that the cues for expression were different between these two enzymes. ALA activity increased as the ratio of dissolved organic nitrogen to inorganic nitrogen increased (Fig 4a). LAP appeared to be responding more to the carbon levels or net production and did appear to be responding to the same cues. This finding suggests that LAP activity may be responding to carbon availability, which is not surprising given that DON and DIN uptake have been shown to be limited by DOC availability (Johnson, Tank and Arango, 2009). These observations support my hypothesis that the ratio of DON to DIN is a relevant parameter for understanding peptidase expression.

The biofilm C:N values were positively correlated to the C:N of the stream water (Fig 4b) and negatively to peptidase activity (Table 5). This may be indicative of the homeostatic relationship described by Sterner & Elser (2002), where the C:N of our biofilm is proportionately changing in response to nutrient C:N. The slope of the line may just be relevant to the epilithic biofilm and may be also be a function of the relative bioavailability of carbon since UV-Vis proxies tended to be the same within the sites, despite changes in DOC concentrations. Furthermore, the correlation of peptidase activity to biofilm C:N would seem to show that peptidase was playing a role in

maintaining key metabolically driven relationships reflected in this elemental ratio. The observed correlations of ecoenzymes with stream chemistry also suggest linkages between microbial metabolism and C and N availability, with peptidase activity mediated by a combination of inorganic and organic N. This supports my hypothesis and evidence from other studies that ecoenzymatic activity reflects water C and N stoichiometry.

