Growing Rocks:

The Effects of Calcium Carbonate Deposition on Phosphorus Availability in Streams

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved January 2015 by the
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ARIZONA STATE UNIVERSITY

May 2015

ABSTRACT

Humans have dramatically increased phosphorus (P) availability in terrestrial and aquatic ecosystems. As P is often a limiting nutrient of primary production, changes in its availability can have dramatic effects on ecosystem processes. I examined the effects of calcium carbonate ($CaCO_3$) deposition, which can lower P concentrations via coprecipitation of phosphate, on P availability in two systems: streams in the Huachuca Mountains, Arizona, and a stream, Río Mesquites, in Cuatro Ciénegas, México. Calcium carbonate forms as travertine in the former and within the microbialites of the latter. Despite these differences, $CaCO_3$ deposition led to lowered P availability in both systems. By analyzing a three-year dataset of water chemistry from the Huachuca Mountain streams, I determined that P concentrations were negatively related to $CaCO_3$ deposition rates. I also discovered that $CaCO_3$ was positively correlated with nitrogen concentrations, suggesting that the stoichiometric effect of $CaCO_3$ deposition on nutrient availability is due not only to coprecipitation of phosphate, but also to P-related constraints on biotic nitrogen uptake. Building from these observations, bioassays of nutrient limitation of periphyton growth suggest that P limitation is more prevalent in streams with active $CaCO_3$ deposition than those without. Furthermore, when I experimentally reduced rates of $CaCO_3$ deposition within one of the streams by partial light-exclusion, areal P uptake lengths decreased, periphyton P content and growth increased, and periphyton nutrient limitation by P decreased. In Río Mesquites, $CaCO_3$ deposition was also associated with P limitation of microbial growth. There, I investigated the consequences of reductions in $CaCO_3$ deposition with several methods. Calcium removal led to increased concentrations of P in the microbial biomass while

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light reductions decreased microbial biomass and chemical inhibition had no effect. These results suggest that $CaCO_3$ deposition in microbialites does limit biological uptake of P, that photoautotrophs play an important role in nutrient acquisition, and, combined with other experimental observations, that sulfate reduction may support $CaCO_3$ deposition in the microbialite communities of Río Mesquites. Overall, my results suggest that the effects of $CaCO_3$ deposition on P availability are general and this process should be considered when managing nutrient flows across aquatic ecosystems.

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CHAPTER 1

INTRODUCTION

As phosphorus is often limiting to primary production (Elser et al. 2007), its supply in an ecosystem can have profound effects on the growth of primary producers, trophic interactions, and other ecological processes. All organisms require phosphorus (P) for growth. Phosphorus is necessary to build biomolecules that store and produce genetic material, form cellular membranes, and transport chemical energy (Sterner and Elser 2002). Supplies of P to the biosphere are governed by the weathering rates of sedimentary and igneous rock (Föllmi 1996). Indeed, largely to support agricultural production, human demand for fertilizer has increased the flux of P from rocks to the biosphere over 400% (Falkowski et al. 2000). This increased supply of P from fertilizer production and application, as well as the mismanagement of wastewater refuse, has led to the eutrophication of lakes and coastal areas and concomitant harmful algal blooms, foul odors, unsafe drinking water, and fish kills (Carpenter et al. 1998, Smith and Schindler 2009, Childers et al. 2011). Therefore, it is imperative to understand the processes that influence P availability in aquatic ecosystems.

Stream ecosystems are particularly relevant to the phosphorus cycle. Conventionally, streams were thought to be passive conduits of materials; this assumption underlies the "watershed" concept in ecosystem ecology that views streams as integrators of terrestrial processes (Bormann and Likens 1967). However, the ability of streams to process and retain nutrients is now widely recognized (House 2003, Mulholland 2004, Alexander et al. 2007, Wollheim et al. 2008). Streams may act as a

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filter for nutrients, lessening nutrient flows to receiving waters that may be vulnerable to eutrophication (Meybeck and Vörösmarty 2005, Alexander et al. 2007). In streams, phosphorus retention is strongly controlled by sediment-water interactions (Meyer and Likens 1979, House 2003).

One type of sediment process that may influence phosphorus cycling in streams is deposition of calcium carbonate ($CaCO_3$). Calcium carbonate deposition is a widespread phenomenon in aquatic systems (Pentecost 2005). When calcium carbonate ($CaCO_3$) precipitates or deposits, it can coprecipitate P in the form of phosphate. Phosphate is either adsorbed on the $CaCO_3$ mineral grains or incorporated into the $CaCO_3$ mineral matrix (Kitano et al. 1978, Ishikawa and Ichikuni 1981, Hu et al. 2014). Coprecipitation of phosphate has been documented for both abiotically and biotically driven $CaCO_3$ deposition (Kitano et al. 1978, Hartley et al. 1997).

The observation that phosphate coprecipitates with $CaCO_3$ in lakes and the observation that photosynthesis may promote $CaCO_3$ precipitation (Hartley et al. 1997) has led some authors to consider this process as a negative feedback on eutrophication (Koschel et al. 1983, Robertson et al. 2007). However, this negative feedback has seldom been considered in biogenic, lithifying $CaCO_3$ deposits. Exceptions to this are the studies of periphyton mats in Florida (Noe et al. 2001, Hagerthey et al. 2011) and of benthic cyanobacterial mats in Belize (Rejmánková and Komárková 2005, Borovec et al. 2010), two systems where $CaCO_3$ deposition and dissolution occur diurnally. Accordingly, coprecipitation is thought to be an important diurnal sink of P within periphyton (Noe et al. 2001, Hagerthey et al. 2011) and cyanobacterial mats (Borovec et al. 2010). Calcium carbonate deposits form in the mats due to the balance of photosynthesis and respiration

(Gleason and Spackman 1974). During the day, $CaCO_3$ precipitates, sequestering phosphate until respiration rates lead to $CaCO_3$ dissolution overnight, releasing any co-precipitated phosphate (McCormick et al. 1997). This pattern suggests that in microbial mats with accumulating $CaCO_3$ deposits, phosphate coprecipitation may be an important long term sink of P (Rejmánková and Komárková 2005). Before $CaCO_3$ deposition in streams can be considered as a buffer against eutrophication, a better understanding of how geochemical and biological processes interact with $CaCO_3$ deposition and phosphorus availability is needed.

Structure and Scope of Dissertation

In my dissertation, I focus on two questions:

Does $CaCO_3$ deposition affect phosphorus availability in streams?

If so, how?

I address these questions with observational and manipulative field experiments involving both travertine $CaCO_3$ deposits and microbialite $CaCO_3$ deposits.

In Chapter 2, I use a natural gradient of $CaCO_3$ deposition rates in streams in southeastern Arizona in the Huachuca Mountains to study the relative influence of different hydrologic and biogeochemical processes on nutrient concentrations. Hydrologic and biogeochemical processes are well known to influence stream water nutrient concentrations and longitudinal transport of nutrients by streams (Alexander et al. 2007). While $CaCO_3$ deposition is thought to be important for controlling phosphorus concentrations in stream water (Avilés et al. 2006, Withers and Jarvie 2008), it has been little considered in headwater streams. Therefore, I used multivariate analysis to

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synthesize a three-year dataset of stream water nutrient concentrations from three streams and compared the resulting components to potential hydrologic and biogeochemical processes and proxies such as stream discharge, temperature, season, and indices of $CaCO_3$ deposition.

In Chapter 3, I consider whether or not reduced phosphorus concentrations due to $CaCO_3$ deposition and coprecipitation of phosphate leads to P limitation of periphyton growth. Multispecies communities, like periphyton, are thought to exhibit nutrient co-limitation of growth (Arrigo 2004, Harpole et al. 2011). Indeed, meta-analyses of stream periphyton responses to nutrient amendments support this statement (Dodds and Welch 2000, Francoeur 2001, Elser et al. 2007). However, observations from $CaCO_3$-depositing aquatic ecosystems suggest that phosphate coprecipitation may promote P-limitation of periphyton growth. To determine if $CaCO_3$ deposition affects phosphorus availability to primary producers, I examined nutrient limitation of periphyton growth across the streams of the Huachuca Mountains using *in situ* nutrient-diffusing substrata. I repeated the experiment in each stream in the fall, spring, and summer to characterize seasonality of responses to nutrient amendments.

Next, in Chapter 4, I investigate the effects of $CaCO_3$ deposition in a lithifying microbial community. Lithifying microbial communities, known as microbialites, have existed on Earth for nearly 3.5 billion years (Hofmann et al. 1999, Riding 2000, Allwood et al. 2009), yet much remains unknown about how the microbial communities have and continue to interact with the mineral deposits that define their structure. I approach this problem by asking an ecological question: how does $CaCO_3$ deposition influence nutrient availability to microbialites? To answer this question, I first considered whether or not

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resource limitation differs between microbial communities associated with microbialites and those not associated with microbialites. Next, I manipulated rates of $CaCO_3$ deposition in the microbialites using several techniques to determine if lithification lowers phosphorus bioavailability. I used the oncoid microbialite communities found in Río Mesquites, Cuatro Ciénegas, México, as my model ecosystem.

In Chapter 5, I return to the Huachuca Mountain streams for an *in situ* experiment testing how $CaCO_3$ deposition influences ecosystem-scale metrics of phosphorus cycling in montane, headwater streams. In streams, $CaCO_3$ deposition can be promoted by photosynthesis of benthic algae (Pentecost 2005, Okumura et al. 2012). Therefore, I used shade structures to reduce photosynthetically driven $CaCO_3$ deposition in a stream with active $CaCO_3$ deposition and compared ecosystem responses to those in a similarly shaded stream without $CaCO_3$ deposition. I monitored several ecosystem attributes in each stream including water physicochemistry, periphyton biomass and carbon, nitrogen, and phosphorus stoichiometry, nutrient spiraling parameters, nutrient limitation of periphyton growth, and leaf litter decomposition.

In the final chapter of the dissertation, I briefly synthesize the main points from each chapter and link the outcomes from the two study systems through my main focal research questions. I discuss the new knowledge revealed from my research and its implications for ecosystem management.

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CHAPTER 2

THE STOICHIOMETRIC IMPACT OF CALCIUM CARBONATE DEPOSITION ON

NITROGEN AND PHOSPHORUS CONCENTRATIONS IN THREE MONTANE

DESERT STREAMS

ABSTRACT

The total and relative availability of nitrogen (N) and phosphorus (P), or N:P

stoichiometry, can influence numerous ecological processes. In streams, N:P

stoichiometry is influenced by different hydrologic and biogeochemical processes which

also affect the downstream transport of these nutrients to receiving waters. Calcium

carbonate ($CaCO_3$) deposition, a geochemical process that can occur in alkaline streams

and other aquatic ecosystems, can lower phosphorus concentrations and, potentially,

decrease phosphorus availability relative to nitrogen availability. I test the role of $CaCO_3$

deposition on stream nutrient chemistry using a three-year dataset of stream

physicochemistry and several metrics of $CaCO_3$ deposition across three streams in the

Huachuca Mountains of southern Arizona, U.S.A. $CaCO_3$ deposition rates varied across

and within streams, with benthic coverage of travertine as high as 70% and rates as high

as 8.3 µg Ca L^{-1} m^{-1}. Mean stream water P concentrations were negatively related to

$CaCO_3$ deposition rates ($CaCO_3$ mass transfer: R^2=0.11, $p<0.01$; $CaCO_3$ reaction rate:

R^2=0.11, $p<0.05$). Multivariate analysis revealed that $CaCO_3$ deposition is also associated

with higher N concentrations, suggesting P limitation of organismal growth may reduce

N uptake in the stream and amplify the stoichiometric signal of $CaCO_3$ deposition.

INTRODUCTION

Nitrogen (N) and phosphorus (P) are two of the most important elements for the composition of life. While the availability of these nutrients in an ecosystem has important consequences for primary production (Elser et al. 2007), the relative availability of these nutrients (N:P stoichiometry) has important implications for the nutritional quality of primary producers (Sterner and Elser 2002) and a variety of ecological processes, including community species composition (Stelzer and Lamberti 2001), efficiency of energy transfer through food webs (Malzahn et al. 2007, Dickman et al. 2008, Davis et al. 2010), and nutrient mineralization rates (Mooshammer et al. 2012) (see Sardans et al. 2012 for a comprehensive review). Nitrogen:P stoichiometry also has important consequences for ecosystem services like carbon sequestration (Falkowski et al. 2000, Hessen et al. 2004) and regulation of water quality (Smith 1983).

Given the ecological effects of nutrient availability and the potential impacts of nutrients on ecosystem services, studies that consider how different processes influence the absolute and relative availabilities of N and P in aquatic ecosystems are imperative (von Schiller et al. 2008, Finlay et al. 2011). In streams, numerous hydrologic and biogeochemical processes can influence N and P cycles (Alexander et al. 2007) and, ultimately, nutrient flows to receiving rivers, lakes, and estuaries via longitudinal transport (Peterson et al. 2001). In this study, I examine how multiple processes affect N and P concentrations and how these processes might uncouple or couple N and P cycles.

Hydrological processes, e.g., floods or droughts, can regulate material transport, and therefore nutrient transport, into streams. During floods or spates, the relationship between discharge and dissolved organic matter (DOM) or particulates is generally

positive; however, the nutrient concentration in the DOM or particulates differs among watersheds and, therefore, can lead to differing relationships between discharge and nutrient concentrations (Meyer et al. 1988). For example, in forested watersheds, spates can transport greater amounts of DOM to the stream (Mulholland and Hill 1997) while in a desert stream, spates can increase inorganic nutrient concentrations (Grimm 1992). Conversely, drought can lead to decreased stream water concentrations of DOM, and therefore organic N and P (Dahm et al. 2003).

Biogeochemical processes, which include abiotic and biotic mechanisms, influence nutrient concentrations in headwater streams as well. Some biological processes, like assimilation or mineralization, tend to couple N and P flows because of the relatively restricted range of N:P ratios present in living biomass (Schade et al. 2011, Mooshammer et al. 2012). Other biological processes, like the microbially-mediated conversion of NO_3^- to N_2 via denitrification, lead to the selective removal of one nutrient over another (Grimm and Fisher 1986). Physical sorption processes can also reduce nutrient concentrations in stream water, although the nutrient-specific effect depends on the sediment characteristics (Meyer and Likens 1979, Triska et al. 1994). Indeed, P, as phosphate, tends to interact more readily with sediment particles than N and, as a result, physical adsorption is thought to be more important in removing P from the water column than N (Dorioz et al. 1989, Avilés et al. 2006).

Phosphate can interact with several sediment components: it can bind with aluminum, adsorb to organic matter, and/or coprecipitate with calcium carbonate ($CaCO_3$) (Chave 1965, Meyer and Likens 1979, House and Donaldson 1986). Phosphate coprecipitation with $CaCO_3$ is considered the most important endogenous process

removing P from the water column in rivers (Avilés et al. 2006). It is also likely

important in reducing P concentrations in headwater streams (Withers and Jarvie 2008).

However, the ability of $CaCO_3$ deposition to remove P from the water column can be

saturated, as coprecipitation reactions depend on the chemical equilibrium between the

sediment and overlying stream water (House and Denison 1997). Understanding how

edaphic processes, like $CaCO_3$ deposition, influence relative nutrient availability will

complement the growing knowledge of how the interactions between hydrologic and

biogeochemical processes influence nutrient availability and flows in aquatic ecosystems.

In this study, I focus on the relative importance of hydrologic, biologic, and

edaphic processes, particularly $CaCO_3$ deposition, in influencing nutrient concentrations

in headwater streams draining the Huachuca Mountains in southeastern Arizona. Despite

geomorphic similarities (Jaeger and Olden 2012), preliminary observations of these

streams suggest they exhibit different rates of travertine deposition (Corman, *unpublished

data*). As travertine is a form of $CaCO_3$, this natural gradient allows for a unique

opportunity to assess the impacts of a geochemical process on the stoichiometry of

potentially limiting nutrients in headwater streams (Fisher et al. 2004). I use estimates of

hydrologic processes (stream discharge), biological processes (temperature and season as

proxies), and edaphic processes (estimates of $CaCO_3$ deposition) to determine the relative

influence of each on stream water nutrient concentrations over three years. Based on the

hypothesis that $CaCO_3$ coprecipitation of phosphate will differentially affect P relative to

N, I predict that I will observe lower absolute P concentrations and higher N:P ratios in

streams with higher rates of carbonate deposition.
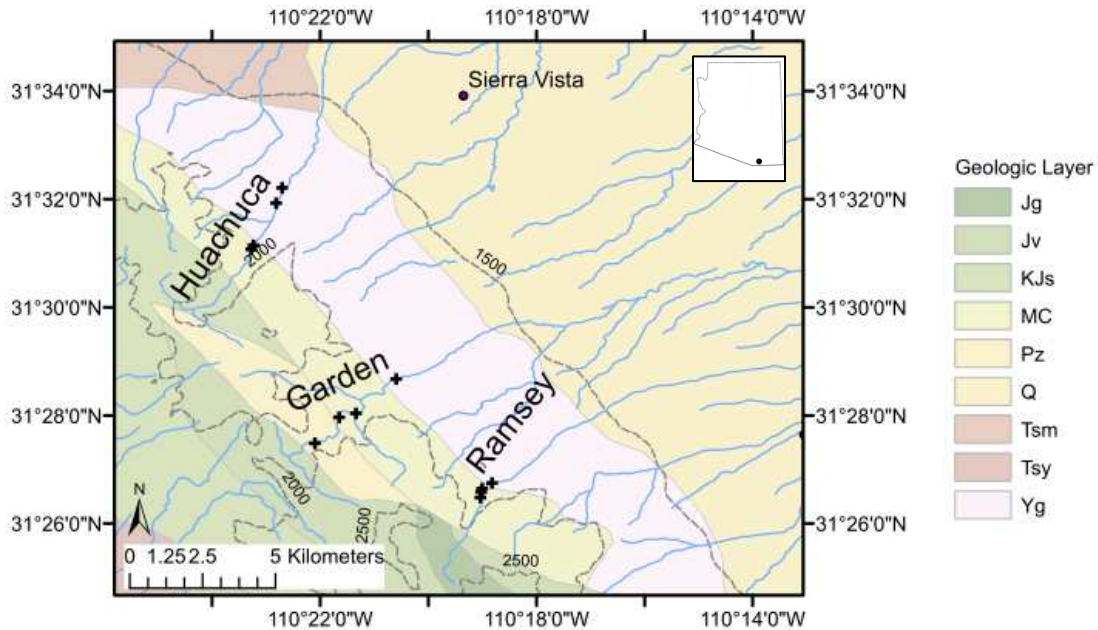
Figure 1. Site map of Huachuca, Garden, and Ramsey Canyon stream sampling locations in the Huachuca Mountains, south-eastern Arizona, U.S.A. Sampling locations are denoted by crosses. Background colors correspond to geologic units. Jg/Yg: granite/granite to diorite, Jv/KJs: sedimentary rocks and volcanics, MC: limestone, Pz: quartzite, Q: surficial alluvium deposits, Tsm/Tsy: sedimentary rocks. Inset shows the state of Arizona and the location of Sierra Vista.

METHODS

Field Site Description

This study was carried out in the Huachuca Mountain Range within the Upper San Pedro River Basin, southeastern Arizona. The Huachuca Mountains are part of the Madrean Sky Island Region, so named for the region's distinct Madrean-affiliated flora and fauna inhabiting the isolated mountain ranges. The peaks of the Huachuca Mountains rise to nearly 2800 m above sea level. The streams in the eastern portion of the Huachuca Mountains are part of the watershed of the San Pedro River, the last major free-flowing,

undammed river in the southwestern US. The climate in this region is semi-arid with about half of the mean annual precipitation of ~80 cm associated with the North American monsoon season and the other half with Pacific fronts in the winter (WRRC 2014). Oak (*Quercus*) and pine (*Pinus*) forests dominate the higher altitudes; grasslands and mesquite (*Prosopis*) desert scrub dominate the lower alluvial fans and river valleys. Broadleaf deciduous trees, including Arizona sycamore (*Platanus wrightii*) and bigtooth maple (*Acer grandidentatum*), dominate the riparian zones along the streams (Brown 1994).

The three streams used in this study, Garden Canyon, Ramsey Canyon, and Huachuca Canyon, are along the northeastern side of the Huachuca Mountains (Figure 1). The streams are spring-fed and perennial above ~1500 m elevation (Jaeger and Olden 2012). Stream channel morphology is characterized by cascade and bedrock reaches in the upper canyons and step-pool, plane bed, and pool-riffle reaches downstream. Substrata are travertine or cobbles and boulders. In some of the reaches, $CaCO_3$ deposition has cemented the channel bed and contributed to travertine step-pool morphology. Stream flow is monitored in each stream by the United States Geological Survey (USGS Gaging Stations: Garden Canyon: 09470800, Huachuca Canyon: 09471310, and Ramsey Canyon: 09470750).

