ABSTRACT

The Importance of Low-Level Nitrogen and Carbon to Periphyton in Alaskan Headwater Streams

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Alaskan headwater streams are ecologically and economically important habitats for juvenile salmonid development. These low-nutrient environments are relatively untouched by anthropogenic effects and contain little marine-derived nutrients due to their distance inland and narrow channels. Reduction in these nutrient sources places high dependence of stream productivity on allochthonous resources supplied by landscape features within the catchment. The current research investigates the importance of low-level nitrogen and carbon (simulating nutrient levels derived from wetlands and alder trees) by observing the effects of these nutrients on microbial biomass, enzyme activity and metabolism. Results indicate that these low-level nutrient sources may be undervalued resources driving stream productivity and that such features should be considered in land management plans within vulnerable stream catchments. The research presented here may be essential in protecting and maintaining habitats that promote healthy salmonid populations in the Kenai lowlands of Alaska and elsewhere.

The Importance of Low-Level Nitrogen and Carbon to Periphyton in Alaskan Headwater Streams

by

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DEDICATION

For my family and friends, who encouraged and supported me throughout this process.

I couldn't have done it without you.

CHAPTER ONE

Introduction

In the Pacific Northwest, recent discussions of salmon conservation strategies have pointed toward a need for nutrient enrichment in spawning sites deplete of marine-derived nutrients (Lackey, 2003). Headwater streams of the Kenai Peninsula lowlands in Alaska are such environments in which little marine-derive nutrients are found, but juvenile salmonids are prevalent (Shaftel et al., 2011). Despite a great lack of previous records of salmonid use in Kenai Peninsula headwater streams, all catchment types in the area supported at least one life-history stage of Dolly Varden (*Salvelinus malma*) and/or coho (*Oncorhynchus kisutch*) salmon in 2012 (King et al., 2012).

The reason for proposed nutrient enrichments in the Pacific Northwest is based on Liebig's Law of the Minimum, which states that growth is limited by the most limiting nutrient required. Thus, salmonid growth should be limited by whichever nutrient is most limiting, and increases in that nutrient should be followed by increases in salmonid growth. Nitrogen (N) and phosphorus (P) are the most common limiting nutrients; nitrogen is needed for protein synthesis, and phosphorus is needed for DNA, RNA and energy transfer (Conley et al., 2009).

For years, freshwater environments have been regarded as mainly P-limited systems because natural mechanisms of atmospheric carbon (C) and N fixation compensate for aquatic C and N deficiencies, while such atmospheric inputs do not exists for P, which is derived mainly from minerals (Schindler, 1977). However, two meta-analyses of nutrient addition studies (one in 653 freshwater environments and the other in

237 freshwater lotic environments) determined separately that N and P may be equally important in limiting freshwater primary producer growth (Francoeur, 2001; Elser et al., 2007). Though C is not commonly believed to limit production, supplements of dissolved organic matter (DOM), mainly organic carbon (OC), to streams in the Hubbard Brook Experimental Forest led to increased uptake of OC, indicating that some systems may even be C-limited (Bernhardt and McDowell, 2008).

Nutrients are controlled by many different abiotic and biotic factors at different geographic locations. In headwater streams of low primary and secondary productivity, like the cold, shaded and relatively pristine streams in Alaska, small changes in nutrients may have large impacts on the trophic structure (Bilby et al., 1996). These low-productivity headwater streams are also heavily dependent on allochthonous inputs of essential nutrients (Richardson et al., 2005). Alaska is dominated by P-rich glacial deposits, permafrost and volcanic ashes, making P unlikely to be limiting in this region (Eicher and Rounsefell, 1957; Hobbie et al., 1999). C and N, however, are much more variable across the landscape, C deriving from wetlands and bluejoint grass (*Calamagrostis canadensis*) leaf litter and N deriving from alder tree (*Alnus spp.*) root nodules containing N-fixing bacteria (Shaftel et al., 2011; Dekar et al., 2012; Shaftel et al., 2012; Walker et al., 2012; Whigham et al., 2012).

Bluejoint grass provides mainly recalcitrant forms of C and is present in almost all catchments across the lowlands, while wetlands emit both recalcitrant and labile forms of C and are associated with more low sloping catchments. C deriving from wetlands tends to be more bioavailable than many other allochthonous sources of DOC (Wiegner and Seitzinger, 2004). Alder trees are found in opposing landscapes with higher slopes

and add bioavailable N to the stream via surface waters, groundwater, and leaf leachate (Bond, 1956; Shaftel et al., 2012). Shaftel et al. (2012) found that alder cover explained 75-96% of variation in NO_x-N in 25 Kenai Peninsula watersheds with alder cover ranging 0-28.2%. Catchments with higher alder cover tended to have higher N concentrations than those without alder. The catchments lacking alder tend to be heavily dominated by wetland cover, which contributes to the higher dissolved organic carbon (DOC) in these reaches (Walker et al., 2012).

Changes in land use and land management in this region may significantly alter the percent alder and wetland cover in watersheds, in turn impacting the amount of terrestrial C and N entering the stream. Alaskan populations of coho salmon (*Oncorhynchus kisutch*) have high interpopulation diversity dominated by genetic drift rather than gene flow between populations (Olsen et al., 2003). This finding led to the recommendation of fine-scale management of the populations in this region, as they may be adapted to very specific stream locations. Therefore, changes in land management surrounding their spawning streams may have major impacts on these populations. Previous studies have found negative impacts on coho salmon as a result of deforestation in other regions of the northwest (Pess et al., 2002), and Kenai Peninsula populations may be even more vulnerable to potential deforestation impacts due to the great influence alder stands have on stream N content. Wetland filling for development may also have severe impacts on the ecosystem, as many stream water chemistry variables are heavily influenced by wetlands in the catchment (Johnston et al., 1990).

Though adult salmonids do not move to small headwater channels, juveniles thrive in the inland waters, protected from many marine predators (Shaftel et al., 2011).

The presence of these fish is directly related to water chemistry, substrate composition, and channel morphology and gradient (King et al., 2012). Additional variance in fish community structure is attributed to macroinvertebrate composition, indicating the importance of trophic interactions for the conservation of juvenile salmonids in Kenai Peninsula headwater streams. Alterations in nutrient levels might have severe impacts on the functioning of these streams, especially the trophic interactions assimilating nutrients into living tissue, a process that begins with the microbial community in the stream.

Microbes are responsible for organic matter decomposition and nutrient transformation and transfer through the food web (Findlay, 2010), thus making them the logical first focus of functional changes due to nutrient manipulations. Periphyton make excellent microbial study organisms in manipulative studies due to their compact spatial distribution and short generation time, leading to rapid responses to environmental changes (Stevenson et al., 1996). Periphyton biomass makes up the basal resources contributing to biomass in higher trophic levels, influencing the entire community. Periphyton biomass estimates may help interpret changes seen in higher trophic level biomass estimates.

Functional responses in periphyton may also indicate important changes in whole stream processing. Extracellular enzyme activity aids decomposition of organic matter, and models have been developed using these enzymes to estimate stream decomposition rates (Sinsabaugh and Linkins, 1990; Sinsabaugh et al., 1992; Asmar et al., 1994; Sinsabaugh and Moorhead, 1994; Sinsabaugh et al., 1994; Sinsabaugh et al., 1994b; Sinsabaugh and Findlay, 1995; Carreiro et al., 2000; Sinsabaugh et al., 2009). These enzymes have also been known to signify limitation of specific nutrients within a habitat

(Hill et al., 2006; Hill et al., 2010; Hill et al., 2012). Thus, differential enzyme production in these microbes can alter ecosystem processes significantly (Allison and Vitousek, 2005).

Additionally, bacteria have one of the highest metabolic rates per unit biomass, contributing significantly to ecosystem metabolism (Chróst, 1989). Changes in metabolism may be the best indicators of acute nutrient enrichment effects on stream ecosystem structure and function (Nelson et al., 2013). Interactions between photosynthetic activity and enzyme activity signify production and recycling of resources needed to support higher trophic level activity. Thus, a better understanding of how periphyton biomass, enzyme activity and metabolism change with nutrient content in the stream may give insight into how and why changes in higher trophic levels might occur.

The research presented here is part of an ongoing project initiated in 2011, entitled "Juvenile Salmon Headwater Rearing Habitat." The main goal of the project as a whole is to develop a model predicting influential habitat characteristics that guide juvenile salmon development in the Kenai Lowland headwater streams of Alaska. Progress on this project indicates that percent alder tree cover and wetland cover in the catchment are major drivers of nutrient content, specifically N and C, in the streams. To validate the predictive model, additions of either N or C were made to two streams naturally low in the supplemented nutrient and naturally high in the unaltered nutrient based on these landscape characteristics and previous water chemistry analyses. The 2013 focus was on determining direct and indirect effects of nutrients on salmonid productivity, movement, and abundance in order to better predict "hotspots" for these species using landscape characteristics. The research presented here investigates the

effects of these allochthonous nutrients on microbial biomass, enzyme activity and metabolism, as changes at the base of the food web could lead to significant changes in higher trophic levels (macroinvertebrates, and ultimately salmonids).

CHAPTER TWO

A Little Goes a Long Way: Low-Level Dissolved Organic Carbon Influences on Periphyton Biomass Accrual, Enzyme Activity and Metabolism in an Alaskan Headwater Stream

Abstract

Alaskan headwater streams are ecologically and economically important habitats for juvenile salmon. Due to low input of marine-derived nutrients, stream communities rely on allochthonous nutrients, such as wetland-derived carbon (C) and alder-fixed nitrogen. An Alaskan headwater stream with high alder cover (high dissolved inorganic nitrogen) but little wetland drainage (low dissolved organic carbon (DOC)) was supplemented for eight weeks with 0.250 mg/L acetate-C, a level likely to be emitted from wetlands in the catchment. Periphyton biomass, enzyme activity and metabolism were monitored in the treatment (TRT) and reference (REF) reaches. After eight weeks, both chlorophyll-a and ash-free dry mass were higher in the TRT than the REF reach. Nitrogenase and beta-glucosidase activity was low and did not change with C addition. Alkaline phosphatase activity was low, but increased in the TRT reach after four weeks. Biomass-specific respiration (R) on individual rocks increased in the TRT reach after eight weeks, while biomass-specific gross primary production (GPP) did not change significantly. Daily R and GPP extrapolated to the stream area both increased in the TRT reach after eight weeks due to the increase in biomass. Based on this study and studies observing responses of aquatic macroinvertebrates and salmon to the C supplement, wetlands should be considered important labile C sources to headwater streams. Further, low-level DOC from wetlands may be an undervalued driver of stream productivity.

Introduction

Though carbon (C) is one of the three main nutrients necessary to sustain life, nitrogen (N) and phosphorus (P) gain much more attention in environmental management practices (Stanley et al., 2012). However, dissolved organic carbon (DOC) frequently influences the bioavailability of both N and P by converting them to their unavailable organic forms (Findlay and Sinsabaugh, 1999; Stanley et al., 2012; Walker et al., 2012). In addition to these changes, organic carbon (OC), often in the form of dissolved organic matter (DOM), regulates water temperature, color (which influences light availability), pH and respiration (Findlay and Sinsabaugh, 2003; Stanley et al., 2012; Walker et al., 2012). Perhaps the most important role, DOM may provide 95% of the organic matter processed for energy in headwater streams (Richardson and Danehy, 2007).

DOC may originate from both autochthonous and allochthonous sources. Autochthonous sources derive from algal exudates released by grazing, lysis, cell death or passive leakage, providing both labile and recalcitrant forms of DOC (Findlay and Sinsabaugh, 2003). Photosynthetically-produced DOC (PDOC) is the most bioavailable autochthonous DOC resource, and approximately 46% of PDOC is incorporated into bacteria (Cole et al., 1982; Findlay and Sinsabaugh, 2003).

Allochthonous energy inputs are influenced by meteorologic inputs in the form of litter and throughfall and geologic vectors of surface and subsurface waters (Fisher and Likens, 1973). Hydrologic flowpaths and soil organic matter (SOM) content in the catchment are the most important factors influencing allochthonous dissolved organic matter (DOM) import into the stream (Mulholland, 1997). Low slopes (slow flowpaths) and high SOM result in longer contact time with C-rich substrate, allowing water to

concentrate higher amounts of DOC and carry them to the stream. Wetlands exist under these conditions, fostering high DOC concentrations due to relatively stagnant waters and leachate from decaying organic matter (Mulholland and Kuenzler, 1979). As a result, the amount of wetland in a stream catchment is one of the best predictors of stream DOC concentration, accounting for 50 to 86% of the variation (Mulholland and Kuenzler, 1979; Eckhardt and Moore, 1990; Dillon and Molot, 1997; Mulholland, 1997; Findlay et al., 1998; Gorham et al., 1998; Aitkenhead et al., 1999; Gergel et al., 1999). In the Hudson River, wetlands caused an enrichment of 5-18% DOC (Findlay et al., 1998), while North Carolina streams containing swamp drainage had 7x higher DOC export than those without swamps in the catchment (Mulholland and Kuenzler, 1979). In catchments with little wetland cover, even the slightest increase in wetland area might have a large impact on stream DOC concentrations (Gorham et al., 1998).

Though C concentrations can be high in many streams, the availability of that C to the food web varies by source and may not reflect total DOC (Sobczak and Findlay, 2002). In a study of 9 rivers in the northeastern United States, on average only 4% of the DOC was bioavailable (Wiegner et al., 2006). Comparatively, pristine cedar bog wetlands in North Carolina contain DOC that is 22% bioavailable (Wiegner and Seitzinger, 2004). The bioavailable fraction of DOC (simple sugars such as glucose and acetate) is the main source of energy for immediate trophic interactions, while recalcitrant DOC (more complex molecules such as cellulose and lignin) may take decades to become available for organisms to use.

Microbes are the first step of C assimilation into the biota of a stream. This assimilation takes place via direct uptake of degraded material, adsorption to particulate

organic matter and ectoenzymatic breakdown of organic matter (Findlay and Sinsabaugh, 2003). Microbes additionally function in conditioning leaf matter for further processing by shredders and provide nutrients directly to scrapers and herbivores (Anderson and Sedell, 1979). Changes in DOM are followed quickly by changes in microbes, making these microscopic organisms the most rapid indicators of changes due to nutrient content in streams (Findlay and Sinsabaugh, 1999). These changes are even more pronounced in oligotrophic environments, such as Alaskan headwater streams (Stets and Cotner, 2008). Thus, a C-addition study was conducted in this environment. The stream chosen contained very little wetland cover in the catchment but very high alder cover, which contributes bioavailable dissolved inorganic N (DIN) to the stream via N-fixation (Shaftel et al., 2012). Based on previous studies examining nutrient regimes in this area (Walker et al., 2012), the chosen stream represents one of the most likely streams in the Kenai Peninsula lowlands to be C-limited.

The aim of this study was to characterize changes in periphyton microbial activity due to realistic wetland-level C supplementation in an alder-dominated watershed. In order to do so, an Alaskan headwater stream on the Kenai Peninsula was enriched with 0.25 mg/L DOC (acetate) during the summer of 2013. Biomass, enzyme activity and metabolism were used to assess the effects of the dosing. Increases in biomass were expected to occur upon C addition if the stream was C-limited before dosing and the addition was great enough to relieve this limitation.

We quantified enzyme activity for assimilation of three main macronutrients: C, N and P, as these enzymes are the best known predictors of decomposition (nutrient cycling) and nutrient limitation (Asmar et al., 1994; Carreiro et al., 2000; Allison and

Vitousek, 2005; Hill et al., 2006; Hill et al., 2010; Hill et al., 2012). Activity of beta-glucosidase (βGLU) for C-limitation, nitrogenase (NA) for N-limitation and alkaline phosphatase (APA) for P-limitation were compared between the dosed and non-dosed reaches. Increases in any of these enzymes likely indicate increasing limitation of their specific nutrients. Alternatively, declines in activity signify relief from limitation of a specific nutrient. βGLU, however is only one of many C-acquiring enzymes, so if no changes occur, perhaps another C-acquiring enzyme may be used more heavily in this system.

We also characterized changes in gross primary production (GPP) and respiration (R) in the periphyton to gain a better understanding of which metabolic activities were impacted most heavily by C limitation at the microbial level. Here we expect to see increases in R due to the utilization of C in the process of R. GPP may increase as a result of bacterial-algal coupling, or it may decline due to algal-bacterial competition. If no change occurs, GPP may not have been C-limited before dosing, or our C addition may not be sufficient to relieve limitation. Collaboration with other researchers link these microbial changes with some of the higher trophic level changes within this stream, such as those in the macroinvertebrate community and ultimately salmonid populations.

Previous studies have investigated the influence of C additions on stream ecosystems (Findlay et al., 2003b; Johnson et al., 2012), and even headwater streams specifically (Bernhardt and Likens, 2002; Wilcox et al., 2005). However, no other studies to our knowledge have combined a long time period (eight weeks) and many trophic levels with realistic (wetland-derived) C dosing levels. This longer time period was expected to be important for observing microbial biomass changes in particular, as

the biomass in this stream is generally very low. With fewer organisms to reproduce, growth will occur more slowly than in an environment with greater biomass. On the other hand, because these streams are so low in nutrients, responses to nutrient additions are expected to be much greater (Bilby et al., 1996). Thus, it is important to add realistic amounts of nutrients in order to simulate the desired impact and not overestimate the result. This study aims to be the most extensive investigation of stream ecosystem changes sensitive to very small and realistic alterations in C supply.

