Investigation of Nutrient Limitation of the Biofilm Community in Acid Mine Drainage Impaired and Remediated Streams

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ABSTRACT

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Investigation of Nutrient Limitation of the Biofilm Community in Acid Mine Drainage Impaired and Remediated Streams

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Acid Mine Drainage (AMD) from pre-regulation mining affects streams in the Appalachian region resulting in acidic waters with high dissolved metal content. Previous studies have shown remediated stream segments have better water quality and biological communities than untreated streams, but these segments have not attained the same biological quality as streams unaffected by AMD. Phosphorus limitation of the biofilm community has been hypothesized as a contributing factor. Nutrient limitation was tested in four stream categories using nutrient diffusing substrates: AMD, transitional, recovered and unimpacted. Chlorophyll *a*, a measure of photosynthetic biomass, was significantly higher in phosphorus treatments. In addition, the phosphorus treatments had lower phosphorus-acquiring enzyme activities compared to the control. The phosphorus with nitrogen treatment showed an increase in polyunsaturated fatty acids, having higher nutritional value for grazers. This study demonstrated that nutrient availability has a substantial impact on the photosynthetic component of biofilms in impaired and remediated streams.

DEDICATION

I would like to dedicate this to my husband, Conner Loudner, and my parents, who have always supported me.

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INTRODUCTION

Algae play an essential role in stream ecosystems. In particular, benthic algae form an association with bacteria and other organisms within a gelatinous matrix establishing a biofilm on most hard surfaces (Bott 1996). Algae in these biofilms are the principal primary producers in wadable streams (Lamberti 1996). Within the biofilm, these algae serve as the main source of carbon (Lambert 1996). The bacteria are clustered around the algal cells and readily take up available algal carbon (McFeters *et al.* 1978, Haack & McFeters 1982). This flow of carbon from algae to bacteria and then to other organisms in the stream is essential to the ecosystem dynamic.

Acid mine drainage (AMD) is a severe environmental problem affecting many streams worldwide. The Appalachian region has been highly impacted by AMD with approximately 10,000 km of streams in the Appalachian region affected (US EPA 1995). AMD occurs when rocks containing pyrite are exposed to air and water, which react to form sulfates, sulfuric acid and iron oxides, causing an increase in acidity (Singer & Stumm 1970). AMD impaired streams can have a pH as low as 3 (Bray *et al.* 2008) and potentially have high solute concentrations of metals, such as iron, aluminum, and copper (Filipek *et al.* 1987). When the pH is elevated (~3.5), iron precipitates as an orange flocculent covering the streambed and rocks, preventing biofilm/algal colonization (Niyogi *et al.* 2002). These AMD effects have caused extreme stress on the biota of streams in the Appalachian region, resulting in decreased species richness and diversity of macroinvertebrates and fish and increased abundance of tolerant species (Rice *et al.* 2002).

The high acidity and dissolved metals associated with AMD affect not only stream community composition, but also stream function. Primary productivity can be affected, as algal biomass and accrual rates are lower (Smucker & Vis 2011) as well as taxonomic richness decreased (Bray *et al.* 2008). In some streams, iron hydroxides coat the stream bottom, lowering biomass due to the unavailability of substrata for algal colonization (Bott *et al*. 2012). This reduction in biomass restricts the amount of energy available to transfer to higher trophic levels (Hogsden & Harding 2012) and may be observed in reduced macroinvertebrate density and diversity (Simmons *et al.* 2005). Fish are generally absent in AMD affected streams and if present, are highly reduced in abundance and diversity (Hogsden & Harding 2012, Rice *et al.* 2002).

Due to the significant and ongoing effects of AMD on chemical, physical and biological components of streams, numerous remediation techniques have been developed (Costello 2003). Remediation typically involves raising the pH to neutral and precipitating metals to allow for downstream biological recovery. Remediation approaches can be categorized as passive or active. Passive treatments do not require continuous inputs of energy and resources and include wetlands, limestone channels, and steel slag leech beds lined with alkaline materials (Gazea *et al.* 1995). Active treatments generally require continuous input of energy or resources (Costello 2003). The primary active treatment systems used in the Appalachian region are alkaline dosers that add calcium oxide to the stream. These dosers are employed when the AMD load is too great for passive treatments to ameliorate or the amount of land required for a passive treatment is not available (Costello 2003).

There has been limited research of biofilms in remediated streams. However, one study has shown gross primary productivity (GPP) and chlorophyll *a* concentrations of a remediated stream to be closer to reference than AMD stream values (Bott *et al*. 2012). Yet, much evidence has accumulated suggesting that biofilms in AMD remediated streams may be limited by nutrients from measured extracellular enzyme activity (EEA). These studies showed an increase in phosphorus-acquiring EEAs in AMD remediated sites compared to the reference streams or sites upstream of the AMD input (Smucker & Vis 2011, Pool *et al.* 2013, Drerup & Vis 2016a). Members of the biofilm community can excrete extracellular enzymes to scavenge nutrients. These enzymes are released when complex molecules are present, simple molecules are absent, and there is a need for the nutrient (Allison & Vitousek 2005). Phosphorus limitation has been inferred from biofilm EEA values of remediated streams (Smucker & Vis 2011, Pool *et al.* 2013, Drerup & Vis 2016a).

In addition to individual EEA values, ecoenzymatic stoichiometry is used to determine the overall enzyme activities of a system. Ecoenzymatic stoichiometry uses the ratios of the total carbon to phosphorus acquiring enzymes and the total carbon to nitrogen acquiring enzymes can elucidate limitation of nutrients or carbon (Moorhead *et al.* 2016). Ecoenzymatic stoichiometry is calculated as a length vector and angle based on the carbon to phosphorus acquiring enzymes plotted against carbon to nitrogen acquiring enzymes. The vector length determines carbon limitation relative to nutrient limitation, with the longer the length, the more carbon limited. The angle establishes if the system is more nitrogen or phosphorus limited, with the higher the vector angle, the more phosphorus limited (Moorhead *et al.* 2016). This method can be very insightful in

nutrient addition studies, to observe the change in enzyme allocation with the addition of the nutrient. Zeglin *et al.* (2007) used ecoenzymatic stoichiometry in a nitrogen-depleted system and found allocation to carbon enzymes when nitrogen was added.

Total fatty acid profiles have been used to determine both total community biomass and community structure (Vestal & White 1989). Fatty acid profiles are considered a proxy for the total community biomass because they represent the active microorganisms (Vestal & White 1989). Some studies have used total fatty acid profiles for ecotoxicological studies of periphyton communities (Lowe *et al.* 1996). Fatty acid profiles showed less variability in the community biomass than other measures of biomass, such as chlorophyll *a* (Lowe *et al.* 1996). Several studies have compared community structure using total fatty acid profiles (Napolitano *et al.* 1994, Lowe *et al.* 1996, Hill *et al.* 2011, DeForest *et al.* 2016). Fatty acid profiles characterize community structure by the types and percentages of fatty acids present, such as saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and highly unsaturated fatty acids (HUFA). Using fatty acid profiles, Deforest *et al.* (2016) accurately separated out streams into their predetermined water quality designations. These fatty acid profiles, such as the percentage of HUFA, PUFA, MUFA, and SAFA, have also been shown to be sensitive to nutrients, such as phosphorus treatments (Hill *et al.* 2011). Drerup & Vis (2016b) used phospholipid fatty acid profiles (PLFA) to compare AMD impacted and AMD remediated streams. They found that PLFA separated out Unimpaired, Remediated and Unimpaired streams into distinct groups. Though Total Fatty Acid profiles are distinct from PLFA profiles used in Drerup & Vis (2016b), they

have potential, as seen with the other studies, for biomonitoring in AMD remediated streams.

The use of nutrient diffusing substrates (NDS) has been a commonly employed method to test nutrient limitation in aquatic systems (Tank *et al*. 2006). NDS consist of a cup with a nutrient enriched agar solution and suitable surface on top of the agar solution for biofilm colonization. If there is a significant increase in biofilm biomass in the nutrient enriched treatment compared to the control that nutrient is considered to be limited (Tank *et al*. 2006). These NDS, sometimes coupled with EEA assays (Scott *et al.* 2009), have shown to be informative in detecting and assessing nutrient limitation in various types of streams (Tank & Dodds 2003).

There is some evidence from studies measuring EEA that doser-remediated streams may be phosphorus limited, similar to AMD-impaired streams (Smucker & Vis 2011, Pool *et al.* 2013). Phosphorus is known to bind to iron and calcium, both of which are readily available in AMD remediated streams. With this characteristic of phosphorus as well as the high phosphorus-acquiring EEAs, it is possible phosphorus is limiting the biofilm communities. This nutrient limitation may hinder biological recovery (Smucker & Vis 2011, Drerup & Vis 2016a). However, there has been little direct testing of biofilm nutrient limitation in AMD (DeNicola & Lellock 2015) and no examination of a recovery gradient downstream of the alkaline treatment. This study seeks to address the question of nutrient limitation by studying streams with commonly used remediation treatments (alkaline doser and limestone leach bed) along the recovery gradient. The types of sites to be examined are ‘transitional’ sites, those that have some recovery, ‘recovered’ sites those that have shown marked improvement, ‘unimpaired’ sites and ‘AMD impaired’

sites. The biofilm communities of NDS were analyzed for biomass, nutrient acquiring enzymes and fatty acid composition. It was hypothesized that phosphorus limitation would be evident in the biofilm from AMD as well as the transitional and recovered sites as compared with unimpaired streams. This limitation of these types would be manifested in lower biomass as measured by chlorophyll *a* and biofilm fatty acid mass, higher nutrient acquiring enzymes and potentially in fatty acid composition.

MATERIAL AND METHODS

Pilot Study

Pilot studies were conducted to test numerous aspects of the experiment. Other experiments have shown that the agar containing phosphorus enriched (+P) and nitrogen and phosphorus enriched (+NP) did not solidify as well as nitrogen enriched (+N) or the control probably due to the phosphorus salt (Lindner 2015). The recipe for these treatments was tested and changed to ensure that the agar has the same consistency in all treatments. A pilot study to determine algal growth was conducted from May 11 to June 1, 2015. The pilot study tested how much algal biomass accumulated over a three-week period for +NP, control, and +P treatments. This study also used varying phosphorus concentrations for the +P treatments to determine the nutrient response threshold. The concentrations of phosphorus utilized were 0.05 M, 0.1 M, 0.5 M, and 1.0 M based on the concentrations used in previous studies (Tank & Dodds 2003, Scrimgeour & Chambers 1997, Rugenski *et al*. 2008). In a previous study, 0.5 M was found to be a threshold such that the high concentration of 1.0 M in the pilot study was considered to be in excess (Scrimgeour & Chambers 1997). Each treatment had three replicates. The NDS were deployed at two streams, an unimpaired and a transitional. These two types of streams were chosen to provide a stream in which there would be much biomass accumulation (unimpaired) and one in which little accumulation was expected (transitional). At the end of the 3-week pilot study, algal dry weight and chlorophyll *a* concentrations were measured on the pre-weighed fretted glass disc. The diffusion rate was tested for the +P and +NP treatments by measuring the remaining phosphorus within the agar. An acid digestion was performed on the agar by adding a 16% 1 N hydrochloric acid solution to

the combusted agar. The solution was placed in a 105º C oven for two hours to allow the samples to undergo a mild acid hydrolysis. The Ascorbic Acid/Colorimeter method was used to measure the phosphorus concentration as described in the phosphorus methods below.

A Two-Way ANOVA was conducted on the chlorophyll *a* concentrations. The pilot study showed significantly less chlorophyll *a* concentration at the unimpaired site than the transitional site ($p<0.001$). This finding was possibly due to the site chosen having a silty substrate as well as very low percentage of open canopy (~20%). Subsequently, this site was not used for the study due to the unsuitable physical environment. At the transitional site, there was a high variability of chlorophyll *a* within a treatment, which potentially affected the statistical power (3 replicates/treatment) such that no significant differences were detected among treatments. However, there was a trend with chlorophyll *a* increasing until reaching the 0.5 M phosphorus concentration and then leveled off for the 0.5 and 1.0 M concentrations. This finding corroborated the results of Scrimgeour & Chambers (1997) who showed 0.5 M phosphorus concentration was the threshold at which the biofilm did not significantly increase in biomass.

The NDS from both sites had phosphorus remaining in the agar after 3 weeks. The cups from the unimpaired site had more particulate phosphorus compared to the transitional site. The cups from the transitional site showed that the higher initial concentrations of phosphorus had less phosphorus remaining at the end of the study. Although this result may seem counter intuitive, it is similar to the findings of Rugenski *et al.* (2008). These researchers concluded that NDS with higher concentrations of phosphorus diffused more phosphorus more quickly than NDS with lower concentrations

of phosphorus. Nevertheless, the goal of the pilot study was to show that phosphorus was still available to the biofilm community after three weeks and not completely finished before the end of the experiment.

Study Sites

Four stream categories were created to examine nutrient limitation along the recovery gradient of AMD remediated streams: AMD impaired, transitional, recovered, and unimpaired. Four sites were chosen for each category. AMD impaired sites were streams heavily impacted by AMD and have not received remediation treatment. To classify sites as transitional or recovered, the Macroinvertebrate Aggregated Index for Streams (MAIS) scores for the past three years were used. The MAIS score ranks a stream as poor, fair, good, and great based on the types of macroinvertebrates present (Johnson 2007). This classification served as an indicator of the recovery gradient downstream of the AMD remediation treatments. The sites classified as transitional have not shown improvement in MAIS score and the score was less than 12. Sites classified as recovered have shown improvement in MAIS score since being monitored and had a score of 12 +/- 1 for the past three years, but are not classified as Exceptional Warm Water Habitat. The 12-15 range in MAIS score represents "good," but is not completely restored to excellent warm water habitat conditions (Johnson 2007). Sites classified as unimpaired have not been previously impacted by AMD and are Excellent Warm Water Habitat. Only of the four unimpaired sites were regularly sampled for MAIS scores, the other three sites were considered exceptional warm water habitat and were Fully Attaining (in regards to other biological indices) according to the EPA. The sites were selected from four watersheds within the Hocking River Drainage Area: Hewett Fork

within Raccoon Creek, Sunday Creek, Monday Creek and Federal Creek. Each of these watersheds is within the Western Allegheny Plateau ecoregion and is also within the Pennsylvanian Allegheny coal basin.