Stream Water Sampling

Stream water was sampled at least every 2 – 3 months from February 2011 – May 2014 (Figure 2). Four reaches were sampled per stream (Figure 1). On dates when parts of the stream were dry, only wet reaches were sampled. At each reach, temperature (^{o}C),

specific conductivity (μS cm^{-1}), and dissolved oxygen (mg L^{-1}) were determined using a YSI 85 (Yellow Springs, OH) and pH using a Beckman-Coulter 255 pH/mV (Beckman Coulter Inc., Brea, CA) hand-held probe. Alkalinity was quantified by Gran titration (APHA 2005). Stream water samples were collected in acid-cleaned HDPE bottles and, when necessary, filtered (pore size = 0.45 um) and/or preserved with acid. Cation concentrations (Ca^{2+}, Mg^{2+}, Na^+, K^+) were analyzed on filtered samples preserved with 2% HNO_3 using a Thermo iCAP6300 Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Waltham, MA, USA). Anion concentrations ($SO_4^=$, Fl^-, Cl^-, Br^-) were assessed using ion chromatography (Dionex ICS-2000, Sunnyvale, CA, USA). Soluble reactive phosphorus (SRP) was quantified spectrophotometrically with the ammonium molybdate colorimeteric method (APHA 2005). Total dissolved phosphorus (TDP) and total phosphorus (TP) were determined on filtered and unfiltered samples, respectively, by persulfate digestion (Solorzano and Sharp 1980) after which phosphorus concentrations were analyzed as described for SRP. Nitrate and ammonium (NO_3^- and NH_4^+) were determined using a Lachat QuikChem 8000 Flow Injection Automated Ion Analyzer (Hach, Lovelend, CO, USA); dissolved inorganic nitrogen (DIN) concentrations were calculated by summing NO_3^- and NH_4^+ concentrations. Total dissolved nitrogen (TDN) was analyzed concomitantly with dissolved organic carbon (DOC) on filtered samples preserved with 2% HCl using a Shimadzu TOC-VN/TN Analyzer. Total nitrogen (TN) was determined by the in-line persulfate/UV oxidation method using a Lachat QC8000. Dissolved organic phosphorus (DOP) and dissolved organic nitrogen (DON) concentrations were calculated as the difference between total dissolved and dissolved inorganic P or N, respectively.
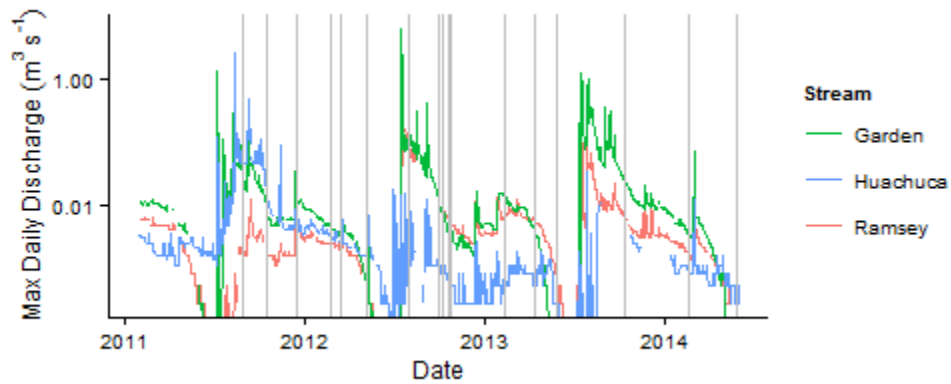
Figure 2. Three-year hydrograph for Garden, Ramsey, and Huachuca Canyon streams, Arizona, 2011 – 2014. Note abscissa is log scale. Grey vertical lines indicate dates when samples were collected.

CaCO₃ Deposition Rate Determinations

I quantified the natural gradient of $CaCO_3$ deposition across the streams using three metrics of calcium carbonate deposition rates: travertine cover, deposition on a natural substrate, and Ca^{2+} transfer reactions. To determine travertine cover, an indication of past $CaCO_3$ depositional patterns, benthic surveys were conducted at each site on 31 August 2011, 30 July 2012, 11 October 2013, and 5 May 2014. A 0.25 m^2 quadrat was thrown haphazardly in the stream at each site in three different locations. The percent cover of travertine was estimated visually at each location. Travertine deposits on leaves were included in the estimations of travertine cover (Figure 3).
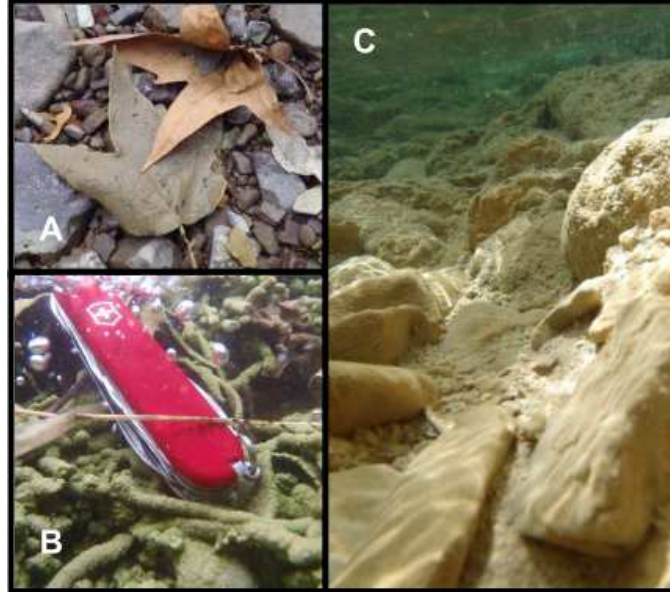
Figure 3. Images of CaCO$_3$ deposits from study streams. (A) A CaCO$_3$-encrusted leaf (on bottom left) as compared to a regular leaf. (B) CaCO$_3$-covered organic material on stream bottom with a pocket knife for scale; photo taken underwater. (C) CaCO$_3$-covered rocks; photo taken underwater. *Photo credits:* Jessica Corman.

I determined net CaCO$_3$ deposition rates over several months in three reaches per stream using leaves as natural substrata. Dried, senescent leaves of Arizona sycamore (*Platanus wrightii*) were collected in December 2010 from trees in the riparian zone of Ramsey Canyon. Leaves were transported immediately to the laboratory at Arizona State University where they were dried at 50°C and then stored in a climate-controlled storage facility until use. In November 2011, I re-dried the leaves and placed them into mesh pecan bags (Gulf Coast Bag and Bagging Company, Houston, TX) containing about 5 g of dried leaves each. I deployed litter bags at each site on 20 November 2011 and retrieved one bag per site at four intervals over the following three months. Three control bags were brought to the field and processed without deployment in the stream to measure the amount of material lost in transport, which I subtracted from all final dry

mass totals. I processed litter bags by rinsing leaves to remove all macroinvertebrates that had colonized them, drying the remaining leaf material at 50 °C, combusting a homogenized subsample at 500 °C to measure ash-free dry mass (AFDM), and, finally, dissolving the remaining material in 2% HNO_3 to analyze Ca^{2+} concentrations as described above. The Ca^{2+} content in leaves from the control bags was used to correct final calcium values of incubated leaves to reflect only Ca^{2+} accumulated on the substrate. The linear change in the amount of accumulated calcium per dry leaf mass over time was used to determine the $CaCO_3$ accumulation rate (mg $CaCO_3$ g^{-1} dry leaf day^{-1}). As the accumulation rate was based on the change in Ca^{2+} per initial dry mass of the leaf, and leaf mass was likely lost during the incubation due to decomposition or physical breakdown, these values serve as conservative estimates of $CaCO_3$ deposition rate.

Finally, $CaCO_3$ deposition or dissolution rates in each stream were determined from Ca^{2+} transfer reactions estimated on each sampling day (Lorah and Herman 1988). Calcium carbonate mass transfer from solution to channel bed (mg L^{-1}) was determined using the change in Ca^{2+} between successive sampling points within a stream. Mass transfers were normalized across streams by dividing the change in Ca^{2+} concentration by the distance between sampling points (reported in units of μg Ca^{2+} L^{-1} m^{-1}). The reaction rate of $CaCO_3$ was determined by dividing the mass transfer by estimates of the reaction time, determined from discharge and stream area, following Lorah & Herman (1988). The reaction rates are reported in units of μg Ca^{2+} L^{-1} s^{-1}. Positive mass transfers or reaction rates indicate $CaCO_3$ precipitation, while negative indicate $CaCO_3$ dissolution. A preliminary analysis of changes to concentrations of conservative ions between sampling points to detect groundwater intrusion showed negligible changes in conservative ion

concentration (Appendix A, Figure S1). Therefore, groundwater flux to the stream is assumed to be negligible and all changes to Ca^{2+} ions are assumed to be due to $CaCO_3$ deposition or dissolution.

Statistical Analyses

All values are reported as means ± 1 standard error unless otherwise noted. To determine univarate differences in basic physicochemical parameters (temperature, pH, specific conductivity, dissolved oxygen) or travertine cover across streams, I used one-way repeated measures ANOVA with date as a repeated factor. To determine seasonality of parameters, season was added as a fixed factor with year as a repeated factor. Due to the biseasonal rainfall pattern in the region, with rains occurring due to monsoon storms in late summer or Pacific fronts in winter, seasons are defined as monsoon (July – September), fall (October – November), winter (December – March), or summer (April – June). To determine rates of $CaCO_3$ accrual on leaves, I used linear regression within each stream and reach.

Next, I determined the variance structure of the stream nutrient chemistry and investigated potential processes that are associated with it. Nutrient data from each stream (DIN, SRP, DON, DOP, TDN, TDP, TN, and TP) were synthesized into multivariate gradients, using principal components analysis (PCA). A complete data matrix is needed for multivariate analysis (Tabachnick and Fidell 2001), therefore, missing values were imputed based on the median value of the nutrient. The median value was used because some of the univariate variables were not normally distributed. Next, distributions of univariate variables were checked for normality; if variables were

not normally distributed, either a log-transformation or square root transformation was performed to achieve normality (McGarigal et al. 2000). Then, univariate variables were normalized to a mean of zero and a variance of one to reduce the effect of different measurement scales and the PCA was performed. The number of meaningful principal components was determined using the broken stick method (Jackson 1993). After performing the PCA, I examined correlations between stream descriptor variables (temperature, $CaCO_3$ deposition, flow) and the PCs to further examine the effects of hydrologic and biogeochemical processes on nutrient concentrations. I chose $CaCO_3$ mass transfer and reaction rate as proxies of $CaCO_3$ deposition instead of travertine cover or $CaCO_3$ deposition on leaves because these proxies were determined at the same temporal scale as the nutrient data (i.e., I was able to calculate mass transfer and reaction rates for every date on which I sampled stream water). For stream discharge at each site, I used the average stream discharge at the gaging station on the day of sampling. The stream gages were located no more than 2.6 km upstream or downstream of the sampling reaches in Garden, Huachuca, and Ramsey Canyon. Although the discharge data from the gages may not indicate the precise discharge at each site, I assume the estimate of flow from the gage can still be a proxy of potential hydrologic influence throughout the year. If a correlation between a stream descriptor variables and a PC was significant, I fit a regression model with the PC as a response to the physicochemical variable.

All statistical tests were performed in R (ver 3.0.2) (R Core Team 2014). ANOVA was performed using the 'nlme' package and post-hoc comparisons were made using Tukey's Honestly Significant Difference test with the 'multcomp' package. I assessed homoscedasticity and normality of residuals visually for each model with a plot of model

residuals vs. fitted values and a normal probability plot, respectively. For the repeated-measures ANOVA, I tested the assumption of sphericity and, if needed, corrected with the Greenhouse-Gaiser estimate of epsilon (Geisser and Greenhouse 1958). PCA was performed with the 'vegan' package and PCA plots were made using the 'ggbiplot' package.

RESULTS

Water Physicochemistry

Discharge ranged from 0 to 6.26 m^3 s^{-1} (Figure 2). Median discharge ranged from 0.003 m^3 s^{-1} in Ramsey to 0.006 m^3 s^{-1} in Huachuca and Garden Canyon. The highest flow events occurred in July during the monsoon season; the highest mean daily discharge on a date that was sampled was 0.069 m^3 s^{-1} in Ramsey Canyon on 30 Jul 2012 (Figure 2).
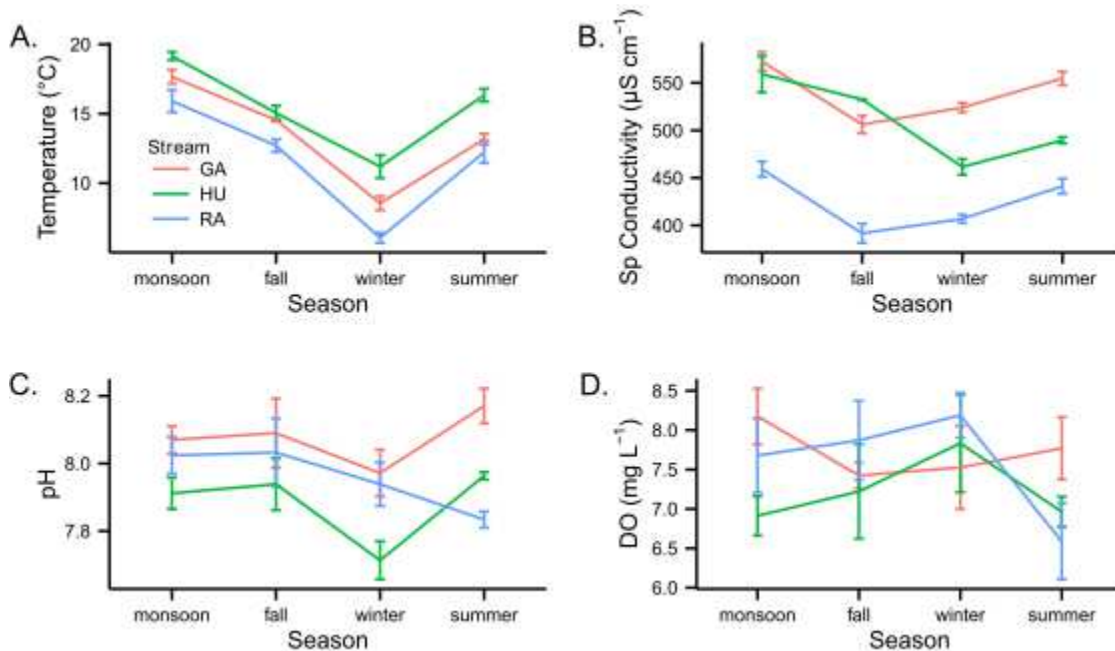
Figure 4. Physicochemical parameters in Garden (GA), Ramsey (RA), and Huachuca (HU) Canyon streams, Arizona, 2011 – 2014, including (A) temperature, (B) specific conductivity, (C) pH, and (D) dissolved oxygen (DO). Error bars represent ± 1 standard error.

Table 1. Cation concentrations in Garden, Huachuca, and Ramsey Canyon streams, Arizona, from 2011 – 2014. Concentrations for calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^+), and potassium (K^+) are reported as the mean and ± 1 standard error (in parentheses) as mg L^{-1} unless otherwise specified.

Stream	Season	Cations			
		Ca^{2+}	Mg^{2+}	Na^+	K^+
Garden	fall	74.8 (0.5)	18.7 (0.2)	3.64 (0.01)	0.46 (0.03)
	monsoon	79.7 (0.8)	18.7 (0.7)	3.47 (0.18)	0.45 (0.00)
	summer	81.2 (1.8)	20.2 (0.2)	4.20 (0.06)	0.48 (0.01)
	winter	78.2 (0.9)	20.2 (0.2)	4.18 (0.03)	0.48 (0.02)
Huachuca	fall	82.9 (0.2)	16.2 (0.2)	6.47 (0.66)	0.54 (0.01)
	monsoon	83.7 (1.0)	15.7 (0.5)	6.11 (0.17)	0.53 (0.02)
	summer	74.3 (0.5)	15.2 (0.2)	6.11 (0.16)	0.65 (0.02)
	winter	80.8 (1.2)	15.5 (0.3)	6.28 (0.03)	0.72 (0.04)
Ramsey	fall	58.2 (0.4)	12.2 (0.0)	3.84 (0.14)	0.50 (0.00)
	monsoon	64.2 (1.2)	12.3 (0.3)	3.49 (0.17)	0.53 (0.02)
	summer	63.4 (0.5)	13.0 (0.2)	4.22 (0.06)	0.65 (0.01)
	winter	61.3 (0.6)	12.4 (0.2)	4.06 (0.06)	0.63 (0.01)

Table 2. Anion concentrations in Garden, Huachuca, and Ramsey Canyon streams, Arizona, from 2011 – 2014. Concentrations for alkalinity (Alk), sulfate ($SO_4^=$), bromide (Br^-), fluoride (Fl^-) and chloride (Cl^-) are reported as the mean and ± 1 standard error (in parentheses) as mg L^{-1} unless otherwise specified.

Stream	Season	Anions				
		Alk (meq L^{-1})	$SO_4^=$	Br^-	Fl^-	Cl^-
Garden	fall	5.43 (0.24)	16.2 (1.0)	0.00 (0.00)	0.86 (0.61)	2.14 (0.17)
	monsoon	5.98 (0.29)	22.2 (1.5)	0.05 (0.04)	1.02 (0.72)	3.28 (0.39)
	summer	5.73 (0.29)	36.0 (2.0)	0.03 (0.01)	2.14 (1.07)	3.30 (0.69)
	winter	5.65 (0.23)	29.9 (3.2)	0.04 (0.01)	0.76 (0.21)	3.08 (0.20)
Huachuca	fall	5.96 (0.18)	13.9 (2.7)	0.00 (0.00)	0.09 (0.06)	3.00 (0.62)
	monsoon	6.01 (0.01)	12.3 (0.5)	0.06 (0.04)	0.92 (0.65)	3.43 (0.20)
	summer	5.38 (0.27)	18.9 (1.3)	0.04 (0.02)	1.32 (0.76)	5.76 (0.95)
	winter	6.04 (0.06)	16.9 (0.8)	0.05 (0.01)	0.95 (0.27)	3.29 (0.23)
Ramsey	fall	4.21 (0.24)	15.5 (1.3)	0.00 (0.00)	0.46 (0.33)	2.23 (0.15)
	monsoon	4.67 (0.07)	12.3 (0.1)	0.05 (0.04)	0.83 (0.59)	2.82 (0.13)
	summer	5.00 (0.13)	18.9 (0.9)	0.03 (0.01)	1.68 (0.84)	3.51 (0.43)
	winter	4.45 (0.15)	17.0 (0.6)	0.04 (0.01)	0.78 (0.24)	2.85 (0.19)

Temperature, specific conductivity and pH differed significantly among streams (temperature: $F_{2,22}=16.77$, $p<0.001$, specific conductivity: $F_{2,22}=56.60$, $p<0.001$, pH: $F_{2,21}=7.91$, $p<0.01$); dissolved oxygen concentrations did not (Figure 4). Temperature was highest in Huachuca Canyon, 15.2 $^{\circ}$C ± 1.0 $^{\circ}$C, compared to Garden Canyon (13.0 $^{\circ}$C ± 1.0 $^{\circ}$C) or Ramsey Canyon (11.3 $^{\circ}$C ± 1.2 $^{\circ}$C). Specific conductivity and pH were highest in Garden Canyon. Temperature and specific conductivity showed significant seasonality (temperature: $F_{2,32}=42.71$, $p<0.001$, specific conductivity: $F_{2,32}=6.97$, $p=0.001$) with values usually highest during the monsoon season (Figure 4). Across all streams, cation concentrations followed the order: $Ca^{2+} \gg Mg^+ > Na^+ > K^+$. Anion concentrations followed the order: $SO_4^{2-} > $ Alkalinity $> Cl^- > Fl^- > Br^-$ (Tables 1 and 2).

CaCO₃ Deposition Rates

Proxies of $CaCO_3$ deposition (travertine cover, deposition on leaves, and Ca^{2+} transfer reactions) indicated that rates were generally higher in Garden Canyon than Huachuca Canyon, and low or undetectable in Ramsey Canyon. However, the spatially-explicit method, $CaCO_3$ deposition on leaves, showed that $CaCO_3$ deposition rates varied within a stream.
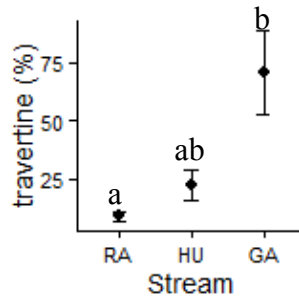
Figure 5. Travertine cover on stream beds in Ramsey (RA), Huachuca (HU), and Garden (GA) Canyon streams, Arizona. Different letters indicate significant differences (Tukey's post hoc test, $p<0.05$). Error bars represent ± 1 standard error.
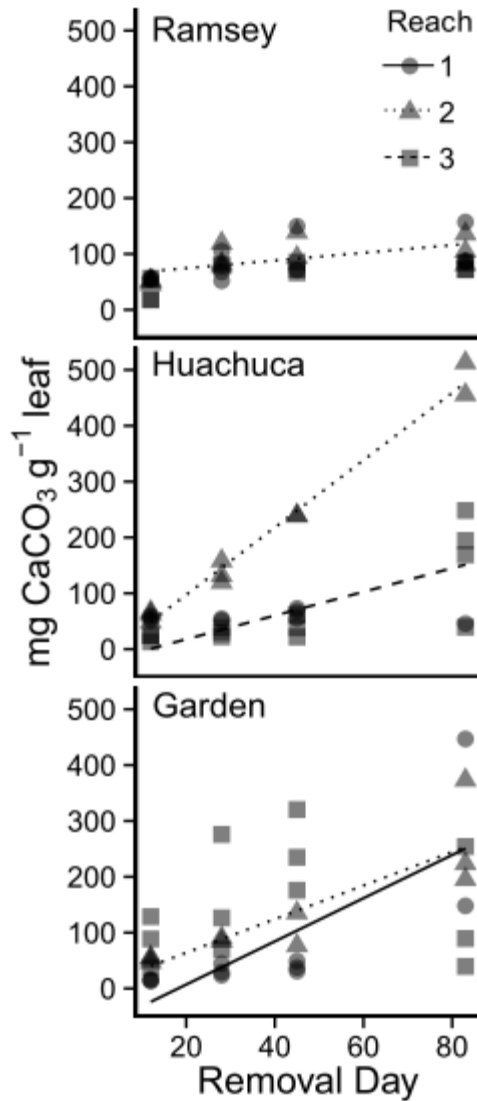
Figure 6. Calcium carbonate (CaCO$_3$) deposition on leaves in Garden, Huachuca, and Ramsey Canyon streams, Arizona, between November 2011 – February 2012. Lines indicate reaches with significant CaCO$_3$ accumulation rates. Points for individual samples are semitransparent to aid with overplotting.

Travertine cover varied significantly among the three streams ($F_{2,40}=16.79$, $P<0.001$; Figure 5). Travertine cover was lowest in Ramsey Canyon stream (9% ± 2%) and higher in Huachuca Canyon stream (23% ± 7%) and Garden Canyon stream (70% ± 18%).