Materials and Methods

Study Site

This study takes place on the lower Kenai Peninsula lowlands to the west of Kachemak Bay in south-central Alaska. Shaftel et al. (2011), Walker et al. (2012), and Dekar et al. (2012) describe in detail the geomorphic setting, vegetation, and climate of the Kenai Peninsula. Briefly, this area contains relatively undeveloped landscapes of mixed spruce, birch, willow and alder forests in combination with fireweed and bluejoint grass meadows. Annual precipitation is ~13-18 cm/yr and average minimum and maximum temperatures range from -8.5 to 16.1˚C. The study stream was chosen based on extensive datasets and models created from previous research in the area (Shaftel et al., 2011; Dekar et al., 2012; King et al., 2012; Kostka, 2012; Shaftel et al., 2012; Walker et al., 2012; Whigham et al., 2012). Kostka (2012) describes the specific stream in more detail. Briefly, Anchor-1203 (ANC-1203) is a first-order tributary of the Anchor River, dominated by cobble substrate with interspersed sand and fine organic matter. This stream was chosen for its high (10.47%) alder cover and low wetland cover, resulting in a

stream with high DIN and low DOC inputs. Despite the vast amount that is known about this stream and others in the area, a whole-stream nutrient manipulation study has never been attempted in this region.

Treatment (TRT) and reference (REF) reaches for the stream were chosen based on proximity, accessibility and, most importantly, similarity in physical environment. Both reaches extend 75 m down the stream channel, with the REF reach ending approximately 80 m upstream of the dosing station and TRT reach beginning immediately downstream of the dosing station. The REF reach had a slope of 5.28% and sinuosity of 1.06, while the TRT reach had a slope of 4.88% and sinuosity of 1.07 (Fig. 1).

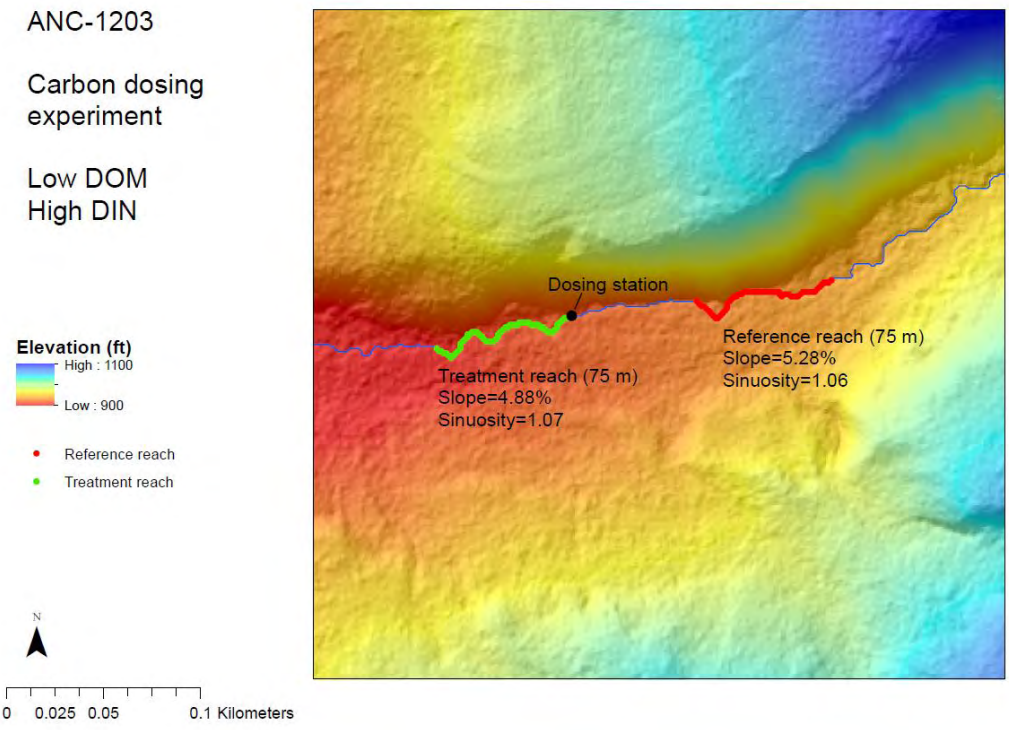

Figure 1. Elevation map for first order tributary of the Anchor River (ANC-1203), a stream on the Kenai Peninsula lowlands with high (10.47%) alder cover and low wetland cover resulting in low DOM and high DIN. The treatment reach was dosed with sodium acetate at a rate of 0.025 L/min from 24 June 2013 to 25 August 2013.

Dosing

We set up a dosing station 2 m from the stream with a carboy of stock solution contained within a ditch lined with a plastic tarp both below and above the stock tank to minimize disturbance and potential leakage of the stock solution to the environment. Polyvinyl chloride (PVC) tubing led from the carboy directly to the stream and the solution was pumped at a rate of 0.025 L/min. We dosed the TRT reach with sodium acetate targeting a concentration of +0.25 mg C/L from 24 June 2013 to 25 August 2013, during the season of peak N inputs to the streams (Shaftel et al., 2012). Johnson et al. (2012) found acetate to be preferentially taken up over formate, making it a more effective labile C source for this study. The dosing level was based on a 2011 determination of bioavailable DOC in the stream (Doyle et al., unpublished data). These data show that although total DOC in the stream was 10-13 mg/L, only about 10% of this total (1.2-1.5 mg/L) was bioavailable. Furthermore, only 0.05-0.15 mg/L was considered rapidly bioavailable (metabolized in <15 days). We added a bromide tracer of 0.05 mg/L to the stock solution to assure proper mixing and to account for dilution due to groundwater inputs and transient storage.

Water Chemistry

We monitored water chemistry in the REF and TRT reaches weekly both before and after dosing the stream. We collected triplicate stream water samples of 250 mL each for analysis of phosphate (PO_4-P), ammonia (NH_4-N), nitrate (NO_3-N), total dissolved phosphorus (TDP), dissolved organic phosphorus (DOP), total dissolved nitrogen (TDN), dissolved organic nitrogen (DON), dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), and bromide (Br^-). We also collected triplicate water

samples of 50 mL each for total nitrogen (TN) and total phosphorus (TP). All samples were frozen and shipped to the lab at Baylor University for measurement on a flow-injection auto-analyzer (Lachat QuikChem 8500 and Series 520 XYZ Autosampler) and a Shimadzu TOC 5-5-analyzer. We collected triplicate water samples of 15 mL each and acidified them with 30 µl sulfuric acid (H_2SO_4) for ammonium (NH_3) analysis on a spectrophotometer. We measured discharge weekly in conjunction with water collections using a salt tracer and an YSI EXO 1 datasonde (Yellow Springs Instruments, Yellow Springs, Ohio).

Sampling Schedule

Samples were collected at 3 stations [upstream (UP), middle (MID), and downstream (DWN)] in both the REF and TRT reaches for all enzyme assays and biomass estimates at each sampling time. Metabolism samples were collected only at the UP station. Pre-dosing enzyme assays were conducted in the REF and TRT reach one wk before dosing began (16-21 June 2013). Due to equipment difficulties, pre-dosing metabolism assays were conducted the day of dosing (24 June 2013). All post-dosing assays were conducted 4 wks (22-24 July 2013) and 8 wks (19-24 August 2013) post-dosing in both the REF and TRT reaches for all assays. Additionally, metabolism was measured at 2 wks (8-13 July 2013) in order to capture any immediate changes. Any enzyme assays that clearly did not respond to the dosing were discontinued after wk 4 in order to conserve both resources and time. Rocks for these assays were still collected in order to maintain sample sizes for biomass estimates.

Biomass

Biomass measurements were taken from all rocks used in enzyme assays. Rocks were placed in a refrigerator after assays were complete and scraped into separate beakers within one wk using paint scrapers and toothbrushes to remove biomass. Scrapings were then diluted to a known volume using deionized water and homogenized using a hand blender if necessary. A subset of the homogenized slurry was filtered onto glass fiber filters (GFF) for chlorophyll *a* (chl-a) and ash-free dry mass (AFDM) analysis. AFDM filters were combusted at 500˚C for two hours and weighed before slurries were filtered through them. All filters from slurries were wrapped in aluminum foil and frozen until analysis. AFDM filters were then dried at 60˚C for >24 hrs, placed in a dessicator, weighed, and combusted at 500˚C for one hour. After combustion, filters were placed in a dessicator to cool, and weighed once more. Organic carbon content was calculated as the material lost in combustion (difference between non-combusted and combusted weights) extrapolated back to the entire slurry volume and normalized to surface area of the rock. Chl-a filters were extracted in 10 mL of ethanol, placed in a 78˚C water bath for 5 minutes, and kept in the fridge in the dark overnight. Absorbance was measured using a spectrophotometer at 665 nm wavelength for chl-a. Values again were extrapolated to the entire slurry volume and normalized to surface area of the rock. All biomass estimate methods were derived from Biggs and Kilroy (2000).

Rock surface area was measured by covering tops of dry rocks in aluminum foil and weighing the aluminum foil. This weight was converted to square centimeters of surface area using a standard curve. Composite slurries of all rocks from a single station on a sampling date for each assay were dried at 60˚C in plastic weigh boats, scraped,

placed in tin capsules and analyzed on an Elemental Analyzer (EA) for C:N of the periphyton. Rocks from metabolism assays were not included in biomass statistical analyses, as these rocks were only collected at the UP station of each reach and may have biased our results toward that location.

Enzymes

Four replicate rocks per assay were collected at each station (UP, MID, DWN) in both the REF and TRT reaches for alkaline phosphatase (APA) and beta-glucosidase (βGLU) assays. βGLU is the most common glucose stereoisomer found in nature and is used to break down cellobiose and cellodextrins in leaf matter into glucose molecules that are readily taken up (Dunn et al., 2014). APA hydrolyzes phosphoric acid monoesters to cleave phosphate groups from organic substrates (Dunn et al., 2014). Enzyme activity of the two microbial exoenzymes was measured fluorometrically. In these fluorometric assays, the acting enzyme (βGLU or APA) metabolizes the substrate added to the sample (methylumbelliferyl-glucopyranoside or methylumbelliferyl-phosphate), forming a fluorescent product (methylumbelliferyl (MUF)) that can then be measured on a fluorometer once the desired nutrient is cleaved (Dunn et al., 2014). The rate of fluorescent increase indicates accumulation of MUF and is used as a measure of enzyme activity. This method is recognized as the best measurement of enzyme activity in stained wetland waters and waters of low activity like those used in this study (Freeman et al., 1995).

Each incubation jar contained 20 mL filtered stream water, 60 mL TRIS buffer (pH 10), a periphyton rock, and 8 mL of substrate (0.5 mM 4-MUF-β-D-glucopyranoside for βGLU or 0.5mM 4-MUF-phosphate for APA). One additional control sample for

each station within each reach (6 total) was used to account for background activity by combining filtered stream water, TRIS and substrate without a rock. We measured fluorescence every 10 minutes after substrate was added, until 30 minutes passed. The slope of the relationship between time and fluorescence was interpreted as the rate of enzyme activity (after subtracting controls). β-GLU and APA were normalized to AFDM, as they are believed to be enzymes produced mainly by bacteria (Chróst, 1989; Cunha et al., 2010).

Six replicate rocks were collected at each station (UP, MID, DWN) in both reaches (TRT and REF) for estimation of nitrogenase activity (NA). Nitrogenase is an enzyme used to break the strong triple bond between atmospheric N atoms in order to produce bioavailable ammonium (NH_4^+) for uptake. We used the acetylene-reduction assay to measure NA, which uses conversion of acetylene to ethylene as a proxy for N-fixation. Incubations occurred in 250 mL Mason jars equipped with septa for syringe access and filled completely with filtered stream water and a periphyton rock. One control jar from each station (6 total) contained filtered stream water only, and one dark jar (6 total) covered with aluminum foil contained filtered stream water and one rock.

All jars (including controls and darks) were inoculated with 40 mL of acetylene gas, generated by adding a few bricks of CaC_2 to 400 mL of deionized water in a 1 L cubitainer equipped with a septum top for syringe access. After water was saturated with acetylene gas, the remaining bubble was removed using a syringe and replaced with filtered stream water from the associated station. Incubations ran overnight (~10-14 hr) in a water bath with constant high light (~325 $\mu E/m^2/s$) and temperature (13-20°C). Though incubation time and temperature ranges were fairly wide, all samples within one

time point were treated identically. Temperatures changed throughout the incubations due to limited temperature-regulation equipment availability.

At the end of the incubation, a subsample of 5 mL of water was taken into a 10 mL syringe and equilibrated with 5 mL of air for one minute, shaking vigorously. The headspace in this syringe was collected in a 4 mL blood serum vacutainer and ethylene produced was measured by an Agilent Technologies 7890A gas chromatograph with a flow rate of 40 mL/min and oven temperature of 85˚C. NA activity was normalized to chl-a concentrations, as it is mainly associated with photosynthetic organisms such as cyanobacteria (Chróst, 1989; Cunha et al., 2010).

Metabolism

Light and dark incubations were used to determine biomass-specific (per μg chl-a per hr) and daily areal estimates (per cm^2 per day) of gross primary production (GPP) and respiration (R). We collected five replicate rocks at the UP station in both the REF and TRT reach. The oxygen change method was used to determine metabolism of periphyton-covered rocks enclosed in 250 mL Mason jars with plastic BOD bottle top adapters fastened using welding putty and sealed with rubber stoppers. Prior to filling jars, filtered stream water from each reach was bubbled with nitrogen gas in order to decrease oxygen levels to ~3 mg/L and prevent oversaturation. One control jar for each station (2 total) contained filtered stream water of the respective station with no periphyton rock in order to account for changes due to non-filtered particles or contamination.

Each rock was incubated under no light (0 $\mu E/m^2/s$), low light (160-180 $\mu E/m^2/s$), and high light (300-350 $\mu E/m^2/s$) conditions in the lab, with a 1 hr acclimation period

between each light treatment. Incubations with no light ran overnight in order to ensure enough change in oxygen occurred due to R. Light treatment incubations continued until a significant change in dissolved oxygen (DO ± 0.5 mg/L) was observed in all replicates except controls (~1-2 hrs).

Biomass-specific net primary production (NPP) rates were calculated by dividing DO change by incubation time in the light, after accounting for the total water volume in the jar. Rates were normalized to chl-a rather than AFDM due to the necessity of chl-a for photosynthesis. Dark incubation rates were used as R estimates, and GPP was calculated by subtracting R rates from NPP rates. These hourly biomass-specific rates (per µg chl-a per hr) were extrapolated to a daily areal scale by multiplying by the average chl-a (per cm^2 of the appropriate Reach and Time) and multiplying GPP by total daylight hrs and R by 24 hrs. Light data was acquired from the Estuarine Reserves Division (ERD), Office of Ocean and Coastal Resource Management (OCRM), National Ocean Service, National Oceanic and Atmospheric Administration (NOAA).

No significant differences in NPP were observed between the low and high light treatments, so only high light measurements were used in analyses. Unfortunately, because of this light-saturating response seen in the low light treatment, we were unable to estimate alpha values of light acclimation in this study. The method used may overestimate GPP, but this error should be small due to the low light saturation of benthic algae in general and specifically in Alaskan light-limited species (Gray and Hill, 1995). Additionally, the longer than average daylengths make the time period of acclimation negligible over the entire day.

Data Analysis

All enzyme assay and biomass accrual data were analyzed using R Statistics Software nlme package for mixed effects models with Time (PRE, 4WK, 8WK) and Reach (REF vs TRT) as fixed effects and Station (UP, MID, DWN) as a random effect. Results focus on the interaction term (Time*Reach), as this indicates a significant difference in the overall pattern of activity between the REF and TRT reach during the course of the dosing. The package phia was used to evaluate post-hoc comparisons of this interaction term based on a Chi-squared distribution of the data. Post-hoc comparisons examined significance between the REF and TRT reaches at any given Time. Metabolism data were analysed using R Statistics Software ANOVA with Time (PRE, 2WK, 4WK, 8WK) and Reach (REF and TRT) as the independent variables, since only the UP station was sampled. The package phia again was used to evaluate post-hoc comparisons of the interaction term Time*Reach, but these tests were based on an F distribution rather than Chi-squared. Again, individual post-hoc comparisons evaluate differences in REF and TRT at a given Time.

Results

Water Chemistry

Water chemistry results indicated that our dosing raised DOC levels to near target concentrations (0.25 mg/L) in the TRT reach. The data are still being processed and interpreted.

Biomass

Chl-a and AFDM showed similar patterns over the course of this study, with little change in TRT reach biomass but significant declines in both metrics in the REF reach (Figs. 2 & 3). The pattern of change in the REF reach was significantly different from the pattern in the treatment reach for both chl-a (Time*Reach $F_{(2,181)}$=4.52, p=0.01) and AFDM (Time*Reach $F_{(2,171)}$=4.54, p=0.01). Chl-a and AFDM were not significantly different between the REF and TRT reaches before dosing (χ^2_1=0.27, p=0.61 and χ^2_1=0.003, p=0.96, respectively). By wk 8 post-dosing, both chl-a and AFDM were significantly greater in the TRT reach than in the REF reach (χ^2_1=9.76, p=0.005 and χ^2_1=13.67, p=0.0007, respectively). In the REF reach, AFDM declined by 62% from pre-dosing to wk 8 and chl-a decline 94% during the same time period. Patterns in C:N ratios did not significantly differ between the REF and TRT reach (Time*Reach $F_{(2,43)}$=0.10, p=0.91).