Hewett Fork, a subwatershed of the Raccoon Creek, has a drainage area of approximately 104 km^2 (Figure 1) and is approximately 80% forested. The primary AMD treatment is an alkaline doser located at river kilometer 17.7 near Carbondale, which was installed in 2004. Within this watershed four sites were utilized with one recovered, two transitional and one unimpaired. Kings Hollow Tunnel (R-HF01), at river kilometer 6.4 was the recovered site. The transitional sites were located adjacent to State Route 356 between river kilometers 11.6 and 11.9, T-HF01 and Waterloo Wildlife area (T-HF02). The unimpaired site (U-HF01) was located adjacent to Carbondale Road at river kilometer 21.6 upstream of the AMD seep.

Monday Creek has a drainage area of approximately 297 km^2 (Figure 1) and is approximately 87% forested. Monday Creek is treated with steel slag leech beds, limestone channels and an alkaline doser at Jobs Hollow at river kilometer 46, which was installed in 2004. Remediation began in Monday Creek in 1995 with a project at Rock Run (Bowman & Johnson 2015). Within this watershed, three sites were utilized with one recovered, one unimpaired, and one impaired. The recovered site (R-MC01) was located near State Route 278 in Carbon Hill at river kilometer 16.7. The unimpaired site (U-MC01) was located in Little Monday Creek adjacent to Gore-Greendale Rd. at river kilometer 1.6. The impaired site (I-MC01) for Monday Creek was Snow Fork located off of state route 685 in Buchtel at river kilometer 3.9.

Sunday Creek has a drainage area of approximately 355.8 km^2 (Figure 1) and is approximately 78% forested. The West Branch and Main Stem of Sunday Creek are both impacted by AMD. West Branch in Sunday Creek has a drainage area of approximately 90 km^2. West Branch of Sunday Creek is treated with limestone channels, limestone leach beds and an alkaline doser at the mouth of Pine Run, which was installed in 2013. Six sites within this watershed were utilized with two recovered, two transitional, and two impaired. The recovered sites were at Cornstill Rd. at river kilometer 9.9 (R-SC01) and at Oakdale Rd. near Moore Rd. at river kilometer 2.9 (R-SC02). The transitional sites were West Branch at county road 31 bridge at river kilometer 16.8 (T-SC01) and Indian Run road at river kilometer 18.3 (T-SC01). The impaired sites were Sunday Creek off of state route 76 at river kilometer 35.2 (I-SC01) and at state route 76 and 155 at river kilometer 37.8 (I-SC02).

Federal Creek has a drainage area of approximately 371 km^2 (Figure 1). Federal Creek does not have a substantial history of mining as the other watersheds in the Hocking River drainage area. Mush Run (U-FC01) was the unimpaired site within Federal Creek, located off of state route 690, just upstream of the confluence with McDougal Branch.

Nutrient Diffusing Substrates (NDS) Preparation

The nutrient-diffusing substrates (NDS) were prepared in a similar manner to the methods provided in Tank *et al*. (2006) (Figure 2). The NDS consisted of a 30 mL plastic cup filled with agar and nutrients depending on the treatment with a fritted glass disk for biofilm colonization. The four treatment types were control, phosphorus enriched (+P), nitrogen enriched (+N), and nitrogen and phosphorus enriched (+NP). The control

treatment had 20 g of agar powder (2% by weight) per liter of water. The nitrogen treatment had 50.6 g of KNO_3 per liter added to the base agar solution for a 0.5 M nitrogen molarity. The phosphorus treatment had 68 g of KH_2PO_4 per liter added to the base agar solution for 0.5 M phosphorus molarity, which was determined by the pilot study. The nitrogen and phosphorus treatment had the same amounts of salt as the individual treatments, but had an extra 10 g of agar powder per liter added to the base solution to keep the solution 2% agar (by weight).

Each site had three replicates for each treatment. Each replicate consisted of six cups per treatment, resulting in a total of 18 cups per treatment per site. The six cups of a replicate were pooled into a single sample. A replicate consisted of two L-bars with three cups per treatment on each L-bar, which were randomly placed along the L-bar. The L-bars were randomly assigned together as a replicate before deployment in the field.

Field Methods

The NDS were deployed the week of June 24, 2015. The NDS were secured in the streams using rebar and zip ties (Figure 2) and the following physical parameters were measured at each stream: current velocity, specific conductance, pH, temperature, stream depth, stream width and canopy cover. The current velocity was measured using the Global Water Flow Probe (Global Water Instrumentation, Inc., College Station, TX). The Multi-Parameter PCTestr™ 35 (Eutech Instruments Pte Lte, Singapore) was used to measure the specific conductance, pH and the temperature. The stream width and depth were measured and the canopy cover delineated using a spherical densitometer (Forestry Suppliers Inc., Bartlesville, OK, USA). Four 20 mL scintillation vials of filtered stream water (0.45µm pores; Millipore®, Billercia, MA) were collected at each stream site for

water chemistry. The filtered water was stored on ice for transport to the lab and immediately placed in a -20ºC freezer until further analyses. In addition, filtered water was collected about half way through the study so that water chemistry was monitored on the first and last day as well as the middle of the study to account for potential changes in the water chemistry over the course of the experiment.

The NDS were retrieved the week of July 20th and were in stream for a total of 25-26 days. On the last day of the study, current velocity, specific conductivity, pH, temperature, stream depth, and stream width were again measured. In addition to the four 20 mL scintillation vials of filtered water for water chemistry, a one 250 mL Nalgene bottle of filtered water was collected at each site for the enzyme assays. Two 1 L Nalgene bottles were collected with unfiltered stream water at each site for the biofilm samples. The NDS were transported back to the lab. In the lab, the biofilm was scrubbed from the discs with a soft bristled brush with the 6 discs within a replicate pooled and stored as a 125 mL sample at 4ºC. Within 24 hours, the sample was homogenized and a 40 mL subsample was placed in a 50 mL centrifuge tube for the enzyme assays, a 5 mL subsample in a 20 mL scintillation vial for preservation. The remainder of the sample to be used for the chlorophyll *a* and total fatty acid analysis was lyophilized for three days in the FreeZone 4.5 (Labconco, Kansas City, MO). The chlorophyll *a* biomass will be expressed as per unit area.

Stream Chemistry Analyses

The filtered water samples were analyzed for nitrate (NO_3^-) (method 8192), total iron (Fe) (method 8008), and sulfate (SO_4^{2-}) (method 8051) using HACH DR/890™ colorimeter (HACH Company, Loveland, CO) with HACH powder pillows (Hach

company, 2009). The nitrate, iron, and sulfate samples were analyzed within 48 hours of collection. The samples for phosphorus remained frozen at -20ºC until the samples were analyzed for soluble reactive phosphorus. The concentration of phosphate was measured using the ascorbic acid/colorimeter method (Stainton *et al.* 1976) using a Genesys™ 20 Visible Spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA).

Chlorophyll *a* Analysis

For each treatment at each site, three analytical replicates of 2-4 mg of freeze-dried sample were analyzed for chlorophyll *a*. The mean of these samples is reported. The samples were soaked in 90% acetone for 18-20 hours as described by EPA method 445.0 (Arar & Collins 1997). The concentration of chlorophyll *a* was determined using a Turner TD-700 fluorometer (Turner Designs, Sunnyvale, CA). To correct for phaeophytin-a, 0.1 N HCl solution was added.

Total Fatty Acid Profiles

Total fatty acids were extracted using methods of Sasser (1990) modified by DeForest *et al.* (2016). The treatment replicates for an individual site were pooled such that there was a single sample for each of the four treatments for each site. Approximately 200-350 mg of freeze-dried material was saponified with a 1.5 ml mixture of NaOH, methanol, and DI water (37.5: 125: 125 g/ml/ml). A 250-µL aliquot of the internal standard 19:0 (33.5 µg C) was added. The sample was placed in a hot water bath at a temperature ranging from 95 to 100° C for five minutes. The sample was then placed in a tray of cold water. This step was repeated once more for exactly 25 minutes in the hot bath. A 3 ml solution of a mixture of 6 N HCl and methanol (135:115 ml/ml) was added and the sample placed in an 80 °C bath for 10 minutes. The samples were placed in

a tray of cold water. A 1.5 ml solution of a mixture of hexane and MTBE (50:50 ml/ml) was added and the sample was left standing until the sample separates into two phases. The bottom phase was removed with a small portion of the top phase. A 3 ml solution of a mixture of NaOH and DI water (3: 250 g/ml) was added and placed in the centrifuge for 5 minutes at 3000 RPM. About 0.5 ml of the top phase was placed in a clean GC sample vial. The vial was placed in a plastic tube and evaporated on the N-EVAP until all liquid had evaporated. A total of 400 µL of Hexane-MTBE was added to the GC vial and capped. The samples were stored at 4 ºC until analyzed, which occurred within 48 hours of the fatty acid extractions. To separate and analyze the fatty acids, gas chromatography was used (HP690 series, Agilent Technologies, Inc, Santa Clara, CA, USA) with an autosampler and controlled using the Microbial Identification System Sherlock Software (v 6.2, MIDI, Inc., Newark, DE, USA). An Agilent HP-ULTRA2 (25 m x 200µm) was used for the GC column with ultra-high purity nitrogen as the carrier gas. The initial temperature was 190 ºC, which increased 10 ºC per minute until 285 ºC for the first ramp. For the second ramp, the temperature increased 60 ºC per minute until 310 ºC. The initial flow was 1.4 mL per minute for 9.5 minutes and then increased at a rate of 3.0 mL per minute for the second temperature ramp. The individual fatty acids were identified from the Sherlock software using a calibration standard (MIDI, Inc.).

Extracellular Enzyme Activity

A 40 mL subsample of each replicate for all treatments and sites was analyzed for extracellular enzyme activity. The subsamples were frozen at -20°C until analysis was performed. The following six enzymes were analyzed for each sample: phosphomonoesterase (MonoP) (M8883), phosphodiesterase (DiP) (B35000G) (Biosynth

International, Inc. Itasca, IL), chitinase (NAG) (M2133), β-glucosidase (GLU) (M3633), β-xylosidase (XYLO) (M7008) and leucine aminopeptidase (LAP) (L2145) (Sigma-Aldrich, St. Louis, MO). Fluorescent methylumbelliferone (MUB)-linked substrates were used to measure MonoP, DiP, NAG, GLU, and XYLO activities in the biofilms and the fluorescent substrate leucine 7-amido-4-methylcoumarin (AMC) to measure LAP activity.

EEA was measured using the methods described in Smucker *et al.* (2009). A 96-well black polystyrene microplates with 300µL wells (Whatman Inc., Florham Park, NJ) arranged in 12 columns by 8 rows was utilized. The rows served as analytical replicates. Column 1 was a blank, which contained 250µL of filtered stream water to account for background fluorescence of the stream water. Column 2 was a reference standard, which contained 200 µL filtered stream water and 50 µL of MUB or AMC. Column 3 was a negative control, which contained 200 µL of filtered stream water and 50 µL of the substrate to account for fluorescence from the substrate. Columns 4-6, 7-9, and 10-12 were used to determine the EEA for each sample. Columns 4, 7, and 10 served as the quench factor for each sample, containing 200 µL of homogenized sample and 50 µL of MUB or AMC to account for fluorescence masked by the sample. Columns 5, 8, and 11 served as the sample control, which contained 200 µL of sample and 50 µL of the filtered stream water to account for the natural fluorescence of the sample. Columns 6, 9, and 12 were the sample assays, which contained 200 µL of sample and 50 µL of the substrate. The plate was incubated in the dark for 20-40 minutes and the fluorescence was read with a Synergy HT microplate reader at an excitation wavelength of 365 nm and an emission wavelength of 455 nm (Synergy HT, BioTek, Winooski, VT). The levels of EEA were

calculated using the fluorescence as described in DeForest (2009). However, the chlorophyll *a* biomass was used as the mass component rather than the ash free dry mass. To avoid deterioration of the standard solutions, fresh solutions were made a few days prior to EEA analysis.

Statistical Analyses

Using the R Statistical Program (R Foundation for Statistical Computing, Vienna, Austria), a mixed-model analysis of variance (ANOVA) with a Bonferroni post hoc test was performed on the stream physical and chemical characteristics to determine any differences among the stream categories. To determine differences among the stream categories and nutrient treatments, a two-way ANOVA was conducted with a Tukey's Multiple Comparison of Means post hoc test. This analysis was performed for the biomass measurements (chlorophyll *a* and biofilm fatty acids), fatty acid composition, individual EEA and ecoenzymatic stoichiometry values. An analysis of covariance (ANCOVA) was conducted to determine if stream chemical and physical characteristics acted as covariates on the biomass measurements (chlorophyll *a* and fatty acid biomass). The EEA values used in the ecoenzymatic stoichiometry were log transformed to meet normality assumptions. A reaction ratio was calculated to determine differences due to the effects of the nutrient treatments from other factors that may have differed from site to site within a stream category. The reaction ratio is the difference of the control from the nutrient treatment divided by the control treatment (nutrient biomass – control biomass/ control biomass). The reaction ratio was calculated for the chlorophyll *a* concentrations, fatty acid biomass, and EEA. A t-test was performed to determine if the nutrient treatments were significantly different from 0. Pearson product moment

correlations were conducted for the chlorophyll *a* biomass with the stream chemical and physical characteristics.