Calcium carbonate accumulation rates on dry leaves varied among and within streams. Calcium carbonate accumulation rates were nearly undetectable in Ramsey Canyon. Only one site exhibited significant rates of $CaCO_3$ deposition, Reach 2. However, the rate was about 75% less than the average at Garden or Huachuca Canyon: 0.70 mg $CaCO_3$ g dry leaf^{-1} day^{-1}. Garden Canyon and Huachuca Canyon had similar average accumulation rates (2.50 mg $CaCO_3$ g dry leaf^{-1} day^{-1} ± 1.06 and 2.78 mg $CaCO_3$ g dry leaf^{-1} day^{-1} ± 1.81, respectively), though linear regressions were only significant in two of the three reaches in each site (Figure 6). Huachuca Canyon also had the highest rate at a single location: 6.17 mg $CaCO_3$ g dry leaf^{-1} day^{-1} in the middle reach, Reach 2.
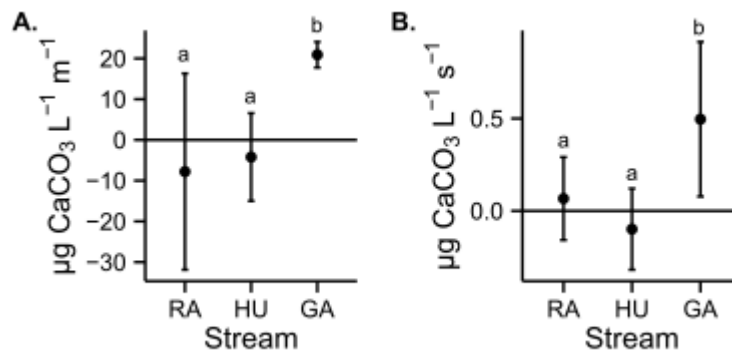
Figure 7. Calcium carbonate ($CaCO_3$) dissolution or deposition rates. (A) $CaCO_3$ mass transfer per stream length and (B) $CaCO_3$ reaction rates varied significantly across Garden (GA), Huachuca (HU), and Ramsey (RA) Canyon streams, AZ, during 2011 – 2014. Values below zero indicate dissolution; values above zero indicate deposition. Different letters indicate significant differences (Tukey's post hoc test, $p<0.05$). Error bars represent ± 1 standard error.

Calcium carbonate deposition, in terms of mass transfer and reaction rates, also varied significantly among streams (mass transfer: $F_{2,28}$=9.308, P<0.001; reaction rate: $F_{2,26}$=8.583, P<0.01) (Figure 7). In Ramsey Canyon, mass transfer of Ca^{2+} was slightly negative, -3.1 µg Ca^{2+} L^{-1} m^{-1} ± 2.6, suggesting some dissolution of $CaCO_3$ along the reach (Figure 7A). In Huachuca Canyon, mass transfer of Ca^{2+} was near zero, -1.7 µg Ca^{2+} L^{-1} m^{-1} ± 1.2, suggesting little deposition or dissolution. Positive mass transfer of Ca^{2+} was observed in Garden Canyon (8.3 µg Ca^{2+} L^{-1} m^{-1} ± 1.3), indicating deposition of $CaCO_3$. Similarly, reaction rates were near zero in Ramsey Canyon (0.07 µg $CaCO_3$ L^{-1} s^{-1} ± 0.22) and Huachuca Canyon (-0.10 µg $CaCO_3$ L^{-1} s^{-1} ± 0.22), but positive in Garden Canyon (0.50 µg $CaCO_3$ L^{-1} s^{-1} ± 0.42) (Figure 7B). Seasonal variation was not detected.

Nutrients

In general, I found stream water P concentrations were highest in Ramsey Canyon and lowest in Garden Canyon (Figure 8). This pattern was reversed for DOC and N concentrations (Figure 9). Dissolved organic compounds represented a substantial proportion of total dissolved nutrient content. For phosphorus, DOP represented over half of TDP concentrations, but with a greater proportion in Garden (79%) compared to Huachuca (67%) or Ramsey (56%) Canyon. For nitrogen, DON also represented over half of TDN concentrations, with the greatest proportions in Huachuca (91%) and Ramsey (75%) compared to Garden (56%) Canyon. Dissolved inorganic N was comprised mostly of NO_3^- across all streams, with NH_4^+ representing 7%, 18%, and 4% of DIN in Ramsey, Huachuca, and Garden Canyon, respectively. The molar ratio of TN to TP (TN:TP) was generally below or similar to the Redfield Ratio, 16:1 (Redfield

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1958), in Ramsey Canon and Huachuca Canyon but exceeded the Redfield ratio in

Garden Canyon (Figure 10).
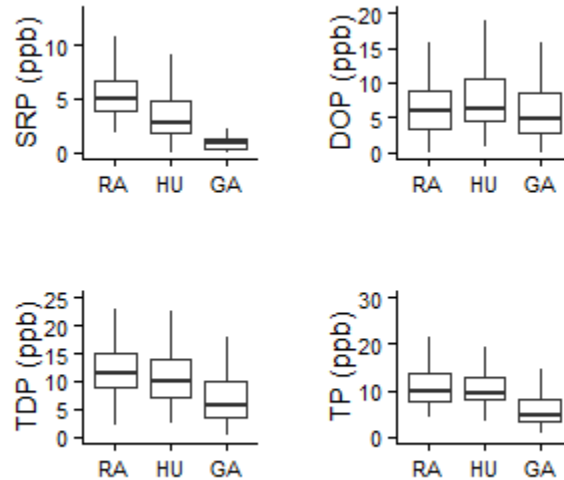
Figure 8. Stream water phosphorus concentrations in Ramsey (RA), Huachuca (HU), and Garden (GA) Canyon streams including soluble reactive phosphorus (SRP), dissolved organic phosphorus (DOP), total dissolved phosphorus (TDP), and total phosphorus (TP). Outliers are removed.
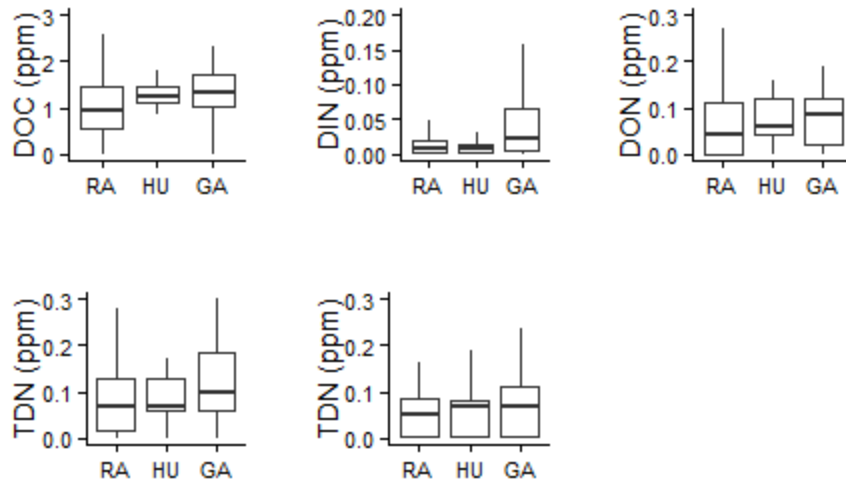
Figure 9. Stream water dissolved organic carbon (DOC) and N concentrations in Ramsey (RA), Huachuca (HU), and Garden (GA) Canyon streams including dissolved inorganic N (DIN), dissolved organic N (DON), total dissolved N (TDN), and total N (TN). Outliers are removed.
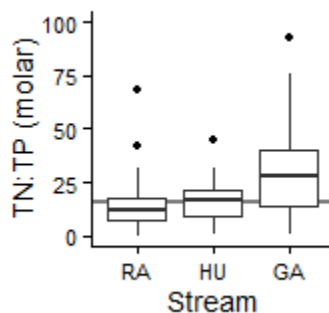
Figure 10. The molar ratio of total nitrogen (TN) and total phosphorus (TP) in Ramsey (RA), Huachuca (HU), and Garden (GA) Canyon streams. Grey horizontal line represents 16:1, the Redfield Ratio.

Multivariate Analyses

Principal components analysis was performed on 41 samples of eight chemical constituents (Table 3). Four of the sampling points were removed prior to the analysis after being identified as univariate outliers. The analysis revealed a significant three-dimensional ordination that explained 74% of the variance (Table 3). The first two principal components, PC1 and PC2, suggested a stoichiometric ordination: nitrogen species had the highest loading on principal component 1 (PC1), while phosphorus species had the highest loadings on PC2 (Table 2, Figure 11). The third principal component, PC3, had negative loadings from DOP and TN (Table 2, see Supplementary Materials for PCA biplots with PC3). Samples, in terms of stream identity, were segregated more along PC2 than PC1, with Garden Canyon samples more negatively related to PC2 than Huachuca or Ramsey Canyon samples (Figure 11). Variation among sample variables along PC1 and PC2 decreased in the warmer seasons (summer and monsoon), however, the overlapping confidence ellipses suggest seasonal variation does not significantly explain differences in PC1 and PC2 (Figure 11).

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Table 3. Loadings on principal components. Structure correlations greater than 0.5 are bolded.

Parameter	PC1	PC2	PC3
DOC	**0.758**		
DIN	0.402	-0.432	
DON	**0.784**	0.411	
TDN	**0.868**		
TN	0.407		**-0.684**
SRP		**0.691**	0.477
DOP	**-0.518**	0.462	**-0.662**
TDP	**-0.504**	**0.778**	
TP		**0.716**	
Variance explained	32.3%	24.7%	16.8%
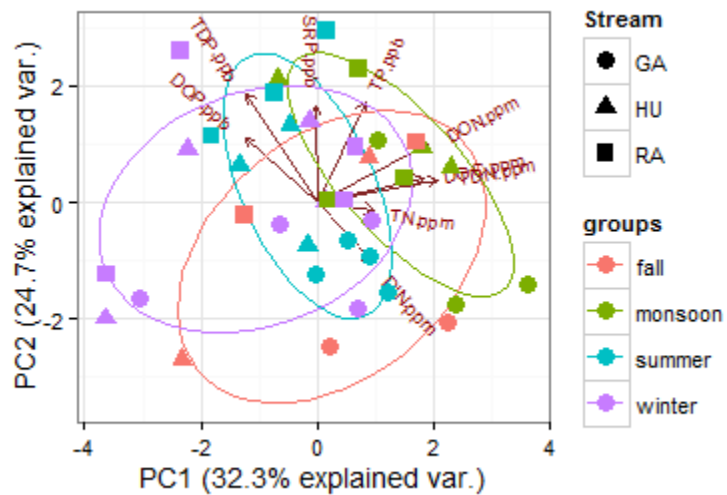
Figure 11. PCA Ordination plot of stream water chemistry samples from Garden (GA), Huachuca (HU), and Ramsey (RA) Canyon streams, AZ, during 2011 – 2014. Confidence ellipses are drawn around samples based on season.

The measured stream descriptor variables correlated distinctly between PC1 and

PC2 or PC3. Temperature, discharge, and $CaCO_3$ reaction rate were strongly positively

correlated to PC1 ($R^2=0.24$, $R^2=0.11$, and $R^2=0.13$, respectively) (Table 4; Figure 12).

Total dissolved N, the main loading variable on PC1, showed a significant linear

relationship with temperature ($R^2=0.24$ and $p<0.01$), an exponential relationship with

discharge ($R^2=0.17$ and $p<0.01$), and a curvilinear relationship with $CaCO_3$ reaction rate

($R^2=0.60$ and $p<0.001$) (see Supplementary Material for figures). $CaCO_3$ mass transfer

and reaction rate were strongly negatively correlated with PC2 ($R^2=0.39$ and $R^2=0.33$,

respectively) and PC3 ($R^2=0.16$, for reaction rate) (Table 4; Figure 12). Total dissolved P,

the main loading variable on PC2, had a significant linear relationship with $CaCO_3$ mass

transfer ($R^2=0.20$ and $p<0.01$) and a significant linear relationship with $CaCO_3$ reaction

rate ($R^2=0.11$ and $p<0.05$) (see Supplementary Material for figures).

Table 4. Pearson correlations between variables and ordination axes. Asterisks indicate
significance at * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

	PC1	PC2	PC3
Log Discharge	**0.41***	-0.21	-0.28
Ca ($\mu g\ L^{-1}\ m^{-1}$)	0.14	**-0.63***	-0.13
Ca ($\mu g\ L^{-1}\ s^{-1}$)	**0.38***	**-0.57**	**-0.47***
Temperature ($^{\circ}C$)	**0.51**	0.15	-0.08
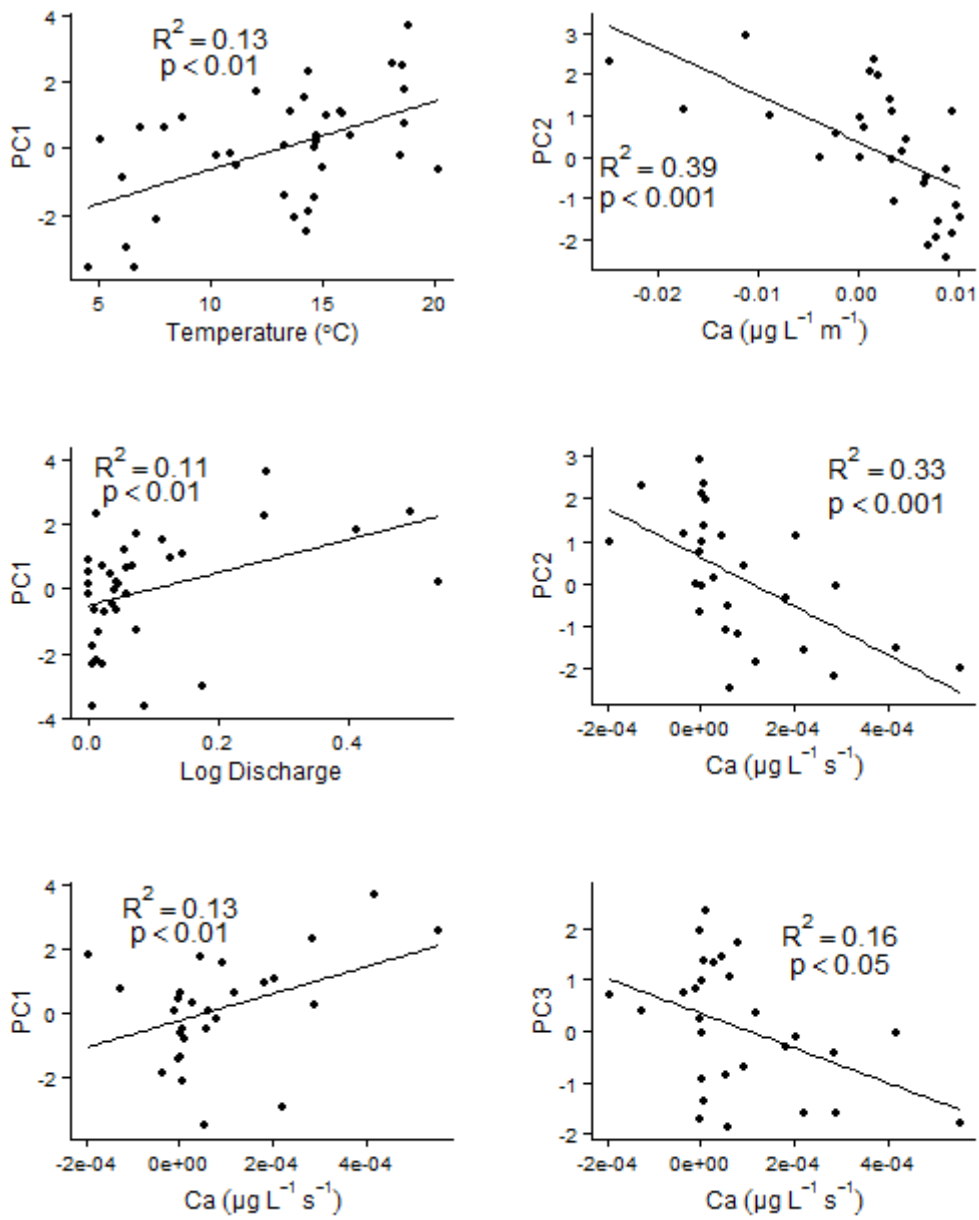
Figure 12. Significant correlations between principal components (PC1, PC2, PC3) and stream descriptor variables.

The reaction rate of $CaCO_3$ deposition was also linked with the stoichiometric ratio of N:P in the stream water. As predicted by my hypothesis, the N:P ratio was positively correlated with $CaCO_3$ deposition rate (Figure 13). Furthermore, deposition of $CaCO_3$ occurred at N:P ratios greater than 30.
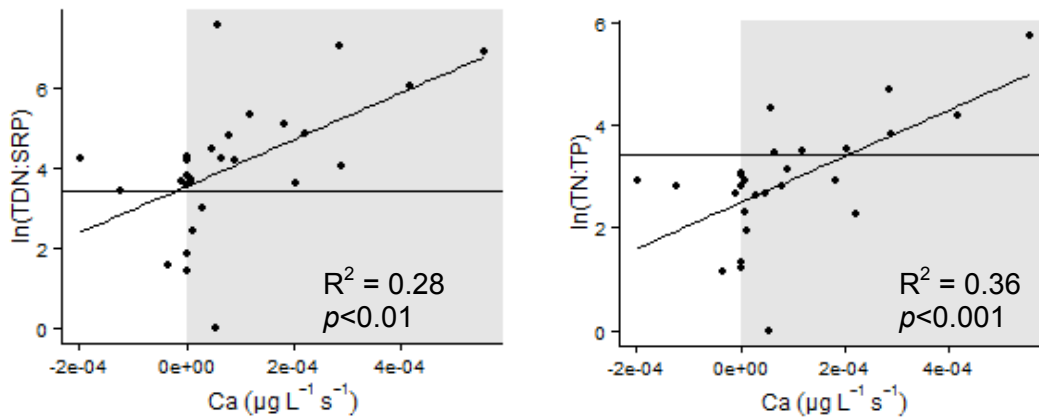
Figure 13. The natural log of the atomic ratio of (A) total dissolved nitrogen (TDN) to soluble reaction phosphorus (SRP) and (B) total nitrogen (TN) to total phosphorus (TP) compared to the reaction rate of $CaCO_3$ deposition across all streams. The horizontal line represents a ratio of 30:1 and the shaded region delineates net positive $CaCO_3$ deposition.

DISCUSSION

While many studies of stream ecosystems have focused on understanding the dynamics of single elements (Mulholland and Webster 2010), my research considers how hydrologic and biogeochemical processes may couple or uncouple the availability and transport of both N and P in streams. The results indicate that $CaCO_3$ deposition is effective in reducing stream water P concentrations and thus exports of P from headwater streams to receiving ecosystems during baseflow conditions. These results expands the role of $CaCO_3$ coprecipitation of phosphate in lowering P availability from nutrient-rich

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streams (Jenkins et al. 1971, Arvin and Jenkins 1985, Jarvie et al. 2005) to include oligotrophic headwater streams. I also found both that the relative amount of phosphorus compared to nitrogen is lower when there is active $CaCO_3$ deposition and that there is a positive correlation between N concentrations and $CaCO_3$ deposition rate, exacerbating the stoichiometric imbalance of N and P related to PO_4 coprecipitation. Discharge and temperature were related to nutrient concentrations, as well, but $CaCO_3$ deposition rates had the most pronounced effects on nutrient availability and stoichiometry of the variables tested.

Discharge and Nutrient Availability

The multivariate analysis of the study streams suggests discharge is linked to N and DOC concentrations, but, unexpectedly, does not strongly influence P concentrations. There are several potential reasons for this apparent hydrologic decoupling of N and P concentrations. First, increases in N or DOC during higher flows may be caused by hydrologically induced sediment suspension (Casey and Farr 1982). As coprecipitated PO_4 is bound in $CaCO_3$ deposits that cement the stream bottom (House 2003), P might not easily be suspended in increased flow events. However, as one of the study streams did not exhibit $CaCO_3$ deposition (Ramsey Canyon), sediment P suspension during higher flows events should have been detected in the dataset. Alternatively, the lack of a correlation between P and discharge could be because watershed P inputs to the stream are low, but N inputs are not, or because the sampling dates did not occur directly during or after a storm event (Figure 2) and, thus, I may have missed an increase in P concentrations following spates. Indeed, based on a literature review, TP and suspended

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sediments consistently increased following spate events in streams across the globe (Meyer et al. 1988). Increased surface (lateral) flow has also increased transport of inorganic nutrients to streams (McDiffett et al. 1989, Grimm 1992). Closer monitoring of these streams will help differentiate whether the mechanism behind the relationships between discharge and nutrient concentrations are due to differences in nutrients in the watersheds or due to the low-frequency sampling scheme.

Biologic demand and nutrient cycling

Biological processes are often seasonal, generating predictable lows in nutrient concentrations in temperate streams in the spring and fall when biological demand is greatest due to low canopy cover (Meyer and Likens 1979, Mulholland 2004). While the study streams are located in a desert, the riparian zone of these reaches is dominated by sycamore, maple, and other deciduous trees (Brown 1994); therefore, seasonality in biological processes is expected to be similar to streams in temperate forests. In the study streams, field observations suggested that autotrophic growth was seasonal, with periphyton biomass greatest in the spring or summer and lowest in the winter. Thus, the lack of a strong effect of seasonality on stream nutrient concentrations in the multivariate analysis (Figure 11) may be because the temporal scale of the sampling was too coarse to capture directly the signal of seasonal variation in biological nutrient uptake within a stream or because other processes, .e.g., temperature or $CaCO_3$ deposition, had greater effects on nutrient cycling.

Temperature was an important variable for explaining nitrogen concentrations in the study streams. The positive relationship between temperature and PC1 and TDN

(Figure 12 and Figure S4) suggests that warmer temperatures stimulated decomposition and N mineralization thereby increasing nitrogen concentrations in the water column (Chergui and Pattee 1990, Brookshire et al. 2011, Ferreira and Chauvet 2011, Duan and Kaushal 2013).

CaCO$_3$ Deposition and Nutrient Availability

The results provide strong support for the role of CaCO$_3$ coprecipitation of phosphate in lowering stream phosphate concentrations. This relationship extends to other forms of phosphorus, as well, e.g., TP and TDP, based on both the coarse inverse relationship between P concentrations and stream identity (Figure 8) and the grouping of SRP, TDP, and TP in the multivariate analysis (Table 2). These results suggest that retention of phosphate due to coprecipitation influences other aspects of in-stream P cycling, changing the form and quantity of P transported to downstream ecosystems (Withers and Jarvie 2008).

By using multivariate analysis, I found an unexpected and positive relationship between CaCO$_3$ deposition and N concentrations. That is, CaCO$_3$ deposition was associated with low P concentrations *and* high N concentrations. Thus, while temperature is likely an important driver for N concentrations in the streams (Figure 12 and Appendix D, Figure S4), it is not the only process influencing N concentrations. If it were, N concentrations would have been greatest in Huachuca Canyon, the stream with the highest temperature (Figure 4), but they were not (Figure 9). Instead, it seems that the main ordinations in the multivariate analysis are driven by CaCO$_3$ deposition.