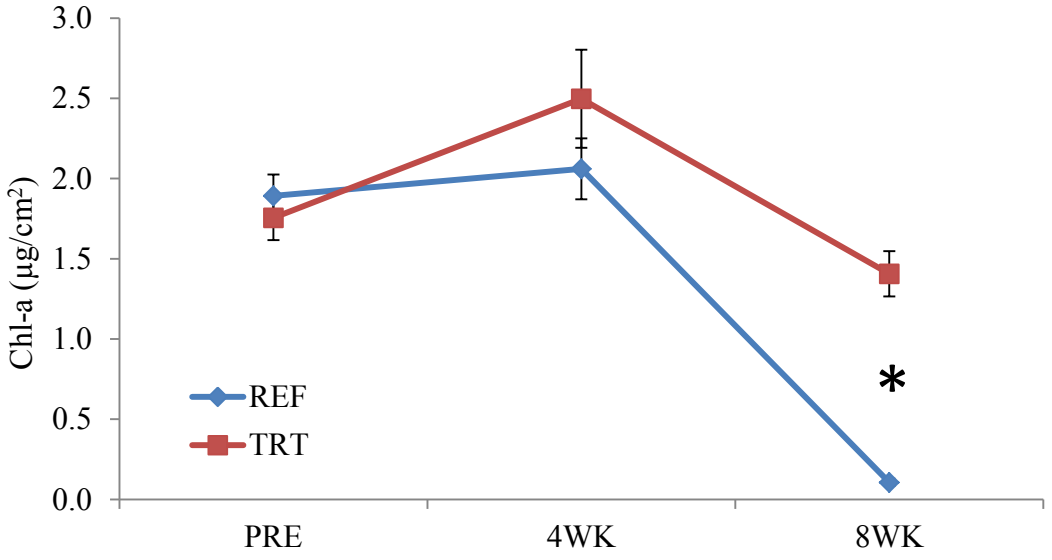

Figure 2. Patterns of mean ± SE chl-a (µg/cm^2) of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(2,181)}$=4.52, p=0.01). Chl-a was significantly greater in TRT than REF at 8WK (χ^2_1=9.76, p=0.005).

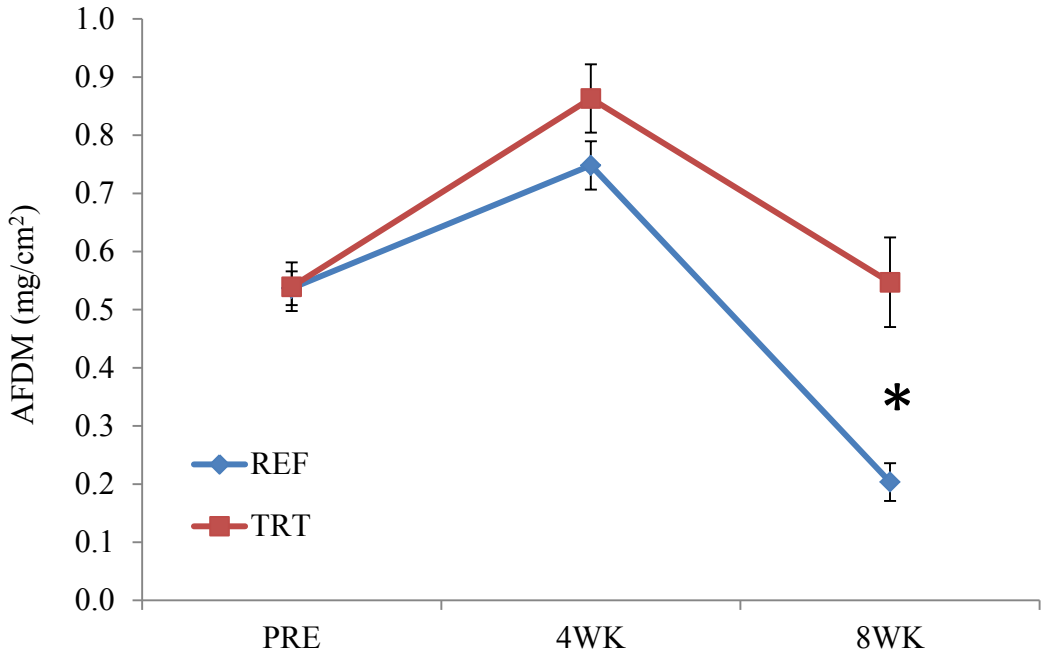

Figure 3. Patterns of mean ± SE AFDM (mg/cm^2) of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(2,171)}$=4.54, p=0.01). AFDM was significantly greater in TRT than REF at 8WK (χ^2_1=13.67, p=0.0007).

Enzymes

NA remained below detection limits before dosing and 4 wks after dosing, and was discontinued after this time. Patterns in βGLU activity did not differ between REF and TRT reaches after 4 wks of dosing (Time*Reach $F_{(1,42)}$=0.06, p=0.81), and this assay was discontinued after this time. Patterns in APA were significantly different between the REF and TRT reach, with the REF remaining unchanged and the TRT increasing significantly (Time*Reach $F_{(2,64)}$=3.35, p=0.04; Fig. 4). APA was significantly higher in the TRT reach relative to REF before dosing (χ^2_1=4.58, p=0.03), but increased significantly more in the TRT reach relative to the REF reach after 4 wks (χ^2_1=26.41, p=5.53x10^{-7}) and remained higher at 8 wks (χ^2_1=29.81, p=1.42x10^{-7}).

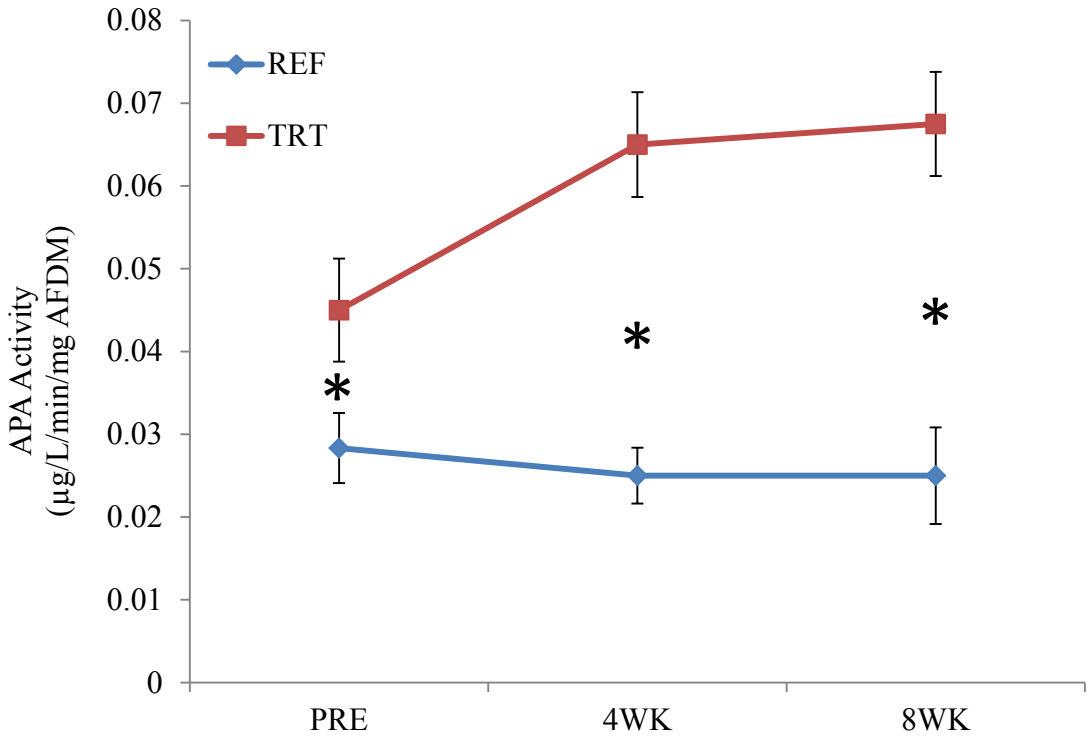

Figure 4. Patterns of mean ± SE APA (µg/L/min/mg AFDM) of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(2,64)}$=3.35, p=0.04). APA was significantly greater in TRT than REF before (χ^2_1=4.58, p=0.03) and after dosing (χ^2_1=26.41, p=5.53x10^{-7}; χ^2_1=29.81, p=1.42x10^{-7}).

Gross Primary Production (GPP)

Hourly biomass-specific GPP ranged from 1.01 to 8.94 µg O_2/µg chla/hr throughout this study, increasing in both reaches as the season progressed. Patterns in GPP did not significantly change between REF and TRT reaches throughout the study (Time*Reach $F_{(3,32)}$=2.13, p=0.12; Fig. 5).

Daily areal GPP patterns were significantly different in the TRT reach relative to REF, with declines in the REF reach and increases in TRT (Time*Reach $F_{(2,24)}$=15.05, p=0.00006; Fig. 6). GPP did not differ between REF and TRT reaches before dosing (F=0.003, p=0.95), but increased significantly more in the TRT reach relative to the REF

reach by wk 4 (F=7.63, p=0.02), and remained greater in the TRT reach by wk 8 (F=57.90, p=2.27x10^{-7}). Propagated error bars in Fig. 6 account for the error associated with average chl-a/cm^2 estimates used to extrapolate to the areal scale, and indicate that though the difference at wk 4 is statistically significant, it may not be meaningful. The difference at wk 8, however, remains significant after accounting for biomass error.

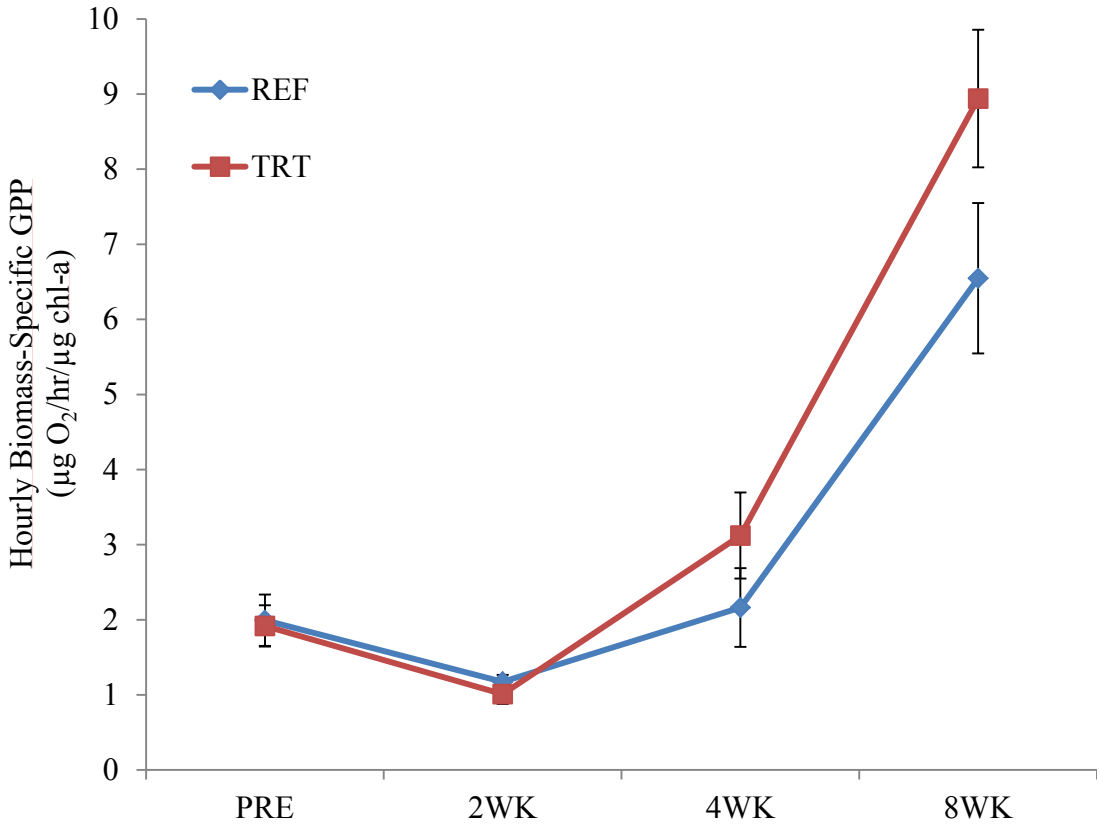

Figure 5. Patterns of mean ± SE hourly biomass-specific GPP (µg O_2/hr/µg chl-a) of periphyton on rocks at ANC-1203 in REF and TRT reach over time were not significantly different (Time*Reach $F_{(3,32)}$=2.13, p=0.12).

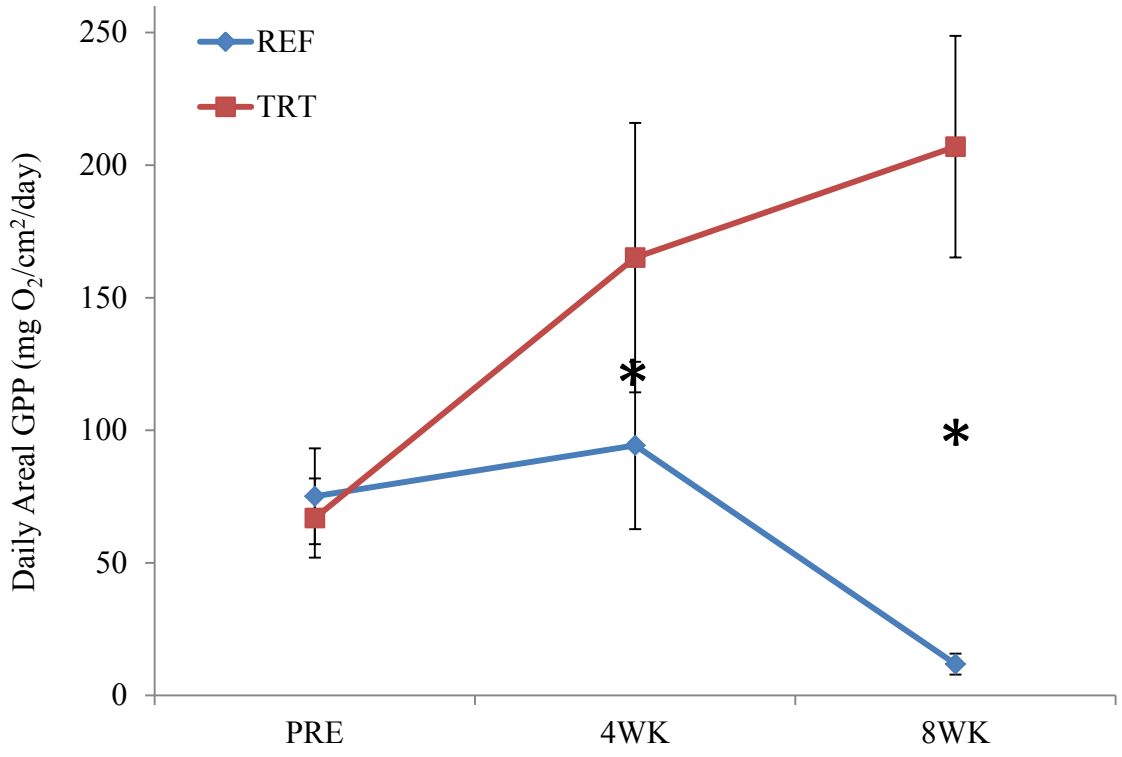

Figure 6. Patterns of mean ± propagated SE daily areal GPP (µg O_2/cm^2/day) of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(2,24)}$=15.05, p=0.00006). GPP was significantly greater in TRT than REF at 4WK (F=7.63, p=0.02) and 8WK (F=57.90, p=$2.27x10^{-7}$). Error bars indicate propagated standard error based on error in chl-a estimates.

Respiration (R)

Hourly biomass-specific R ranged from -0.17 to -2.90 µg O_2/µg chla/hr throughout the study, increasing in both reaches over time. Patterns of change in R differed significantly between the REF and TRT reaches, with R increasing significantly more in the TRT reach (Time*Reach $F_{(3,32)}$=12.93, p=0.00001; Fig. 7). R was not significantly different between REF and TRT reaches before dosing (F_1=0.005, p=1.00), but R increased significantly more in the TRT reach relative to the REF reach by wk 8 (F_1=57.00, p=$5.37x10^{-8}$).

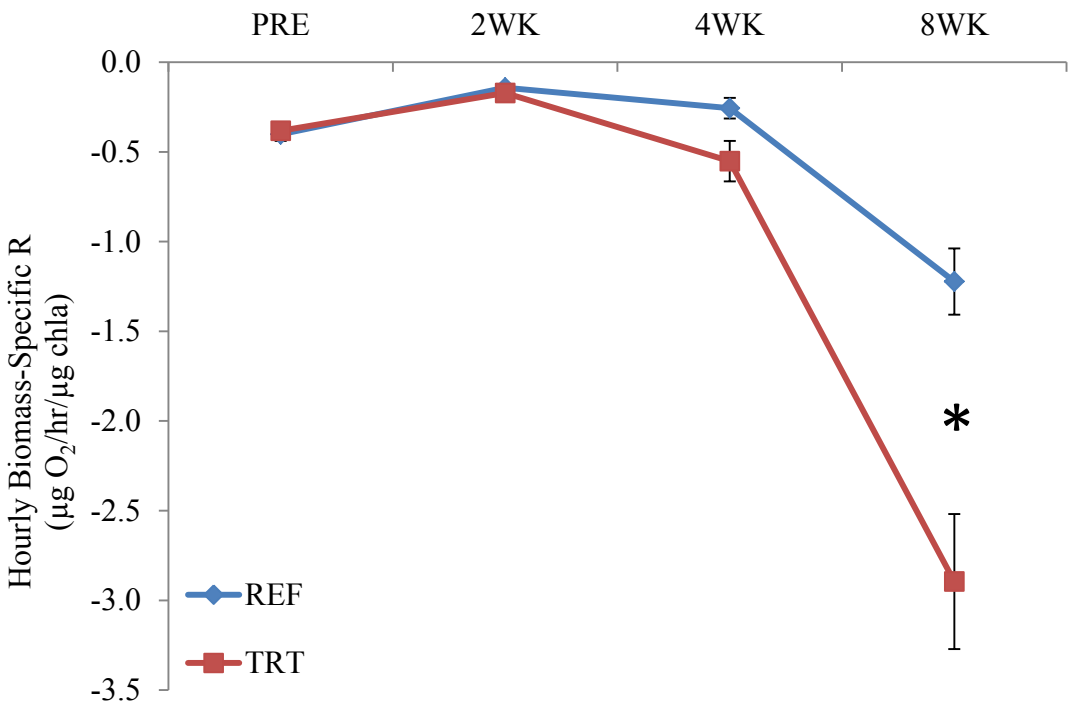

Figure 7. Patterns of mean ± SE hourly biomass-specific R (µg O_2/hr/µg chl-a) of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(3,32)}$=12.93, p=0.00001). R was significantly greater in TRT than REF at 8WK (F_1=57.00, p=5.37x10^{-8}).