RESULTS

Due to several storm events during the study, some NDS at some locations were covered by sediment. At AMD Impaired site (I-MC02) and Unimpaired site (U-FC02) the NDS could not be used further in the study. Sites T-SC01, I-SC01, R-SC01, and U-FC01 each had one replicate for each treatment that could not be used. At some sites one of two L-bars was not usable leaving half a replicate (three disks per treatment). These replicates were still used, but the calculations were adjusted to three disks per replicate.

Numerous physical and chemical characteristics were measured at each stream site (Table 1). Of the characteristics measured, only stream temperature and sulfate concentration were significantly different among stream categories (Table 2). Stream temperature was significantly different among stream categories ($p = 0.006$). Impaired (mean 17ºC) was significantly less than Recovered and Unimpaired (mean 21ºC and 20ºC, respectively) with Recovered and Unimpaired not significantly different ($p > 0.024$, Table 2). Transitional was not significantly different from Impaired or Recovered and Unimpaired. There was a significant difference in the sulfate concentrations among the categories ($p = 0.024$). The sulfate concentration for Unimpaired (mean 66 mg/L) was significantly lower than the Impaired (mean 236 mg/L) ($p = 0.031$, Table 2). The Transitional and Recovered were not significantly different from each other or from Unimpaired or the Impaired. Some measured characteristics were expected to be different, but were not including pH, specific conductance, iron concentration, and soluble reactive phosphorus concentration. The mean pH for Impaired, Transitional, Recovered and Unimpaired were 6.2, 7.3, 7.3 and 7.4, respectively (Table 2). The pH was not significant different among categories, potentially due to the broad range in Impaired

(5.8 – 7.1) (Table 1). The mean specific conductance for Impaired, Transitional, Recovered and Unimpaired were 877, 591, 525, 426, respectively (Table 2) and did not significantly differ among stream categories potentially due to the broad ranges in Impaired (665 – 1002 μS/cm^2) and Transitional (348 – 824 μS/cm^2) (Table 1). The mean iron concentrations for the Impaired, Transitional, Recovered and Unimpaired were 9.7, 0.1, 0.2, and 0.1 mg/L, respectively (Table 2). The iron concentrations were not significantly different, potentially due to the Impaired having a broad standard deviation (mean=9.7 s.d.=10.4 mg/L) (Table 1). There was very little variation in the concentrations of soluble reactive phosphorus within and among stream categories with the mean concentrations for the Impaired, Transitional, Recovered and Unimpaired being 0.02, 0.01, 0.01, and 0.02 μg/L, respectively (Table 2).

Two measures of biomass were analyzed in this study: chlorophyll *a*, a measure of the photosynthetic biomass and total fatty acid biomass, a measure of whole community (autotrophic and heterotrophic) biomass. The chlorophyll *a* concentrations for Transitional, Recovered, and Unimpaired stream categories had mean nutrient treatment ranges of 0.35-0.65, 0.27-0.47, and 0.17-0.28 mg/m^2 respectively (Table 3) and these stream categories did not significantly differ from each other (Figure 3, Table 4). The mean treatment chlorophyll *a* concentration for Impaired ranged from 0.05 to 0.09 mg/m^2 (Table 3). This stream category showed significantly lower ($p < 0.05$) chlorophyll *a* than the other categories (Figure 3). The reaction ratios showed significant differences among the nutrient treatments, with the +P and +NP treatments having a higher amount of chlorophyll *a* compared to the +N treatments ($p < 0.005$, Table 4), as well as having one to six-fold more chlorophyll *a* than the control treatments ($p < 0.002$, Figure 4). The fatty

acid biomass was not significantly different among the stream categories or among the nutrient treatments, with a range of 2.22 to 4.04 mg C/m^2 among the stream categories (Tables 3, 4, Figure 5). There were no significant differences in the reaction ratios (Table 4, Figure 6).

Although there were no significant differences in the fatty acid biomass among stream categories or nutrient treatments, there were significant differences observed in the types of fatty acids among the stream categories and nutrient treatments. The PUFA and HUFA percentages were combined, since HUFA is a subcategory of PUFA, and there was a significant stream category effect as well as significant treatment effect ($p < 0.007$, Table 4, Figure 7). Transitional, Recovered and Unimpaired (mean PUFA/HUFA 4.7% – 12.8%, 6.2% – 9.9%, and 2.7% – 10.8%, respectively) were not significantly different from each other. These three stream categories were significantly different from Impaired (mean PUFA/HUFA 0.6% – 2.9%) ($p < 0.007$, Table 3, Figure 7). A correlation analysis showed that there were no significant effects of stream temperature on the percent PUFA ($p = 0.35$), although there was a trend of increased percent PUFA with increasing temperature. There was a significant treatment effect (Table 4), in which the +NP treatments had significantly higher percentages of PUFA/HUFA compared to the control ($p < 0.002$) and the +N treatments ($p < 0.02$). However, there were no other significant differences among the treatments. The percentages of SAFA had both significant stream category effects as well as nutrient treatment effects (Table 4). There were higher percentages in Impaired, (mean nutrient treatment 84.3% – 90.7%), compared to Transitional, Recovered and Unimpaired with the mean nutrient treatment 56.2% – 74.3%, 64.2% – 77.3%, and 62.3% – 88.0%, respectively (Figure 7, Table 3, $p <$

0.002). There were no significant differences among Transitional, Recovered and Unimpaired categories. There was a nutrient effect in which the +P and +NP treatments had lower percentages of SAFA than the +N and control treatments ($p< 0.01$). The percentage of MUFA in the biofilm communities had both stream category as well as nutrient effects (Table 4). Transitional and Recovered streams (mean nutrient treatment 20.3% – 30.6% and 16.3% – 26.3 %, respectively) were significantly different from Impaired with mean nutrient treatment 7.0% – 12.7% ($p < 0.005$, Table 3, Figure 7). Transitional and Recovered were not significantly different from the Unimpaired nor was Unimpaired significantly different from Impaired. There were significantly higher percentages of MUFA in the +P and +NP treatments compared to the +N and control treatments ($p < 0.03$). The +P and +NP treatments were not significantly different from each other nor were the control and the +N treatments significantly different from each other.

Each enzyme activity analyzed showed a significant difference among the stream categories ($p < 0.015$, Table 4), but treatment effects were not discernable, with the exception of the DiP and GLU activities. DiP and GLU showed effects of stream category as well as nutrient treatment effects (Table 4). The +P and +NP treatments had significantly lower DiP activities compared to the control treatment ($p < 0.01$, Table 3). There was no significant difference between the +P, +NP or the +N treatments nor was the +N treatment significantly different from the control. The +NP treatments had significantly higher GLU activities compared to the control and +N treatments (Table 3). The +P and +NP treatments were not significantly different from each other nor were the +P, +N and control treatments significantly different from each other. Reaction ratios

were used to determine the nutrient effects of the biofilm community. The MonoP activity showed a significant decrease ($p = 0.02$) in the +P treatment compared to the +N treatment and was less than the control, although not significant (Figure 8). The DiP activity showed a one- to two-fold decrease in the +P and +NP treatments compared to the +N treatment ($p < 0.05$) and were significantly less than the control treatment ($p < 0.005$, Figure 9). The NAG activity showed no nutrient treatment effects (Table 4), but among the stream categories, Recovered had a significantly higher NAG activity reaction ratio compared to Impaired and Unimpaired ($p < 0.05$, Figure 10). The LAP activity showed similar patterns as the NAG activity. There was no nutrient treatment effect (Table 4), but Recovered had up to ten-fold higher LAP activity compared to the Unimpaired ($p = 0.04$, Figure 11). There was no nutrient treatment effect among the treatments for the XYLO activity, though there was a stream category effect, with Unimpaired having significantly less XYLO activity than Transitional ($p = 0.03$, Figure 12). This finding was the only significant difference among the categories. The GLU activity showed stream category effects, nutrient treatment effects, and an interaction effect between stream category and nutrient treatment (Table 4). Transitional had a higher GLU activity compared to the Impaired, Recovered and Unimpaired ($p < 0.02$, Figure 13). Impaired, Recovered and Unimpaired were not significantly different from each other. The +NP treatment had a higher GLU activity compared to the +N treatment ($p < 0.05$) and higher activity compared to the control ($p < 0.05$). GLU was the only enzyme that saw a stream category by nutrient affect. Transitional +NP was significantly higher than the Unimpaired and Recovered +NP treatments ($p < 0.035$).

Ecoenzymatic stoichiometry was used to examine the overall allocation of enzymes. The ecoenzyme analysis showed significant differences both in the length vector and angle vectors. Significant differences were present in the vector length with Impaired having greater lengths compared to Recovered and Unimpaired ($p < 0.01$, Table 4, Figure 14). Recovered had significantly shorter lengths than Transitional ($p < 0.016$), though the Unimpaired and Transitional were not significantly different from each other. Impaired was not significantly different from Transitional nor were Recovered and Unimpaired significantly different from each other. There were significant differences among the treatments, with the +P and +NP treatments having significantly greater lengths than the +N and control treatments ($p < 0.001$, Figure 14). The +P and +NP treatments were not significant from each other nor were the control and +N treatments significantly different from each other. The angle vectors did not have significant stream category effects, but did have significant nutrient treatment effects (Table 4). There were significant differences in the angle vector, with the +P treatment having significantly lower angle than the +N treatment ($p < 0.002$). The +NP treatment had significantly lower angle than the +N and control treatments ($p < 0.001$, Figure 15). The control was not significantly different from the +N or +P treatments nor was the +P and +NP treatments significantly different from each other.

Correlations and ANCOVAs were utilized to examine chlorophyll *a* biomass and fatty acid biomass with the stream environmental variables. Chlorophyll *a* biomass was negatively correlated ($p = 0.001$) with stream depth, iron concentration ($p = 0.01$) and soluble reactive phosphorus concentration ($p = 0.006$) and positively correlated with pH ($p = 0.01$). The ANCOVA supported the relationship between chlorophyll *a* biomass and

stream depth ($p = 0.02$) and pH ($p = 0.04$) as was seen in the correlations. However, neither of these variables were significant across stream categories and would not explain possible stream category differences. The fatty acid biomass reaction ratios also showed relationships with pH ($p = 0.03$) and stream width ($p = 0.04$).

DISCUSSION

The chlorophyll *a* biomass reaction ratios showed a significant increase in the +P and +NP treatments. The increase in those treatments confirms that the photosynthetic component of the biofilm is primarily limited by phosphorus and most likely secondarily limited by nitrogen (Tank & Dodds 2003, Tank *et al.* 2006). DeNicola & Lellock (2015) did a similar study examining nutrient limitation along the gradient of an AMD impacted stream. The AMD impacted stream in DeNicola & Lellock (2015) was treated using passive treatments and sulfate was used to determine the AMD impact gradient. DeNicola & Lellock (2015) used reaction ratios to ameliorate differences due to canopy cover and found significant increases in the +NP treatments compared to the control and the +P treatments were intermediary between the control and +NP, but not significantly different either one. They determined that AMD impacted streams were co-limited by phosphorus and nitrogen based on significant increase in the chlorophyll *a* biomass in the +NP treatments (DeNicola & Lellock 2015).

There were no significant differences in the fatty acid biomass among the stream categories or nutrient treatments. Cashman *et al.* (2013) examined light levels and nutrient additions on mature biofilm communities by placing mature biofilms (~30 days growth) in open and closed canopy portions of streams as well as adding nutrients in the form of plant food fertilizer. This study found that nutrient additions did not significantly affect the total fatty acid biomass. They believe the reason for the insignificant response in the biomass was due to the streams being primarily limited by light. Lowe *et al.* (1996) discovered that the total fatty acid biomass to be a less sensitive measure of biomass, in

which no significant changes were observed while other measures (e.g. chlorophyll *a* and cell density) showed significant changes in biomass. Although these other studies also showed total fatty acid biomass not differ among treatments, in this study it was expected that a difference in the Impaired compared to the other stream categories would be detected due to the assumption that Impaired streams would have lower biomass per unit area compared to the other stream categories (Drerup & Vis 2016b). Grazers, though not examined in this study, are a possible explanation for no change detected in the overall biomass. The lack of change may also be indicative of a change within microbial community.

Although there were no significant increases in the fatty acid biomass, there were potential important changes in the nutritional composition of the biofilm community, as seen in the fatty acids profiles. The percentages of fatty acids types varied depending on the nutrient treatment in the stream categories. In the +NP treatments, there was an increase in the percentage of PUFA and HUFA present in the biofilm. Cashman *et al.* (2013) also showed that nutrient additions increased the percentages of PUFA present. When examining lipid biomarkers across a water quality gradient, DeForest *et al.* (2016) discovered that essential fatty acids were only detected in the higher quality streams. PUFA are known to have a high nutritional value and are primarily produced by photosynthetic organisms (Brett & Muller-Navarra 1997, Torres-Ruiz *et al.* 2010). This nutritional value is due to the use of PUFAs for fluidity of cell membranes, as well as use in growth and reproduction of other aquatic organisms (Torres-Ruiz *et al.* 2007). Torres-Ruiz *et al.* (2010) found that net-spinning caddisflies are unable to produce essential fatty acids, even when given the precursor fatty acid, and rely on the algae they eat for

essential fatty acids. Brett *et al.* (2000) showed that the zooplankton community growth rates were negatively affected, when the biofilm community was phosphorus limited. With the increase in percentages of PUFA/HUFA in nutrient replete conditions, there would most likely be a positive effect for aquatic organisms within the remediated streams, such as macroinvertebrates that are dependent on the biofilm community.

The +P and +NP treatments had a decrease in the percentage of SAFA and an increase in the percentage of MUFA. The decrease in amount of SAFA could be explained due to the allocation of more fatty acids to PUFA/HUFA than SAFA. Both SAFA and MUFA are a lower nutritional quality to aquatic organisms than PUFA. SAFA and MUFA are used for storage, especially in nutrient limiting conditions (Napolitano 1994, Hill *et al.* 2011). Hill *et al.* (2011) showed that both SAFA and MUFA decreased with the addition of phosphorus, while in the current study only SAFA decreased with phosphorus addition.