The positive correlation between N concentrations and CaCO$_3$ deposition may be an indirect outcome of biological responses to reduced P availability. CaCO$_3$ deposition was strongly related to N:P ratios (Figure 13) and when CaCO$_3$ deposition was positive, molar N:P ratios in the water were generally greater than 30, a threshold indicative of potential P limitation of primary producers (Downing and McCauley 1992). A recent study of nutrient cycling in different montane headwater streams suggests that stoichiometric constraints on biological nutrient uptake can be detected from ecosystem-scale measurements of nutrient cycling (Schade et al. 2011). Schade and co-authors show that streams dominated by heterotrophic processes exhibit a strict coupling of nutrient uptake (2011). Hence, under P limitation, the ability of the biota, particularly heterotrophic microbial communities, to sequester and process N may be reduced, allowing N to reach higher concentrations in the water column. High N:P ratios have been documented in other CaCO$_3$-depositing streams (Elser et al. 2005, Marks et al. 2006) and wetlands (Noe et al. 2001); I suggest that in all of these systems with high rates of CaCO$_3$ deposition, biological N demand and, therefore, biological N uptake, are lower because the organisms are P-limited.

Comparisons with other CaCO$_3$-depositing Streams

Calcium carbonate-depositing streams are found across the world, including in Australia (e.g., Drysdale et al. 2002), Asia (e.g., Lu et al. 2000, Liu et al. 2010), Europe (e.g., House and Denison 1997, Merz-Preiß and Riding 1999, Auqué et al. 2013), and North America (e.g., Jacobson and Langmuir 1970, Herman and Lorah 1987, Malusa et al. 2003). Compared with other headwater streams, CaCO$_3$ deposition rates found in the

study streams of the Huachuca Mountains are low (e.g., 0.5 μg $CaCO_3$ L^{-1} s^{-1} in Garden Canyon) (Figures 6 – 7). For instance, in Falling Springs Creek, VA, calculated $CaCO_3$ deposition rates range from 3.3 – 93 μg $CaCO_3$ L^{-1} s^{-1} (Herman and Lorah 1987) while in Fossil Creek, Arizona, USA, deposition rates range from 1.2 – 39 μg $CaCO_3$ L^{-1} s^{-1} (Malusa et al. 2003). Therefore, I expect the stoichiometric feedback between $CaCO_3$ deposition and nutrient cycling may be even stronger in other $CaCO_3$-depositing streams. Also, by using multiple proxies of $CaCO_3$ deposition rate, this study found strong spatial variability in $CaCO_3$ deposition within stream reaches (Figure 6). Understanding this variability may have important implications for how $CaCO_3$ deposition influences ecological processes within a stream.

$CaCO_3$ deposition is potentially related to many ecological processes in streams. For example, $CaCO_3$ encases leaf litter, which is thought to decrease rates of detrital decomposition in $CaCO_3$-depositing reaches of streams (Casas and Gessner 1999, Martínez et al. 2014). $CaCO_3$ deposition is also related to increased rates of leaf litter decomposition as travertine dams increase turbulence and therefore physical breakdown of leaf material (Carter and Marks 2007, Milisa et al. 2010). $CaCO_3$-depositing reaches have also been found to host higher biodiversity of macro-organisms (Marks et al. 2006, Carter and Marks 2007), but lower overall macroinvertebrate abundance (Carter and Marks 2007, Martínez et al. 2014), although the reason is unknown. Additionally, some organisms interact directly with $CaCO_3$ deposits. Amphipods in a stream in southern Germany use $CaCO_3$ as body armor to reduce predation pressure (Ruff and Maier 2000). Microbes, as well, have long interacted with $CaCO_3$ deposits, both promoting and inhibiting lithification (Dupraz and Visscher 2005). When biofilms interact with $CaCO_3$

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deposits, phosphate coprecipitation rates may be lower than expected, suggesting $CaCO_3$ formation may not always result in decreased P bioavailability (Jarvie et al. 2002). Indeed, at longer time scales, dissolution of $CaCO_3$ deposits may serve as a P source to periphyton (Hagerthey et al. 2011). In future research, it will be important to distinguish under what conditions $CaCO_3$ deposition acts as a permanent versus temporary sink of P (Ensign and Doyle 2006).

CONCLUSION

The total and relative amounts of nutrients available in an ecosystem have important consequences for numerous ecological processes. Different hydrologic and biogeochemical processes are associated with changes in nutrient concentrations in streams (Alexander et al. 2007) and my results suggest the importance of $CaCO_3$ deposition in regulating the N:P stoichiometry of stream water nutrient concentrations. Furthermore, while organisms in streams are often found to be co-limited by N and P (Dodds and Welch 2000, Francoeur 2001, Elser et al. 2007), my results suggest $CaCO_3$ deposition may be a geochemical scenario in which primary P limitation may prevail. Streams with active $CaCO_3$ deposition are geographically widespread. In a comprehensive assessment of travertine, Pentecost (2005) describes over 100 streams with actively depositing $CaCO_3$ distributed across six continents. It should be noted that the ability of $CaCO_3$ coprecipitation of phosphate to lower P concentrations in a stream loses effectiveness at higher PO_4 concentrations, as phosphate may inhibit $CaCO_3$ formation (House 1990, Neal 1999). Stream beds are not pure $CaCO_3$ substrates, and the

presence of other minerals, organic matter, or biofilms may moderate expected relationships between $CaCO_3$ deposition rates and phosphate coprecipitation (Jarvie et al. 2002). Therefore, kinetic experiments with sediment sorption capacity may be useful in translating these results to other streams (Jarvie et al. 2005, Demars 2008). My data add to accumulating research on $CaCO_3$-depositing streams that document the unique effects of $CaCO_3$ on ecosystems and highlight the potential for managing $CaCO_3$ to minimize downstream P flows, but not N flows, to receiving environments.

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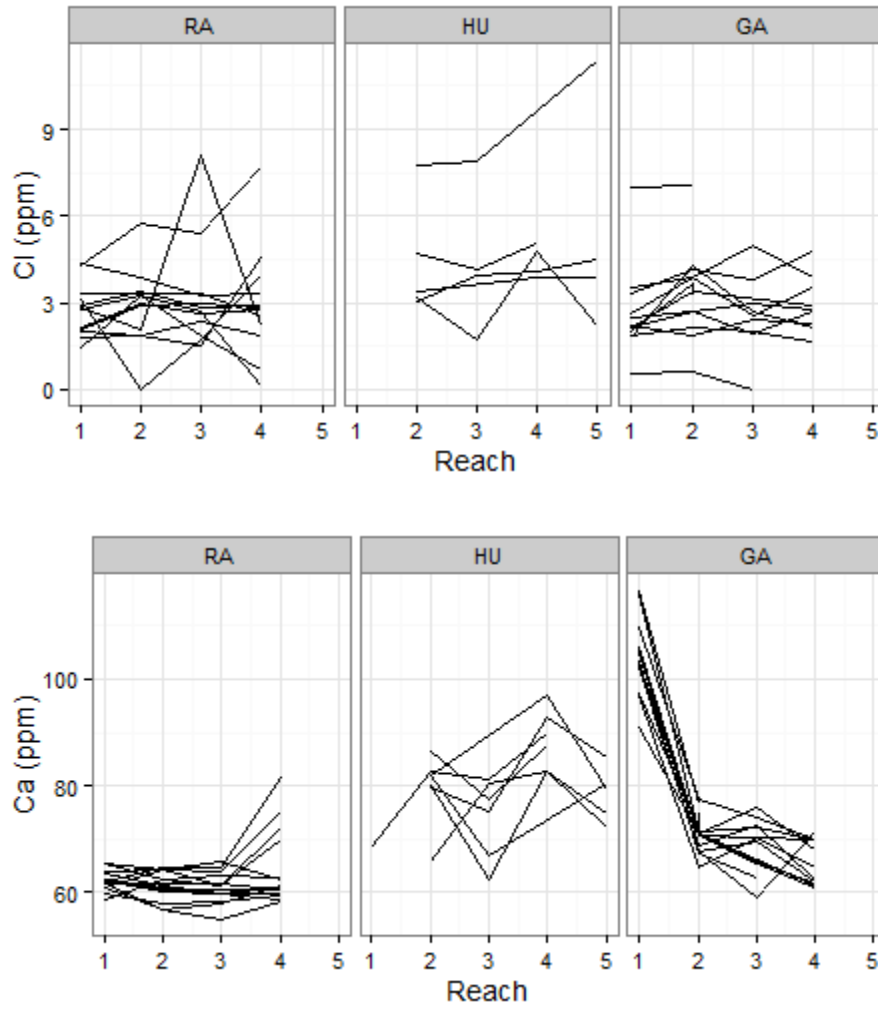
Figure S1. (A) Conservative ions like chloride (Cl) show little change across the sampling reach compared to the (B) reactive ion calcium (Ca). Lines represent different sampling days.
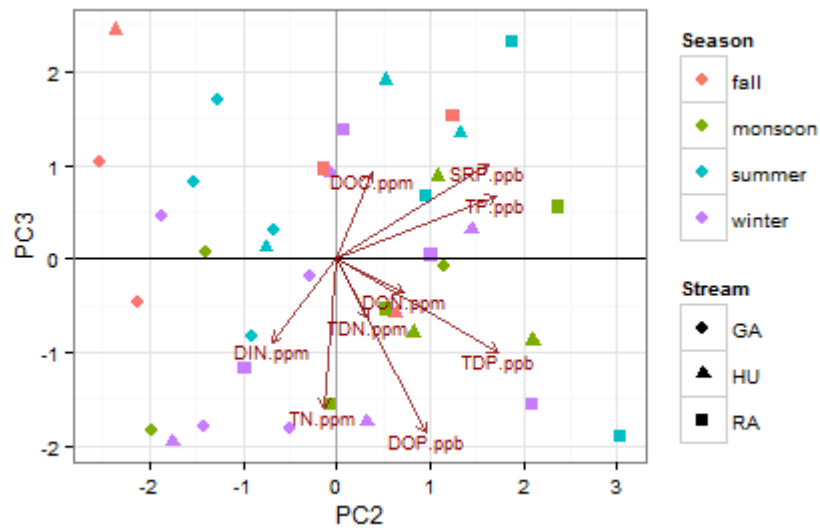
Figure S2. PCA Ordination plot of stream water chemistry samples from Garden (GA), Huachuca (HU), and Ramsey (RA) Canyon streams, AZ, during 2011 – 2014, showing PC2 (23.7% explained variance) and PC3 (18.2% explained variance).
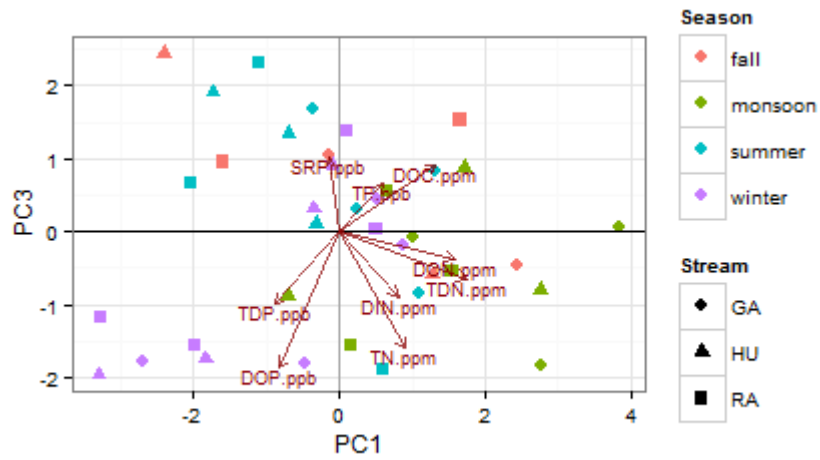
Figure S3. PCA Ordination plot of stream water chemistry samples from Garden (GA), Huachuca (HU), and Ramsey (RA) Canyon streams, AZ, during 2011 – 2014, showing PC1 (32.3% explained variance) and PC2 (23.7% explained variance).
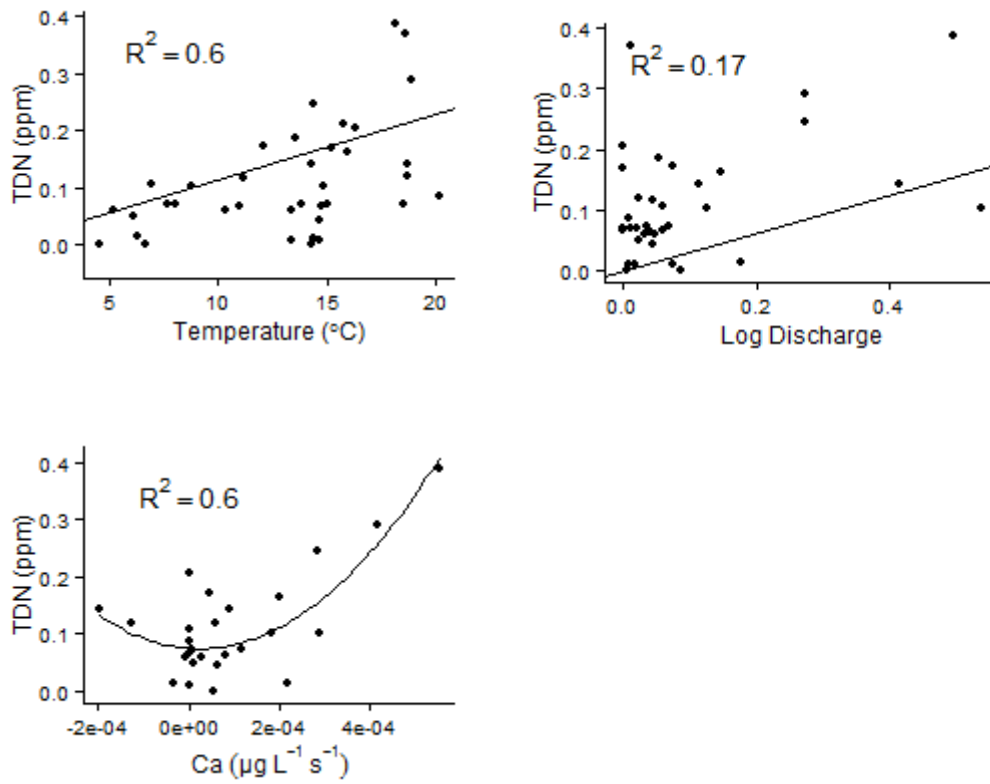
Figure S4. Total dissolved nitrogen (TDN), the main component of PC1, is significantly correlated with stream temperature, discharge, and reaction rate of $CaCO_3$.
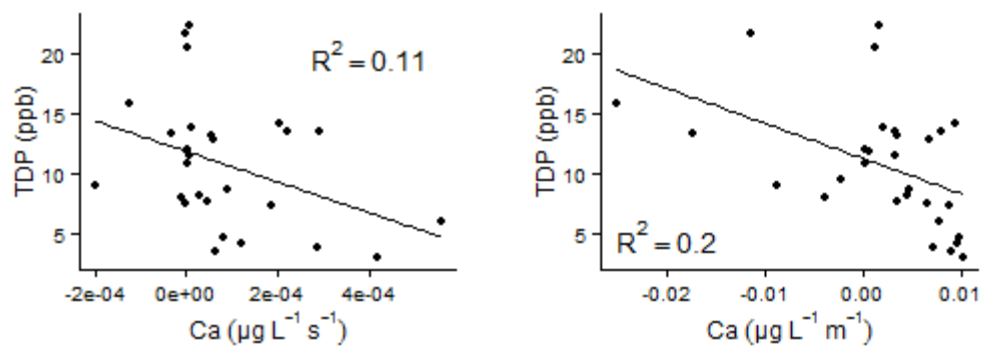
Figure S5. Total dissolved phosphorus (TDP), the main component of PC2, is significantly correlated with mass transfer and reaction rate of calcium carbonate.

CHAPTER 3

NUTRIENT LIMITATION OF PERIPHYTON IN STREAMS ACROSS A GRADIENT
OF CALCIUM CARBONATE DEPOSITION RATE

ABSTRACT

Empirical research on nutrient limitation of periphyton growth has challenged the paradigm of single-element nutrient limitation in stream ecosystems. However, if geological or geochemical conditions exist that disproportionately influence the concentration of one nutrient over another, single-element nutrient limitation of periphyton growth may still occur. As calcium carbonate ($CaCO_3$) deposition lowers stream water concentrations of phosphorus relative to nitrogen, it may be such a geochemical process which leads to single-element nutrient limitation. To test the hypothesis that $CaCO_3$ deposition causes phosphorus (P) limitation, I used nutrient-diffusing substrata (NDS) to monitor nutrient limitation of periphyton in three streams in southeastern AZ that exhibit a gradient of $CaCO_3$ deposition rates. In support of my hypothesis, I found that P limitation was more likely to occur in the streams with $CaCO_3$ deposition. However, stimulation of Chl a accrual by P enrichment occurred in the $CaCO_3$-depositing streams only in the spring (stream x season interaction: $F_{4,14}=5.31$, $p<0.05$), suggesting that periphyton in these streams are primarily light-limited. I also found positive correlations of nitrogen (N) limitation with temperature ($R^2=0.24$, $p<0.01$), suggesting N demand is stimulated by warmer conditions. Overall, this research

suggests that the ability of $CaCO_3$ deposition in an ecosystem to cause single-element

nutrient limitation may be dependent on other limiting factors (e.g., light) being met.

INTRODUCTION

Like all organisms, the growth of the microbes and algae that comprise periphyton is influenced by the availability of necessary resources. In a stream, seasonal changes in light or temperature can influence periphyton growth and, therefore, demand for nutrients to support growth (Francoeur et al. 1999, Wold and Hershey 1999). However, if nutrient demand is not met because nutrient supply in an ecosystem is low, periphyton growth may still be constrained (Sanderson et al. 2009) and/or their nutrient content may change (Cross et al. 2005, Qin et al. 2007, Hill et al. 2011, Liess et al. 2012). This may have important ecosystem effects as periphyton can be a disproportionately large source of energy to organisms in upper trophic levels compared to other autochthonous or allochthonous sources (McCutchan and Lewis 2002). Also, nutrient imbalances between periphyton and their consumers can influence trophic dynamics due to effects of poor stoichiometric food quality (Frost and Elser 2002, Elser et al. 2005).

By extending Liebig's Law of the Minimum for monoculture crops to multispecies communities, ecologists initially viewed nutrient limitation of periphyton growth as being controlled by a single nutrient (Danger et al. 2008, Harpole et al. 2011). However, recent meta-analyses have uncovered the ubiquitous role of multiple nutrients in constraining growth. In a comparison of stream nutrient amendment experiments, Francoeur (2001) found that it was common for periphyton growth in the same stream to be stimulated by the addition of multiple nutrients independently. In two other, independent meta-analyses, Dodds and Welch (2000) and Elser and co-authors (2007) found combined additions of nitrogen (N) and phosphorus (P) lead to the greatest increases in periphyton growth, suggesting that NP co-limitation is the dominant form of

nutrient limitation in stream benthic communities. Indeed, Arrigo (2005) and Harpole et al. (2011) argue that production in multispecies communities, as in periphyton, is expected to be co-limited due to factors like differential resource requirements across species or individual-specific modifications of nutrient allocation in responses to nutrient stress.

Yet, despite the predominance of co-limitation of periphyton growth, low nutrient supply, particularly in relation to other resources, is still often associated with single nutrient limitation. For example, low absolute nitrogen concentrations (<20 μM dissolved inorganic nitrogen, DIN, and <11 μM total nitrogen, TN) predicted N limitation of periphyton growth in studies by Keck and Lepori (2012) and Dodds et al. (2002), respectively. In their meta-analysis, Keck and Lepori (2012) went on to show that it was actually low nitrogen concentrations relative to phosphorus (as soluble reactive phosphorus, SRP) that best predicted the probability that periphyton were N-limited. These authors also found that P limitation was best predicted by total phosphorus (TP) availability. These observations suggest that a nutrient may reach a point at which its availability is low enough to limit growth across numerous species in a community, despite the biological complexity in the community as described in Arrigo (2005) and Harpole et al. (2011). Therefore, factors that influence the nutrient concentrations of stream water may still play an important role in regulating nutrient limitation of periphyton growth.

Nutrient availability in a stream is influenced by numerous factors. One of these factors is the geologic or geochemical condition (Gibbs 1970), e.g., calcium carbonate ($CaCO_3$) deposition (House 2003). Calcium carbonate deposition commonly occurs in

alkaline, groundwater-fed streams that have high concentrations of calcium (Ca^{2+}).

Indeed, $CaCO_3$ deposition has been documented in streams across the globe (Pentecost 2005). Importantly, it can influence phosphorus concentrations in the overlying water. When $CaCO_3$ is deposited, it adsorbs phosphate, a process known as phosphorus coprecipitation (Ishikawa and Ichikuni 1981, House and Donaldson 1986, Hartley et al. 1997). In lakes, phosphorus coprecipitation has been suggested as a negative feedback on eutrophication. In this scenario, nutrient-stimulated photosynthesis shifts alkalinity which promotes $CaCO_3$ precipitation; the $CaCO_3$ binds phosphate via coprecipitation and rapidly sinks. Therefore, the phosphorus, which is now associated with the sedimented $CaCO_3$, is no longer available to stimulate photosynthesis in the water column (Koschel et al. 1983, Robertson et al. 2007, Hamilton et al. 2009). In streams, $CaCO_3$ deposition can increase phosphorus retention in the sediments (Reddy et al. 1999, House 2003) and lead to lower phosphorus concentrations in stream water (see Ch. 2). Yet, phosphorus deposited in sediments in streams has been suggested to remain accessible to the periphyton (Schlesinger and Bernhardt 2013) even though phosphorus co-precipitated in wetland $CaCO_3$ deposits remains inaccessible to biological communities as long as the $CaCO_3$ is not dissolved (Noe et al. 2001, Borovec et al. 2010, Hagerthey et al. 2011). Research is needed to determine if coprecipitation of phosphorus with $CaCO_3$ deposition leads to phosphorus limitation of stream periphyton growth.