Daily areal R patterns were significantly different between the REF and TRT reach throughout the study, with increases in the TRT reach and decreases in the REF reach, mirroring changes in daily areal GPP and changes in biomass (Time*Reach $F_{(2,24)}$=34.66, p=8.37x10^{-8}; Fig. 8). R was not significantly different between REF and TRT reaches before dosing (F=0.06, p=0.80), but R increased significantly more in the TRT reach relative to the REF reach by wk 4 (F=5.62, p=0.05) and remained greater by wk 8 (F=120.99, p=2.22x10^{-10}). Once again, the propagated error shown in Fig. 8 indicates that the differences at wk 4 may not be meaningful, but the differences by wk 8 appear to remain significant when accounting for biomass error.

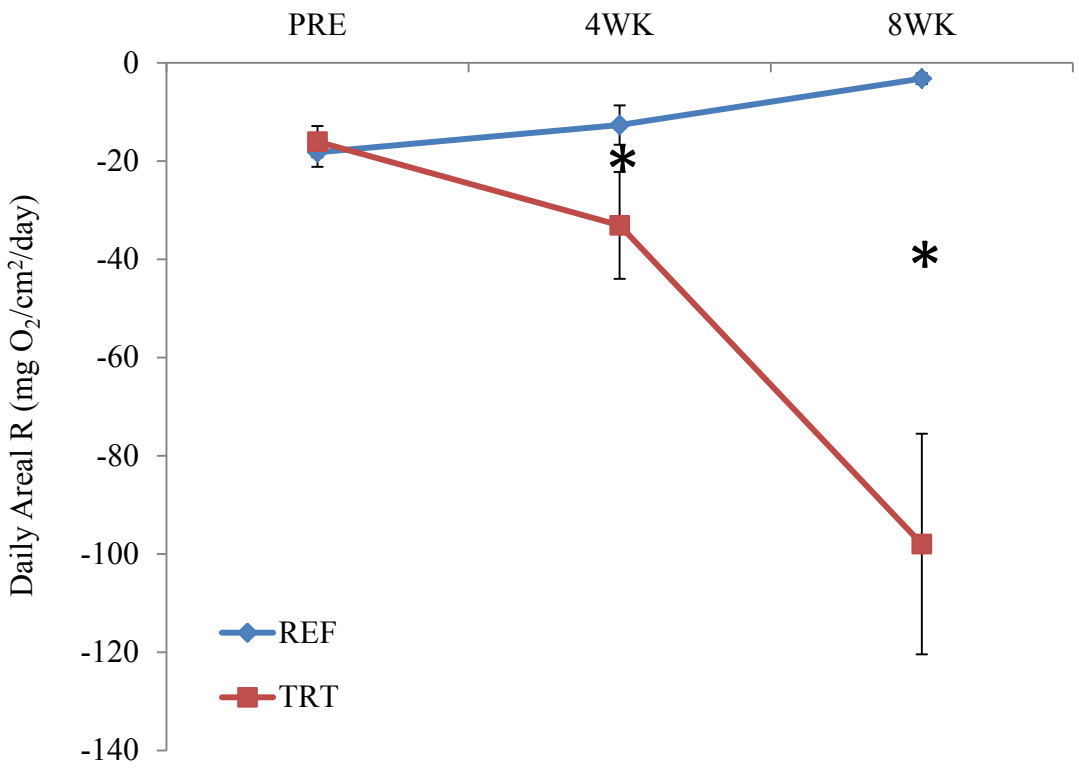

Figure 8. Patterns of mean ± propagated SE daily areal R (µg O_2/hr/µg chl-a) of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(2,24)}$=34.66, p=8.37x10^{-8}). R was significantly greater in TRT than REF at 4WK (F=5.62, p=0.05) and 8WK (F=120.99, p=2.22x10^{-10}). Error bars indicate propagated standard error based on error in chl-a estimates.

GPP:R

The pattern of hourly biomass-specific ratio of GPP:R change was significantly different between the REF and TRT reach, with the TRT reach GPP:R being significantly lower than REF (Time*Reach $F_{(3,32)}$=5.68, p=0.003; Fig. 9). GPP:R was not significantly different between the two reaches before dosing (F=0.06, p=0.79), but decreased significantly more in the TRT reach relative to the REF reach by 2 wks (F=20.14, p=0.0003), and continued to be different after 4 wks (F=22.37, p=0.0002) and 8 wks (F=17.93, p=0.0004).

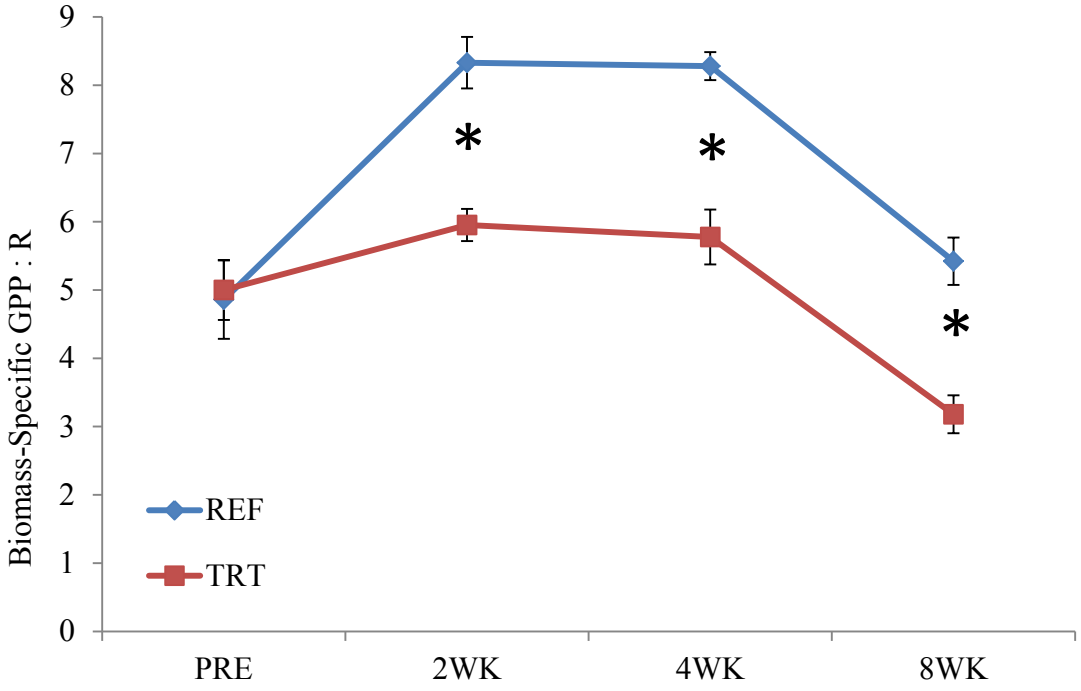

Figure 9. Patterns of mean ± SE hourly biomass-specific GPP:R of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(3,32)}$=5.68, p=0.003). GPP:R was significantly greater in REF than TRT at 2WK (F=20.14, p=0.0003), 4WK (F=22.37, p=0.0002) and 8WK (F=17.93, p=0.0004).

The patterns of daily GPP:R over the study were also significantly different between the REF and TRT reaches, the TRT reach declining relative to the REF (Time*Reach $F_{(2,24)}$=7.03, p=0.004; Fig. 10). GPP:R was not significantly different between the two reaches before dosing (F=0.03, p=0.80), but decreased significantly more in the TRT reach relative to the REF reach by wk 4 (F=23.92, p=0.0002), and remained significant by wk 8 (F=11.54, p=0.005).

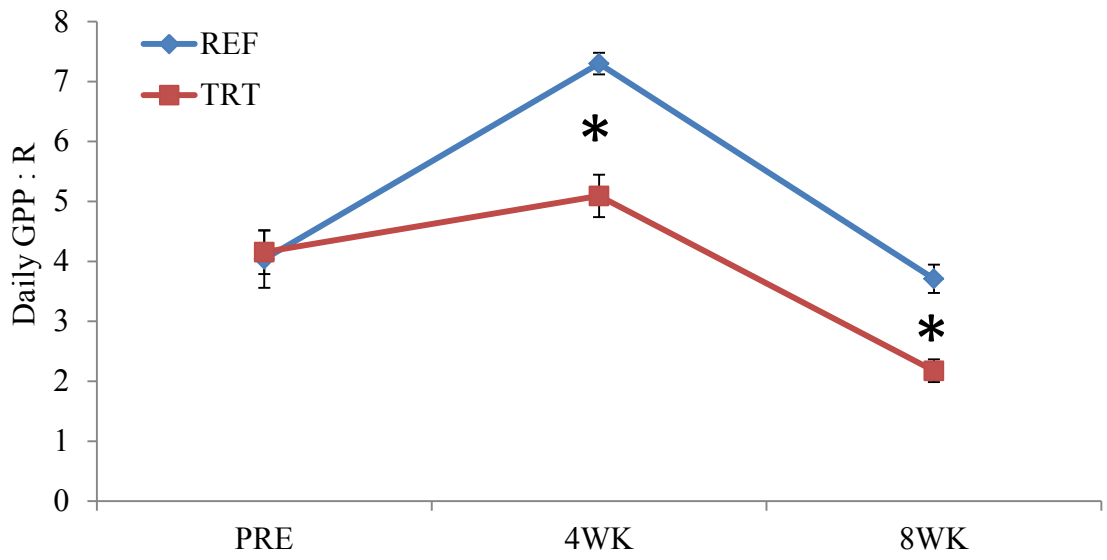

Figure 10. Patterns of mean ± SE daily GPP:R of periphyton on rocks at ANC-1203 in REF and TRT reach over time were significantly different (Time*Reach $F_{(2,24)}$=7.03, p=0.004). GPP:R was significantly greater in REF than TRT at 4WK (F=23.92, p=0.0002) and 8WK (F=11.54, p=0.005).

Discussion

Biomass

Periphyton biomass accumulation in streams is dependent on five main variables: nutrients, disturbance, grazing, temperature, and light (Francoeur et al., 1999). In the present study periphyton biomass, in both autotrophic (chl-a) and heterotrophic (AFDM) organisms, was greater in the DOC TRT reach relative to the REF reach. Biomass in the REF reach declined significantly over the study period, while biomass in the TRT reach remained constant.

AFDM, an estimate of total autotrophic and heterotrophic biomass, was preserved in response to DOC. Increased bacterial production has been seen in previous studies using various DOC sources (Bott et al., 1984; Findlay et al., 1993; Bernhardt and Likens, 2002; Sobczak and Findlay, 2002; Hasegawa et al., 2005; Klug, 2005; Wilcox et al.,

2005; Judd et al., 2006; Kritzberg et al., 2006; Stets and Cotner, 2008; Tanentzap et al., 2014). Bernhardt and Likens (2002) observed an increase in filamentous bacteria (*Sphaerotilus* sp.) specifically with their acetate addition of 5-7 mg/L. Other studies reported changes in bacterial community composition and function (Judd et al., 2006). Still others observed no change at all in bacterial abundance or diversity (Johnson et al., 2012). Fungi and macroinvertebrates have also been known to increase in abundance with supplemented DOC (Wilcox et al., 2005).

Autotrophic biomass (approximated by chl-a) was preserved as well in the present study. Previously, algal biomass responses varied greatly in relation to DOC additions, declining in concentration in some studies (Stets and Cotner, 2008), while increasing in others (Kiffney et al., 2000; Rier and Stevenson, 2002). Algal growth may have been partly enhanced by the increase in bacterial abundance, which explained 9-29% of variation in algal biomass and production in 51 Canadian streams (Carr et al., 2005). This positive relationship between algal and bacterial abundance is stronger under oligotrophic conditions (like those in Alaskan headwater streams) and was described previously as a mutual relationship dependent on the polysaccharide matrix in which the cells live and recycle nutrients (Rier and Stevenson, 2002; Carr et al., 2005; Scott et al., 2008). The recycling of these nutrients by the bacteria fuels further primary production, allowing for increases in algal biomass (Wetzel, 1993). Therefore, the C supplement, which fueled respiration in this study, likely caused an increase in nutrient cycling and a subsequent increase in microbial biomass in the stream.

C supplementation has been previously hypothesized to cause decoupling of this algae-bacteria relationship due to a decreased dependence of bacteria on algal exudates

(Scott et al., 2008). However, Rier and Stevenson (2002) believe bacteria remain dependent on algae as a colonization substrate even when their DOC needs are met by allochthonous resources. Thus, as DOC increases, bacterial biomass increases, which supports more nutrient recycling within a shared polysaccharide matrix, providing more nutrients to both algae and bacteria, explaining the increase in both AFDM and chl-a in the TRT reach relative to REF.

Our declining trend in both AFDM and chl-a in the REF reach was observed previously in a study of a 6^{th} order stream in Switzerland during the month of August (Naegeli and Uehlinger, 1997). The sharp decline in both chl-a and AFDM by wk 8 in the REF reach can be partially explained by increased shading due to growth of riparian vegetation, but more likely resulted from increased grazing pressure in the stream. If shading was the main cause of declines in biomass, these declines should have been seen in both the REF and TRT reach, as algae cannot photosynthesize when light is limiting, regardless of nutrient availability. Previous studies have shown that, in many cases nutrient additions made little to no difference in biomass accrual when light was a limiting factor (Larned and Santos, 2000; Mallory and Richardson, 2005; Von Schiller et al., 2007; Hill and Fanta, 2008).

Grazing pressure, on the other hand, may have been overcome in the TRT reach by increased biomass accrual in response to the C supplement, while in the REF reach biomass accrual may have been much slower, causing declines in the total biomass/cm^2 as grazing rates became faster than biomass accrual rates. Mallory and Richardson (2005) observed declines in chl-a and AFDM of 50 to 66% due to the presence of grazers in their streams, and AFDM biomass in the present study also declined approximately

62% in the REF reach from pre-dosing to 8 wks. Declines in chl-a were much greater (94%) during the same time frame, indicating that shading may have had some effect on the decline at least in the autotrophic portion of the periphyton. Though periphyton C:N ratios did not change in response to the C addition in this study, others have seen that periphyton nutrient ratios do not necessarily reflect stream nutrient ratios (Francoeur et al., 1999).

Our C supplement displayed a preservation effect on the biomass in our TRT reach, as biomass did not increase relative to pre-dosing, but remained constant rather than declining like the REF reach. Preservation of periphyton biomass likely will result in increased biomass in higher trophic levels, as previous studies have seen tight coupling of algal and bacterial biomass and the herbivores that consume them (Rosemond et al., 1993; Tanentzap et al., 2014).

Enzymes

Enzyme activity analysis in this study was unable to detect any true nutrient limitation throughout the summer. In a previous study, bacterial exoenzyme production responded more clearly to C additions than bacterial growth and R responded (Findlay et al., 2003b). Further, glucosidases, specifically βGLU, reacted faster and exhibited stronger responses to C additions than any other enzymes (Foreman et al., 1998; Findlay et al., 2003b). However, bacteria are capable of utilizing many different forms of OC, and as such produce a wide variety of C-acquiring enzymes (Carr et al., 2005). Perhaps our study did not capture the most prevalent enzyme used to cope with C limitation in this particular habitat.

Production of these different enzymes also depends on community composition, as not all bacteria can produce all enzymes, making it difficult to predict whether changes in limitation or species composition are driving changes in activity (Kirchman et al., 2004). In a previous study, βGLU activity actually increased in response to algal DOC additions (Findlay et al., 1997), indicating that activity may not directly correlate with C limitation. Additionally, enzyme activity is dependent upon the availability of both the labile ("simple") and recalcitrant ("complex") nutrient sources (Allison and Vitousek, 2005). If not enough cellulose is present to be broken down by βGLU, less of the enzyme will be produced so that the energy needed to produce it is not wasted. This scenario is unlikely, however, given the fact that these streams are dominated by *Calamagrostis canadensis* (bluejoint grass) along their banks, providing ample supply of cellulose molecules. The lack of βGLU activity does not necessarily indicate a lack of C-limitation in the system. Other responses to DOC found in this study, such as biomass accrual and metabolic changes, indicate that C was limiting periphyton production before dosing.

DOC also stimulates N and P uptake by bacteria (Stets and Cotner, 2008) while converting these nutrients to their unavailable organic forms (Findlay and Sinsabaugh, 1999; Stanley et al., 2012; Walker et al., 2012), leading one to believe that enzymes indicating limitation in these nutrients might increase upon C addition. NA activity remained non-existent throughout the summer, likely indicating that alder trees supply highly saturating levels of bioavailable N to the stream. The increased uptake and conversion to organic forms of this nutrient due to C additions did not appear to deplete

the highly abundant stores that were constantly being replenished by inflowing groundwater.

APA, on the other hand, did significantly increase in the TRT reach relative to the REF reach, indicating a slight decline in P availability relative to demand. However, this change was very slight and may not be biologically significant. Increased periphyton biomass may have caused a larger P demand in the TRT reach than in the REF reach, without a subsequent increase in P supply (Stevenson et al., 1996). P usually is abundant in lower quantities than is demanded by an ecosystem, thus causing bacteria to outcompete the larger and less efficient algal cells for this limiting resource (Klug, 2005). However, bacteria usually rely on algae as a major C source, creating incentive for bacteria to reduce their hold on the P stores when it is limiting. In environments where allochthonous DOM inputs are high, the dependence of bacteria on this autochthonous algal DOC may be reduced, leading to decoupling of the loop and higher bacterial P uptake (Klug, 2005). Increased bacterial uptake in addition to the constant algal uptake could cause a slight decline in total P stores in the system, leading to increased APA activity.