The phosphorus acquiring enzyme activities measured in this study showed different patterns among nutrient treatments. The +P treatment had significantly less MonoP activity compared to the control; however, the effect was not significant in the +NP treatment. This effect of MonoP activity has been shown in studies of soils (Olander & Vitousek 2000). Olander & Vitousek (2000) had +N, +P and +NP treatments in soils and showed a significant decrease in MonoP activity in the +P treatments, but not the +NP treatments compared to the control. The DiP activity also decreased when phosphorus was added. In this study, there was a significant effect in both the +P and +NP treatments compared to the control. There was a stronger response in the DiP activity compared to the MonoP activity, suggesting that the DiP is more sensitive to

nutrient changes than MonoP. This greater sensitivity has been demonstrated in other studies that measured enzyme activities in soil (DeForest *et al.* 2011, Shaw & DeForest 2013). The decrease in phosphorus acquiring enzyme activities with the addition of phosphorus is a direct measure of phosphorus being a limiting nutrient. Although MonoP has shown to be less sensitive than DiP, MonoP did show trends of lowered activity in the +P and +NP treatments, alongside the significant decrease of activity in the +P and +NP treatments in DiP.

Four additional enzymes were measured, two nitrogen and two carbon acquiring. There were no significant differences in the nitrogen acquiring enzyme (NAG and LAP) activities with the addition of nutrients. In regards to NAG, this may be a factor of substrate availability rather than nutrient availability. NAG breaks down chitinase, which may not be abundant in these streams. XYLO and GLU were used to measure the carbon acquiring enzyme activities. There was no difference among the nutrient treatments for either and very little XYLO activity in general. This lack of XYLO activity is most likely due to XYLO's role in breaking down allochthonous sources of carbon, generally from terrestrial sources (Romani & Sabater 2000). With the season of the study being early to mid-summer, leaves are not an abundant carbon source in the stream. There was a higher GLU activity in the +NP treatments. GLU breaks down autochthonous sources of carbon, such as those from algae (Romani & Sabater 2000). The increase of GLU shows that there are more in-stream carbon sources available for the biofilm community, as seen with the increase of algal biomass (increase in chlorophyll *a*). This increase in carbon acquiring enzyme activity has been shown to occur when a limiting nutrient is added; the community then needs to acquire the next limiting resource. Zeglin *et al.* (2007) observed

an increase in GLU activity when nitrogen was added to nitrogen limited terrestrial systems.

Ecoenzymatic stoichiometry provided a means to examine the carbon to nitrogen to phosphorus acquiring enzyme activities since the need for each of these elements is interdependent. Many studies have used ecoenzymatic stoichiometry to assess nutrient limitation in both aquatic and terrestrial systems (Hill *et al.* 2012, Hill *et al.* 2014, Waring *et al.* 2014). Hill *et al.* (2012) sampled the biofilm and stream sediment in over 2100 streams and used ecoenzymatic stoichiometry to compare the microbial enzyme activities. They determined stream biofilms to be phosphorus limited, evident by high phosphatase enzyme activities, and stream sediments to be more carbon and nitrogen limited. Hill *et al.* (2014) showed fens to be phosphorus limited with the higher phosphorus-acquiring enzyme activities than nitrogen and carbon acquiring enzyme activities in comparison with upland soils. Comparison of ecoenzymatic stoichiometry of the biofilms with nutrient addition and without nutrient addition is very insightful for resource changes in the biofilm community. The +P and +NP had relatively longer lengths than the control and +N, revealing that the biofilms are more carbon limited when phosphorus is added. Zeglin *et al.* (2007) had a similar finding when studying the nitrogen and carbon acquiring enzyme activities in nitrogen limited soils. With the addition of nitrogen, the activities for carbon and phosphorus enzymes increased (Zeglin *et al.* 2007). In my study, the +NP had lesser angles than the control and +N treatments, indicating less phosphorus limitation. This result showed that with the addition of phosphorus, the biofilm did not invest as much energy into acquiring phosphorus. Considering the angle and length vectors together, the control and +N treatments were

more nutrient limited than carbon limited and were more phosphorus limited than nitrogen limited. The +P and +NP treatments are less phosphorus limited and are more carbon limited than nutrient limited. When phosphorus is added, the biofilms shifted from being phosphorus limited to carbon limited.

Stream category effects were observed in many of the characteristics measured. The Impaired stream category in comparison to the other categories always showed an effect, as was clear with the chlorophyll *a,* percent of fatty acids and all enzyme activities. This finding was expected with the chlorophyll *a* and has been observed in previous studies in AMD impaired streams (Smucker & Vis 2011). In my study, Transitional, Recovered and Unimpaired stream categories did not show many differences in the characteristics measured among them. An effect would have been expected among the Transitional, Recovered, and Unimpaired stream categories. Drerup & Vis (2016a) observed Unimpaired streams to have the highest chlorophyll *a* biomass, AMD impaired streams with the lowest and AMD remediated streams not significantly different from either. This pattern was not observed between the Unimpaired and AMD remediated streams, potentially due to grazers, which were not taken into account in this study. There also may have been differences among these stream categories, but the effects may not have been as drastic as resulting in non-significance of the differences. There may be other factors affecting recovery as well, such as spate frequency.

AMD remediated streams, both Transitional and Recovered, as well as the AMD Impaired and Unimpaired streams showed an increase in chlorophyll *a* biomass with the addition of phosphorus. None of the stream categories showed an increase in the fatty acid biomass with the nutrient treatments; however, there were profound changes in the

fatty acid composition. The PUFA percentage increased in Transitional, Recovered, and Unimpaired sites and SAFA percentage decreased in the +NP treatments with these biofilms having a better nutritional quality for organisms, such as macroinvertebrates. With the addition of phosphorus, the phosphorus acquiring enzyme activities decreased, but there was no change in nitrogen acquiring enzyme activities with nutrient addition. Since there was no biomass increase or a response of the enzyme activities in the +N treatments, it would appear that the AMD remediated streams are not primarily limited by nitrogen. When phosphorus was added, there was an increase in carbon acquiring enzyme activities suggesting that the overall biofilm community shifted towards carbon limitation rather than phosphorus limitation, which is clearly evident from the ecoenzymatic stoichiometry results. All factors measured point to AMD remediated streams, including Transitional and Recovered, as well as the AMD Impaired streams being limited by phosphorus. However, the Unimpaired streams showed the same trends as the AMD remediated streams. The evidence, seen with the chlorophyll *a* biomass, enzyme activities and fatty acid composition show that the addition of phosphorus has a positive impact on the biofilm communities. Without the increased fatty acid biomass, it cannot conclusively be said that these streams are phosphorus limited. However, the biofilms may have a different composition, which may be important for nutritional quality and other organisms in the food web.

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Table 1: Mean physical and chemical characteristics measured at each stream site. Significant differences were determined using Bonferroni post hoc Test ($p<0.05$) indicated by superscript letters. Measurements with the same letter are not significant different. BDL= below detection limit, SRP=soluble reactive phosphorus.

Status	Watershed	ID	Drainage Area (km^2)	pH	Conductivity ($\mu S/cm^2$)	Temperature (ºC)	Canopy Cover (% open)	Wet Width (m)	Depth (cm)	Current Velocity (m/s)	Iron (mg/L)	Sulfate (mg/L)	Nitrate (mg/L)	SRP (µg/L)
Impaired	SC	I-SC01	27	7.1	965	18^{a}	1.82	6.5	17.7	0.23	6.73	247^{a}	0.13	0.03
Impaired	SC	I-SC02	23	6.5	1002	16^{a}	85.28	6.3	8	0.37	21.22	280^{a}	0.01	0.03
Impaired	MC	I-MC01	64	5.8	665	19^{a}	8.58	9.1	14.5	0.29	1.08	180^{a}	0.10	0.01
Transitional	HF	T-HF01	52	7.1	348	20^{ab}	10.92	5.7	31.5	0.36	0.15	110^{ab}	0.04	0.01
Transitional	HF	T-HF02	43	6.9	383	21^{ab}	32.5	4.5	20.5	0.38	0.13	100^{ab}	0.05	0.01
Transitional	SC	T-SC01	27	7.6	824	19^{ab}	5.46	6.0	13.7	0.18	0.10	267^{ab}	0.08	0.01
Transitional	SC	T-SC02	23	7.7	808	19^{ab}	17.16	3.4	14.3	0.44	0.03	157^{ab}	0.09	0.02
Recovered	HF	R-HF01	72	7.4	299	20^{b}	2.86	5.8	24.2	0.63	0.27	50^{ab}	0.06	0.02
Recovered	MC	R-MC01	195	6.6	533	22^{b}	18.46	10.2	18.0	0.34	0.09	98^{ab}	0.09	0.01
Recovered	SC	R-SC01	58	7.6	684	21^{b}	47.84	6.1	13.0	0.22	0.11	175^{ab}	0.05	0.01
Recovered	SC	R-SC02	89	7.6	584	21^{b}	21.06	8.1	8.0	0.11	0.16	100^{ab}	0.04	0.02
Unimpaired	HF	U-HF01	21	7.2	314	21^{b}	8.84	5.7	11.8	0.37	0.14	95^{b}	0.04	0.02
Unimpaired	MC	U-MC01	62	6.9	562	21^{b}	8.32	5.5	15.7	0.48	0.17	85^{b}	0.09	0.02
Unimpaired	FC	U-FC01	34	8.1	403	19^{b}	3.12	3.6	8.0	0.29	0.02	18^{b}	BDL	0.03

Table 2: Mean (standard deviation) physical and chemical characteristics for each stream category. Significant differences were determined using Bonferroni post hoc Test ($p<0.05$) indicated by superscript letters. Measurements with the same letter are not significant different. SRP=soluble reactive phosphorus.

Variable	AMD Impaired	Transitional	Recovered	Unimpaired
pH	6.2 (1.1)	7.3 (0.4)	7.3 (0.5)	7.4 (0.6)
Conductivity ($\mu S/cm^2$)	877 (185)	591 (261)	525 (163)	426 (126)
Temperature (ºC)	17 (1.7)^{a}	20 (0.7)^{ab}	21 (0.5)^{b}	20 (1.3)^{b}
Canopy Cover (% Open)	32 (46.4)	17 (11.7)	23 (18.7)	7 (3.2)
Wet Width (m)	7.3 (1.6)	4.9 (1.2)	7.5 (2.1)	4.9 (1.1)
Depth (cm)	13.4 (4.9)	20.0 (8.3)	15.8 (6.9)	11.8 (3.8)
Current Velocity (m/s)	0.30 (0.07)	0.34 (0.11)	0.32 (0.22)	0.38 (0.10)
Iron (mg/L)	9.7 (10.4)	0.1 (0.05)	0.2 (0.08)	0.1 (0.08)
Sulfate (mg/L)	236 (51)^{a}	158 (76)^{ab}	106 (52)^{ab}	66 (42)^{b}
Nitrate (mg/L)	0.08 (0.06)	0.06 (0.03)	0.06 (0.02)	0.04 (0.04)
SRP (µg/L)	0.02 (0.01)	0.01 (0.001)	0.01 (0.002)	0.02 (0.005)

Table 3: Mean (standard error) biofilm characteristics for the nutrient treatments for each stream category. C= control, +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched. MonoP = Phosphomonoesterase, DiP = Phosphodiesterase, NAG = chitinase, LAP = Leucine Aminopeptidase, GLU = β-glucosidase, XYLO = β-xylosidase, PUFA = polyunsaturated fatty acid, HUFA = highly unsaturated fatty acid, MUFA monounsaturated fatty acid, SAFA = saturated fatty acid.

Characteristic	Impaired				Transitional				Recovered				Unimpaired			
	C	+N	+P	+NP	C	+N	+P	+NP	C	+N	+P	+NP	C	+N	+P	+NP
Chlorophyll a (mg/m^2)	0.05 (0.02)	0.06 (0.01)	0.07 (0.01)	0.09 (0.01)	0.38 (0.08)	0.35 (0.07)	0.63 (0.11)	0.49 (0.09)	0.31 (0.08)	0.27 (0.07)	0.36 (0.08)	0.47 (0.13)	0.17 (0.03)	0.18 (0.04)	0.26 (0.04)	0.28 (0.06)
Fatty Acid Biomass (mg C)	3.05 (0.76)	3.18 (1.60)	2.52 (0.52)	2.59 (0.60)	2.49 (0.35)	3.08 (0.57)	2.22 (0.83)	4.09 (0.80)	3.50 (0.93)	3.96 (1.04)	3.35 (0.82)	4.04 (0.71)	3.77 (1.54)	2.56 (1.49)	3.27 (0.95)	2.83 (1.21)
MonoP (nm/hr/ mg chl a)	16931.9 (9391.1)	16597.4 (9289.1)	4730.7 (1576.7)	4048.4 (1099.1)	1215.9 (255.2)	1887.1 (308.0)	486.0 (132.3)	828.0 (132.2)	2689.2 (1143.9)	3156.9 (703.3)	1441.1 (497.2)	1264.3 (339.3)	1120.8 (265.2)	3514.7 (1743.8)	809.2 227.3)	759.2 (200.2)
DiP (nm/hr/ mg chl a)	2975.6 (1471.6)	1802.6 (697.1)	137.2 (27.7)	138.6 (32.2)	229.9 (36.6)	333.8 (60.9)	22.3 (4.4)	26.9 (2.5)	278.0 (102.2)	377.8 (165.4)	58.4 (18.9)	46.1 (10.9)	372.3 (151.2)	418.9 (155.6)	46.5 (8.4)	38.9 (7.3)
NAG (nm/hr/ mg chl a)	507.4 (118.9)	385.4 (99.1)	349.2 (37.3)	459.7 (73.5)	39.8 (15.7)	57.4 (18.9)	52.6 (14.9)	85.8 (24.7)	118.9 (75.7)	125.1 (49.9)	128.2 (51.9)	194.8 (66.6)	60.3 (11.9)	72.8 (25.8)	75.9 (15.7)	56.4 (12.7)
LAP (nm/hr/ mg chl a)	1189.8 (344.3)	2297.2 (904.0)	1233.6 (339.7)	2727.3 (939.2)	911.5 (384.2)	972.2 (294.4)	521.4 (128.6)	944.4 (180.9)	2018.9 (1218.4)	1312.7 (510.9)	2222.3 (1009.0)	2077.4 (618.4)	1122.7 (329.6)	1086.8 (440.7)	699.9 (132.9)	711.1 (117.1)
GLU (nm/hr/ mg chl a)	851.3 (99.2)	1112.8 (184.0)	1389.6 (452.9)	1695.5 (436.7)	182.0 (111.7)	167.1 (56.6)	310.8 (52.3)	568.9 (62.7)	361.9 (274.2)	222.9 (76.9)	346.1 (131.1)	455.9 (124.7)	137.5 (22.9)	194.6 (64.6)	213.1 (26.1)	283.5 (117.2)
XYLO (nm/hr/ mg chl a)	39.9 (7.3)	41.1 (10.6)	52.1 (11.9)	65.0 (16.3)	10.9 (4.5)	6.5 (1.7)	10.3 (1.9)	20.7 (4.5)	22.4 (13.1)	12.2 (4.8)	19.0 (8.1)	24.5 (7.9)	9.6 (2.3)	15.6 (9.7)	10.5 (2.5)	10.8 (2.3)