The aim of this study is to determine if nutrient limitation is associated with $CaCO_3$ deposition. I assessed nutrient limitation in three streams that vary by extent of $CaCO_3$ deposition (see Ch. 2). To determine nutrient limitation of periphyton growth, I deployed nutrient diffusing substrata (NDS) in several reaches in each stream in fall,

spring, and summer and monitored responses of chlorophyll *a* biomass (Chl *a*). I predicted that P-limitation of periphyton will be strongest in the streams with active $CaCO_3$ deposition.

METHODS

Study Sites

The Huachuca Mountains are located in southeastern Arizona between the southern Rocky Mountain and the Sierra Madre Occidental cordilleras. The mountains are part of the Madrean Sky Islands, a region of NW-SE trending isolated ranges that are separated by arid valleys. The climate in the region is semi-arid with the majority of the ~80 cm annual precipitation occurring either in the summer during high-intensity thunderstorms associated with the North American monsoon season or in the winter during Pacific frontal storms. The mountains support a pine (*Pinus*) and oak (*Quercus*) forest, while the riparian zones of the streams support a mixed broadleaf deciduous forest with Arizona sycamore (*Platanus wrightii*), bigtooth maple (*Acer grandidentatum*), and velvet ash (*Fraxinus velutina*) (Brown 1994). Many of the plant and animal communities in the region are highly diverse (Bowers and McLaughlin 1996, Tsai et al. 2007, Bogan et al. 2013).

For this study, I chose three small, northeastern-facing headwater canyon streams in the Huachuca Mountains: Garden Canyon, Huachuca Canyon, and Ramsey Canyon (see Ch. 2, Figure 1). I performed the experiments in regions above 1500 m, where all but the bottom-most site is thought to be perennial (E. Moody, *unpublished data*) (Jaeger and

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Olden 2012). The bedrock of the Huachuca Mountains is composed of granite, limestone, and other sedimentary geologic units. Even though they are only 11 km apart, the streams differ markedly in one respect: rates of $CaCO_3$ deposition (see Ch. 2). Active $CaCO_3$ deposition is found in Garden and Huachuca Canyon, but not in Ramsey Canyon. The spatial extent of $CaCO_3$ deposition is greater in Garden Canyon, though reach-scale $CaCO_3$ deposition rates were higher in Huachuca Canyon during the study period (see Ch. 2).

Experimental Design

To determine nutrient limitation of periphyton communities, I placed nutrient diffusing substrata (NDS) in three (summer) or four (spring, fall) reaches in each stream. Sites are referenced by their stream name ("GA" for Garden Canyon, "HU" for Huachuca Canyon, and "RA" for Ramsey Canyon) and reach number (1 to 4 from upstream to downstream). Only three reaches (1 to 3) were used in the summer due to stream desiccation at each of the bottom-most reaches (Reach 4). The experiment was repeated in each stream in three seasons (date deployed in parenthesis): fall (30 September 2012), late winter/early spring (hereafter, "spring"; 22 Feb 2012), and summer (12 June 2013). The abundance of chlorophyll *a* on NDS was used as an indicator of photoautotrophic periphyton biomass but I note that biomass does not always scale with primary production (Tank and Dodds 2003). Concomitant with NDS experiments, I determined stream water physicochemical properties from each reach.

Stream Physicochemistry

At each site, I measured pH (Beckman-Coulter 255 pH/mV, Beckman Coulter Inc., Brea, CA), temperature, and specific conductivity (YSI 85, Yellow Springs, OH) prior to NDS deployment. I also collected water samples for nutrient analysis; unfiltered samples were used for total concentrations and filtered samples (0.45 μm Supor membrane, Acrodisc) were used for dissolved concentrations. Water samples for dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were preserved at pH < 2 with concentrated hydrochloric acid and analyzed on a Shimadzu TOC-VN/TN Analyzer. The remaining water was frozen until analysis. Nitrate (NO_3^-), ammonium (NH_4^+), and TN were determined using a Lachat QuikChem 8000 Flow Injection Automated Ion Analyzer (Hach, Lovelend, CO, USA); the in-line persulfate/UV oxidation method was used for TN. Dissolved organic nitrogen (DON) was calculated as the difference between TDN and NO_3^- and NH_4^+. Soluble reactive phosphorus (SRP) was determined using the ammonium molybdate colorimetric method (APHA 2005) using a spectrophotometer with a 10-cm pathlength quartz cell. Total dissolved phosphorus (TDP) and TP were determined by persulfate digestions (Solorzano and Sharp 1980), after which phosphorus concentrations were analyzed as SRP. Dissolved organic phosphorus (DOP) was calculated as the difference between TDP and SRP.

Nutrient Diffusing Substrata

Nutrient diffusing substrata (NDS) were assembled following the methods of Tank et al. (2006), which are described briefly. Nutrient treatments were nitrogen (N; as KNO_3), phosphorus (P; as KH_2PO_4), nitrogen + phosphorus (NP), or control (C; no

nutrient amendment). 2% agar solutions were made with 0.5 moles L^{-1} of the respective nutrient; 3% agar solutions were used for the NP treatment to ensure that the agar solidified. Agar solutions were poured into plastic cups (Poly-Cons®; Madan Plastics, Crawford, New Jersey, USA) and fritted glass disks (Leco Corporation, St. Joseph, Michigan, USA) were placed in a hole drilled out in the lid of each cup once the solution had solidified. Four (in the spring) or five (in the summer and fall) replicates of each NDS treatment were incubated at each site for three weeks. Upon removal, glass disks were wrapped in aluminum foil and placed on ice until transport to the lab where they were frozen until analysis for chlorophyll *a* (Chl *a*). Chl *a* was analyzed fluorometerically or spectrophotometrically following a 20 h extraction with cold, 90% buffered acetone. Chl *a* concentrations reported here are phaeophytin-corrected values.

I classified nutrient amendment interactions based on the definitions of Harpole et al. (2011) for different types of treatment responses reported from nutrient enrichment experiments: simultaneous, independent, serial, and antagonistic limitation and absolute antagonism. Briefly, simultaneous, independent, serial, and antagonistic limitations occur when the NP treatment response is the greatest, but are differentiated by the N, P, and/or C treatment responses. Simultaneous co-limitation occurs when N, P, and C treatment responses are equal; independent co-limitation occurs when N and P treatment responses are equal, but greater than the C response; serial limitation occurs when one nutrient treatment is greater than C, but not the other; antagonistic co-limitation occurs when the sum of the N and P treatment response is greater than the NP treatment response. Absolute antagonism occurs when the NP treatment response is less than the N or P

response alone. I classified single nutrient limitation as a nutrient response in which the single and NP treatment responses were equal, but greater than the C response.

Statistical Analysis

To determine nutrient limitation of periphyton growth, I analyzed Chl *a* responses to nutrient amendments with a one-way ANOVA within each reach during each season with treatment as the fixed effect (4 levels: C, N, P, and NP). If the data from a reach did not meet the assumptions of normality, a Kruskall-Wallis test was performed instead. When a significant treatment response was found, post hoc comparisons were made using either a Tukey's Honestly Significant Difference test or a Mann-Whitney test for the parametric or non-parametric model, respectively.

To determine potential causes of nutrient limitation, responses to nutrient treatments were compared across season and stream by taking the normalized response of each treatment at each reach:

$$LN(Chl_{treatment}/Chl_{control}),$$

where the $Chl_{treatment}$ and $Chl_{control}$ are the mean Chl *a* concentrations of the replicates at the end of the incubation in the nutrient (either N, P, or NP) or control treatment, respectively. The normalized response of each treatment was used in a two-way ANOVA model with stream (levels: Garden, Huachuca, and Ramsey) and season (levels: spring, summery, fall) as factors. A type III sum of squares ANOVA model was used due to the unbalanced design of the test (the number of reaches per stream differed by season). Prior to analysis, assumptions of normality and homogeneous variance were checked. Outliers were removed based on Studentized residuals > 3 standard deviations from the mean and

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Cook's D > 0.25. Finally, I used Pearson correlation to test for predictors of nutrient limitation based on stream water physicochemistry data: temperature, DOC, NO_3^-, NH_4^+, DON, TDN, TN, SRP, DOP, TDP, and TP and the ratios of DOC:DON, DOC:DOP, DON:DOP, TDN:TDP, TN:TP, DIN:SRP, and DIN:TP. Then, I used regression analyses to determine relationships between physicochemistry data and normalized Chl *a* responses to nutrient amendments. All statistical tests were performed in R (ver 3.0.2) (R Core Team 2014); ANOVA was performed using the 'car' package. All results are reported as the mean ± 1 standard error, unless otherwise specified.

RESULTS

During the study period, nitrogen and phosphorus concentrations across Garden, Huachuca, and Ramsey Canyon streams were low (median TN = 82.9 µg N L^{-1}; median TP = 10.9 µg P L^{-1}) (Table 1). Phosphate concentrations tended to be higher in Ramsey Canyon (median SRP = 7.0 µg P L^{-1}) than in Garden (median SRP = 2.0 µg P L^{-1}) or Huachuca (median SRP = 3.1 µg P L^{-1}) Canyon while nitrate concentrations tended to be higher in Garden (median NO_3^- = 20.3 µg N L^{-1}) and Huachuca (median NO_3^- = 16.0 µg N L^{-1}) Canyon than in Ramsey Canyon (median NO_3^- = 6.8 µg N L^{-1}) (Table 1).

Nutrient diffusing substrates were successfully collected from most of the reaches. However, some of the NDS were unable to be analyzed due to stream drying or animal interference (Supplementary Material, Figure S1). In the spring, NDS were disturbed in GA1, GA2, and RA4, and so were not included in the analysis. In the fall, I was unable to analyze NDS from GA4 because the stream had dried to an extent that

Table 1. Seasonal physicochemical and nutrient characteristics of Garden (GA), Huachuca (HU), and Ramsey (RA) Canyon streams, AZ, including dissolved organic carbon (DOC), ammonium (NH_4^+), nitrate (NO_3^-), dissolved organic nitrogen (DON), total dissolved N (TDN), total N (TN), soluble reactive phosphorus (SRP), dissolved organic phosphorus (DOP), total dissolved P (TDP), and total P (TP). Nutrient ratios are molar ratios. Values are shown as averages across reaches (± 1 standard error). Units are $\mu g\ L^{-1}$ unless otherwise marked.

Stream	Season	Temp (^{o}C)	Sp Cond ($\mu S\ cm^{-2}$)	pH	DOC ($\mu g\ L^{-1}$)	NH_{4+}-N	NO_3^--N
Garden	Fall	15.3 (0.5)	562 (38)	8.06 (0.07)	1.4 (0.1)	6.0 (0.6)	60 (22)
	Spring	9.2 (0.8)	533 (34)	8.22 (0.03)	1.3 (0.1)	3.6 (0.6)	14 (5.0)
	Summer	14.7 (0.4)	534 (62)	8.24 (0.01)	2.1 (1.0)	5.3 (4.3)	20 (20)
Huachuca	Fall	16.5 (2.2)	568 (36)	7.91 (0.08)	0.8 (0.3)	6.0 (0.0)	31 (9.0)
	Spring	14.2 (0.7)	506 (13)	7.88 (0.17)	1.3 (0.1)	2.5 (0.5)	6.1 (1.5)
	Summer	18.5	482	7.99	0.6	2.0	16
Ramsey	Fall	13.8 (0.2)	440 (13)	7.98 (0.13)	1.2 (0.2)	6.1 (0.9)	20 (10)
	Spring	5.6 (0.5)	413 (3)	8.03 (0.19)	1.1 (0.1)	4.4 (2.4)	6.8 (2.2)
	Summer	14.4 (0.4)	500 (22)	7.70 (0.41)	0.5 (0.1)	1.5 (0.3)	1.0 (1.0)

Table 1, con't.

		DON	TDN	TN	SRP	DOP	TDP	TP
GA	Fall	50 (18)	104 (28)	85 (52)	5.5 (4.1)	3.5 (1.1)	8.9 (4.3)	6.9 (3.7)
	Spring	51 (6.3)	68 (8.8)	44 (18)	0.9 (0.3)	3.6 (0.7)	4.5 (0.9)	6.7 (1.6)
	Summer	40 (26)	67 (33)	87 (11)	2.1 (1.1)	5.6 (1.6)	7.6 (2.0)	7.4 (0.4)
HU	Fall	160	185	91	3.1 (0.4)	3.3 (2.0)	6.4 (1.6)	11.5 (7.7)
	Spring	60 (6.6)	66 (6.5)	42 (12)	4.0 (1.2)	5.8 (1.0)	9.8 (1.2)	13.4 (3.2)
	Summer	60	70	62	1.9	5.3	7.2	8
RA	Fall	55 (26)	76 (35)	192 (94)	6.0 (0.8)	4.5 (0.9)	9.8 (1.4)	10.9 (3.6)
	Spring	42 (8.2)	55 (7.3)	57 (21)	9.0 (2.0)	3.0 (0.5)	11 (2.0)	14.7 (2.8)
	Summer	10 (7.1)	13 (7.5)	83 (32)	7.0 (1.8)	6.3 (0.7)	13 (1.8)	12.8 (2.3)

Table 1, con't.

		DOC:DON	DOC:DOP	DON:DOP	TDN:TDP	TN:TP
GA	Fall	29 (10)	6530 (4089)	36 (5)	47 (11)	104 (40)
	Spring	29 (3)	1123 (146)	44 (9)	42 (10)	43 (7)
	Summer	61 (40)	1013 (389)	47 (25)	41 (15)	26 (4)
HU	Fall	4	733 (201)	136	101	21
	Spring	28 (4)	688 (112)	27 (5)	18 (4)	16 (2)
	Summer	12	288	24	23	17
RA	Fall	16 (5)	985 (337)	49 (8)	32 (6)	91 (35)
	Spring	31 (4)	853 (132)	35 (7)	12 (2)	13 (0)
	Summer	616 (351)	220 (21)	4 (2)	3 (2)	21 (6)

NDS were not fully submerged by the end of the experiment. In the fall and the summer, NDS were not deployed in the bottom three reaches of Huachuca Canyon due to a dry stream bed. Overall, 24 tests of nutrient limitation were successfully completed across the three streams and three seasons.

Table 2. Summary of nutrient limitation tests across streams in the Huachuca Mountains, Arizona. Bolded treatments indicate that the response was significant at $p<0.05$ while "n.s." indicates that nutrient limitation was not detected. Superscripts indicate the type of nutrient limitation response, where ^{aa} = absolute antagonism and ^{s} = serial co-limitation. Dashes, "--", indicate that the test was not performed.

Stream	Site	Season		
		Fall	Spring	Summer
Garden	1	**NP^s**	--	**N^{aa}**
	2	**N^{aa}**	--	*n.s.*
	3	N^{aa}	P^{aa}	**NP^s**
	4	--	N	--
Huachuca	1	N	**P**	NP^s
	2	--	**P**	--
	3	--	P	--
	4	--	*n.s.*	--
Ramsey	1	N	*n.s.*	**N^{aa}**
	2	NP^s	*n.s.*	**N**
	3	N^{aa}	N^{aa}	**N^{aa}**
	4	**N^{aa}**	--	--

Nutrient Limitation within Sites

Nearly half of the tests for nutrient limitation (10 out of 24) showed significant responses to nutrient amendments; another ten tests showed signs of nutrient limitation, but the results were not significant at $p<0.05$ (Table 2). Significant nutrient limitation was found in at least one stream each season (Appendix, Table S1). In the fall, I detected significant nutrient limitation in Garden (GA1: Kruskal-Wallis H=7.99, d.f.=3, $p<0.05$;

GA2: ANOVA $F_{3,16}=14.21$, $p<0.001$) and Ramsey Canyon (RA4: ANOVA $F_{3,16}=13.95$, $p<0.05$). In the summer, there were signs of nutrient limitation, as either N or NP co-limitation, at every reach (Table 1), although responses were significant only in Garden (GA1: ANOVA $F_{3,16} = 5.63$, $p<0.01$; GA3: ANOVA $F_{3,16} = 10.26$, $p<0.001$) and Ramsey Canyon (RA1: Kruskall-Wallis H=9.125, d.f.=3, $p<0.05$; RA2: ANOVA $F_{3.16}=5.65$, $p<0.01$, RA3: ANOVA $F_{3,16}=11.7$, $p<0.001$). In the spring, I detected significant nutrient limitation in two reaches in Huachuca Canyon (HU2: Kruskall-Wallis H=5.94, d.f.=3, $p<0.05$; HU3: Kruskall-Wallis H=8.78, d.f.=3, $p<0.05$), but noted only a trend towards positive responses of Chl *a* to nutrient additions in Garden and Ramsey Canyon (Table 2). Phosphorus amendments led to increases in Chl *a* concentrations in the spring in both of the $CaCO_3$-depositing streams, Garden and Huachuca Canyon, although the increases were only significant in Huachuca Canyon. In both streams, P additions more than doubled the Chl *a* concentrations compared to the control treatment. In Huachuca Canyon, phosphorus additions increased Chl *a* concentrations on average 722% in reaches 1 – 3. In Garden Canyon, P additions increased Chl *a* concentrations 442% in reach 3.

Chl *a* biomass on the control NDS differed by reach. In the reaches where there was a significant response to nutrient additions, Chl *a* was greater (2.46 ± 0.17 µg/cm^2) than in the reaches where there was not a significant response to nutrient additions (1.46 ± 0.15 µg/cm^2).

Overall, nutrient availability tended to constrain periphyton growth, although the type of nutrient limitation varied by season and stream (Table 2; Figures 1 – 3). Periphyton in Garden and Ramsey Canyon showed consistent signs of NP co-limitation,