Metabolism

Hourly biomass-specific GPP rates ranged from 1.01 to 8.94 μg O_2/μg chla/hr throughout this study. This range is slightly larger than that found in another Alaskan stream metabolism study, which displayed a range of 0.35 to 6.14 μg O_2/μg chla/hr (Arscott et al., 1998). Hourly biomass-specific R rates also displayed a slightly larger spread in our study, from -0.14 to -2.90 μg O_2/μg chla/hr compared to -0.101 to -1.18 μg O_2/μg chla/hr in the previous study (Arscott et al., 1998). Arscott et al. (1998) found that

riffle epilithon communities exhibited higher rates of GPP and R than pool epilithon. Though all of our sites were riffles, perhaps the high variability in flow over time at these small headwater streams contributed to our larger range of measurements for both GPP and R. Headwater streams are fairly heterogeneous in nature, and despite infrequent disturbances, these disturbances have lasting impacts on the affected areas (Richardson et al., 2005). Any disturbances due to varying flow may have affected metabolic activity in the stream.

Hourly biomass-specific GPP and R increased in both REF and TRT reaches throughout the study period, possibly due to increasing water temperature over the course of the summer. Increases in temperature at low temperature environments have caused more dramatic increases in both of these processes than temperature increases in higher temperature environments (Staehr and Sand-Jensen, 2006).

Gross primary production (GPP). Though production is correlated with chl-a concentration (Kalff, 1967), which increased in response to DOC, hourly biomass-specific GPP did not change in the TRT reach relative to the REF reach. This lack of increase in hourly biomass-specific GPP may be due to shading both in the stream and within the periphyton itself over time. In a long term study of GPP and R in a headwater stream in Tennessee, 84% of the variation in GPP was explained by light levels alone (Roberts et al., 2007). Since our metabolism assays were conducted under saturating light levels, light-limitation is likely not what prevented increases in GPP in our chamber assay, though it could have impacted GPP on a whole-stream scale. "Self-shading" within the periphyton caused by increased thickness of the biofilm sometimes causes decreased production in response to biomass accrual (Stevenson et al., 1996). However,

increased grazing pressure may reduce the effects of light limitation on these biofilms (Stevenson et al., 1996). Interaction between light-limitation reducing GPP and grazing enhancing GPP may have resulted in no net change in overall activity.

Though biomass-specific production can respond negatively to biomass increases due to self-shading, total areal GPP almost always increases when biomass increases (Stevenson et al., 1996). Accounting for the increase in average chl-a/cm^2 in the TRT reach relative to REF resulted in a significant increase in daily stream GPP per reach area in the TRT relative to the REF reach. Johnson et al. (2012) found that whole stream GPP did not respond to C additions, but algal biomass was not estimated as part of this study, it was conducted in a higher nutrient environment and the C addition lasted only 5 days. Additionally, whole stream GPP may be affected differently than periphyton GPP, as other photosynthesizers (phytoplankton and aquatic macrophytes) are included in whole stream estimates.

Caution should be taken when considering the GPP estimates in this study, as many indirect effects that may have been caused by natural DOM additions were not considered in this study. As stated previously, DOM influences many aspects of stream water chemistry other than C availability to the biota (Stanley et al., 2012). One of the most notable influences that DOM has is its ability to alter optical properties of the water, diminishing light penetration to the streambed (Stanley et al., 2012). The acetate supplement that we provided in this study did not have the optical properties of natural DOM that would have stained the waters and potentially reduced light availability for photosynthesis. Thus, our estimates of hourly biomass-specific and daily areal GPP may

have been exaggerated under our light-saturating conditions compared to rates that would be seen in the natural environment.

Respiration (R). Hourly biomass-specific R significantly increased in the TRT reach with C additions. Respiration, as all metabolic activities, is limited by the amount of substrate (in this case, labile C) available for the reaction (Wang et al., 2003). Thus, adding more bioavailable C to the stream resulted in increased respiratory activity of individual cells in the periphyton. Since R is not limited by light, as is GPP, varying light conditions throughout the study would not have prevented R activity. Due to significant increases in chl-a in the TRT reach relative to the REF reach, the increase in R was even greater when extrapolated to daily areal measurements.

In previous studies, C additions of varying compositions have resulted in increased R by 60 to 600% (Foreman et al., 1998; Strauss and Lamberti, 2000; Bernhardt and Likens, 2002; Strauss and Lamberti, 2002; Findlay et al., 2003b; Wilcox et al., 2005; Johnson et al., 2012). Total OC in Australian soils explained 75-81% of variance in microbial R (Wang et al., 2003). The non-accounted for variation was attributed to soil chemistry, microbial biomass, and moisture. In a study of Scottish woodland and pasture soils, soil DOC was positively related to soil microbial R and explained 85% of variance in this activity (Fang and Moncrieff, 2005). In the same study, microbial biomass alone sufficiently explained 95% of the variation in soil microbial R. Thus, our DOC addition, which stimulated increases in microbial biomass, fueled large increases in R in the TRT reach of our stream. DOC provided not only a substrate for R, but increased productivity in the biomass, resulting in further increased activity.

GPP:R. GPP:R ratios in this system declined significantly in response to DOC due to the larger increases in both hourly and daily R relative to increases in GPP in the TRT reach. Allochthonous resources provide energy to the stream without taking energy out of the stream to support their growth, unlike energy-consuming autochthonous resource production (Fisher and Likens, 1973). However, allochthonous resources do reduce both oxygenation and light penetration in the stream, causing potential stress to both producers and consumers (Fisher and Likens, 1973). Fisher and Likens (1973) value "maturity" of an ecosystem by its "efficiency" to process allochthonous resources. Thus, an ecosystem with a smaller GPP:R ratio (closer to 1:1) may be more efficient at processing these energy-providing resources and therefore may be more mature. Increasing autotrophy in a system may actually slow the developmental process.

Our GPP:R ratios in this study reflect the benthic epilithon only and do not take into account other organisms such as plankton, macrophytes, macroinvertebrates and fish, which all contribute significantly to whole-stream GPP:R ratios. In a study of four Texas reservoirs, planktonic production made up 80% of production in the whole reservoir, but planktonic R accounted for only 33% of the whole reservoir R (Huang, 2006). Further, in a pre-alpine, 6^{th}-order gravel-bed river in Switzerland, periphyton consisting mainly of algae contributed only 4-19% to whole-stream R (Naegeli and Uehlinger, 1997). Hyporheic R, on the other hand, may be a very significant contributor to whole stream R (Naegeli and Uehlinger, 1997). Sampling only a subset of an ecosystem may under- or overestimate the actual GPP:R ratio of that system's entirety (Huang, 2006; Forbes et al., 2012). In this case, since most headwater streams are regarded as heterotrophic environments, our positive GPP:R ratios found in this study may overestimate production

throughout the stream. A whole-stream estimate of GPP:R ratios likely would find significant contributions to R from some of the larger heterotrophic organisms, such as macroinvertebrates and salmonids. A concurrent whole-stream metabolism study was conducted to complement these periphyton-specific metabolism measurements.

Conclusions

Based on this study and concurrent studies observing major responses of periphyton, aquatic macroinvertebrates and salmon to relatively small, realistic DOC supplements, DOC may be an undervalued driver of stream productivity. Thus, resources providing such a product should be considered when managing these ecosystems. Further, wetlands should be considered important C sources to headwater streams, as the amount of labile DOC that they provide has shown tremendous changes in the stream community. Bacterial and algal biomass, R and GPP all increased in this stream upon C addition, indicating higher productivity of the periphyton and signifying relief from a resource limitation stress. This information should be used to support protection of wetlands in land management plans.

Previous studies have found alteration in composition, concentration and bioavailability of stream DOC and DOM as a result of land use changes in the watershed (Findlay and Sinsabaugh, 1999; Findlay et al., 2001). Specifically, Findlay and Sinsabaugh (1999) pointed the blame towards hydrologic flowpath manipulations and wetland destruction. In the Pine Barrens of New Jersey, the bioavailability of DOC in cedar bog wetlands was reduced by 10% in polluted bogs compared to pristine bogs (Wiegner and Seitzinger, 2004), providing further evidence of the unidentified impacts land use changes might have on the environment. This catchment scale issue is most

often handled at only a stream channel scale (Stanley et al., 2012), which leads to ineffective restoration efforts. Stanley et al. (2012) proposes riparian restoration and wetland management as a compromise between protecting the entire watershed and protecting only the stream itself. Thus, management of landscape features such as wetlands in the catchments of Alaskan headwater streams may help maintain healthy, natural ecosystems, support higher productivity and reduce the need for artificial nutrient supplementation to these streams.

CHAPTER THREE

Low-Level Nitrogen Impacts on Periphyton in Alaskan Headwater Streams

Abstract

Alaskan headwater streams are ecologically and economically important habitats for juvenile salmon. Due to low input of marine-derived nutrients, stream communities rely on allochthonous nutrients, such as wetland-derived carbon and alder-fixed nitrogen. An Alaskan headwater stream with high wetland drainage (high dissolved organic carbon) but little alder cover (low dissolved inorganic nitrogen (DIN) was supplemented for eight weeks with 0.15 mg/L DIN in order to assess the importance of this resource. Periphyton biomass, enzyme activity and metabolism were monitored in the treatment and reference reaches throughout the summer. Data were highly variable among and within stream reaches before dosing began, making results challenging to interpret. Only biomass exhibited clear differences after dosing, but changes occurred in the reference reach rather than treatment reach, indicating dosing may not have been the cause. No significant and interpretable responses were observed in enzymes or metabolism. Though strong recommendations for landscape management cannot be made from the current study, a C addition study in an opposing landscape high in alder cover indicated a C-limited system free of N-limitation, supporting the importance of this landscape feature for stream nutrient dynamics. Future studies are recommended in order to better understand the potential importance of alder-derived nitrogen.

Introduction

Nitrogen (N) is an essential nutrient for protein and nucleic acid synthesis, and may also regulate different functional aspects of development and growth, such as gene activation and sexual processes (Sigee, 2005). Though N and phosphorus (P) have recently been stated as being equally limiting across ecosystems (Elser et al., 2007), N-limitation alone is believed to be uncommon without simultaneous P-limitation in freshwater environments (Francoeur, 2001; Rabalais, 2002). The dominance of P-limitation in these ecosystems is partially due to the ability of some organisms to fix atmospheric carbon (C) and N, replenishing dwindling stocks (Schindler, 1977). No such mechanisms exist for P acquisition, leading to a dependence of organisms on terrestrial inputs of P (deriving mostly from minerals in rocks), release of P buried in sediment, and recycling via the biota (Sigee, 2005).

However, the Kenai Peninsula in Alaska is infrequently P-limited due to high P contributions from volcanoes, glaciers and permafrost (Eicher and Rounsefell, 1957; Hobbie et al., 1999). Thus, of all freshwater systems, streams in this region are some of the most likely environments to display N-limitation without P-limitation. Some streams on the peninsula, however, also have additional N sources in the form of alder trees in their catchments. These alders maintain a symbiotic relationship with N-fixing bacteria in their root nodules, and the N fixed by bacteria makes its way to the stream via groundwater, surface waters and leaf litter (Bond, 1956; Shaftel et al., 2012). So, how do the streams without this extra boost of N compare? Previous studies have shown that catchments with low flow-weighted slopes contain high proportions of wetlands which contribute to DOC to the stream, but have very little alder cover (Shaftel et al., 2012;

Walker et al., 2012). As wetlands provide DOC to these streams and P is naturally high from terrestrial inputs, perhaps alder-derived N contributions could be a significant resource to reduce N-limitation and fuel stream productivity.

In order to assess the above prediction that alder in Alaskan headwater stream catchments may relieve N-limitation, we looked at changes in periphyton microbial activity in response to low-level dissolved inorganic N (DIN) supplements to a stream with high wetland cover and low alder cover (high C, low N). Previous research found that DIN, which is bioavailable, may be limiting to algal productivity, while the organic fraction has little impact (Francoeur et al., 2003). Periphyton was chosen as our indicator of change due to its rapid reaction rate, discrete spatial distribution, and its role as the assimilator of nutrients into the biota of a stream community (Stevenson et al., 1996; Findlay, 2010).

Periphyton activity indicators used in this study include biomass accrual, enzyme activity, and metabolism. Increases in biomass upon N addition would indicate relief from a previously N-limited environment. Specific nutrient-acquiring enzymes (beta-glucosidase, nitrogenase and alkaline phosphatase) were selected, due to their importance in organic matter decomposition and nutrient recycling and primarily to signify limitation of C, N and P, respectively (Asmar et al., 1994; Carreiro et al., 2000; Allison and Vitousek, 2005; Hill et al., 2006; Hill et al., 2010; Hill et al., 2012). Increases in any of these enzymes after N addition would indicate increases in limitation of the specific nutrient, while decreases would indicated relief from limitation. Gross primary production (GPP) and respiration (R) were also assessed in order to gain a sense of energy input and recycling changes due to the N supplement. Increases in both

components were expected upon N addition if these processes were limited due to lack of N. This project was conducted concurrently with studies measuring changes in higher trophic levels, so that the contribution of microbial responses to the macroinvertebrates and salmonids in the stream might be observed.

This study aims to be the most extensive view of long-term, whole-stream, low-level DIN manipulation effects on a stream ecosystem. Due to the low biomass in Alaskan headwater streams, a longer time period was used in order to better capture increases in biomass due to our nutrient addition. Changes will occur slower due to the smaller amount of cells replicating. Alternately, this low biomass, which is caused by an environment low in nutrients, is likely to respond more drastically to nutrient additions due to their relative starvation (Bilby et al., 1996). As such, realistic responses will only be observed if nutrient additions are low enough to simulate real increases in nutrients due to landscape features such as wetlands. Higher additions may provide unrealistic results in the study.

Many whole-stream nutrient additions have focused on a combination dosing of N and P (Hillebrand and Kahlert, 2001; Rier and Stevenson, 2002; Romanı et al., 2004; Mineau et al., 2013; Nelson et al., 2013), and cannot interpret changes due solely to N additions. Of those studies focusing only on N, all have either dosed at much higher levels (Mosisch et al., 1999; Francoeur et al., 2003; Von Schiller et al., 2007) or much shorter time periods (Mosisch et al., 1999; Larned and Santos, 2000; Francoeur et al., 2003; Von Schiller et al., 2007; Mineau et al., 2013). Thus, to our knowledge, the current combination of in-situ low-level single-nutrient N supplementation over a long study period has never been attempted in streams.

Materials and Methods

Study Site

This study takes place on the lower Kenai Peninsula lowlands to the west of Kachemak Bay in Alaska. Shaftel et al. (2011), Walker et al. (2012), and Dekar et al. (2012) describe in detail the geomorphic setting, vegetation, and climate of the Kenai Peninsula. Briefly, this area contains mostly relatively undeveloped landscapes of mixed spruce, birch, willow and alder forests in combination with fireweed and bluejoint grass meadows and wetlands. Annual precipitation is ~13-18 cm/yr and average minimum and maximum temperatures range from -8.5 to 16.1˚C.

The study stream was chosen based on extensive information gathered and models created from previous research in the area (Shaftel et al., 2011; Dekar et al., 2012; King et al., 2012; Kostka, 2012; Shaftel et al., 2012; Walker et al., 2012; Whigham et al., 2012). Kostka (2012) describes the specific stream in more detail. Briefly, Stariski-171 (STAR-171) is a first-order tributary of the Stariski River dominated by cobble and gravel substrate with interspersed fine organic matter. This stream was chosen based on its high wetland cover, associated with low flow-weighted slopes (FWS), and absence of alder cover (0%), leading to a high dissolved organic matter (DOM) and low DIN content in the stream. Despite the vast amount that is known about this stream and others in the area, a whole-stream nutrient manipulation study has never been attempted in this region.

Treatment (TRT) and reference (REF) reaches for the stream were chosen based on proximity, accessibility, and, most importantly, similarity in physical environment. Both reaches extend 75 m down the stream channel, with the REF reach ending approximately 80 m upstream of the dosing station and TRT reach beginning

immediately downstream of the dosing station. The REF reach had a slope of 0.99% and sinuosity of 1.24, while the TRT reach had a slope of 1.15% and sinuosity of 1.25 (Fig. 11).

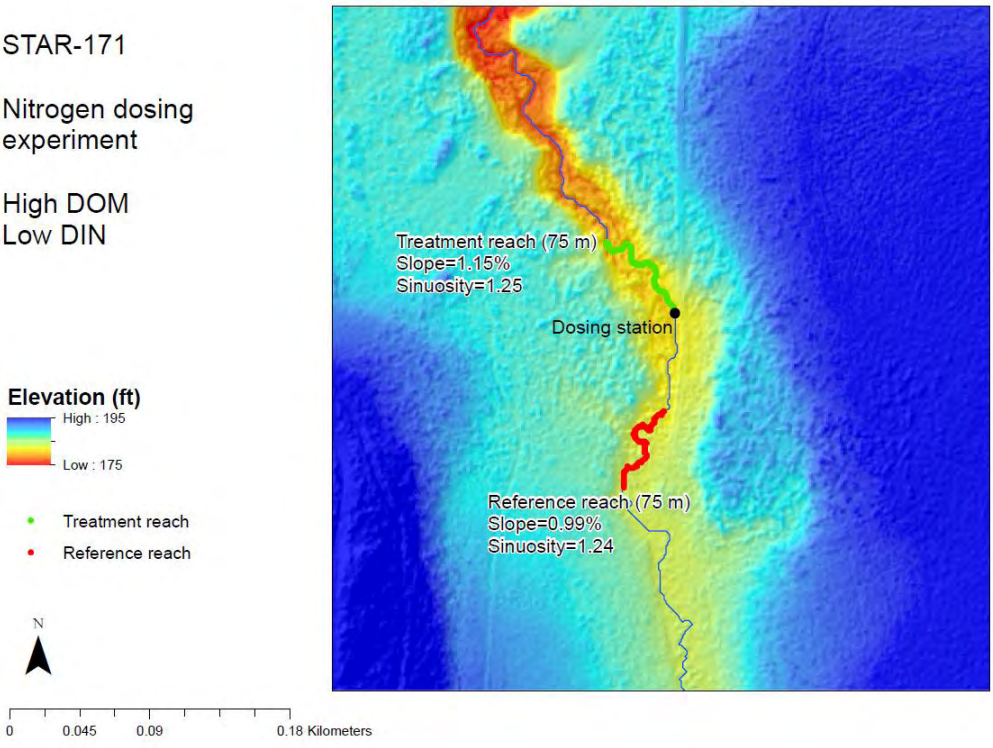

Figure 11. Elevation map for a first order tributary of the Stariski River (STAR-171), a stream on the Kenai Peninsula lowlands, Alaska, with no alder cover and high wetland cover resulting in high DOM and low DIN. The treatment reach was dosed with 0.15 mg/L DIN (nitrate and ammonium) 17 June 2013 to 18 August 2013.