Table 3: continued

Percent PUFA + HUFA	1.6 (0.2)	2.3 (0.7)	0.6 (0.3)	2.9 (0.7)	4.7 (1.7)	6.4 (2.5)	9.3 (1.7)	12.8 (1.1)	6.4 (2.8)	6.2 (2.1)	8.0 (1.8)	9.9 (1.2)	2.7 (0.8)	5.3 (1.3)	9.0 (3.6)	10.8 (3.4)
Percent MUFA	8.9 (2.3)	7.0 (1.0)	10.8 (2.0)	12.7 (2.6)	20.3 (3.4)	24.5 (5.4)	34.2 (6.1)	30.6 (2.5)	16.3 (5.8)	19.1 (6.0)	26.3 (0.9)	25.5 (1.2)	9.1 (3.3)	15.3 (4.9)	27.9 (7.4)	26.9 (5.5)
Percent SAFA	89.2 (2.6)	90.7 (0.5)	88.6 (1.9)	84.3 (3.7)	74.3 (4.9)	68.9 (7.4)	56.3 (7.8)	56.2 (3.0)	77.3 (8.5)	74.8 (8.1)	65.8 (2.2)	64.2 (1.8)	88.0 (3.3)	79.1 (5.8)	62.5 (11.3)	62.3 (8.6)

Table 4: Analysis of Variance (ANOVA) for biofilm characteristics among stream categories and nutrient treatments for the corrected data (First column) and the reaction ratios (second column). d.f. = degrees of freedom, MS = mean squares, *F* = F value, *P* = p-value. MonoP = Phosphomonoesterase, DiP = Phosphodiesterase, NAG = chitinase, LAP = Leucine Aminopeptidase, GLU = β-glucosidase, XYLO = β-xylosidase, PUFA = polyunsaturated fatty acid, HUFA = highly unsaturated fatty acid, MUFA monounsaturated fatty acid, SAFA = saturated fatty acid.

	Corrected Data				Reaction Ratio			
Characteristic	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Chlorophyll a								
Category	3	1.0627	19.14	$2.18e^{-10}$	3	3.621	3.66	0.015
Treatment	3	0.1746	3.15	0.0273	2	7.830	7.91	$6.49e^{-04}$
Category x Treatment	9	0.0359	0.65	0.7552	6	0.763	0.77	0.595
Fatty Acid Biomass								
Category	3	121.2	40.41	0.571	3	0.6586	1.71	0.187
Treatment	3	177.8	59.27	0.405	2	0.7657	1.98	0.155
Category x Treatment	9	406.6	45.18	0.654	6	0.3302	0.86	0.538
% PUFA + HUFA								
Category	3	111.48	8.26	$2.15e^{-04}$	-	-	-	-

Table 4: continued								
Treatment	3	74.76	5.54	0.00283	-	-	-	-
Category x Treatment	9	9.86	0.73	0.679	-	-	-	-
% MUFA								
Category	3	718.2	10.71	$2.67e^{-05}$	-	-	-	-
Treatment	3	425.0	6.34	0.00128	-	-	-	-
Category x Treatment	9	31.0	0.46	0.89055	-	-	-	-
% SAFA								
Category	3	1399.7	10.88	$2.33e^{-05}$	-	-	-	-
Treatment	3	810.8	6.30	0.00133	-	-	-	-
Category x Treatment	9	67.9	0.53	0.84564	-	-	-	-
MonoP (chlorophyll a)								
Category	3	$6.04e^{+08}$	9.92	$6.19e^{-06}$	3	9.44	0.87	0.4579
Treatment	3	$1.52e^{+08}$	2.49	0.0632	2	45.76	4.23	0.0174
Category x Treatment	9	$8.11e^{+07}$	1.33	0.227	6	12.48	1.15	0.3374
DiP (chlorophyll a)								
Category	3	$1.02e^{+07}$	8.54	$3.18e^{-05}$	3	0.240	0.61	0.609
Treatment	3	$6.22e^{+06}$	5.23	0.00192	2	21.125	53.98	$<2e^{-16}$
Category x Treatment	9	$3.18e^{+06}$	2.68	0.00690	6	0.254	0.65	0.691
NAG (chlorophyll a)								
Category	3	$9.42e^{+05}$	36.24	$<2e^{-16}$	3	22.756	4.17	0.00807
Treatment	3	$1.68e^{+04}$	0.65	0.588	2	8.733	1.60	0.207
Category x Treatment	9	$1.29e^{+04}$	0.50	0.876	6	0.800	0.15	0.989
LAP (chlorophyll a)								
Category	3	$1.29e^{+07}$	3.58	0.0158	3	18.512	2.96	0.0361
Treatment	3	$1.13e^{+06}$	0.31	0.8155	2	12.078	1.93	0.151
Category x Treatment	9	$2.10e^{+06}$	0.58	0.8109	6	7.359	1.18	0.325
GLU (chlorophyll a)								
Category	3	$7.98e^{+06}$	26.31	$2e^{-13}$	3	161.43	8.67	$3.64e^{-05}$
Treatment	3	$9.58e^{+05}$	3.16	0.0269	2	180.05	9.68	$1.45e^{-04}$

Table 4: continued								
Category x Treatment	9	$1.94e^{+05}$	0.64	0.7627	6	43.99	2.36	0.0355
XYLO (chlorophyll a)								
Category	3	9778	18.68	$6.77e^{-10}$	3	37.21	3.30	0.0262
Treatment	3	873	1.67	0.178	2	33.00	2.92	0.0613
Category x Treatment	9	245	0.47	0.893	6	6.39	0.57	0.7557
Ecoenzyme Length								
Category	3	0.3217	14.15	$8.64e^{-08}$	-	-	-	-
Treatment	3	0.6671	29.34	$8.56e^{-14}$	-	-	-	-
Category x Treatment	9	0.0560	2.46	0.0138	-	-	-	-
Ecoenzyme Angle								
Category	3	14.2	0.81	0.493	-	-	-	-
Treatment	3	473.7	26.92	$6.19e^{-13}$	-	-	-	-
Category x Treatment	9	29.4	1.67	0.106	-	-	-	-

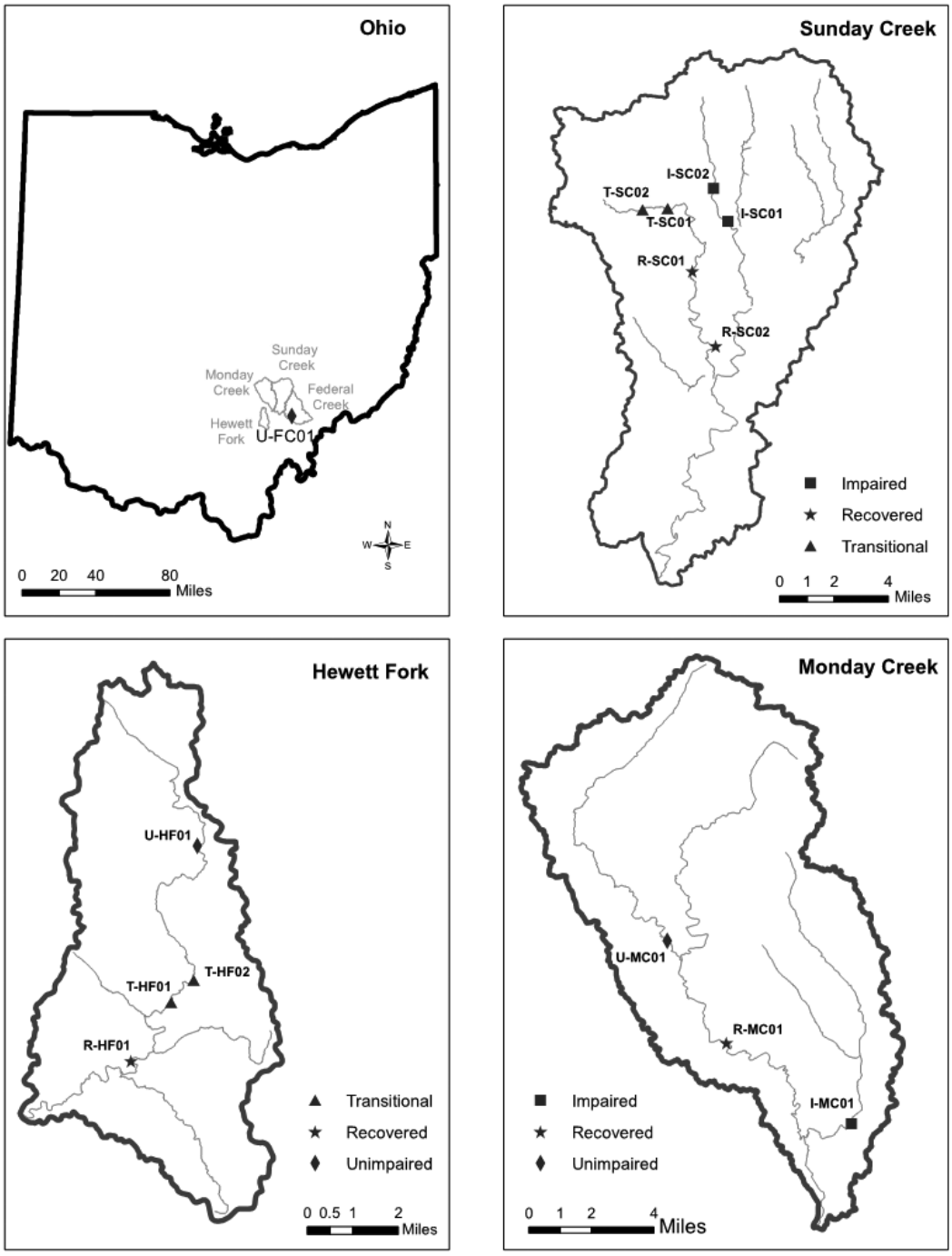

Figure 1: (Top Left) Map of Ohio with the watersheds in which samples were collected outlined.

(Top Right) Sample locations within Sunday Creek. (Bottom Left) Sample Locations within Hewett Fork. (Bottom Right) Sample locations within Monday Creek. The sites are categorized as follows: square = Impaired, triangle = Transitional, star = Recovered, diamond = Unimpaired.

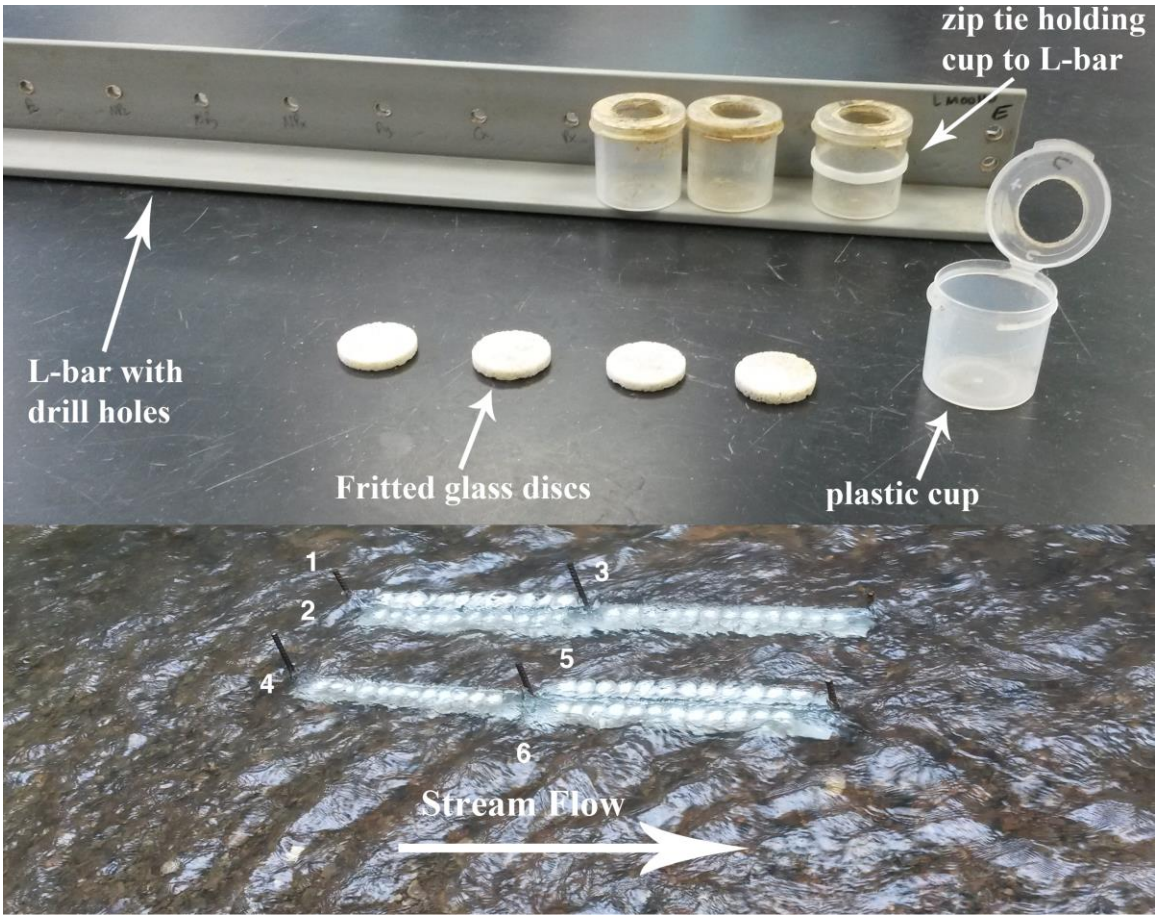

Figure 2: (Top) Components of nutrient diffusing substrates (NDS).