except in the spring. Periphyton in Huachuca Canyon never showed signs of co-limitation. Only two types of nutrient interactions were detected: serial limitation and absolute antagonism, with the latter being far more common than the former. In all but one instance, absolute antagonism occurred due to phosphorus antagonism (NP treatment response was less than the N treatment response). There were also several instances of negative responses (nutrient response < control response) of Chl *a* to nutrient treatments (e.g., N in spring in HU1 and P in summer in RA1-3).
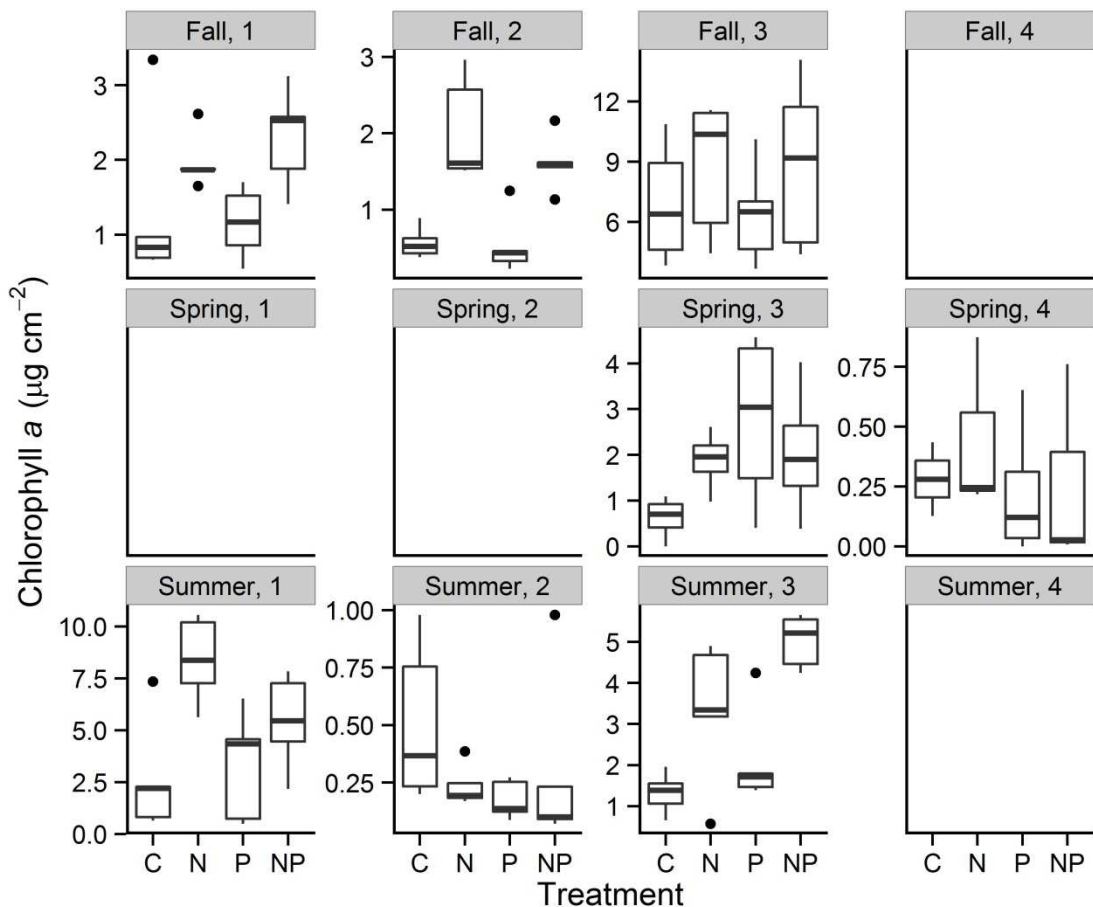
Figure 1. Areal mass of chlorophyll *a* on substrata at the end of the nutrient amendment experiment in Garden Canyon by season (row) and stream reach (panel within row as indicated by the number). Blank graphs indicate experimental combinations that were lost or not performed. Error bars represent ± 1 standard error.
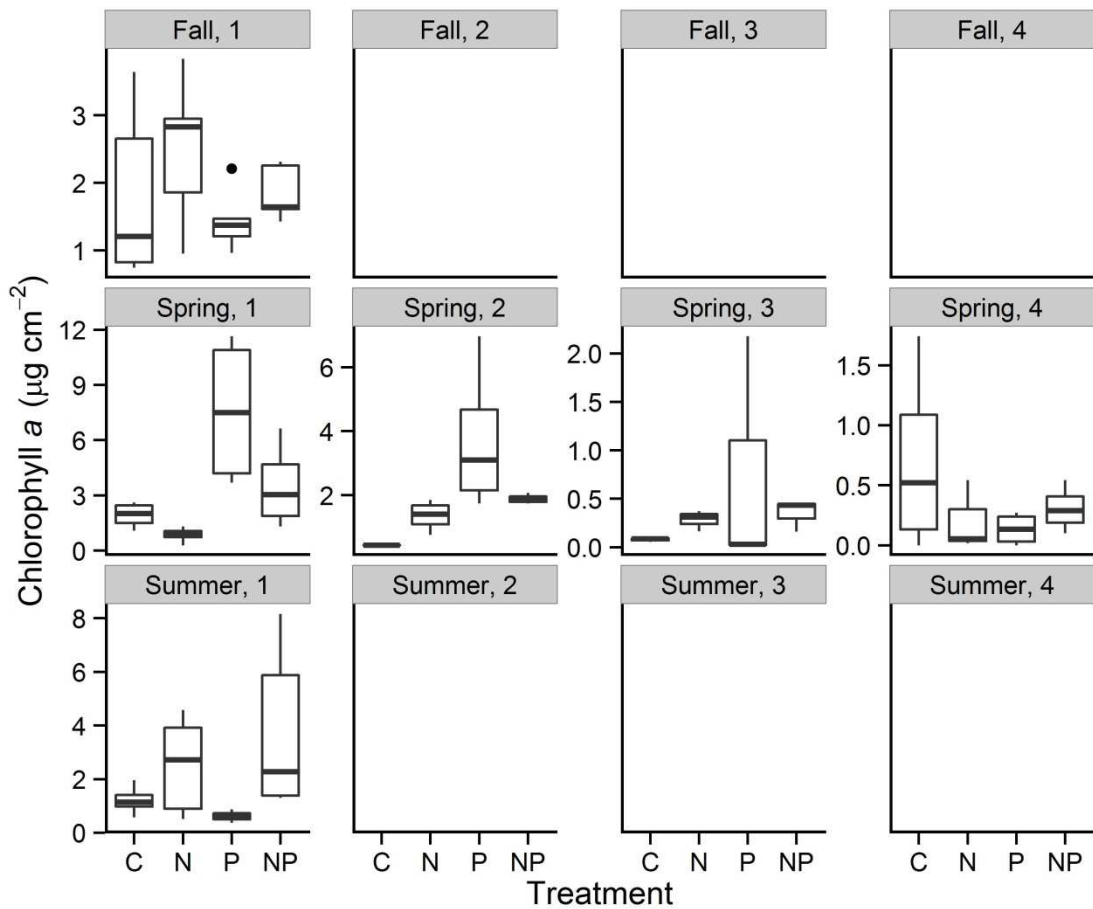
Figure 2. Areal mass of chlorophyll *a* on substrata at the end of the nutrient amendment experiment in Huachuca Canyon by season (row) and stream reach (panel within row as indicated by the number). Blank graphs indicate experimental combinations that were lost or not performed. Error bars represent ± 1 standard error.
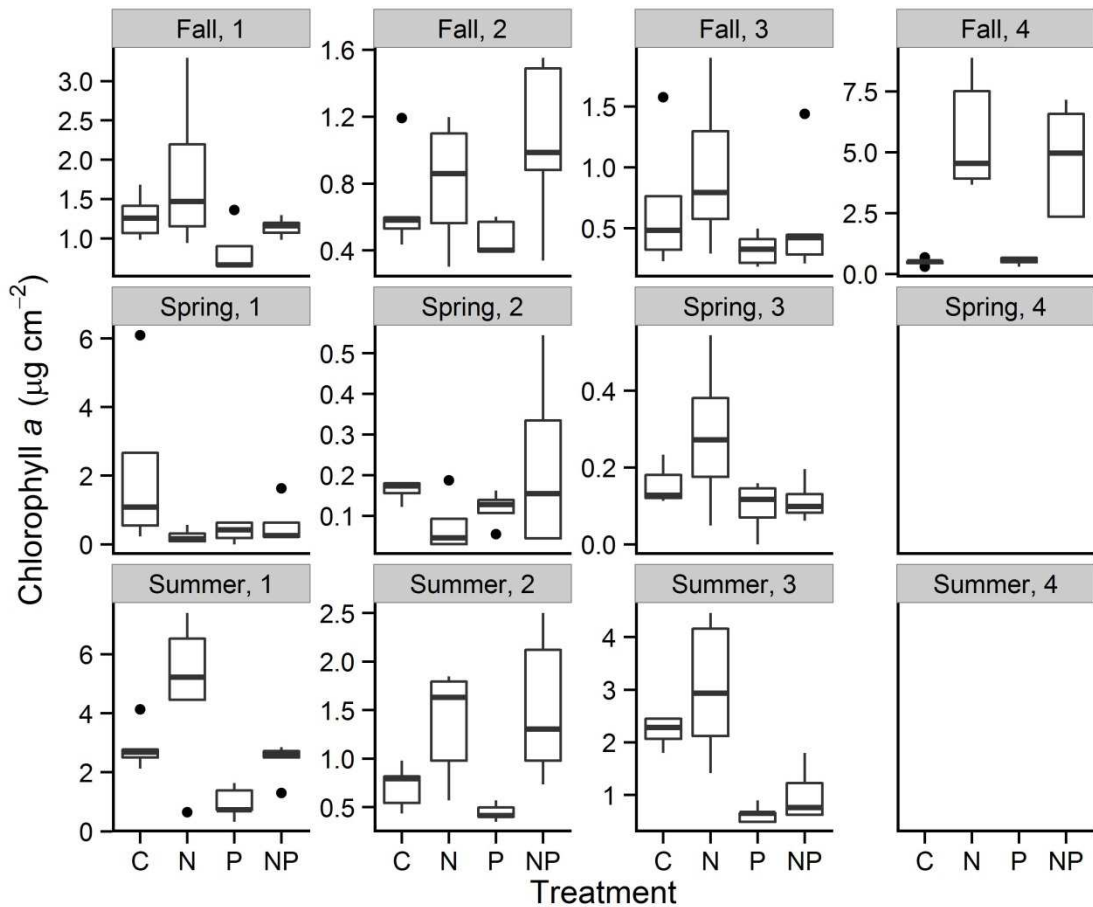
Figure 3. Areal mass of chlorophyll *a* on substrata at the end of the nutrient amendment experiment in Ramsey Canyon by season (row) and stream reach (panel within row as indicated by the number). Blank graphs indicate experimental combinations that were lost or not performed. Error bars represent ± 1 standard error.
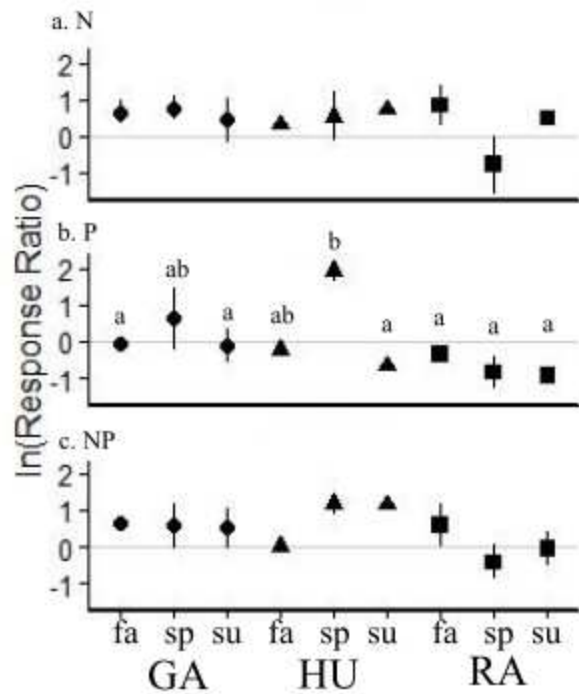
Figure 4. Proportional change in chlorophyll *a* concentration on (a) N, (b) P, and (c) NP-enriched substrates compared to control substrates (LN($Chl_{treatment}/Chl_{control}$)) across streams (GA: Garden Canyon, HU: Huachuca Canyon, RA: Ramsey Canyon) and seasons (fa: fall, sp: spring, and su: summer). Letters indicate significant differences based on Tukey's Post Hoc Comparisons ($p<0.05$). Error bars ± 1 se. Shapes indicate stream (• = GA, ▲ = HU, ▪ = RA).

Linking Nutrient Limitation across Sites

Chlorophyll *a* responses to nutrient amendments differed across streams and seasons. For instance, the response ratio of Chl *a* to P amendments was related to stream and season (Stream x Season interaction: $F_{4,14}=3.61$, $p<0.05$) (Figure 4). In the spring, P amendments increased Chl *a* concentrations in the $CaCO_3$-depositing streams (Garden and Huachuca Canyon), but not in the stream without $CaCO_3$ deposition (Ramsey Canyon). Neither stream nor season predicted Chl *a* response to N or NP amendments. However, based on the Pearson correlation test, the response ratios of Chl *a* to N and NP

amendments were significantly related to temperature (r=0.51 and r=0.42, respectively) (Figure 5). No other physicochemical parameter (Table 1) was significantly related to nutrient response ratios.
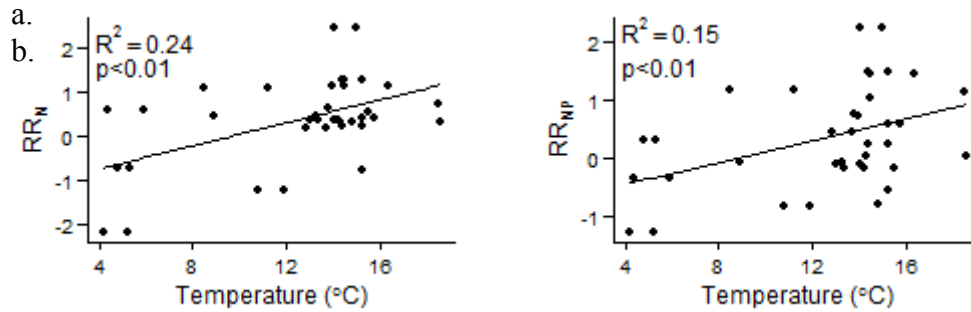
Figure 5. Proportional change in chlorophyll *a* concentration on (a) N-enriched substrates and (b) NP co-enriched substrates relative to control substrates (LN(Chl$_{treatment}$/Chl$_{control}$)) versus stream water temperature across three study streams.

DISCUSSION

This study documents strong seasonal shifts in nutrient limitation across streams with different $CaCO_3$-depositing regimes. Therefore, while $CaCO_3$ deposition influences phosphorus retention in a stream (Reddy et al. 1999, House 2003), my results suggest that it also affects the biological community by enhancing phosphorus limitation of periphyton growth. This phenomena is also seen in wetland carbonate ecosystems (Noe et al. 2001, Borovec et al. 2010, Hagerthey et al. 2011). My results also suggest that the influence of $CaCO_3$ on nutrient limitation is seasonally dependent, as the signal of phosphorus limitation was strongest in the spring when light limitation of autotrophic growth was likely minimal (Hill and Knight 1988, Larned and Santos 2000).

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Null and Antagonistic Responses of Periphyton

As in many other systems (Bernhardt and Likens 2004, Harpole et al. 2011, King et al. 2014), including oligotrophic streams (Sanderson et al. 2009), null or antagonistic responses of Chl *a* to nutrient additions were common in this study. "Null" responses of Chl *a* to nutrient treatments could be considered a weak, positive response that is statistically insignificant or a neutral response. The former likely reflects the low statistical power of the NDS method when less than 8 – 10 replicates per treatment are used (Francoeur 2001). Indeed, statistical power for the analysis within reach was consistently low (<0.8), even for statistically significant results (Appendix, Table S1). However, neutral responses in this study were associated with low Chl *a* concentrations on the control NDS, suggesting that another resource, e.g., light, was primarily limiting of periphyton growth when null responses were found (Mosisch et al. 2001).

Antagonistic responses of Chl *a* to nutrient additions, found in 25% of these experiments, have several potential causes that are not easily differentiated: (1) nutrient availability not actually increasing due to adsorption or by altering the availability of another nutrient, (2) increased herbivory, (3) biodiversity shifts, or (4) toxicity (Harpole *et al*. 2011 and references therein). While the data do not allow differentiation of the mechanism(s) responsible for these observations, several of the mechanisms appear more likely than others. Since $CaCO_3$ actively deposits in Garden and Huachuca Canyon (see Ch. 2), one might predict a role for mechanism (1) due to coprecipitation of phosphate with $CaCO_3$ on the surface of the NDS, preventing phosphorus from actually reaching periphyton cells. This possibility would predict that negative responses of Chl *a* to the P treatment should occur primarily in the $CaCO_3$-depositing streams and not in Ramsey

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Canyon, which does not exhibit CaCO₃ deposition. Instead, I found absolute antagonism in Ramsey Canyon, as well as Garden Canyon, suggesting absorption is unlikely the cause of nutrient antagonism in these streams. Increased rates of herbivory or treatment-specific shifts in periphyton community composition during the three-week incubation that result in overall lower rates of biomass accumulation are both possible, but further experimental work is needed to determine if these ecological interactions are influencing periphyton responses in these streams. Phosphorus antagonism, as found in this study, is common in other studies of nutrient limitation of periphyton growth (e.g., Hill and Knight 1988, Tank and Dodds 2003, King et al. 2014, Sanderson et al. 2009), suggesting that toxicity is likely mechanism for the antagonistic responses. Indeed, recent research on microbiological cultivation techniques suggests that autoclaving agar with phosphate, as is the standard technique for NDS construction, can generate compounds on plates that are harmful to bacterial growth (Tanaka et al. 2014). However, if the phosphorus treatment was toxic (Tanaka et al. 2014) or impaired growth due to low biotic phosphorus requirements (Bothwell 1985), I would not expect to see frequent positive responses of Chl *a* to phosphorus additions; nevertheless, such responses were relatively common (Table 1, Figures 1-3). Therefore, I suggest increased herbivory or shifts in biodiversity are the most likely mechanisms causing nutrient antagonism in these streams.

Seasonality of Nutrient Limitation

The interaction between nutrient treatment and season across streams suggests that periphyton nutrient demand is seasonal in these canyon streams. Light availability is often the primary factor controlling growth, and therefore nutrient demand, of primary

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producers in stream periphyton (Larned and Santos 2000, Mosisch et al. 2001, Tank and Dodds 2003). Despite the fact that the Huachuca Mtns are in the desert, the riparian zone of these montane streams support a deciduous forest (Brown 1994). Therefore, light availability and photosynthesis in the streams is likely greatest prior to leaf emergence in the spring (Hill et al. 2001). Indeed, the strong response of Chl *a* to P amendments in the spring suggests nutrient demand, and thus nutrient limitation, is greatest when light limitation is alleviated.

The strong correlation between water temperature and the degree of N or NP limitation of Chl *a* accrual suggests that nutrient demand by the periphyton is also controlled by a physiological response to temperature. Indeed, this pattern is common in temperate streams with similar temperature ranges (Biggs et al. 1998, Francoeur et al. 1999, Hoellein et al. 2010), suggesting temperature regulation of periphyton nitrogen demand is a widespread phenomenon. Temperatures differed substantially between streams, with temperatures almost always highest in Huachuca Canyon regardless of season (Table 1). Therefore, given the relationship between temperature and N across streams (Figure 5), the repeated observations of P limitation in Huachuca Canyon support the strong role of phosphorus coprecipitation with $CaCO_3$ deposition in determining nutrient limitation of periphyton growth.

$CaCO_3$ Deposition and Phosphorus Bioavailability

Calcium carbonate deposition can influence stream ecosystems through several mechanisms. Calcium carbonate deposits form dams that influence flow regimes (Fuller et al. 2011) which can increase rates of leaf litter decomposition (Carter and Marks 2007,

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Milisa et al. 2010). Conversely, $CaCO_3$ deposits cement leaf litter, decreasing decomposition rates due to lowered accessibility of the substrate to the decomposers (Casas and Gessner 1999, Martínez et al. 2014). When precipitation of $CaCO_3$ occurs in the water column, reduced light penetration lowers photosynthetic production (Strong 1978). Calcium carbonate deposition is also linked to increased biodiversity (Carter and Marks 2007) and shifts in trophic interactions (Vanderploeg et al. 1987, Ruff and Maier 2000), although the mechanisms for these patterns are not well known. Data from this study suggests another mechanism by which $CaCO_3$ deposition can influence ecological interactions: geochemical sequestration of a limiting nutrient.

Phosphorus limitation of periphyton growth is not common (Tank and Dodds 2003). As this study shows, streams with $CaCO_3$ deposition may be an exception to the paradigm that phosphorus does not limit periphyton growth in streams (Schlesinger and Bernhardt 2013, Grimm and Fisher 1986a, Grimm and Fisher 1986b): $CaCO_3$ deposition leads to phosphorus limitation during seasons with greatest autotrophic demand. Furthermore, phosphorus availability, in addition to light, is a main factor affecting rates of gross primary production of streams (Peterson et al. 2001). Therefore, $CaCO_3$ deposition may ultimately control rates of gross primary production in stream ecosystems in calcareous environments, though more research is needed to test this hypothesis. More research is also needed to see if carbonate-induced phosphorus limitation extends to the heterotrophic (microbial and herbivores) communities and, therefore, to ecosystem respiration and net ecosystem productivity and trophic energy flows. Further research may suggest $CaCO_3$ can influence decomposition rates through physical (via changes in

flow regimes and/or barring access to leaf litter) as well as geochemical (via phosphorus coprecipitation) mechanisms.

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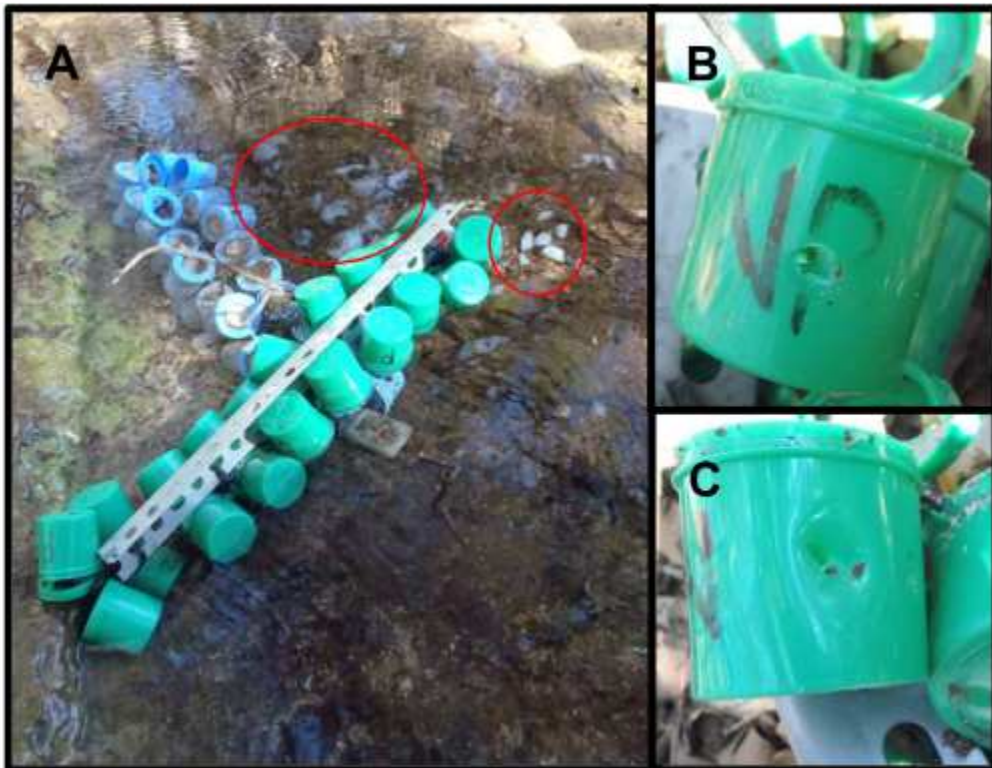
Figure S1. Example of animal damage to nutrient diffusing substrata (NDS) during experimental incubation. (A) NDS were moved in the stream; agar pieces in the water (highlighted by red circles) suggest the animal(s) may have eaten the agar. (B) and (C) Bite marks on the NDS cups. *Photographs by Jessica Corman.*

Table S1. Statistical analysis of chlorophyll *a* response to nutrient amendments at each reach where Test = statistical test performed (ANOVA = 1-way ANOVA, KW = Kruskal-Wallis), Int. = nutrient interaction (aa = absolute antagonism, s = serial), and DF = degrees of freedom. Power analysis was performed using observed effect sizes.