Dosing

We set up a dosing station 2 m from the stream with a carboy of stock solution contained within a ditch lined with a plastic tarp both below and above the stock tank to minimize disturbance and potential leakage of the stock solution to the environment. Polyvinyl chloride (PVC) tubing led from the carboy directly to the stream and the solution was pumped at a rate of 0.025 L/min. We dosed the TRT reach with DIN of two

parts nitrate, one part ammonium for a concentration of +0.15 mg/L from 17 June 2013 to 18 August 2013. We added a bromide tracer of +0.05 mg/L to the stock solution to assure proper mixing and to account for dilution due to groundwater inputs and transient storage.

Water Chemistry

We monitored water chemistry in the REF and TRT reaches weekly both before and after dosing the stream. We collected triplicate stream water samples of 250 mL each for analysis of phosphate (PO_4-P), ammonia (NH_4-N), nitrate (NO_3-N), total dissolved phosphorus (TDP), dissolved organic phosphorus (DOP), total dissolved nitrogen (TDN), dissolved organic nitrogen (DON), dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), and bromide (Br^-). We also collected triplicate water samples of 50 mL each for total nitrogen (TN) and total phosphorus (TP). All samples were frozen and shipped to the lab at Baylor University for measurement on a flow-injection auto-analyzer (Lachat QuikChem 8500 and Series 520 XYZ Autosampler) and a Shimadzu TOC 5-5-analyzer. We collected triplicate water samples of 15 mL each and acidified them with 30 µl sulfuric acid (H_2SO_4) for ammonium (NH_3) analysis on a spectrophotometer. We measured discharge weekly in conjunction with water collections using a salt tracer and a YSI EXO 1 datasonde (Yellow Springs Instruments, Yellow Springs, Ohio).

Sampling Schedule

The study was conducted during the summer of 2013, which is the most common season for freshwater systems to be N-limited (Conley, 2000). All biomass estimates and

enzyme activity assays were conducted using rocks from 3 stations [upstream (UP), middle (MID), downstream (DWN)] in the REF and TRT reaches. Metabolism assays were conducted only at the UP station in both reaches. Pre-dosing assays were conducted in the REF and TRT reach one wk before dosing began (10-14 June 2013). Post-dosing assays were conducted 4 wks (14-20 July 2013) and 8 wks (11-17 August 2013) post-dosing in both the REF and TRT reaches for all assays. Additionally, metabolism was measured at 2 wks (1-6 July 2013) in order to capture any immediate responses to dosing. Al enzyme assays that clearly showed no change in activity after wk 4 were discontinued in order to save time and resources. Rocks for these assays were still collected in order to maintain sample sizes for biomass estimates.

Biomass

Biomass measurements were taken from all rocks used in enzyme assays. Rocks were placed in a refrigerator after assays were complete and scraped into separate beakers within one wk using paint scrapers and toothbrushes to remove biomass. Scrapings were then diluted to a known volume using deionized water and homogenized using a hand blender if necessary. A subset of the homogenized slurry was filtered onto glass fiber filters (GFF) for chlorophyll *a* (chl-a) and ash-free dry mass (AFDM) analysis. All filters from slurries were wrapped in aluminum foil and frozen until analysis.

AFDM filters were combusted at 500˚C for two hours and weighed before slurries were filtered through them. AFDM filters were then dried at 60˚C for >24 hrs, placed in a dessicator, weighed, and combusted at 500˚C for one hour. After combustion, filters were placed in a dessicator to cool, and weighed once more. Organic carbon content was calculated as the material lost in combustion (difference between non-combusted and

combusted weights) extrapolated back to the entire slurry volume and normalized to surface area of the rock. Chl-a filters were extracted in 10mL of ethanol, placed in a 78˚C water bath for 5 minutes, and kept in the fridge in the dark overnight. Absorbance was measured using a spectrophotometer at 665 nm wavelength for chl-a. Values again were extrapolated to the entire slurry volume and normalized to surface area of the rock. All biomass estimate methods were derived from Biggs and Kilroy (2000).

Rock surface area was measured by covering tops of dry rocks in aluminum foil and weighing the aluminum foil. This weight was converted to square centimeters of surface area using a standard curve. Composite slurries of all rocks from a single station on a sampling date for each assay were dried at 60˚C in plastic weigh boats, scraped, placed in tin capsules and analyzed on an Elemental Analyzer (EA) for C:N of the periphyton. Rocks from metabolism assays were not included in biomass statistical analyses, as these rocks were only collected at the UP station of each reach and may have biased our results toward that location.

Enzymes

Four replicate rocks were collected at each station (UP, MID, DWN) in both the REF and TRT reaches for alkaline phosphatase (APA) and beta-glucosidase (βGLU) assays. βGLU is the most common glucose stereoisomer found in nature and is used to break down cellobiose and cellodextrins in leaf matter into glucose molecules that are readily taken up (Dunn et al., 2014). APA hydrolyzes phosphoric acid monoesters to cleave phosphate groups from organic substrates (Dunn et al., 2014). Enzyme activity of the two microbial exoenzymes was measured fluorometrically. In these fluorometric assays, the acting enzyme (βGLU or APA) metabolizes the substrate added to the sample

(methylumbelliferyl-glucopyranoside or methylumbelliferyl-phosphate), forming a fluorescent product (methylumbelliferyl (MUF)) that can then be measured on a fluorometer once the desired nutrient is cleaved (Dunn et al., 2014). The rate of fluorescent increase indicates accumulation of MUF and is used as a measure of enzyme activity. This method is recognized as the best measurement of enzyme activity in stained wetland waters and waters of low activity like those used in this study (Freeman et al., 1995).

Each incubation jar contained 20 mL filtered stream water, 60 mL TRIS buffer (pH 10), a periphyton rock, and 8 mL of substrate (0.5 mM 4-MUF-β-D-glucopyranoside for βGLU or 0.5mM 4-MUF-phosphate for APA). One control sample for each station within each reach (6 total) was used to account for background activity by combining filtered stream water, TRIS and substrate. We measured fluorescence every 10 minutes after substrate was added, until 30 minutes passed. The slope of the relationship between fluorescence and time was interpreted as the rate of enzyme activity (after subtracting controls). β-GLU and APA were normalized to AFDM, as they are believed to be enzymes produced mainly by bacteria (Chróst, 1989; Cunha et al., 2010).

Six replicate rocks were collected at each station (UP, MID, DWN) in both reaches (TRT and REF) for estimation of nitrogenase activity (NA). Nitrogenase is an enzyme used to break the strong triple bond between atmospheric N atoms in order to produce bioavailable ammonium (NH_4^+) for uptake. We used the acetylene-reduction assay to measure NA. In this assay, acetylene is converted to ethylene by the nitrogenase enzyme, and ethylene production is used as a measure of enzyme activity. Incubations occurred in 250 mL Mason jars equipped with septa for syringe access and filled

completely with filtered stream water and one periphyton rock. One control jar from each station (6 total) contained only filtered stream water, and one dark jar from each station (6 total) covered with aluminum foil contained filtered stream water and one rock.

All jars (including controls and darks) were inoculated with 40 mL of acetylene gas, generated by adding a few bricks of CaC_2 to 400 mL of deionized water in a 1 L cubitainer equipped with a septum top for syringe access. After water was saturated with acetylene gas, the remaining bubble was removed using a syringe and replaced with filtered stream water from the associated station. Incubations ran overnight (~10-14 hr) in a water bath with consistent high light (~325 $\mu E/m^2/s$) and temperature (13-20°C). Though incubation time and temperature varied, all samples from one sampling event were treated identically. Due to equipment restrictions, temperature was not able to be constant, and varied throughout the incubation.

At the end of the incubation, a subsample of 5 mL of water was taken into a 10ml syringe and equilibrated with 5 mL of air for one minute, shaking vigorously. The headspace in this syringe was collected in a 4 mL blood serum vacutainer and ethylene produced was measured by an Agilent Technologies 7890A gas chromatograph with a flow rate of 40 mL/min and oven temperature of 85˚C. NA activity was normalized to chl-a concentrations, as it is mainly associated with photosynthetic organisms such as cyanobacteria (Chróst, 1989; Cunha et al., 2010).

Metabolism

Light and dark incubations were used to determine biomass-specific (per µg chl-a per hr) and daily areal estimates (per cm^2 per day) of gross primary production (GPP) and respiration (R). We collected five replicate rocks at the UP station in both the REF and

TRT reach. The oxygen change method was used to determine metabolism of periphyton-covered rocks enclosed in 250 mL Mason jars with plastic BOD bottle top adapters fastened using welding putty and sealed with rubber stoppers. With the exception of the pre-dosing assay, filtered stream water from each reach was bubbled with nitrogen gas before filling jars in order to decrease oxygen levels to ~3 mg/L and prevent oversaturation. One control jar for each station (2 total) contained filtered stream water of the respective station with no periphyton rock in order to account for changes due to non-filtered particles or contamination.

Each rock was incubated under no light (0 $\mu E/m^2/s$), low light (160-180 $\mu E/m^2/s$), and high light (300-350 $\mu E/m^2/s$) conditions in the lab, with a 1 hr acclimation period between each light treatment. Incubations with no light ran overnight in order to ensure enough change in oxygen occurred due to R. Light treatment incubations continued until a significant change in dissolved oxygen (DO ± 0.5 mg/L) was observed in all replicates except controls (~1-2 hrs). All assays were completed in ~24 hrs with the exception of the pre-dosing assay, which extended 3 days due to the higher oxygen levels at the start, and subsequent need for further reduction via dark incubations. This assay was additionally conducted with rocks from all 3 stations (UP, MID, DWN) in each reach, adding to sampling time. The data from these other stations will not be presented here, as this sampling method was not continued through the other sampling times.

Biomass-specific net primary production (NPP) rates were calculated by dividing DO change by incubation time in the light, after accounting for the total water volume in the jar. Rates were normalized to controls and then to total chl-a rather than AFDM due to the necessity of chl-a for photosynthesis. Dark incubation rates were used as R

estimates, and GPP was calculated by subtracting R rates from NPP rates. These hourly biomass-specific rates (per μg chl-a per hr) were extrapolated to a daily areal scale (per cm^2 per day) by multiplying by the average chl-a (per cm^2 of the appropriate Reach and Time) and multiplying GPP by total daylight hrs and R by 24 hrs. Light data was acquired from the Estuarine Reserves Division (ERD), Office of Ocean and Coastal Resource Management (OCRM), National Ocean Service, National Oceanic and Atmospheric Administration (NOAA)).

No significant differences in NPP were observed between the low and high light treatments, so only high light measurements were used in analyses. Unfortunately, because of this light-saturating response seen in the low light treatment, we were unable to estimate alpha values of light acclimation in this study. The method used may overestimate GPP, but this error should be small due to the low light saturation of benthic algae in general and specifically in Alaskan light-limited species (Gray and Hill, 1995). Additionally, the longer than average daylengths make the time period of acclimation negligible over the entire day.

Data Analysis

All enzyme assays and biomass accrual were analyzed using R Statistics Software nlme package for mixed effects models with Time (PRE, 4WK, 8WK) and Reach (REF vs TRT) as fixed effects and Station (UP, MID, DWN) as a random effect. Results focus on the interaction term (Time*Reach), as this indicates a significant difference in the overall pattern of activity between the REF and TRT reach during the course of the dosing. The package phia was used to evaluate post-hoc comparisons of this interaction term based on a Chi-squared distribution of the data. Post-hoc comparisons examined

significance between the REF and TRT reaches at any given Time. Metabolism data were analysed using R Statistics Software ANOVA with Time (PRE, 2WK, 4WK, 8WK) and Reach (REF and TRT) as the independent variables, since only the UP station was sampled. The package phia again was used to evaluate post-hoc comparisons of the interaction term Time*Reach, but these tests were based on an F distribution rather than Chi-squared. Again, individual post-hoc comparisons evaluate differences in REF and TRT at a given Time.

Results

Water Chemistry

Water chemistry results indicated that our dosing raised DIN levels to near target concentrations (0.15 mg/L) in the TRT reach. The data are still being processed and interpreted.

Biomass

Patterns in both chl-a and AFDM were significantly different between the REF and TRT reaches (Reach*Time $F_{(2,245)}$=8.03, p=0.0004 and Reach*Time $F_{(2,254)}$=6.54, p=0.002, respectively; Figs. 12 & 13). Chl-a was not significantly different between the REF and TRT reach before dosing (χ^2=3.94, p=0.09) or after 4 wks (χ^2=0.31, p=0.58). After 8 wks, REF chl-a increased and was significantly greater than TRT chl-a, which did not change (χ^2=35.57, p=7.40x10^{-9}). AFDM, on the other hand, was significantly different between the REF and TRT reach before dosing, with REF having greater biomass than TRT (χ^2=8.26, p=0.004). This difference became more significant after 4 wks (χ^2=29.76, p=9.77x10^{-8}) and 8 wks (χ^2=51.08, p=2.67x10^{-12}) as REF biomass

increased and TRT biomass remained constant. Patterns in C:N ratios were not significantly different between the REF and TRT reach (Reach*Time $F_{(2,50)}$=1.25, p=0.30).

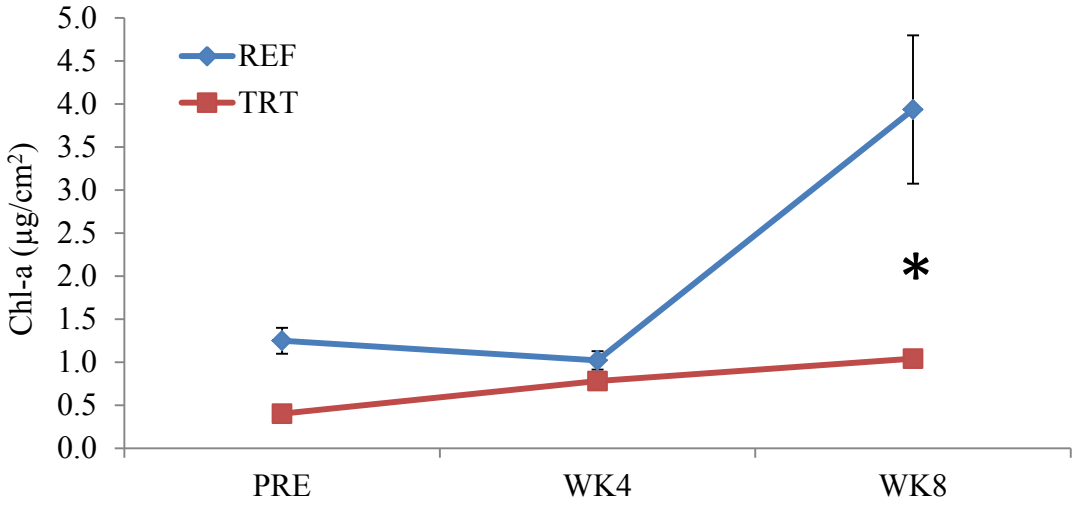

Figure 12. Patterns of mean ± SE chl-a (µg/cm^2) of periphyton on rocks at STAR-171 in REF and TRT reach over time were significantly different (Reach*Time $F_{(2,245)}$=8.03, p=0.0004). Chl-a was significantly greater in REF than TRT at 8WK (χ^2=35.57, p=7.40x10^{-9}).

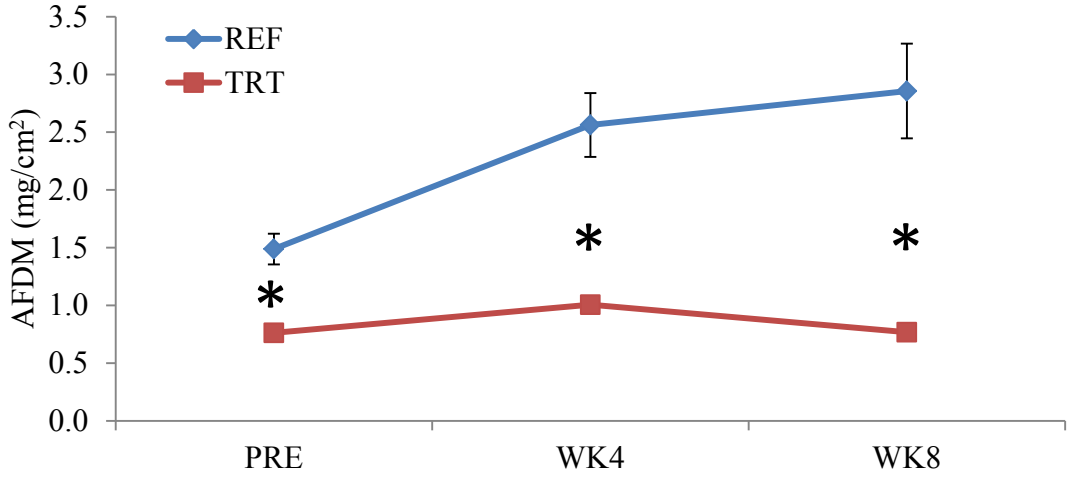

Figure 13. Patterns of mean ± SE AFDM (mg/cm^2) of periphyton on rocks at STAR-171 in REF and TRT reach over time were significantly different (Reach*Time $F_{(2,254)}$=6.54, p=0.002). AFDM was significantly greater in REF than TRT both before (χ^2=8.26, p=0.004) and after dosing (χ^2=29.76, p=9.77x10^{-8}; χ^2=51.08, p=2.67x10^{-12}).