(Bottom) NDS on L-bars within stream. Each white number represents one L-bar in the stream and is half a stream replicate.

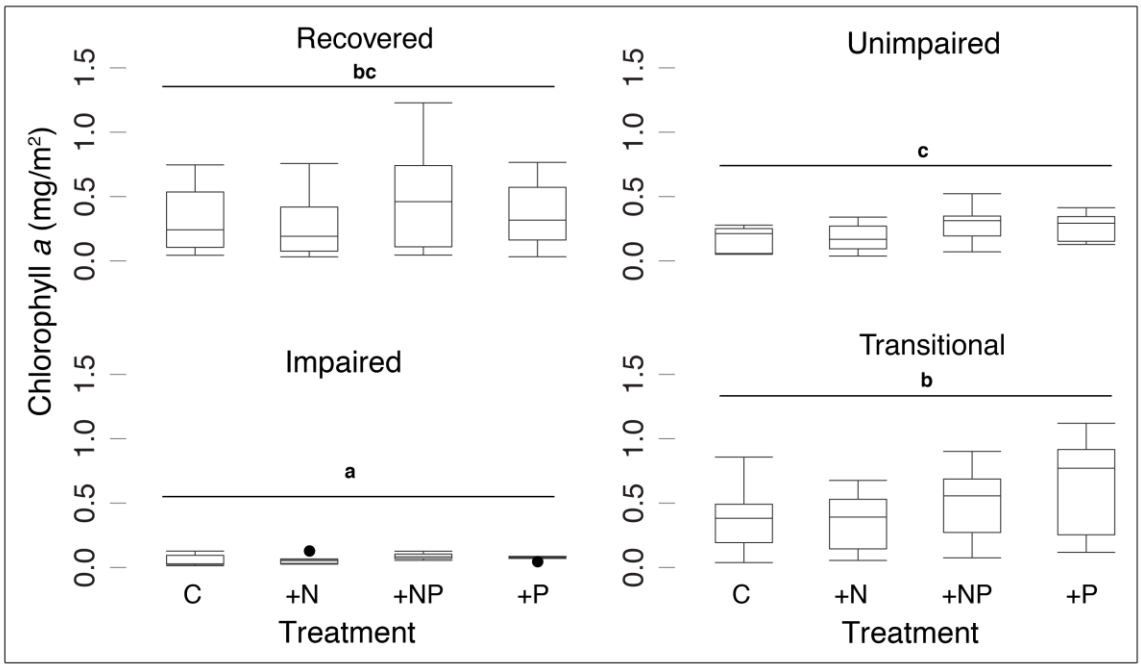

Figure 3: Chlorophyll *a* (mg/m^2) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. C = control, +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

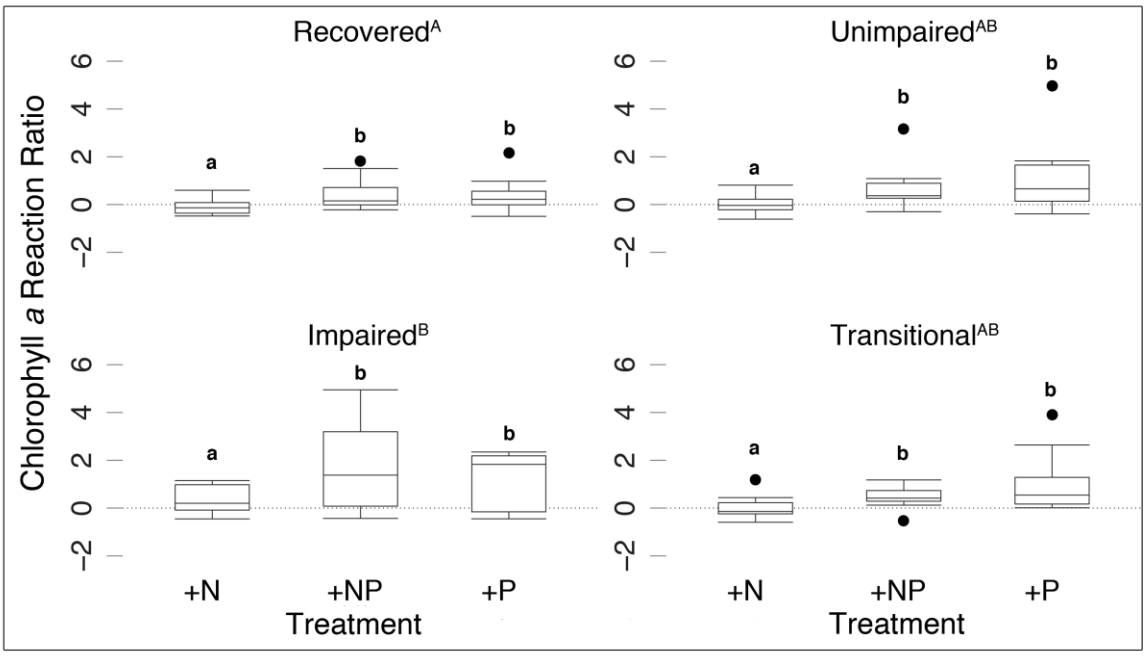

Figure 4: Chlorophyll *a* reaction ratio (treatment biomass-control biomass/control biomass) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

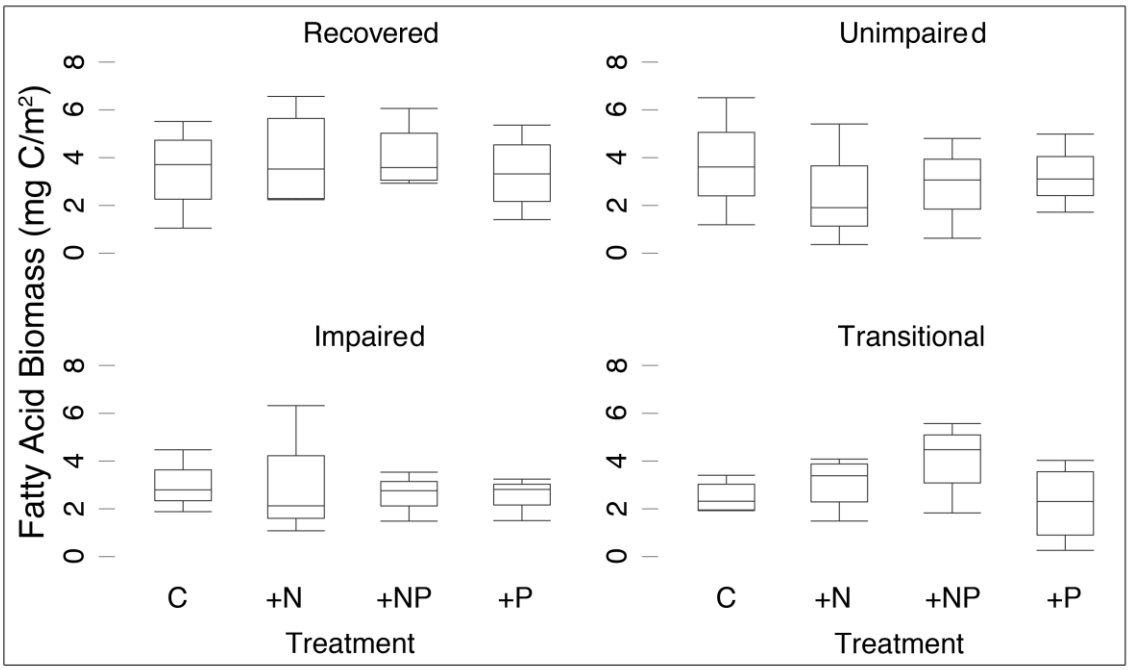

Figure 5: Fatty Acid biomass (mg/m^2) grouped by stream Category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. There were no significant differences. C = control, +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

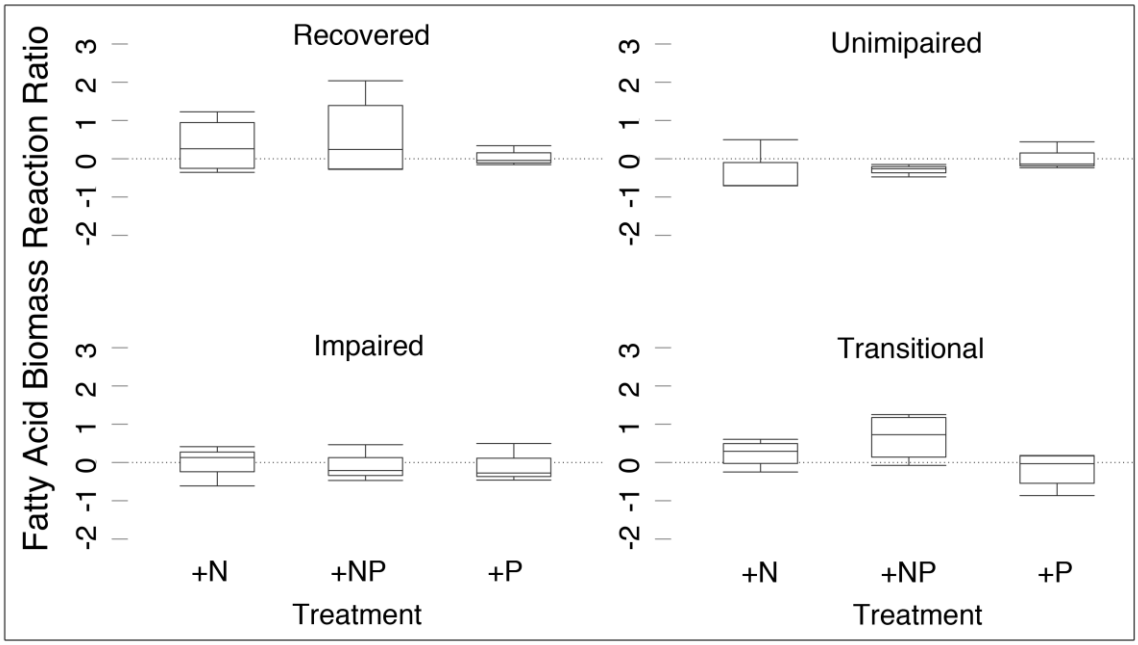

Figure 6: Fatty Acid biomass reaction ratio (treatment biomass-control biomass/control biomass) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by open circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. There were no significant differences. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

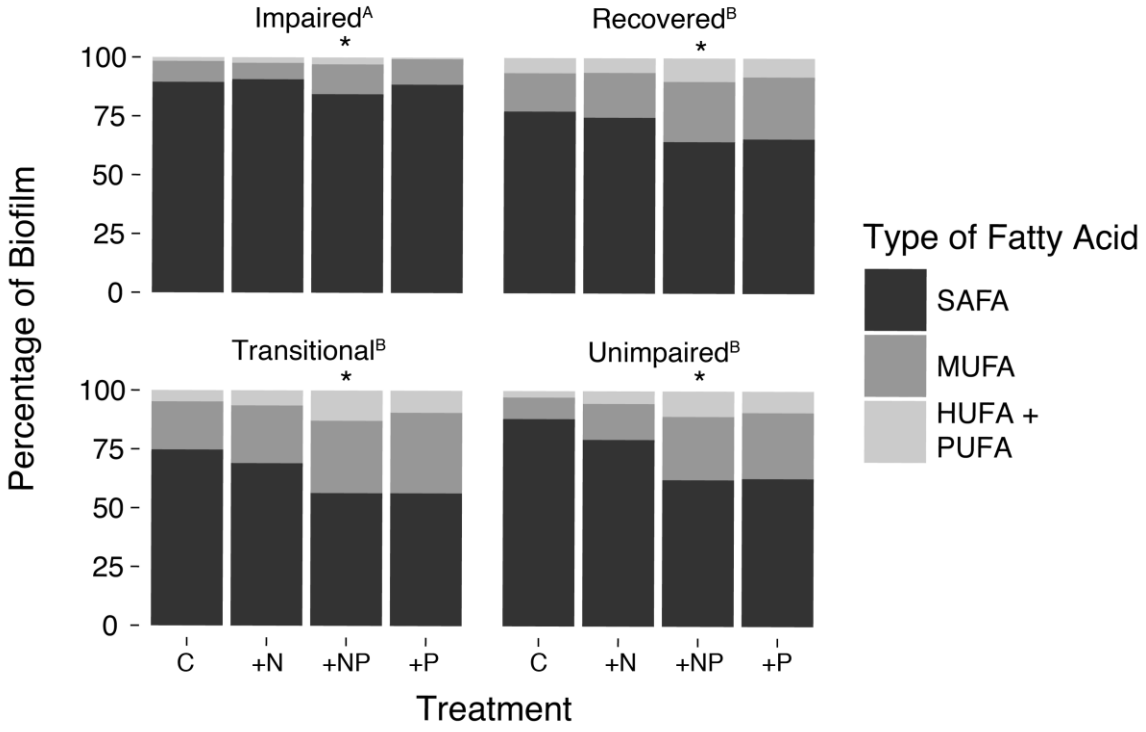

Figure 7: Percentage of fatty acid categories grouped by stream category. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Bars that share a letter are not significantly different from each other. The asterisk (*) indicates significant treatment effect, upper case letters indicate stream category effect. SAFA = Saturated Fatty Acids, MUFA = Monounsaturated Fatty Acids, PUFA = Polyunsaturated Fatty Acids, HUFA = Highly Unsaturated Fatty Acids. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