Stream	Season	Reach	Test	Limiting Nutrient	Int.	Test Statistic	DF	P-value	Power
Garden	Fall	1	KW	N	s	7.99	3	0.046	0.18
		2	ANOVA	N	aa	14.21	3,16	<0.001	0.67
		3	ANOVA	(N)	aa	0.73	3,16	0.55	0.06
		4	—						
	Spring	1	—						
		2	—						
		3	ANOVA	(P)	aa	1.83	3,12	0.195	0.13
		4	KW	(N)		4.57	3	0.206	0.06
	Summer	1	ANOVA	N	aa	5.63	3,16	0.008	0.37
		2	KW	*n.s.*		4.79	3	0.188	0.10
		3	ANOVA	NP	s	10.26	3,16	<0.001	0.58
Huachuca	Fall	1	ANOVA	(N)		1.15	3,16	0.359	0.08
		2	—						
		3	—						
		4	—						
	Spring	1	ANOVA	P		5.94	3,12	0.01	0.37
		2	KW	P		8.78	3	0.03	0.28
		3	KW	(P)		3.92	3	0.27	0.07
		4	KW	*n.s.*		1.67	3	0.644	0.10
	Summer	1	ANOVA	(NP)	s	3.12	3,16	0.056	0.20
		2	—						
		3	—						
Ramsey	Fall	1	KW	(N)		6.69	3	0.082	0.20
		2	ANOVA	(NP)	s	2.34	3,15	0.115	0.16
		3	KW	(N)	aa	4.71	3	0.194	0.10
		4	ANOVA	N	aa	13.95	3,16	<0.001	0.67
	Spring	1	KW	*n.s.*		4.74	3	0.192	0.11
		2	KW	*n.s.*		2.45	3	0.480	0.08
		3	ANOVA	(N)	aa	1.92	3,11	0.185	0.14
		4	—						
	Summer	1	KW	N	aa	9.13	3	0.028	0.42
		2	ANOVA	N		11.70	3,16	<0.001	0.37
		3	ANOVA	N	aa	5.65	3,16	0.008	0.62

"—" indicates that test was not performed, parentheses indicate trend towards nutrient limitation, "n.s." indicates the test was not significant

CHAPTER 4

GROWING ROCKS IN A STREAM: INCREASES IN RESOURCE AVAILABILITY

CAUSE SHIFTS IN BIOGEOCHEMICAL PROCESSES AND BACTERIAL

COMMUNITIY COMPOSITION IN A LITHYFYING MICROBIALITE

ABSTRACT

Lithified, microbial structures (microbialites) have been present on Earth for billions of

years. Understanding how these lithified structures form, particularly for calcium

carbonate ($CaCO_3$) structures, is one of the most hotly debated topics in geochemistry

and geobiology. When $CaCO_3$ deposits, it may coprecipitate phosphorus rendering this

nutrient less bioavailable to the microbes. Therefore, microbes associating with $CaCO_3$

may be doing so to gain access to a resource necessary for growth. In this study, I

compared phosphorus limitation of microbial communities associated with and not

associated with $CaCO_3$ deposition. Then, I used a mesocosm study to manipulate rates of

$CaCO_3$ deposition in microbialites to determine if lithification reduces microbial access

to phosphorus. All work was performed in Río Mesquites, Cuatro Ciéngeas, where

microbialites form as spheroid "oncoids." I found that phosphorus additions significantly

increased rates of gross primary production ($F_{2,13}=77.0$, $p<0.001$), net primary production

($F_{2,13}=129.6$, $p<0.0001$), and ecosystem respiration ($F_{2,13}=6.44$, $p<0.05$) in the oncoids,

while phosphorus addition had no effect on photoautotrophic production in the non-

$CaCO_3$ associated microbial communities. Growth of the non-$CaCO_3$ associated

phototrophs was only marginally stimulated when nitrogen and phosphorus were added

simultaneously ($F_{1,36}=3.98$, $p=0.053$). In the experiment with oncoids, resource additions

led to some shifts in Proteobacteria, Bacteroidetes, and Cyanobacteria, but mostly, had

little effect on the bacterial community composition. I also found that calcification rates

increased significantly with organic carbon additions ($F_{1,13}=8.02$, $p<0.05$). Next, in the

experiment where I lowered rates of $CaCO_3$ deposition by decreasing calcium

concentrations in the water, microbial biomass accumulation rates increased both in terms

of organic carbon ($F_{4,48}=5.23$, $p<0.01$) and phosphorus ($F_{6,48}=13.91$, $p<0.001$). These

results provide strong evidence in support of the role of lithification in controlling

nutrient limitation of microbialite communities.

INTRODUCTION

Stromatolites have been present on Earth for nearly 3.5 billion years (Hofmann et al. 1999, Riding 2000, Allwood et al. 2009). The microbial communities that formed stromatolitic fossils are thought to have played a crucial role in biogeochemical cycles throughout Earth's history (Dupraz and Visscher 2005). Most notably, the rise in oxygen (O_2) ~2.3 Gya was likely the result of these microbial consortia supported by photosynthetic organisms that fix carbon dioxide (CO_2) into organic matter and produce O_2 (Bekker et al. 2004, Canfield 2005, Dupraz and Visscher 2005). However, our understanding of these early microbial communities – or, potentially, other signs of microbial life in the rock record – is hampered by the inability to interpret whether the processes that form the laminated lithified structures of stromatolites are predominantly abiotic or biotic (Grotzinger and Rothman 1996, Reid et al. 2000, Gómez et al. 2014). Such laminations can be produced by different mechanisms: (1) the trapping and binding of sediment (Riding 2000, Altermann 2008), (2) "organomineralization" from mineral precipitation due to increases in alkalinity or pH as a by-product of microbial metabolic processes (Dupraz and Visscher 2005), or (3) the promotion of calcium carbonate ($CaCO_3$) precipitation in extracellular polymeric substances (EPS; Arp et al. 1998, Dupraz et al. 2009). Understanding the relative importance of these processes is one of the most hotly debated topics in geochemistry and geobiology (Kandianis et al. 2008). Studies of extant lithifying microbial communities ("microbialites") provide a unique opportunity to determine what factors influence the mechanisms involved in lithification (Garcia-Pichel et al. 2004, Bissett et al. 2008).

Microbes may associate with lithifying surfaces to gain necessary resources. For instance, lithified surfaces can provide a novel habitat for colonization (e.g., Fouke et al. 2000). Depending on how lithification occurs, it can generate protons as a byproduct that may be used by photosynthetic organisms to assimilate nutrients or bicarbonate (McConnaughey and Whelan 1997). Alternatively, microbes may interact with lithification because the lithification process competes with microbes for nutrients by sequestering key elements in forms that are not readily accessible. Indeed, calcium carbonate is able to co-precipitate phosphorus as phosphate (PO_4), a nutrient that often limits organismal growth (Elser et al. 2007). Thus, by sequestering PO_4 from the water column, $CaCO_3$ deposition may act as either a sink or source of phosphorus to microbes, depending on whether or not the microbial communities are able to access the co-precipitated PO_4. Research on subterranean microbial communities suggests the former: bacterial communities preferentially grow on $CaCO_3$ minerals with phosphorus versus those without (Jones and Bennett 2014). However, research on periphyton and cyanobacterial mats suggests the latter: $CaCO_3$ deposition is thought to promote phosphorus-limitation of primary production (Noe et al. 2001, Rejmánková and Komárková 2005, Borovec et al. 2010, Hagerthey et al. 2011).

Several studies have considered if microbial growth in microbialites with mineral compositions dominated by $CaCO_3$ is phosphorus-limited (e.g., Rosen et al. 1996, Elser et al. 2005, Elser et al. 2006, Valdespino-Castillo et al. 2014). After observing the high nitrogen to phosphorus ratios in the water of Lake Clifton, Western Australia, Rosen et al. (1996) suggested that the growth of microbialites living in that system was phosphorus-limited. In a series of nutrient enrichment bioassay experiments, Elser and

co-authors showed that primary production of oncoid microbialites in Río Mesquites, México, was limited by phosphorus availability and that microbes readily incorporated increased phosphorus into their biomass (Elser et al. 2005, Elser et al. 2006). Additionally, in a genetic survey from Alchichica soda lake, México, Valdespino-Castillo and co-authors (2014) concluded that its microbialites, but not its bacterioplankton, were phosphorus-limited due to the greater number of alkaline phosphatase genes found in the microbial consortia associated with the microbialites. The convergent observations of phosphorus limitation of $CaCO_3$-based microbialites suggests this phenomenon may be a common feature of these microbialites. However, it remains unknown if lithification itself plays a role in causing the limitation.

In this study, I build on the earlier work of Elser and colleagues (2005, 2006) in Cuatro Ciénegas, México, to study the interactions between nutrient availability and lithification in the living microbial communities of Río Mesquites. In this spring-fed desert stream, microbialites are found as unattached spheroids known as "oncoids." As suspended sediment loads in Rio Mesquites are low, formation of these oncoids is thought to be microbially mediated, as opposed to being formed by the trapping and binding of allochthonous carbonates (Garcia-Pichel et al. 2004). Previous work indicated that oncoid primary production is phosphorus-limited, while respiration is not (Elser et al. 2005). In this study, I performed three experiments to determine the extent of phosphorus limitation in the microbial communities of Río Mesquites and whether or not lithification may be its cause. I began by testing for phosphorus limitation of microbialite and non-microbialite-associated microbial communities. For the microbialites ("Experiment 1"), I used *in situ* mesocosms to determine if microbial metabolic rates (photosynthesis and

aerobic respiration) are limited by organic carbon and/or phosphorus and how shifts in resource availability interact with calcification rates. Furthermore, I monitored for changes in bacterial community composition in response to resource availability. To test for nutrient limitation of non-microbialite-associated microbes ("Experiment 2"), I used *in situ* bioassays with inorganic nutrient amendments and monitored changes in chlorophyll *a* concentrations as a proxy for photoautotroph biomass. Finally, to determine if lithification causes phosphorus limitation ("Experiment 3"), I manipulated rates of $CaCO_3$ deposition through abiotic (i.e., strontium addition, removal of Ca^{2+} ions) and biotic (i.e., decreased light availability) treatments and monitored changes in nutrient content of microbial biomass. I found phosphorus limitation of growth in Río Mesquites in the microbialites, but not the non-lithifying microbial communities ("periphyton"), and found evidence that lithification is the cause of this phosphorus limitation.

METHODS

Field Site Description

This study was conducted in Río Mesquites, located in the Natural Protected Area of Cuatro Ciénegas, Coahuila, MX. The Cuatro Ciénegas Basin (CCB) is known for its biodiversity with the highest rate of endemism in flora and fauna in North America, a characteristic that extends to the microbial community (Souza et al. 2006). CCB is a complex karstic system in which the underlying Cretaceous limestone formations are being actively dissolved to form >300 springs, pozas, sinkholes, streams, and other aquatic features (Wolaver et al. 2008). The valley floor (on average 740 m above sea

level, asl) is surrounded by mountains reaching >3,000 m asl. The endorheic basin has

long been isolated from marine environments, although many of its microbes are closely

related to marine taxa (Souza et al. 2006). Río Mesquites is one of the larger aquatic

features in the valley. This perennial stream, fed by thermal springs, is between $1-35$ m

wide and up to 4 m deep with a mean annual temperature of 26 $^{\circ}$C (Elser et al. 2005).

Dissolved inorganic phosphorus concentrations are low (0.59 µM P as soluble reactive

phosphorus, SRP), but dissolved inorganic nitrogen concentrations are relatively high

(20.7 µM N; data from $1998-2003$), resulting in strong potential for P limitation of

primary production (Elser et al. 2005).

Environmental Characterization

All experiments were performed in the summer (June to August) of either 2009

(Experiment 1), 2011 (Experiment 3), or 2012 (Experiment 2). Physicochemical

parameters in Río Mesquites were assessed concomitantly with the experiments.

Parameters were assessed in four to six reaches per sampling event. Sampling events took

place once during Experiment 1 and twice during Experiments 2 and 3. Stream water

measurements and collections were made ~0.5 m below the water surface. Temperature,

specific conductivity, and dissolved oxygen (DO) were measured using a YSI 85

temperature-oxygen meter (Yellow Springs Instruments Inc., Yellow Springs, OH, USA)

and pH was measured using a Beckman-Coulter 255 pH/mV meter (Beckman Coulter

Inc., Brea, CA, USA). For total nutrient analysis, stream water samples were unfiltered.

For dissolved nutrient analysis, stream water samples were filtered in the field through a

0.2-µm Supor membrane filter (Pall Corporation, Port Washington, NY, USA). All water

samples were preserved immediately (by 2% HCl for dissolved organic carbon, DOC, and total dissolved nitrogen, TDN; by 2% HNO_3 for cation concentration and trace element concentrations; or by freezing for all remaining analytes) and transported to Arizona State University (ASU) for analysis. Cation concentrations (Ca^{2+}, Mg^{2+}, Na^+, K^+) were assessed using a Thermo iCAP6300 Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Waltham, MA, USA). Anion concentrations ($SO_4^=$, Fl^-, Cl^-, Br^-) were analyzed using ion chromatography (Dionex ICS-2000, Sunnyvale, CA, USA). Trace element concentrations (V, Mn, Fe, Cu, Zn, As, Mo, Cd) were determined using a Thermo X-Series Quadrupole ICP Mass Spectrometer (ICP-MS; Waltham, MA, USA). Concentrations of DOC and TDN were determined using a Shimadzu TOC-VN/TN Analyzer while concentrations of inorganic nitrogen species, nitrate (NO_3^-) and ammonium (NH_4^+), were determined using a Lachat QuikChem 800 Flow Injection Ion Analyzer (Hach, Loveland, CO, USA). Dissolved inorganic phosphorus was analyzed as soluble reactive phosphorus (SRP) using the ammonium molybdate colorimeteric method and a spectrophotometer (APHA 2005). A persulfate digestion was used to convert total dissolved phosphorus (TDP) and total phosphorus (TP) to phosphate for analysis as SRP (Solorzano and Sharp 1980). Dissolved organic N (DON) and P (DOP) concentrations were calculated as the difference between total dissolved forms and dissolved inorganic forms.

Experiment 1. Resource Limitation of Microbialites

To determine the influence of resource availability on the oncoid-associated microbial communities of Río Mesquites, twenty-one oncoids of similar size were chosen

haphazardly from the stream for use in the field experiment on 16 Jul 2009. Three of the oncoids were sampled to characterize the microbial community at the start of the experiment (hereafter, "river" oncoids), as described below. Prior to experimentation, oncoid size (determined by water displacement) and mass was determined. The average size and weight of the oncoids was 16.7 cm^3 ($\pm$7.3) and 31.8 g ($\pm$7.9). River oncoids were returned to Río Mesquites after sampling; the remaining oncoids were used for the experiment.

Each oncoid was placed into a 2-L clear, sealed plastic container ("mesocosm") filled with unfiltered Río Mesquites water; mesocosms were incubated *in situ* on the bottom of the stream for the duration of the 16-day experiment except during water changes (see description below). Oncoids were secured inside mesocosms with aquarium silicon glue. Resource treatments were applied following a 2 x 3 full factorial design with variables being phosphorus (P) and organic carbon (C) addition and with three replicate mesocosms per treatment. Previous monitoring of Río Mesquites suggests that phosphate concentrations (as SRP) are generally around 0.6 μM P (Elser et al. 2005). Therefore, phosphorus additions in this experiment were to made to approximately double (LP) or triple (HP) ambient river concentrations by adding phosphorus, as KH_2PO_4, in spikes of +0.5 μM P (LP) or +1.0 μM P (HP). Organic carbon was added in a spike of +106 μM C using 3:1 (v/v) mixture of glucose and acetic acid. This concentration was chosen so that organic carbon additions would be above or at the Redfield Ratio, 106:1 of C:P (Redfield 1958), when combined with LP or HP treatments. Untreated mesocosms served as controls. In summary, the six treatments consisted of a control ("Ctrl") and amendments

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of organic carbon ("C"), LP, C and LP together ("CLP"), HP, and C and HP together ("CHP").

A randomized block design was used to incubate each of three treatment-replicate mesocosms in a tray at the river bottom. Water temperature was monitored continuously at the river bottom using a HOBO Pendant® temperature logger (Onset Corporation, Bourne, Massachusetts, USA). Water was changed twice daily in each mesocosm, at 07:30 and 19:00 h (interval ranged from 10.5 to 12.5 h between water exchanges). At every water change, the corresponding resource treatment was added. Treatments were added from concentrated stock solutions so that additions were 2 mL or less. For each water change, fresh water was retrieved from the bottom of the river approximately 1 m upstream of the incubation location using a horizontal Van Dorn. Sides of the mesocosms containers were monitored for algal growth and scrubbed when necessary.

Photosynthesis and respiration

At the end of the 16-day experiment, I determined rates of net primary production (NPP), gross primary production (GPP), and respiration (R) following the light-and-dark mesocosm method of Elser et al. (2005). After the water change on the final day of sampling, DO levels were measured in the mesocosms using a YSI 85 probe and containers were sealed between readings to minimize air exchange. Then, I measured NPP by recording concentrations of DO after 30, 75, and 120 minutes. Mesocosms were incubated in the stream between measurements to maintain ambient temperature and light conditions. Additionally, three control mesocosms containing stream water only were used to correct for oxygen production/consumption by planktonic organisms. To assess possible effects of incubation on oncoid metabolism, I also prepared three "river" oncoid

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mesocosms containing oncoids freshly retrieved directly from the stream bottom and of similar size to those used in the experiment. Following NPP determination, mesocosms were transferred to the field station to assay respiration rates. Each mesocosm was placed in the dark in a temperature-controlled room. DO concentration was recorded initially and then again after 4 and 20 hrs. Rates of NPP and R (in terms of O_2 generation and consumption) were calculated for each mesocosm by fitting a line to observations made at each time interval. For both NPP and R, slopes decreased between the penultimate and final DO recordings, and so these recordings were removed from the analysis. GPP was calculated by adding the respiration rate to the NPP rate for that mesocosm.

Calcium uptake

At the end of the experiment, I assessed net calcification rates in the experimental and river oncoids by measuring the bulk change of Ca^{2+} concentration following the methods of Garcia-Pichel et al. (2004). Oncoids were placed in larger mesocosms (4 L) with fresh Río Mesquites water amended with the appropriate nutrient treatment. Larger containers were used so that water pumps could be placed in the mesocosms to provide continuous circulation to reduce the establishment of boundary layers. Mesocosms were placed under constant illumination for 24 h at approximately ambient river water temperatures. Water samples were collected initially and after 24 h and analyzed for Ca^{2+} as described above. The difference in the Ca^{2+} concentration between these two values was used to determine calcification rate.

DNA Extraction

Digitate, dendroidal outwardly projecting "florets" from each oncoid were collected at the end of the experiment for DNA extraction. Samples were frozen at -20°C

until transport to ASU and then stored at -80°C until DNA extraction. Prior to DNA extraction, the CaCO$_3$ matrix was dissolved as described in Wade and Garcia-Pichel (2003). Florets were placed into individual sterile glass beakers with disodium ethylenediaminetetraacetic (Na$_2$EDTA) buffer at pH 5. The samples were placed under vacuum overnight. The samples were then centrifuged at 5,000 x g and 4°C for 10 minutes and the supernatant completely removed. DNA was extracted from the floret biomass using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol, with the following exception. Prior to the salt-ethanol-wash, the silica-bound DNA was washed three times with 500 μL of 6M guanidine thiocyanate to remove potential PCR inhibitors. The success of DNA extraction was checked via gel electrophoresis and the concentration of DNA quantified via absorbance at 260 nm using a spectrophotometer. DNA was successfully extracted from two florets each from the experimental and river oncoids.

PCR and 16S rRNA amplicon sequencing and processing

The V4 region of 16S rRNA was polymerase chain reaction (PCR)-amplified from each DNA preparation using the primers 515F and 806R (Caporaso et al. 2012) barcoded for each sample. PCR was performed in triplicate reactions with the Platinum® Taq High Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA, USA). PCR reactions contained approximately 1 ng DNA, 1X High Fidelity PCR Buffer, 2 mM MgSO$_4$, 200 μM dNTP Mix, 400 μg mL^{-1} acetylated BSA, 250 nM of each primer, and 1.5 units of enzyme. The following thermal cycling parameters were used to amplify the 16S rRNA genes: 94°C for 2 minutes to denature DNA, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s to anneal primers, and 68°C for 2 minutes, ending in a 5 minute extension at

68$^\text{o}$C. Following PCR, the products were gel-purified via the Wizard© SV Gel and PCR

Clean-Up kit (Promega, Madison, WI, USA) following the manufacturer's protocol. The

purified PCR products were then quantified via fluorometry (Qubit dsDNA High

Sensitivity Assay, Invitrogen, Carlsbad, CA, USA), pooled to equimolar concentrations,

and prepared as a single library for 2x250 bp paired-end sequencing on the Illumina

MiSeq (Illumina, San Diego, CA, USA) at the DNASU Genomics Core Facility at ASU.

The 16S rRNA amplicon library was sequenced along with samples for metagenomic and

other amplicon analyses with less than 10% of the lane devoted to amplicons.

Sequences were quality checked using FastQC (ver. 0.10.1) (Andrews 2014) and

paired with PANDASeq (Masella et al. 2012), resulting in 184,688 reads. Following the

guidelines of Kozich et al. (2013), reads were demultiplexed, trimmed of barcodes and

primers, and analyzed in mothur (Schloss et al. 2009). Sequences were aligned to the

Silva (ver. 119) (Quast et al. 2013), chimera-checked using uchime (Edgar et al. 2011),

clustered into operational taxonomic units (OTUs) at a 0.03 cutoff, and classified

according to Greengenes 13_8 (DeSantis et al. 2006). OTUs not present in at least two

samples were removed. After processing, there were 162,514 reads with library sizes

ranging from 3,269 to 19,063 sequences. Libraries were subsampled to the smallest size

for calculation of the number of observed OTUs, ACE richness, Inverse-Simpson Index,

Simpson Evenness Index, and coverage using mothur. Non-subsampled OTU abundances

and taxonomy information were imported into R (R Core Team 2014) via phyloseq

(McMurdie and Holmes 2013) for statistical analysis using DESeq2 (Anders and Huber

2010) as described below.

Sequences were deposited in the National Center for Biotechnology Information
(NCBI) GenBank database (BioProject ID PRJNA271031). The accession numbers for
each oncoid type or treatment are river (KP462893-KP473330), control (KP536579-
KP562679), C (KP518490- KP536578), LP (KP607985-KP634883), HP (KP562680-
KP607984), CLP (KP488780-KP518489), and CHP (KP473331-KP488779).

*Experiment 2. Nutrient Limitation of Non-microbialite Associated Photoautotrophic
Microbial Communities*

I assessed nutrient limitation of growth of photoautotrophic microbial
communities not directly associated with oncoids by assessing biofilm growth on
inorganic substrata amended with nitrogen and/or phosphorus (nutrient-diffusing
substrata, NDS). Biofilm growth was measured as the accumulation of chlorophyll *a* (Chl
a) on the substrata. NDS were constructed following the methods of Tank et al. (2006).
Briefly, NDS were made of 60 mL plastic cups (Poly-Cons®; Madan Plastics, Crawford,
NJ, USA) filled with agar and topped with a fritted glass disk (Leco Corporation, St.
Joseph, MI, USA). Agar was amended with either 0.5 M nitrogen ("N", as NH_4Cl), 0.5 M
phosphorus ("P", as KH_2PO_4), 0.5 M nitrogen and 0.5 M phosphorus together ("NP"), or
remained unamended ("Ctrl", for a control). Four replicates of each treatment were
attached to a metal L-bar and incubated at each of the three sites for 19 days beginning on
2 June 2012. After 19 days, the glass disks were removed and placed immediately in the
dark on ice until return to the field lab. Glass disks were frozen until processed for Chl *a*
accrual no more than 1 month after collection. Chlorophyll *a* was extracted with 90%

magnesium carbonate-buffered acetone and analyzed using either a fluorometer or spectrophotometer; fluorometry was used to quantify Chl *a* concentrations when below detection limit of spectrophotometry (1 μg cm^{-2}).