Enzymes

Patterns in NA were not significantly different between the REF and TRT reach (Reach*Time $F_{(2,82)}$=1.37, p=0.26), but activity in the REF reach was consistently higher than in the TRT reach (Reach $F_{(1,82)}$=40.76, p<0.0001; Fig. 14). Patterns in βGLU activity were not significantly different between the REF and TRT reach throughout the study (Reach*Time $F_{(1,42)}$=0.16, p=0.69). Activity was consistently higher in the TRT reach relative to REF (Reach $F_{(1,42)}$=13.34, p=0.0007).

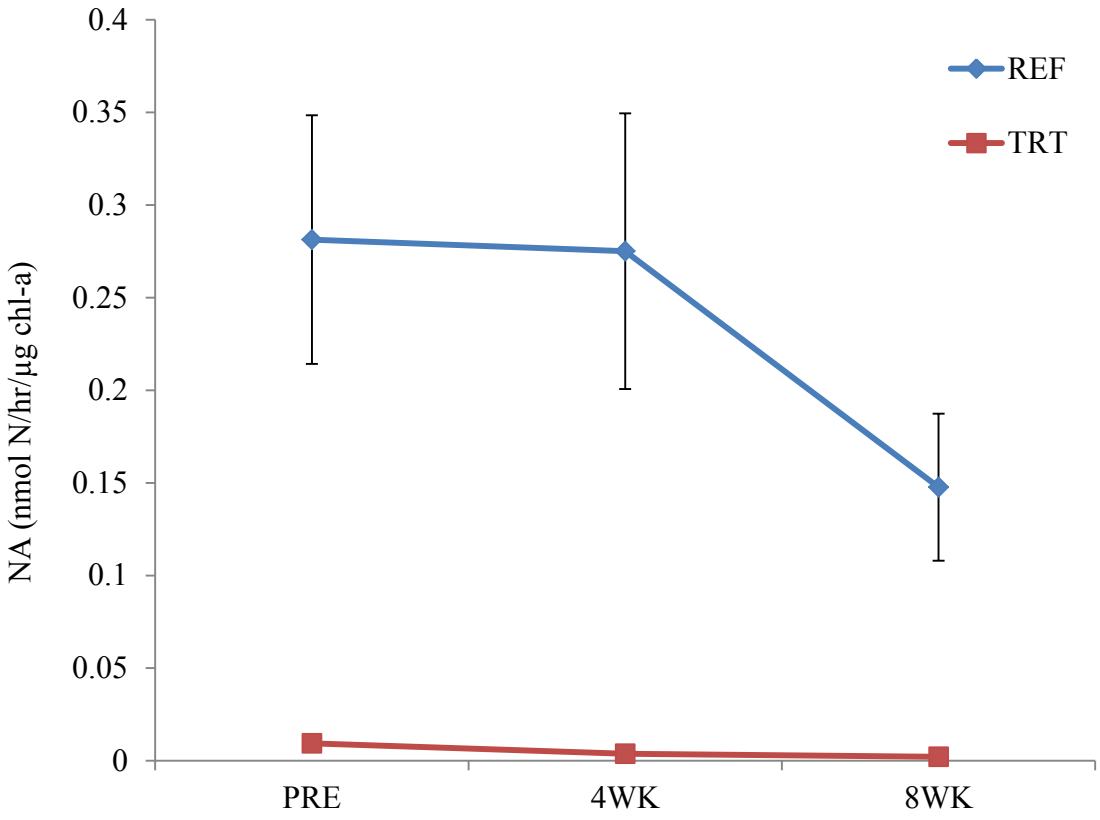

Figure 14. Patterns of mean ± SE NA of periphyton on rocks at STAR-171 in REF and TRT reach over time were not significantly different (Reach*Time $F_{(2,82)}$=1.37, p=0.26), but total activity was significantly higher in the REF relative to TRT throughout the study (Reach $F_{(1,82)}$=40.76, p<0.0001).

Patterns in APA were significantly different between the REF and TRT reaches (Reach*Time $F_{(2,64)}$=4.88, p=0.01; Fig. 15). In the TRT reach, decline in APA activity remained relatively constant and slower than in the REF reach, but total activity was never significantly differed from REF (χ^2_1=4.73, p=0.08; χ^2_1=5.00, p=0.08; χ^2_1=0.06, p=0.80).

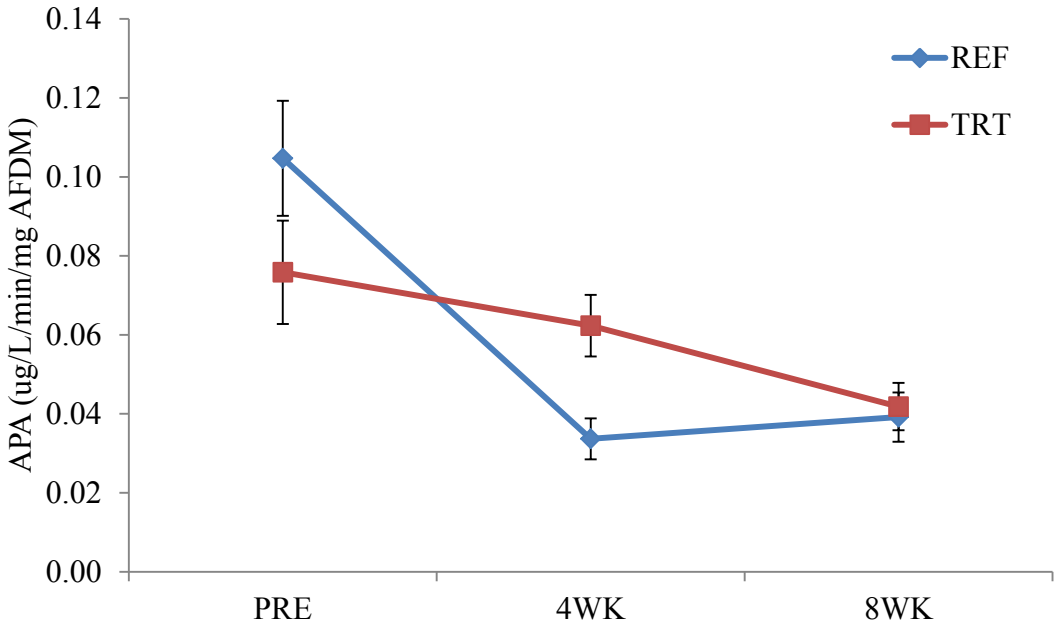

Figure 15. Patterns of mean ± SE APA of periphyton on rocks at STAR-171 in REF and TRT reach over time were significantly different (Reach*Time $F_{(2,64)}$=4.88, p=0.01), but total activity was never significantly different between the two reaches at any time.

Gross Primary Production (GPP)

Patterns in hourly biomass-specific GPP were not significantly different between REF and TRT reaches (Reach*Time $F_{(3,32)}$=1.19, p=0.33; Fig. 16). Daily areal GPP patterns were not significantly different either (Reach*Time $F_{(2,24)}$=0.77, p=0.48; Fig. 17), but daily areal GPP in the REF reach was significantly different than in the TRT reach overall (Reach $F_{(1,24)}$=6.25, p=0.02).

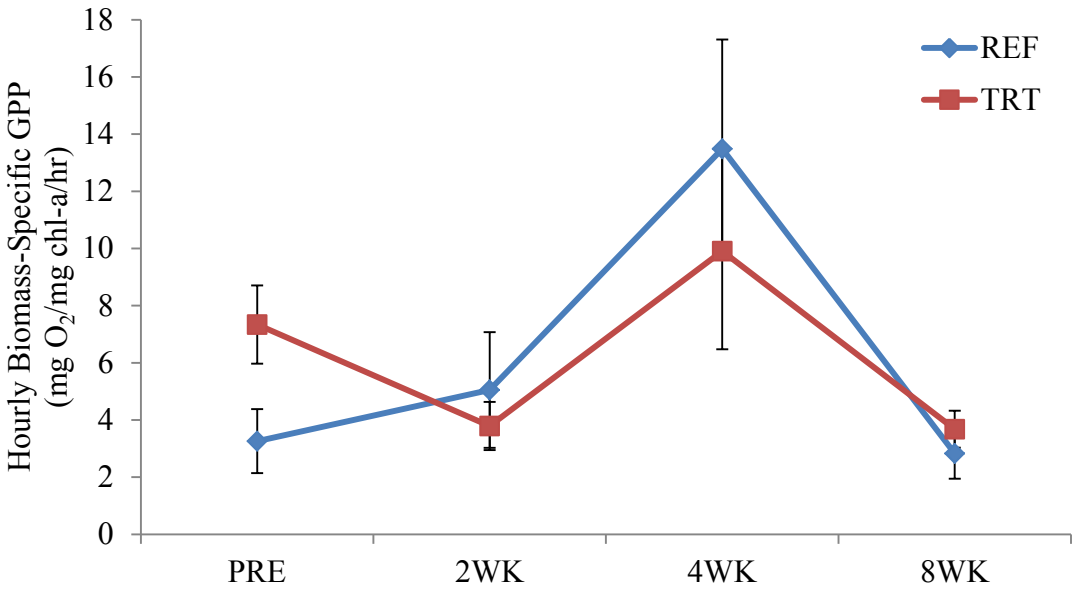

Figure 16. Patterns of mean ± SE hourly biomass-specific GPP of periphyton on rocks at STAR-171 in REF and TRT reach over time were not significantly different (Reach*Time $F_{(3,32)}$=1.19, p=0.33).

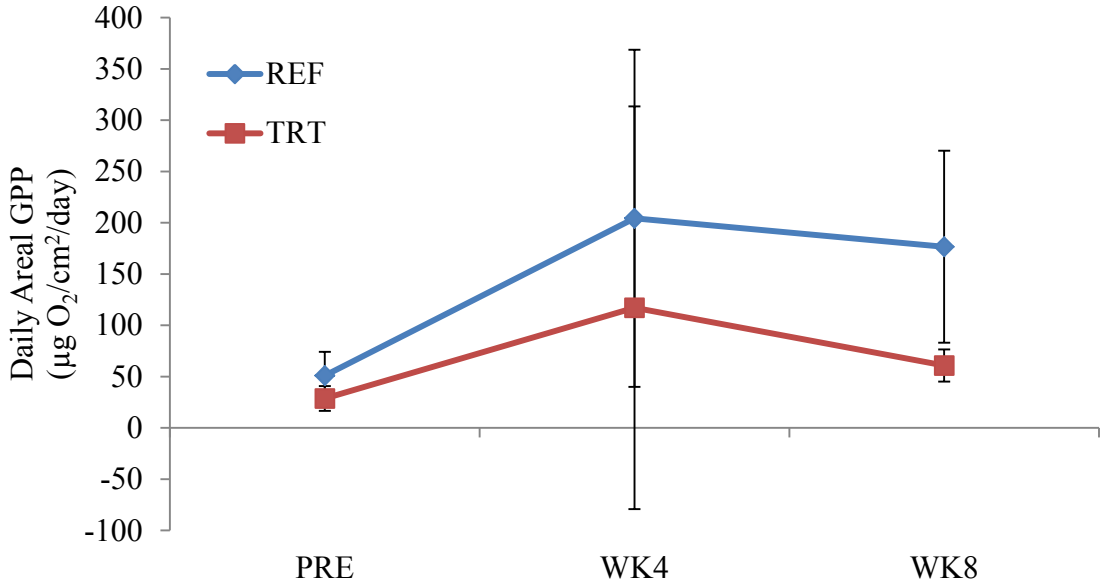

Figure 17. Patterns of mean ± SE daily areal GPP of periphyton on rocks at STAR-171 in REF and TRT reach over time were not significantly different (Reach*Time $F_{(2,24)}$=0.77, p=0.48), but the REF reach was significantly different than the TRT reach overall (Reach $F_{(1,24)}$=6.25, p=0.02). Error bars indicate propagated standard error based on error in chl-a estimates.

Respiration (R)

Patterns in hourly biomass-specific R were significantly different between REF and TRT (Reach*Time $F_{(3,32)}$=6.59, p=0.001; Fig. 18). However, the only time when there was a significant difference between REF and TRT was before dosing began, when R was much greater in the TRT reach than REF reach ($F_{(1,32)}$=19.22, p=0.0005). Daily areal R patterns were not significantly different between the REF and TRT reach (Reach*Time $F_{(2,24)}$=1.74, p=0.20; Fig. 19).

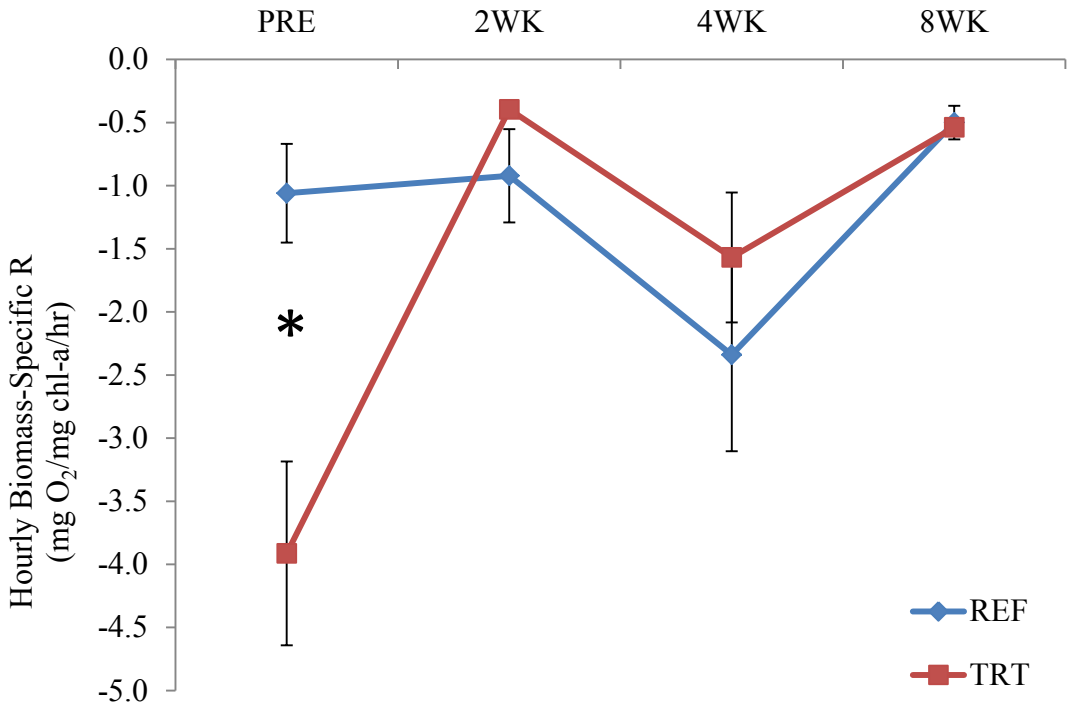

Figure 18. Patterns of mean ± SE hourly biomass-specific R of periphyton on rocks at STAR-171 in REF and TRT reach over time were significantly different (Reach*Time $F_{(3,32)}$=6.59, p=0.001), but total activity was only significantly different before dosing ($F_{(1,32)}$=19.22, p=0.0005).

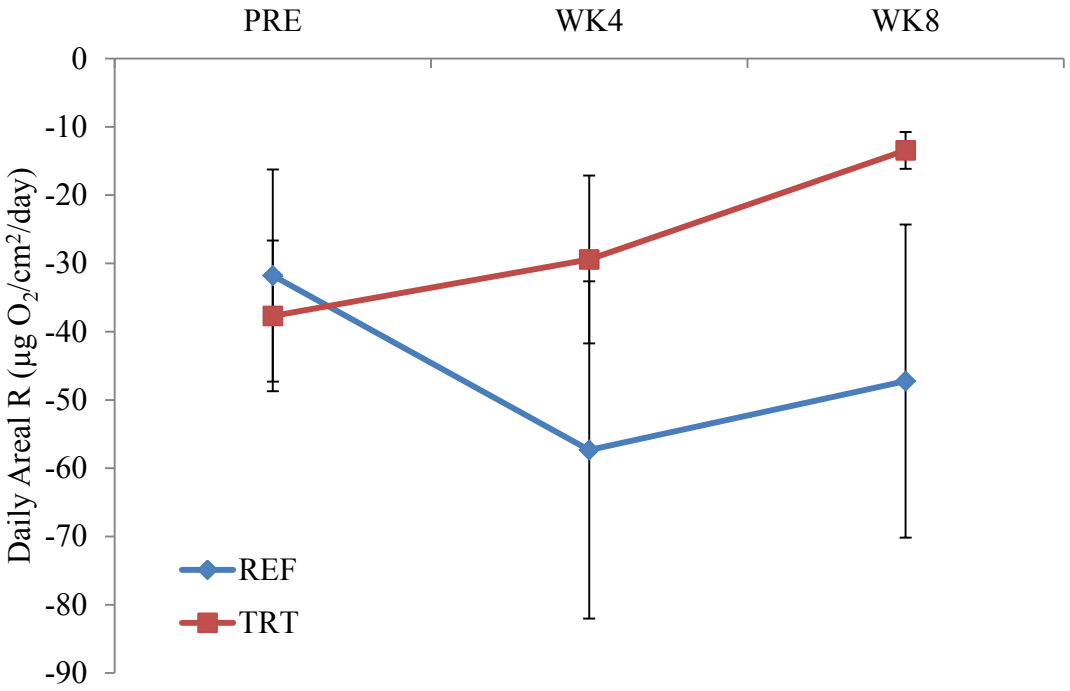

Figure 19. Patterns of mean ± SE daily areal R of periphyton on rocks at STAR-171 in REF and TRT reach over time were not significantly different (Reach*Time $F_{(2,24)}$=1.74, p=0.20). Error bars indicate propagated standard error based on error in chl-a estimates.