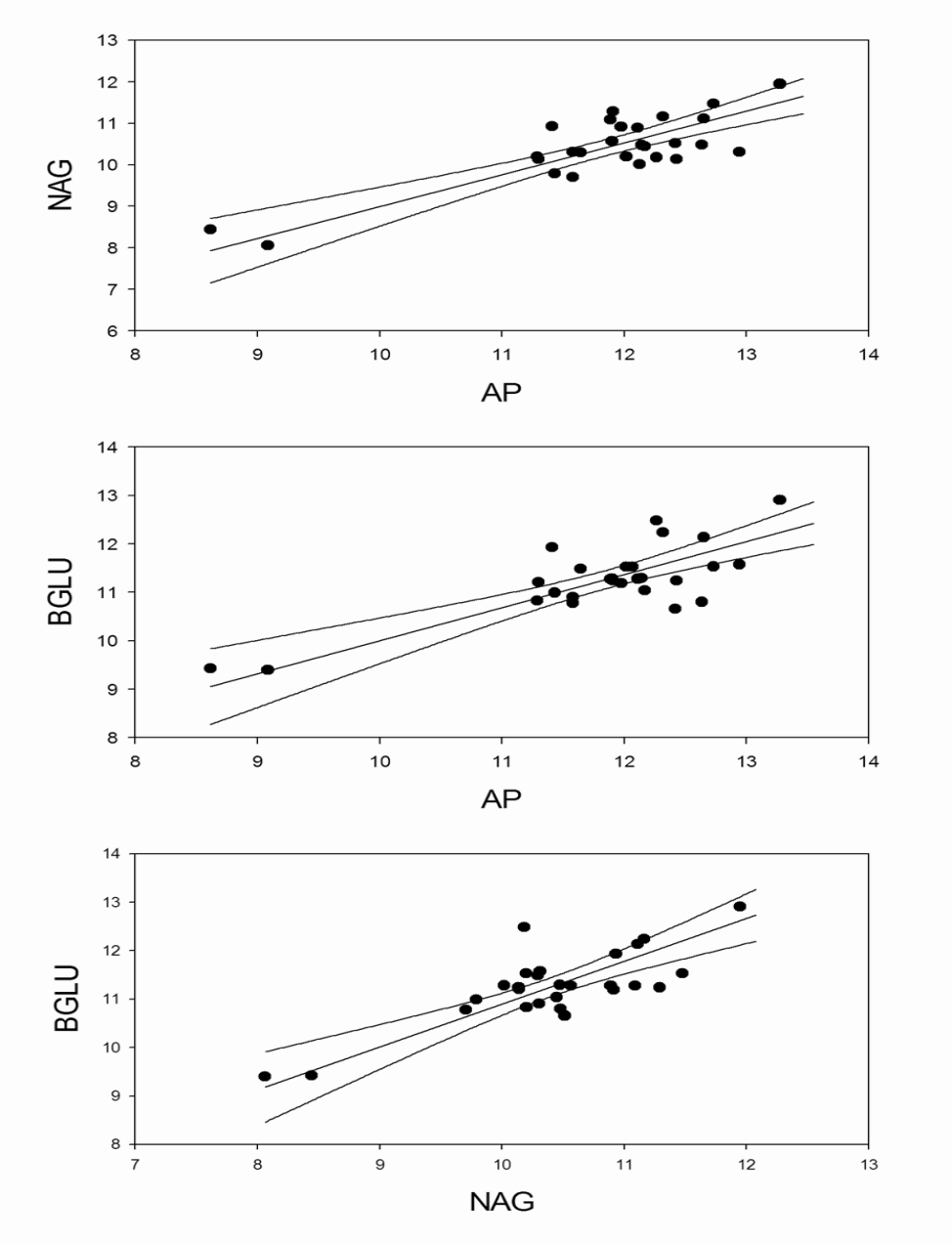

Figure 5. Type II linear regression comparing *ln* of enzyme activity with 95% upper and lower limit confidence intervals shown in the figures (n=25). (a) NAG to AP (r^2 = 0.67, slope= 0.77, p<0.01), (b) BGLU to AP (r^2=0.61, slope= 0.68, p<0.01) and (c) BLGU to NAG (r^2= 62, slope=0.88, p<0.01)

However, the increases in biofilm C:N may also be indicative of increasing algal biomass instead of microbial biomass and therefore higher C:N ratios. Algae demonstrate a higher ratio for C:N (approx. 16:1) than microbes (7:1), which suggests that the biofilm C:N may also be affected by a combination of factors controlling the relative percentages of algae, fungi and microbes in the biofilm (Sterner and Elser 2002; Cleveland and Liptzin, 2007; Artigas, 2008). In freshwater, algal biomass and microbial respiration will tend to be positively correlated and the microbial assemblage is partially dependent on algal exudates for nutrients (Carr, Morin and Chambers, 2005; Scott *et al.*, 2008).

My data also showed relationships (Fig. 5a-c) similar to relationships presented by Sinsabaugh et al. 2009, where sediment and soil data were used to demonstrate that certain ecoenzymatic activities will approximate a 1:1 ratio, when looking at a large data set. They suggested that these ratios are representative of the equilibrium between the incorporation of biomass and the metabolic effort required, as a function of resources availability. Additionally, our estimated slopes of BGLU to AP (0.68) (Fig. 5b) and BLGU (Fig. 5c) to NAG (0.88) are close to and within the confidence intervals of the slopes identified in Sinsabaugh et al. (2010) as 0.65 and 0.95, respectively. When making this comparison, we are assuming that productivity per g C is the same across our sites, since in the Sinsabaugh et al. (2010) study they do normalize to production for comparison between different types of ecoenzymatic communities. My data, albeit from a much smaller sample set, adds additional support for the relationship between ecoenzymatic

activities and the metabolic theory of ecology, that ecoenzymatic activity will reflect resources constraints and biological demand. It could be argued that deviations from the slope (i.e. flatter or steeper slope) will be reflective of differences in stoichiometry of biomass (i.e. biofilm community composition) or the relative recalcitrance of nutrients. This study does not clarify the mechanisms and drivers behind the expression of these ecoenzymes and there are additional environmental factors that may be responsible for the variability behind my relationships. To the best of my knowledge, very few studies have addressed ecoenzymatic activity on naturally occurring epilithic biofilms and this is the first in the Lake Superior region. My results show that ecoenzyme activity and epilithic biofilm C:N are correlated with 1) changes in nutrient concentrations across a broad range, 2) the relative concentrations of organic and inorganic nitrogen, and 3) the stoichiometric ratios of water-column nutrients. In conclusion, my investigation offers additional insights into the response of biofilm ecoenzymes to fluvial chemistry and contributes to the idea of using ecoenzymes as indicators of microbial assemblage response to the stoichiometry of available nutrients in streams.

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