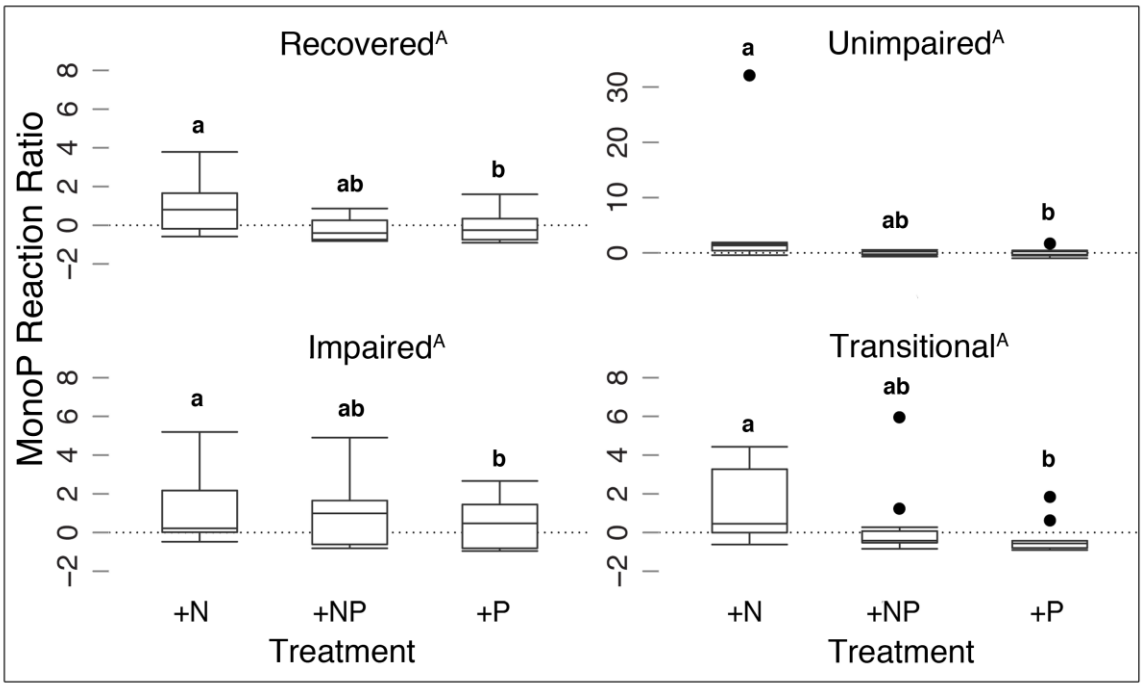

Figure 8: Phosphomonoesterase (MonoP) activity reaction ratio (treatment activity-control activity/control activity) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

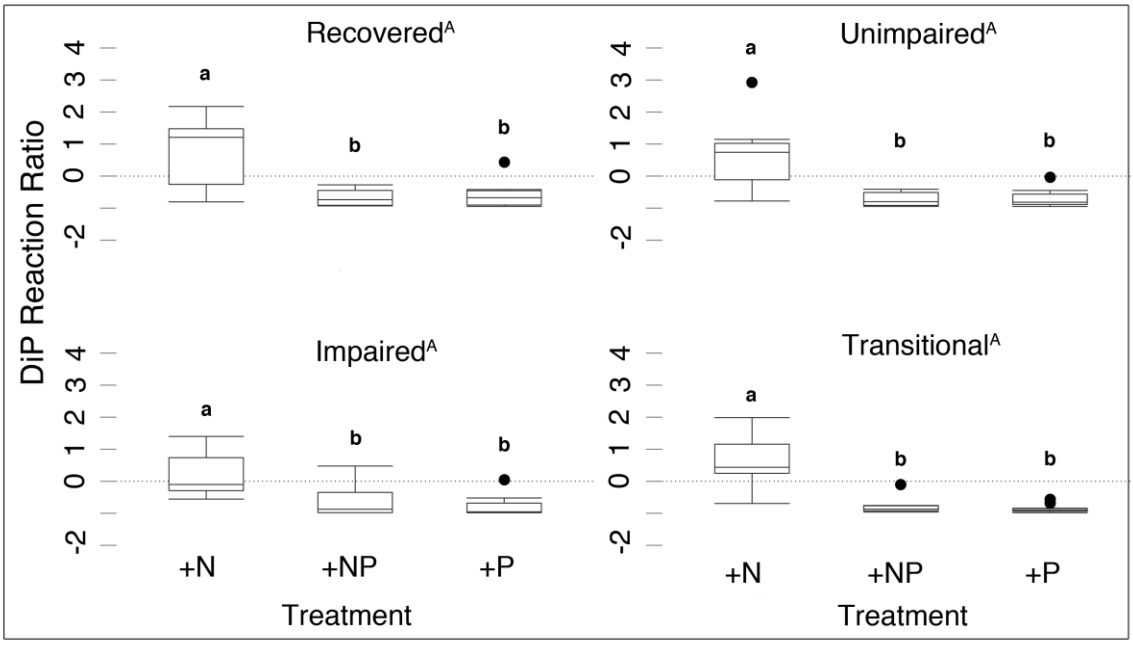

Figure 9: Phosphodiesterase (DiP) activity reaction ratio (treatment activity-control activity/control activity) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by open circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

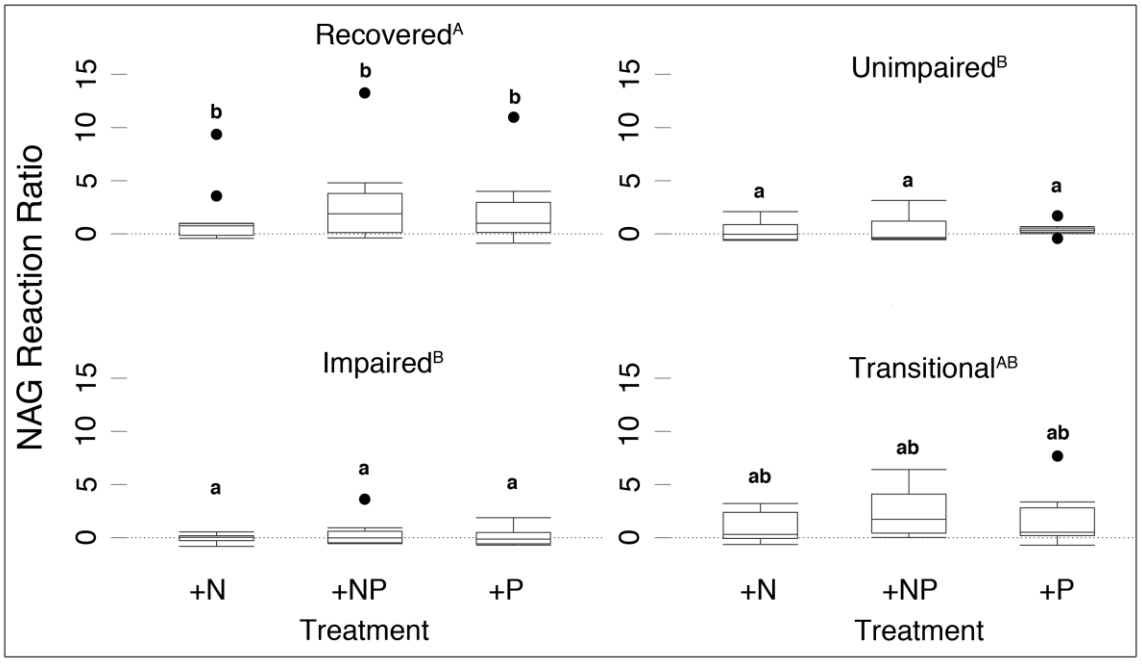

Figure 10: Chitinase (NAG) activity reaction ratio (treatment activity-control activity/control activity) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

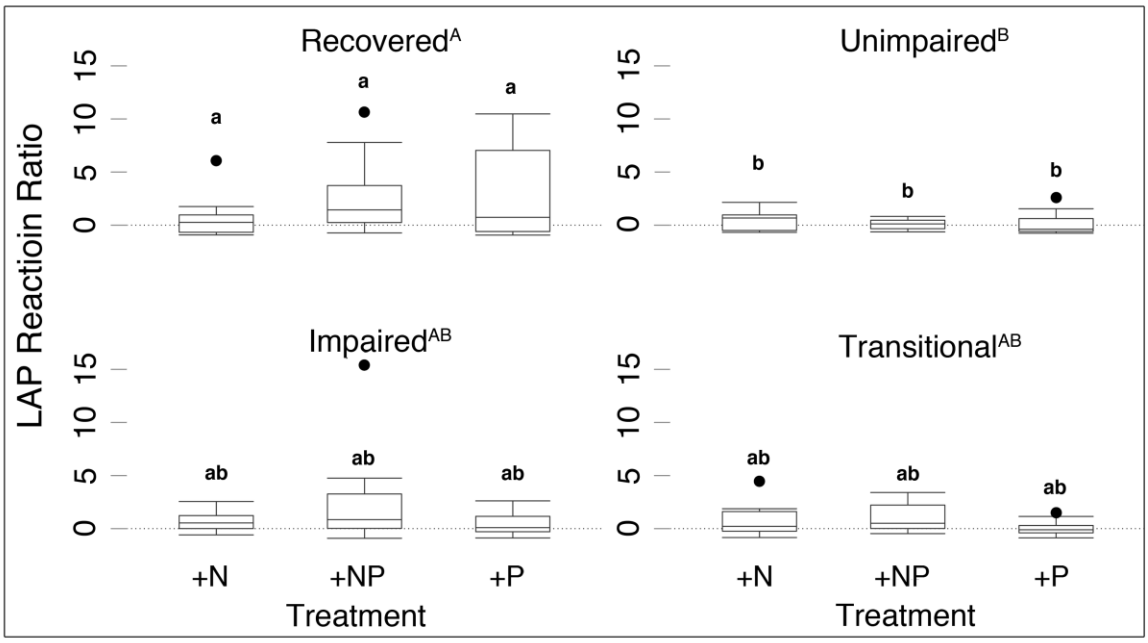

Figure 11: Leucine Aminopeptidase (LAP) activity reaction ratio (treatment activity-control activity/control activity) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

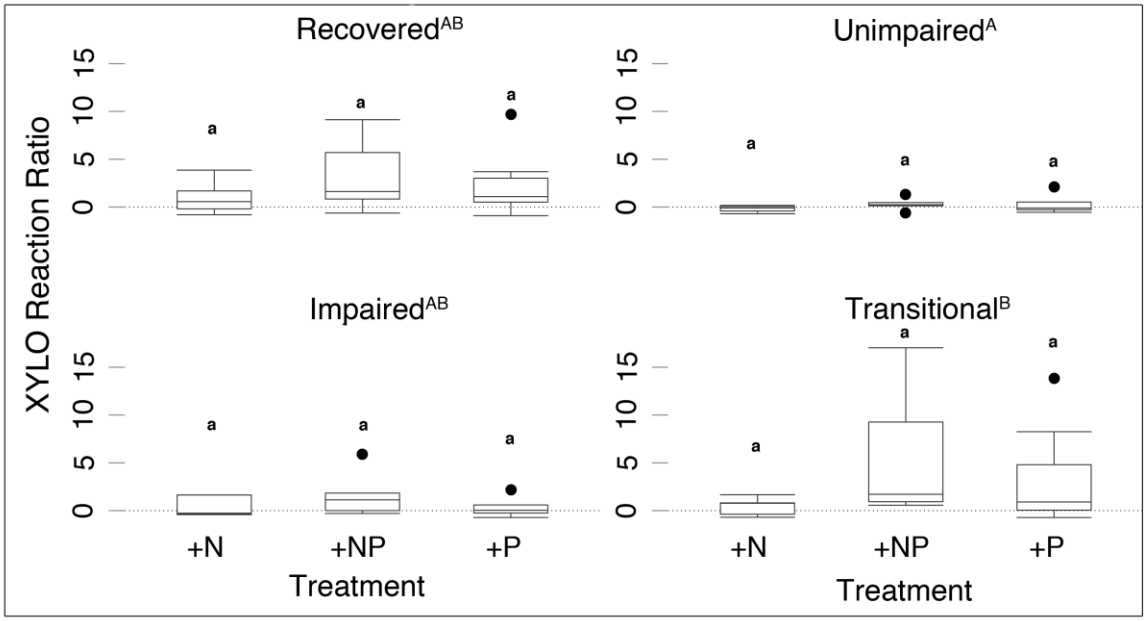

Figure 12: β-Xylosidase (XYLO) activity reaction ratio (treatment activity-control activity/control activity) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