GPP:R

Patterns in hourly GPP:R were significantly different between the REF and TRT reach (Reach*Time $F_{(3,32)}$=4.75, p=0.008; Fig. 20). However, a significant difference between REF and TRT was only seen at 2wk ($F_{(1,32)}$=12.29, p=0.005). Daily GPP:R patterns were also significantly different (Reach*Time $F_{(2,24)}$=4.46, p=0.02; Fig. 21), but the only significant difference was seen before dosing began ($F_{(1,24)}$=6.82, p=0.05).

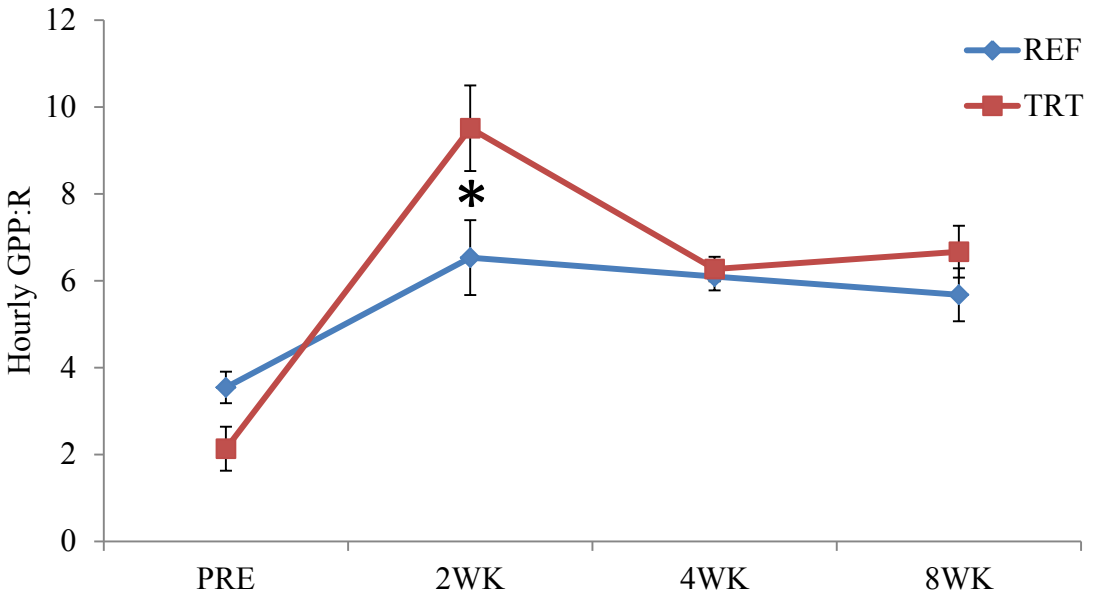

Figure 20. Patterns of mean ± SE hourly GPP:R of periphyton on rocks at STAR-171 in REF and TRT reach over time were significantly different (Reach*Time $F_{(3,32)}$=4.75, p=0.008), but total activity was only significantly different at 2wks ($F_{(1,32)}$=12.29, p=0.005).

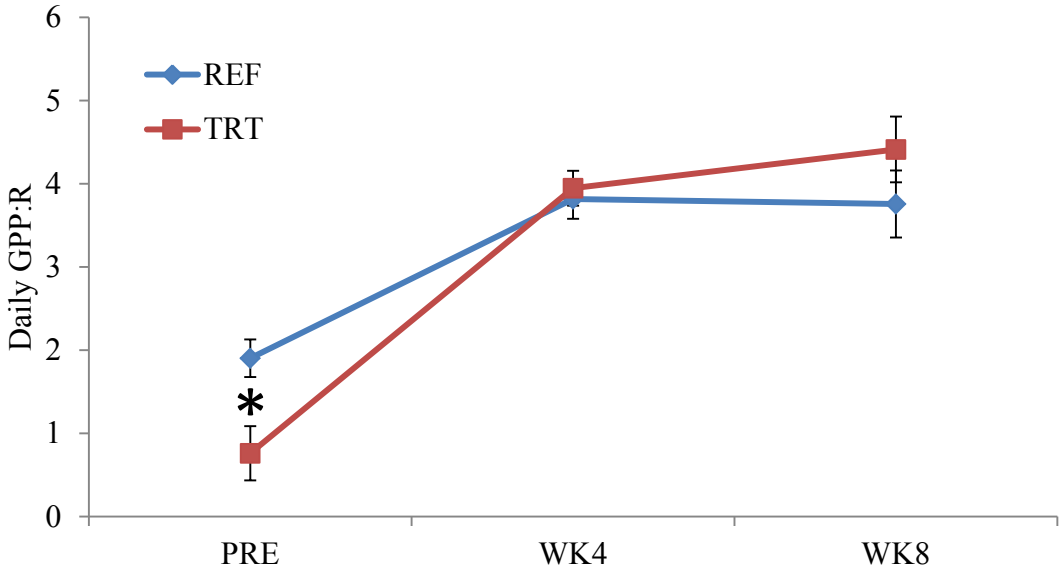

Figure 21. Patterns of mean ± SE daily GPP:R of periphyton on rocks at STAR-171 in REF and TRT reach over time were significantly different (Reach*Time $F_{(2,24)}$=4.46, p=0.02), but total activity was only significantly different before dosing ($F_{(1,24)}$=6.82, p=0.05).

Discussion

Biomass

We saw no changes in periphyton biomass or C:N ratios we believe to be due to N additions in the present study. In a study of seasonal effects on 12 New Zealand headwater streams, both nutrient limitation and periphyton biomass accrual reached a maximum during the summer months and minimum during winter (Francoeur et al., 1999), making our summer study period the most likely time to see differences in biomass accrual in these streams due to N supplements. However, changes in biomass occurred in our REF rather than the TRT reach, which may indicate that other factors are more heavily influencing biomass accrual, and that these factors are changing both in time and space. In a study of 620 stream stations in the United States National Stream Water Quality Monitoring Networks, it was determined that nutrients can be used to predict biomass much more easily in lakes (explaining 69-76% of variance) as opposed to streams (explaining only 40%), which could help explain why N did not affect biomass in the present study (Dodds, 2002). Other studies have indicated that algal biomass responses to nutrient additions may vary significantly depending on the ecoregion, ambient nutrient levels, and metric used to estimate biomass, but that in general algal biomass did increase with nutrient supplements (Stevenson et al., 2006; Nelson et al., 2013).

N is rarely limiting by itself, but rather is co-limiting with P (Francoeur, 2001; Rabalais, 2002). However, the Kenai Peninsula is rich in P, as mentioned above (Eicher and Rounsefell, 1957; Hobbie et al., 1999), making P-limitation unlikely, and our APA assay did not indicate a strong P-limitation in this system. Stevenson et al. (2006) found

ammonium (NH_4^+) to be a slightly better indicator of algal biomass than other stream nutrients, and this particular nutrient was abundant in anomalously high concentrations during this study period compared to previous years. Rosemond et al. (1993), on the other hand, observed that increases in algal biomass are much more significant when both N and P are added. Another study showed no correlation between streamwater N:P and periphyton N:P (Francoeur et al., 1999), indicating that perhaps streamwater nutrients are not the only factors affecting periphyton nutrient acquisition and growth. So, from where does the other 60% of variance in stream benthic algal biomass that is unexplained by nutrients derive?

Periphyton biomass accumulation in streams is dependent on many variables, including nutrients, disturbance, stream gradient, latitude, substrate, grazing, temperature, light and land use (which may influence many of these factors) (Stevenson et al., 1996; Francoeur et al., 1999; Dodds, 2002). A study using NDS found N to be more important than light for chl-a growth, but not for AFDM in streams (Mosisch et al., 1999), and others found that N correlates strongly with algal biomass (Francoeur et al., 2003; Luttenton and Lowe, 2006). A whole-stream fertilizer study in Swedish lakes found that nutrients (N and P) and grazers were equally important variables affecting biomass accrual (Hillebrand and Kahlert, 2001).

However, many other studies found that light was the most important variable influencing periphyton biomass accrual (Kiffney and Bull, 2000; Larned and Santos, 2000; Von Schiller et al., 2007; Hill and Fanta, 2008). A study in Canadian headwater streams found light to have more effect on biomass accrual than grazing (Kiffney and Bull, 2000). Previous studies using NDS (Von Schiller et al., 2007) and experimental

tubs (Larned and Santos, 2000) have found that light availability was more important to biomass accrual than N levels, and that N had little to no effect on stream periphyton biomass. In particular, heavily shaded areas saw no effect due to nutrient enrichment, while only partially-shaded periphyton increased in biomass in response to nutrients (Larned and Santos, 2000). Another study using artificial streams with varying P and light levels determined that 67% of the variance in periphyton biomass was explained by light, and P explained only 14% of additional variance (Hill and Fanta, 2008). Varying light levels in our reaches due to shading from riparian vegetation and differences in the depth of the water may help explain the lack of nutrient enrichment effect, though it is not clear why biomass accrual increased in the REF reach and not the TRT reach. Perhaps the TRT reach was more heavily shaded than the REF reach, allowing greater algal growth in the REF reach. Unfortunately, light data was not captured for each reach separately.

Enzymes

Though patterns in APA were significantly different between the REF and TRT reaches, activity was not significantly different between the REF and TRT reach at any time point. On the other hand, βGLU patterns did not differ between the two reaches, but activity was significantly higher in the TRT reach than the REF both before and after dosing. A study in southern California grassland soils found the same result with no response in enzyme activity with increased N (Alster, 2012). However, a study in forest soil found no changes in ligninolytic enzymes, but increases in cellulase activity (such as βGLU) in response to N additions (Carreiro et al., 2000).

There are a variety of possible reasons for why βGLU and APA did not react to N additions. βGLU is only one of many enzymes that bacteria produce in order to degrade a variety of C compounds (Carr et al., 2005). It is possible that the most utilized C-acquiring enzyme in this habitat was not captured in this study. Additionally, in order for it to be worth the energy of producing an exoenzyme, recalcitrant forms of the desired nutrient must be more abundant than labile forms (Allison and Vitousek, 2005). Even if C or P became more limiting, βGLU and APA would not be produced if they had no substrate to break down. Finally, changes in enzyme activity may be caused by changes in species composition rather than nutrient limitation (Kirchman et al., 2004). Previous studies have found significant changes in periphyton community composition with nutrient additions (Mulholland and Rosemond, 1992), but species composition was not measured in this study, making these two options difficult to differentiate. If the community became dominated by species well-adapted to the high N environment, exoenzymes would be less utilized to support growth. Perhaps our N additions were not significant enough to cause a shift in nutrient limitation, but we cannot be certain based on the data collected in this study.

NA did not change in either the REF or TRT reach throughout the study, but these reaches were significantly different both before and after dosing. NA has previously been found to correlate negatively with N concentrations (Carreiro et al., 2000). The high variability in NA throughout the stream may be due to spatial heterogeneity in periphyton nutrient limitation. This type of nutrient heterogeneity has been seen previously in wetland periphyton (Scott et al., 2005). Our significant difference in NA before dosing may have resulted from differences in depth and flow between the two

reaches. Increased flow reduces boundary layer thickness, allowing for faster nutrient assimilation into periphyton (Stevenson et al., 1996). Since our REF reach was slightly slower and deeper than the TRT reach, the periphyton in this reach may have already been more nutrient-starved than the TRT reach before dosing began. With such low starting NA in the TRT reach, it would have been impossible to see any further reduced activity in response to the N supplement. Perhaps if we had dosed our REF reach, which appeared to be more N-limited before dosing, we would have seen significant reductions in NA, but we cannot make any strong conclusions about N-limitation from the results of this study.

Metabolism

Metabolism results suggest that the REF and TRT reaches were not as similar as they appeared before dosing began, and that N additions had little effect on the TRT reach. While some studies have found nutrients to affect GPP and R in variable ways across ecosystems (Nelson et al., 2013), others show strong positive relationships between DIN and periphyton GPP and DOM processing, which drives R (Francoeur et al., 2003; Mineau et al., 2013). GPP:R tends to increase with increasing N and P due to slight enhancement of GPP relative to R (Nelson et al., 2013). However, Rosemond et al. (1993) found that these increases in productivity are slight when N is added without P. Though nutrient additions tend to cause more dramatic effects in oligotrophic environments like this site (Bilby et al., 1996), our low overall increase in N may not have been great enough to induce significant responses in metabolism.

However, much like biomass accrual, many variables may influence microbial productivity and R besides nutrients, and DOM processing usually is co-limited by both

N and P (Mineau et al., 2013). Light and grazing are the most well-understood, and most consistently observed influences on productivity (Mulholland and Rosemond, 1992), and our small increases in N availability may have taken only a secondary role in controlling the metabolic activity of these periphyton. The significant difference in hourly biomass-specific R and daily GPP:R between the two reaches before dosing began is another indicator that spatial heterogeneity in the stream may be high, and the two reaches that we chose were not similar enough for comparison before dosing.

Conclusion

The differences in many characteristics of the two reaches before dosing make inferences from this study challenging. Spatial heterogeneity is a major issue when dealing with before-after-control-impact (BACI)-type study designs, as differences between the REF and TRT reaches before dosing make comparisons afterward difficult. We were unable to decipher any true responses from the N addition due to large differences in NA, βGLU and R between the reaches before dosing. These differences indicate that the two reaches may not have been functionally similar before N was added. A possible seep located between the REF and TRT reaches may have been partially responsible for this difference if the seep contributed significant amounts of nutrients to the TRT reach below. However, we did not measure the water chemistry of this seep, nor did it appear to affect the water chemistry of the TRT reach before dosing. Other metrics, like GPP and biomass, were similar between the reaches before dosing, but differences in biomass after the N addition may have been a function of other differences between the two reaches. For example, the shallower, faster flow of the TRT reach likely created a periphyton community that was more susceptible to scouring due to high flow periods.

Sloughing of periphyton in the TRT reach might help explain the lower biomass in this reach.

Though we did not see significant effects in the chosen assays in response to our nitrogen addition, many factors may have contributed to this conclusion. Epilithic periphyton, though very essential to the ecosystem, is just one component of the stream community, and other components may have been affected more by the enrichment than were these microbes. Phytoplankton can often be more nutrient limited than periphyton due to its lack of algal-bacterial coupling and nutrient-rich polysaccharide matrix that are characteristic of periphyton (Stevenson et al., 1996). In flowing waters, this difference can be enhanced by the reduced thickness of the boundary layer, allowing for faster nutrient uptake capabilities of periphyton (Stevenson et al., 1996). Thus, examination of the more nutrient-limited phytoplankton rather than periphyton may have uncovered results of nutrient supplementation not observed in this study.

Previous studies have found that more than just stream N level increases as a result of increased alder cover in the catchment. Increased alder cover also leads to increases in detritus and macroinvertebrate transport, as well as increased shading of the stream, which may alter temperature and metabolic activity (Wipfli and Musslewhite, 2004). Thus, our addition of N without the other characteristics associated with increased alder cover may not have induced true results to what a real change in alder cover might cause, even if our two reaches had been similar beforehand. The potential value of alder to headwater stream ecosystems should not be overlooked based on results of this study, as differences in certain factors confounded comparisons. Concurrent study in an opposing landscape with high alder and low wetland cover indicated C-limitation in the

stream, and high N inputs from the alder likely contributed to this chemical environment. Without high N inputs, additions of C likely would not have resulted in the observed increases in productivity. More information is needed in order to develop successful management strategies for this ecologically and economically important habitat.

CHAPTER FOUR

Conclusion

This study examined headwater stream microbial responses to naturally realistic, low-level nutrient supplements in a relatively pristine, low-nutrient habitat of great ecological and economic importance to the state of Alaska. These nutrient additions were proposed to mimic the low-level contributions of bioavailable carbon (C) and nitrogen (N) from dominant landscape features of potential conservation concern. Wetlands in the catchment are known to provide both labile and recalcitrant dissolved organic C, while alder trees, through their symbiotic relationships with N-fixing bacteria in their root nodules, provide labile dissolved inorganic N to the stream community (Bond, 1956; Mulholland and Kuenzler, 1979; Shaftel et al., 2012; Walker et al., 2012).

Both of these landscape features are in potential danger of land development, as neither is desirable in urbanized settings. Wetlands have been historically known as wastelands of little value because they are not buildable land; as such, many have been filled with sediment or otherwise polluted (Mitsch and Gosselink, 2007). Alder trees, on the other hand, grow in dense thickets which impede movement and visibility, creating a nuisance on developed land. Thus, these two potentially essential nutrient sources to an already low-nutrient environment need to be understood in order to better manage their abundances.

Results of this study indicate that nutrients in the low concentrations derived from wetland and alder may be important drivers of stream productivity, but that simple

relationships between nutrients and functionality are unlikely. Many factors influence the activities investigated here, such as light availability, temperature, pH, and flow, some of which may be altered along with C and N when landscape features change. For example, light availability is often reduced in the presence of DOC, which stains waters brown and may decrease photosynthesis. Additionally, increased alder cover might reduce light penetration to the stream, reducing temperature which in turn affects metabolic activities. There are limitations to the study performed here, as it manipulated only one of many characteristics associated with landscape changes.

Nonetheless, we did see drastic changes in the stream community in response to low-level DOC additions realistic to the contributions of wetlands in the catchment of a stream. Biomass accrual, photosynthesis and respiration all increased in response to DOC, indicating that this resource is essential for productivity. Though these same results were not seen in our DIN addition study, presence of alder can accurately predict at least 75% of variance in N levels in Alaskan headwater steams (Shaftel et al., 2012). Without the high levels of N in our stream with the C addition, wetland C inputs likely would not have affected stream productivity as heavily. Both nutrients are essential for sustaining life, and are important for the ecosystem. Thus, though we saw changes in response to C but did not see these responses to our similarly low-level N additions, both alder and wetlands may be important riparian features affecting the stream community. The findings in this study should be used and expanded upon in the future to help understand and evaluate the contributions that wetlands and alder have on Alaskan headwater streams. This information may be essential in formulating best-management

practices in future landscape development, which in turn may play a major role in Alaskan salmon conservation practices.

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