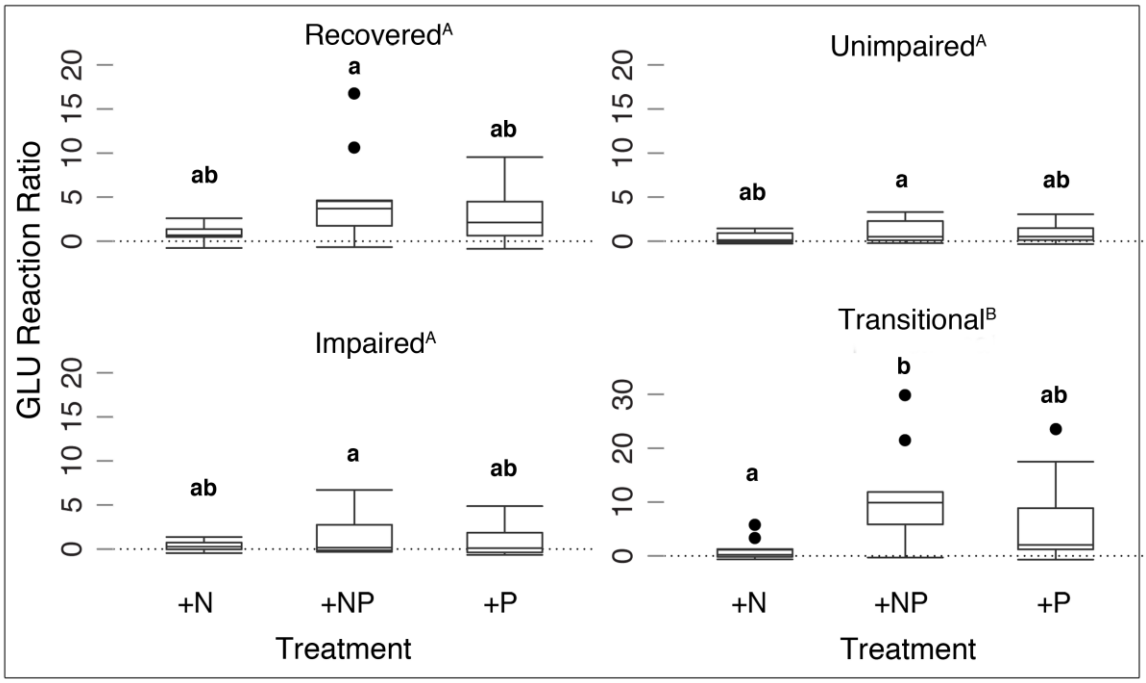

Figure 13: β-Glucosidase (GLU) activity reaction ratio (treatment activity-control activity/control activity) grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. The black dotted line represents the control. +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

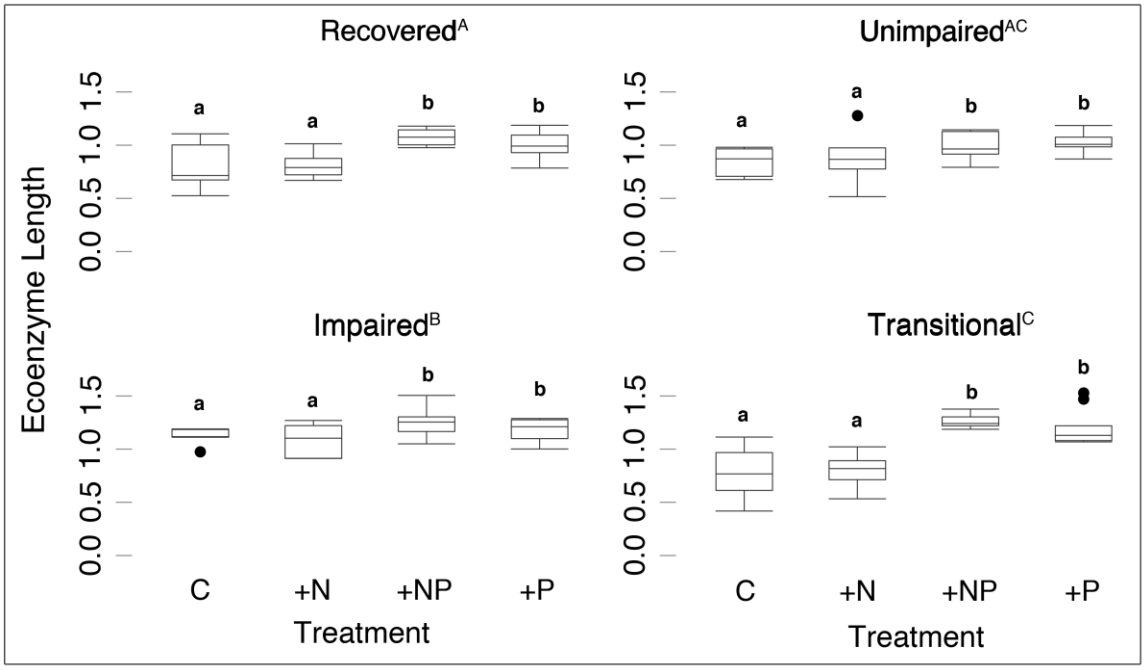

Figure 14: Vector lengths determined by the ratios of the natural log transformed carbon to phosphorus acquiring enzymes activities and the carbon to nitrogen acquiring enzymes grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. C = control, +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

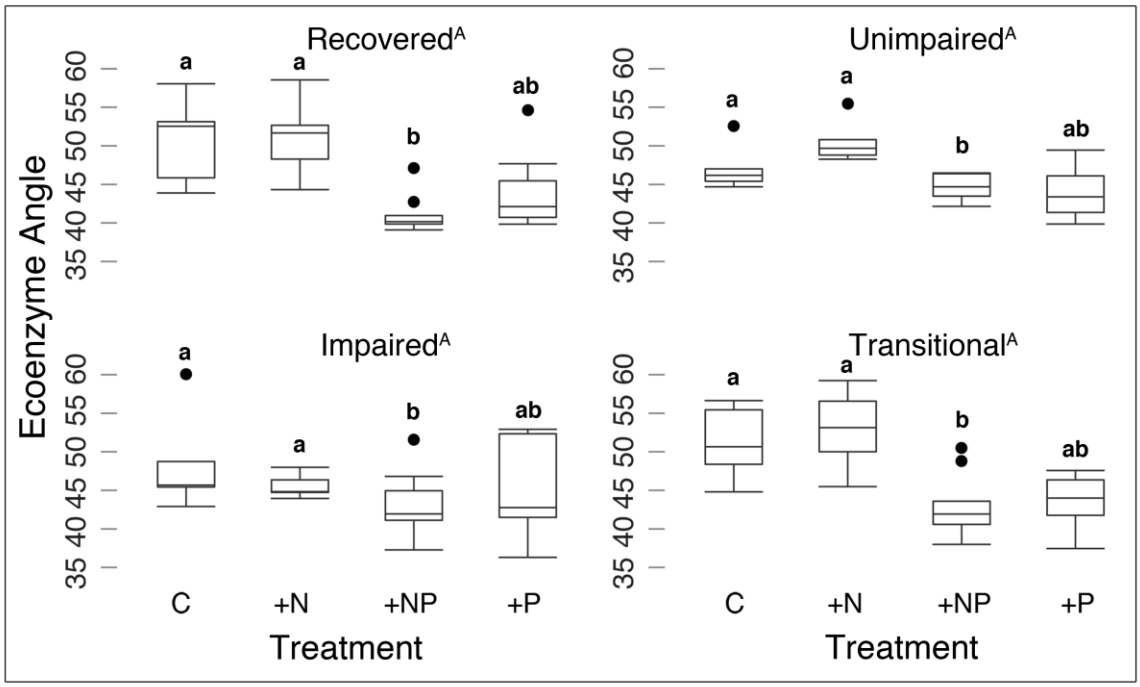

Figure 15: Vector angle determined by the ratios of the natural log transformed carbon to phosphorus acquiring enzymes activities and the carbon to nitrogen acquiring enzymes grouped by stream category.

The line represents the median value with the box the lower (25%) and upper (75%) quartiles. Minimum and maximum values indicated by whiskers and outliers indicated by open circles. Significant differences ($P<0.05$) were determined by a Tukey's post hoc test. Boxes that share a letter are not significantly different from each other. Lower case letters indicate nutrient treatment effect; upper case letters indicate stream category effect. C = control, +N = nitrogen enriched, +P = phosphorus enriched, +NP = nitrogen and phosphorus enriched.

APPENDIX A: PHYSICAL AND CHEMICAL CHARACTERISTICS MEASURED AT EACH SITE.

Measurements taken at the beginning, middle and end of study. BDL= below detection limit. * = MAIS score from 2014 rather than 2015.

Status	Watershed	ID	MAIS	Drainage Area (km^2)	pH	Conductivity ($\mu S/cm^2$)	Temperature (^oC)	Canopy Cover (% open)	Wet Width (m)	Depth (cm)	Current Velocity (m/s)
Impaired	Sunday (SC076)	I-SC01	10	27	7.2 6.8 7.3	966 860 1069	15 20 16	2	6.3 - 6.7	15 23 15	0.24 0.34 0.12
Impaired	Sunday (SC080)	I-SC02	9	23	6.3 6.7 6.5	1050 828 1128	14 20 14	85	6.1 - 6.5	9 10 5	0.43 0.29 0.39
Impaired	Monday (SF00290)	I-MC01	TBA	64	4.1 5.0 5.8	765 651 578	19 18 20	9	9 - 9.2	11.5 15 17	0.21 0.47 0.20
Transitional	Raccoon (HF075)	T-HF01	13	52	7.2 7.2 7.0	396 369 278	21 20 21	11	5.35 - 6.1	29.5 25 40	0.32 0.35 0.41
Transitional	Raccoon (HF090)	T-HF02	11	43	7.0 6.9 6.9	412 417 320	20 20 21	33	4.2 - 4.7	15.5 19 27	0.68 0.16 0.29
Transitional	Sunday (WB003)	T-SC01	6	27	7.6 7.3 7.8	903 713 855	19 20 19	5	5.8 - 6.1	7 17 17	0.27 0.12 0.14
Transitional	Sunday (WB51)	T-SC02	9*	23	7.8 7.5 7.8	910 638 877	19 20 19	17	2.7 - 4.1	10 21 12	0.50 0.57 0.26

Recovered	Raccoon (HF045)	R-HF01	14	72	7.4 7.3 7.5	362 288 248	21 20 21	3	5.65 - 5.9	16.5 25 31	0.68 0.65 0.55
Recovered	Monday (MC00300)	R-MC01	18	195	6.4 6.6 6.8	507 560 532	21 21 23	18	11.3 - 9.1	13 20 21	0.44 0.37 0.21
Recovered	Sunday (WB002)	R-SC01	12	58	7.3 7.5 7.9	742 631 678	21 21 21	48	6.1 - 6.1	8 20 11	0.29 0.22 0.15
Recovered	Sunday (WBSCRM1.8)	R-SC02	18*	89	7.3 7.5 8.0	478 631 644	19 21 22	21	9.1 - 7.1	9 8 7	0.16 0.12 BDL
Unimpaired	Raccoon (HF137)	U-HF01	15	21	7.3 6.8 7.4	323 318 302	21 20 21	9	5.5 - 5.8	15.5 8 12	0.59 0.21 0.31
Unimpaired	Monday (LM00110)	U-MC01	TBA	62	6.5 6.7 7.6	580 569 538	21 21 22	8	5.3 - 5.7	14 18 15	0.54 0.44 0.47
Unimpaired	Federal (MS1)	U-FC01	TBA	34	8.2 - 8.0	380 - 425	18 - 21	3	- - 3.6	11 - 5	0.49 - BDL

APPENDIX B: MEAN CONCENTRATION (STANDARD DEVIATION) OF SOLUBLE REACTIVE PHOSPHORUS (SRP) REMAINING IN AGAR SOLUTION AFTER THREE-WEEK PILOT STUDY.

Status	Treatment	SRP (µg/L)
Transitional	+P 0.05M	289.8 (41.1)
Transitional	+P 0.1M	402.6 (35.5)
Transitional	+P 0.5M	1168.2 (1009.9)
Transitional	+P 1.0M	0.05 (0.4)
Transitional	+NP 0.5M	0.06 (0.06)
Unimpaired	+P 0.05M	236.0 (12.8)
Unimpaired	+P 0.1M	421.7 (122.7)
Unimpaired	+P 0.5M	2039.6 (275.4)
Unimpaired	+P 1.0M	OVER LIMIT
Unimpaired	+NP 0.5M	2270.5 (487.7)

APPENDIX C: TYPES OF FATTY ACIDS USED IN EACH CATEGORY: SAFA, MUFA, PUFA, AND HUFA. SAFA = Saturated Fatty Acid, MUFA = Monounsaturated Fatty Acid, PUFA = Polyunsaturated Fatty Acid, HUFA = Highly Unsaturated Fatty Acid.

SAFA	MUFA	PUFA+ HUFA
c10:0	12:1w8	15:4w3c
a11:0	i14:1w7c	15:3w3c
c11:0	14:1w8c	16:4w3c
a12:0	14:1w7c	16:3w6c
c12:0	14:1w5c	16:2DMA
a13:0	i15:1w9c	18:3w6c
c13:0	i15:1w6c	18:4w3c
i14:0	15:1w8c	18:2w6c
a14:0	15:1w7c	19:4w6c
c14:0	15:1w5c	19:3w6c
i15:0	alc16:1w7c	20:4w6c
a15:0	16:1w8c	20:5w3c
c15:0	16:1w7c	20:2w6c
i15:0DMA	16:1w6c	21:3w6c
15:0 DMA	16:1w5c	21:3w3c
alc16:0N	16:1w9cDMA	22:6w3c
i16:0	16:1w7cDMA	22:5w3c
a16:0	i17:1w10c	22:2w6c
c16:0	i17:1w9c	24:3w6c
10Me16:0	17:1w8c	24:3w3c
16:0DMA	17:1w7c	
i17:0	17:1w5c	
a17:0	17:1w4c	
cy17:0w7c	18:1w9c	
c17:0	18:1w8c	
10Me17:0	18:1w7c	
17:0DMA	18:1w5c	
i18:0	10Me18:1w7c	
c18:0	18:1w9cDMA	
i19:0	18:1w5cDMA	
a19:0	19:1w6c	
cy19:0w9c	20:1w9c	
cy19:0w7c	20:1w8c	
cy19:0w6c	21:1w9c	
c19:0	21:1w5c	

i20:0	21:1w4c	
c20:0	21:1w3c	
c21:0	22:1w8c	
i22:0	22:1w6c	
c22:0	22:1w3c	
c23:0	23:1w5c	
c24:0	23:1w4c	
	24:3w3c	
	24:1w9c	

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