Experiment 3. Influence of Lithification on Nutrient Availability

The third experiment was conducted to determine the effect of CaCO$_3$ deposition on phosphorus availability. As in the first experiment, I performed this experiment using oncoids collected from Río Mesquites placed into mesocosms. I manipulated rates of CaCO$_3$ deposition using several different methods. First, as CaCO$_3$ deposition in these microbialites is thought to be controlled by photosynthetically mediated increases in pH (Garcia-Pichel et al. 2004), I reduced light availability to the oncoids to lessen photosynthetically induced CaCO$_3$ deposition. However, as phototrophs are an important component of the microbial community and their growth would also be modified by shading, I also used two abiotic treatments to lower rates of CaCO$_3$ formation: addition of strontium (Sr) and removal of Ca^{2+} ions. Strontium acts as a chemical inhibitor of CaCO$_3$ deposition (Wasylenki et al. 2005) while decreases in Ca^{2+} concentrations will reduce CaCO$_3$ reaction rates (Stumm and Morgan 2012).

Experimental mesocosms were designed to mimic environmental conditions in Río Mesquites, but were maintained *ex situ* to allow for a longer experimental duration (six weeks). Mesocosms were constructed from clear, 6-L plastic containers. Mesocosms were filled with 5 L of unfiltered water from Río Mesquites; stream water was changed every other day. Water was circulated continuously in each mesocosm by using a submersible water pump. Water temperature within the mesocosms was regulated by

placing mesocosms into water baths. Water baths were kept near 23°C by using a water chiller and submersible pump to circulate the water. Five water baths were used; one replicate mesocosm of each treatment was placed into each bath in a randomized block design. The entire experimental apparatus was outside to allow for adequate illumination, but shade cloth was placed over each bath to reduce incident sunlight to levels similar to those at the bottom on Río Mesquites. Uniformly sized oncoids were collected randomly from Río Mesquites and weighed and measured prior to being placed into the mesocosms on 20 May 2011. One oncoid was suspended in the center of each mesocosms using dental floss. The average size of the oncoids (determined by water displacement) was 102.1 ± 4.0 cm^3. Temperature, specific conductivity, DO, and pH were monitored in the mesocosms every other day. The experiment ran until 24 June.

Experimental manipulations were applied continuously for each treatment. Light removal was achieved by covering mesocosms with black electrical tape and placing aluminum foil loosely on top. Foil was removed only briefly during water replacements. Chemical inhibition was achieved by adding enough Sr (as SrCl) at each water change to reach 1 mM SrCl or 5 mM SrCl, concentrations high enough for calcite inhibition to occur (Wasylenki et al. 2005), but not necessarily high enough for toxic effects on microbial communities (Mei et al. 2006). Calcium removal was achieved by running stream water through a custom-made water softener prior to adding it to the mesocosms. The resin exchanged Ca^{2+} for Na^+ (SpectraPure Inc., Tempe, AZ); preliminary analyses found Ca^{2+} removal to be 90%. Resin exchange efficiency was monitored throughout the experiment by measuring Ca^{2+} concentrations before and after water softening.

<u>Oncoid carbon, nitrogen, and phosphorus contents</u>

At the beginning, middle (3 weeks), and end (6 weeks) of the experiment, I sampled the oncoids for C, N, and P contents as a proxy for nutrient acquisition. For the initial sample, a random subset of five oncoids was sampled; for the middle and end samples, all oncoids were sampled. Oncoid microbial biomass was collected by removing multiple 4 – 5 mm long dendritic florets using ethanol-cleaned metal forceps. The outer ~1 mm of each floret, hereafter referred to as "microbial biomass," was gently scraped away using an ethanol-cleaned metal spatula. A different quadrant of the oncoid was used for each sampling event. All samples were stored at $-20^{o}C$ until transport to ASU. At ASU, samples were dried for 48 h at $60^{o}C$ and ground with a mortar and pestle. From each ground sample, sub-samples were removed for C, N, and P analyses. Nitrogen content was determined using a CHN analyzer (Perkin Elmer 2400, Akron, Ohio, USA). Organic carbon and total phosphorus content were quantified using the methods described in Elser et al. (2005). Briefly, samples were combusted at $550^{o}C$ to assess organic carbon content by loss on ignition and then the remaining ash was extracted in 5 N H_2SO_4 and analyzed for phosphate using spectrophotometry (APHA 2005).

Statistical Analyses

<u>Experiment 1</u>

I tested for treatment effects on GPP, NPP, R, biomass C, N, and P contents and ratios, and Ca^{2+} uptake using a mixed model analysis of variance (ANOVA) with two fixed factors (organic carbon, phosphorus) and one random factor (block). *Post hoc* comparisons were made using Tukey's honestly significant difference (HSD) test. A

Kruskal-Wallis test was used to determine if any richness (number of OTUs observed and ACE richness estimator) or diversity (Inverse Simpson Index and Simpson Evenness Index) indices changed significantly with incubation of the oncoids in mesocosms or with resource addition. To determine if experimental incubation or resource addition resulted in significant changes in the abundance of particular OTUs, pairwise comparisons of OTUs present in river and control samples and control and treatment (C, CLP, CHP, LP, and HP) samples were conducted using DESeq2 (Anders and Huber 2010). DESeq2 was used to implement a Negative Binomial Wald test with a parametric fit of the dispersions. An OTU was considered significantly differentially abundant between samples if it exhibited a log2-fold change greater than 2, a false discovery rate less than 0.05, and a consistent change between replicates.

<u>Experiment 2</u>

I tested for significant changes in chlorophyll *a* on NDS across sites and within sites using a mixed model ANOVA with two fixed factors (nitrogen, phosphorus) and one random factor (site) and a two-way ANOVA with two fixed factors (nitrogen, phosphorus) within each site, respectively.

<u>Experiment 3</u>

First, I tested for physicochemical differences between water baths during the experiment. I analyzed each physicochemical parameter (i.e., temperature, pH, specific conductivity, and DO) using a repeated measures ANOVA with mesocosm as the subject. Changes in nutrient cycling in response to the experimental treatment were tested two ways: rate of accumulation and final percent composition. For the former, I tested for differences in the rate of accumulation of biomass C and P using analysis of covariance

(ANCOVA) with treatment as the categorical variable and blocking for effects of water bath. For the latter, I tested for differences in total C, N, or P or ratios of C:N, C:P, or N:P in the microbial biomass at the end of the experiment using one-way ANOVA while blocking for water bath.

All data are reported as the average (± standard error) unless otherwise noted. Assumptions of ANOVA tests, normality and homoscedasticity of variance, were checked with visual examination of residuals and using Levene's test, respectively. If the assumptions were not met, data were transformed to meet assumptions using either inverse or log transformations of the dependent variable, with the exception of the richness and diversity indices when a non-parametric analysis was performed. Non-transformed data are shown for better interpretation. All statistical tests were implemented in R (ver 3.0.2) (R Core Team 2014).

RESULTS

Physicochemical Characteristics of Río Mesquites

There were no significant differences between physicochemical variables across years, except for 2009 when water samples were contaminated. Therefore, the 2009 water chemistry data were removed from the analysis. Meter data (i.e., temperature, DO, specific conductivity, and pH) were averaged across 2009, 2011, 2012 and water chemistry data were averaged across 2011 and 2012, only.

The water of Río Mesquites was characterized by high conductivity (>2.5 mS cm^{-1}) and low nutrient concentrations (TN = 65 µM, TP = 0.86 µM) (Table 1). Both anions

Table 1. Physicochemical parameters of Río Mesquites, Cuatro Ciénegas, México, in summer 2011 and 2012 (General Descriptor parameters) or in summer 2009, 2011, and 2012 (all other parameters). Parameters include dissolved oxygen (DO) and specific conductivity (Sp Cond). Nutrient values include the concentrations of nitrate (NO_3^--N), ammonium (NH_4^+-N), dissolved organic nitrogen (DON), total dissolved N (TDN), total N (TN), soluble reactive phosphorus (SRP), dissolved organic P (DOP), total dissolved P (TDP), and total P (TP) and the molar ratios of TDN to TDP (TDN:TDP) and TN to TP (TN:TP). Also included are major ions (sulfate, SO_4^{2-}; chloride, Cl^-; bromide, Br^-, fluoride, Fl^-; sodium, Na^+; calcium, Ca^{2+}; potassium, K^+; magnesium, Mg^{2+}) and biologically relevant trace elements (As, arsenic; Cu, copper; Fe, iron; Mn, manganese; Mg, magnesium; Mo, molybdenum; V, vanadium; Zn, zinc). Mean values (and standard errors, SE) are calculated based on within-year averages of water samples collected at multiple locations. Standard errors are omitted for values based on a single sampling year.

Parameter	Mean (SE)	Parameter	Mean (SE)
General Descriptor		*Major Ions (mM, unless otherwise noted)*	
Alkalinity (meq L^{-1})	3.23 (0.45)	SO_4^{2-}	14 (2)
pH	7.31 (0.1)	Cl^-	14 (10)
Temperature (^{o}C)	31.1 (0.9)	Fl^-	0.53 (0.36)
DO (mg L^{-1})	4.2 (0.2)	Br^- (μM)	2.7 (0)
Sp Cond (μS cm^{-1})	2764 (81)	Ca^{2+}	8.7 (0.1)
Carbon and nutrients (μM or molar ratio)		Na^+	5.5 (0.9)
DOC	22 (10)	Mg^{2+}	4.6 (0.1)
NO_3^--N	69 (8)	K^+	0.17 (0.03)
NH_4^+-N	0.75 (0.33)	Si^{4+} (μM)	89
DON	<0.1 (<0.1)	*Trace Elements (nM)*	
TDN	36 (26)	^{51}V	71 (32)
TN	65	^{55}Mn	4.8 (3.2)
SRP	0.07 (0.01)	^{56}Fe	20 (10)
DOP	0.39 (0.11)	^{65}Cu	35 (21)
TDP	0.45 (0.11)	^{66}Zn	57 (12)
TP	0.86 (0.37)	^{75}As	120 (53)
TDN:TDP	77 (50)	^{95}Mo	349 (162)
TN:TP	169	^{114}Cd	0.3 (<0.1)

and cations contributed to the high conductivity. Anion concentrations were dominated by $SO_4^=$ and Cl^-, while cation concentrations were dominated by Ca^{2+} and, to a lesser extent, Na^+ and Mg^{2+}. Nitrogen in Río Mesquites was almost entirely in the form of NO_3^-;

DON concentration was negligible. Conversely, phosphorus in Río Mesquites was usually in the form of DOP (99% ± 37%). This led to molar N:P ratios of TDN and TDP or TN and TP that are over ten times greater than the Redfield ratio of 16:1 (Redfield 1958) (Table 1).

Experiment 1. Resource Limitation of Microbialites

<u>Experimental controls</u>

During the experiment, the diurnal water temperature in Río Mesquites fluctuated from $32.3 \pm 0.5^{\circ}C$ during the day to $27.8 \pm 0.4^{\circ}C$ during the night. Prior to performing the statistical analyses on experimental effects, Welch's T-tests were performed to check for significant differences between the river oncoids and unenriched Ctrl oncoids for each of the following response variables: GPP, NPP, R, and calcium uptake. The river and the Ctrl oncoids were not significantly different for these response variables; therefore, data from river oncoids were combined with those for the Ctrl oncoids for the statistical analyses for oncoid metabolism and Ca^{2+} uptake.

<u>Photosynthesis and Respiration</u>

Oncoid photosynthesis and aerobic respiration rates were phosphorus-limited. Phosphorus additions significantly increased rates of GPP ($F_{2,13}=77.0$, $p<0.001$), NPP ($F_{2,13}=129.6$, $p<0.0001$), and R ($F_{2,13}=6.44$, $p<0.05$; Figure 1). Increases were most pronounced for GPP, with rates of GPP 65% and 216% higher in the LP and HP treatments, respectively (Figure 1). Organic carbon addition appeared to dampen the responses of microbial GPP and NPP to P additions (Figures 1a and 1b; $F_{1,13}=4.89$, $p<0.05$; $F_{1,13}=14.57$, $p<0.01$, respectively), although the interaction term was only

significant for NPP ($F_{2,13}$=5.10, p<0.05; Figure 1). In the absence of P, organic carbon addition tended to increase respiration (Figure 1c). However, the only resource addition that resulted in a significant increase in respiration rate relative to the control was HP.
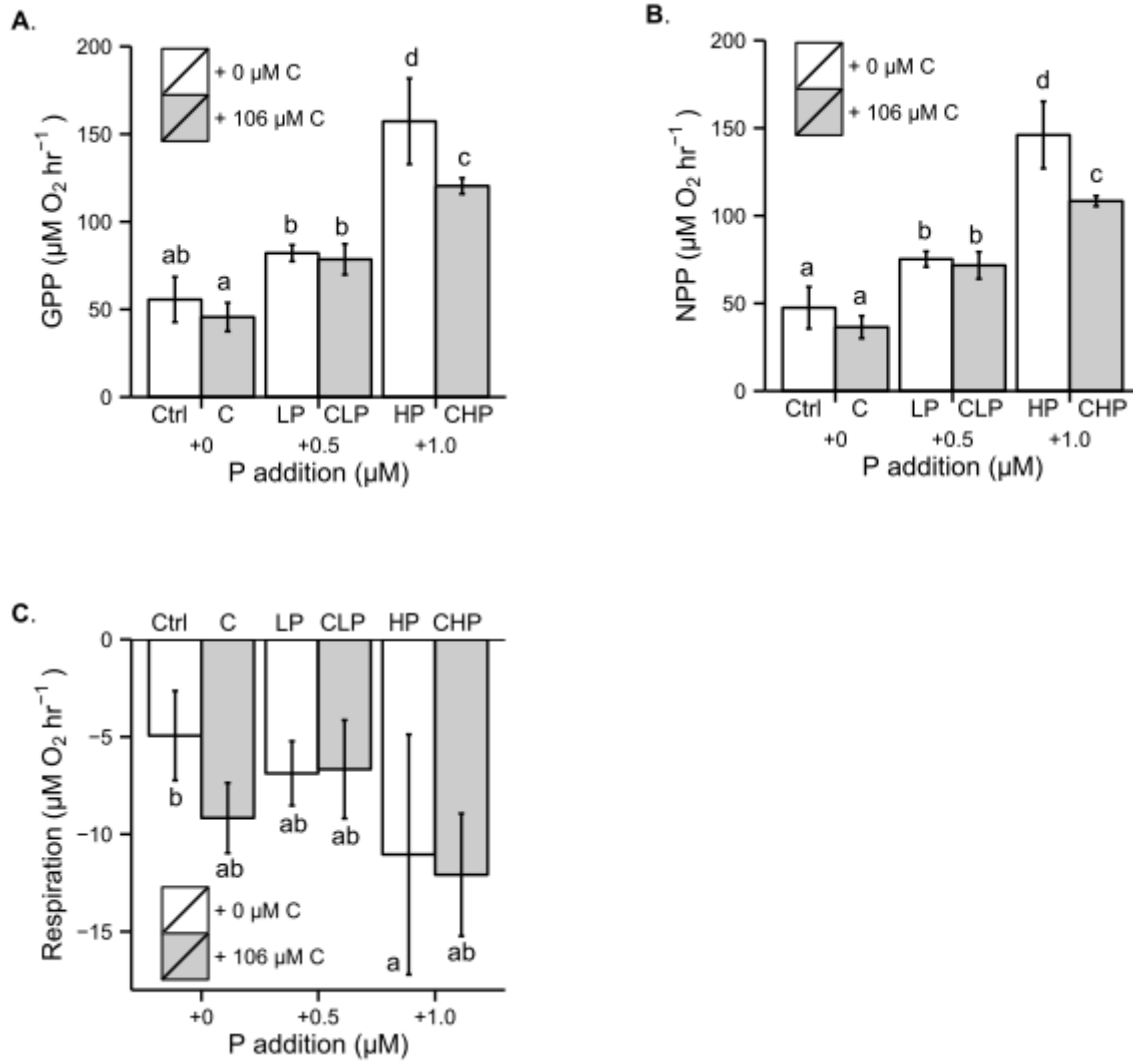
Figure 1. Metabolic responses of oncoid microbialite communities to organic carbon (C) and phosphorus (P) additons. (A) Gross primary production, GPP. (B) Net primary production, NPP. (C) Respiration, R. Error bars indicate ± 1 standard deviation (n = 3). Letters denote significantly different treatments (Tukey's HSD, p<0.05). Note the difference in scale on panel (C).

<u>Calcium Uptake</u>

Organic carbon addition strongly increased calcification rates ($F_{1,13}=8.02$, $p<0.05$), but phosphorus addition had no effect (Figure 2). Without organic carbon, average calcification rates were highly variable with values of 12.2 ± 71.4 µM $CaCO_3$ hr^{-1}; with organic carbon, average calcification rates were 113 ± 61.2 µM $CaCO_3$ hr^{-1}.
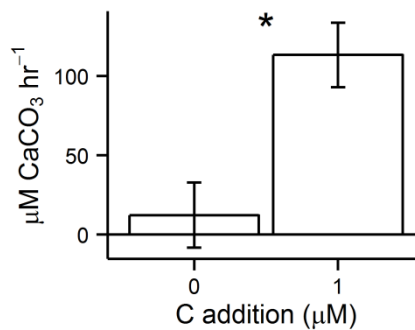
Figure 2. Calcification rates respond positively to organic carbon additions ($p<0.05$, indicated by an *), but not phosphorus (phosphorus data not shown). Error bars indicate ± standard error.

<u>Microbial community composition responses</u>

At the phylum level, the composition of the microbial community did not shift drastically with incubation of oncoids in mesocosms or with any of the resource additions. No indices of community richness or diversity detected a significant response of overall community composition to nutrient enrichment (Table 2). Across all oncoids, the most abundant phyla in the microbial community were Proteobacteria (49.3 ± 5.0 %), Bacteroidetes (19.8 ± 7.1 %), Cyanobacteria (7.5 ± 5.2 %), Planctomycetes (5.4 ± 1.0 %), and Firmicutes (4.6 ± 1.7 %) (Figure 3). Archaea were a minor component of the

microbial community, comprising less than 0.4% of sequences in any given sample. The

relative abundance of Cyanobacteria was greater in the control oncoids than in either

treatment or river oncoids (19.5 ± 0.7 % versus 5.5 ± 1.2 %).

Proteobacteria made up a substantial proportion of the microbial community;

within the Proteobacteria, the Deltaproteobacteria order represented 4.5 – 14% of all

bacteria in the oncoids (Figure 3). The C and HP treatments seemed to reduce abundance

of Deltaproteobacteria and, particularly, of the Desulfobacterales (Figure 4).

Table 2. Richness and diversity estimates and coverage for "river" and experimental
oncoids. 16S rRNA libraries were subsampled to 3,269 sequences. Values reported are
the average (± 1 S. D.) of two replicates.

Oncoid	OTUs	ACE Richness	Inverse Simpson Index	Simpson Evenness	Coverage
River	921 (22)	2798 (34)	91 (17)	0.099 (0.021)	0.837 (0.001)
Control	791 (3)	2287 (29)	76 (2)	0.096 (0.003)	0.864 (0.001)
C	877 (101)	2550 (199)	142 (32)	0.161 (0.017)	0.849 (0.019)
CLP	873 (44)	2501 (260)	120 (25)	0.137 (0.022)	0.849 (0.010)
CHP	797 (35)	2127 (217)	135 (9)	0.170 (0.004)	0.868 (0.009)
LP	836 (36)	2334 (109)	98 (0.1)	0.117 (0.005)	0.859 (0.008)
HP	826 (45)	2344 (249)	104 (30)	0.125 (0.029)	0.858 (0.010)
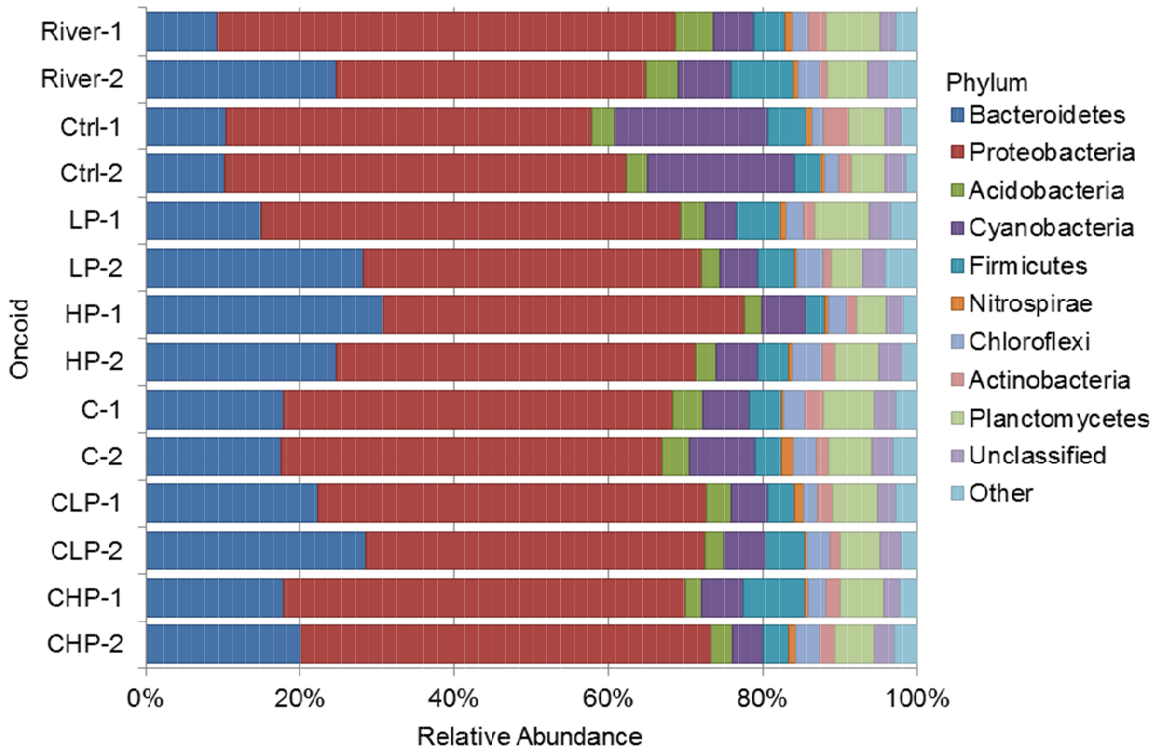
Figure 3. Relative abundance of bacterial phyla in oncoid microbialites of Río Mesquites after enrichment with organic carbon (C) or phosphorus (P). Relative abundances are displayed for each of two replicate oncoids. Microbial communities were sampled directly from the river ("River") or following the resource addition experiment.
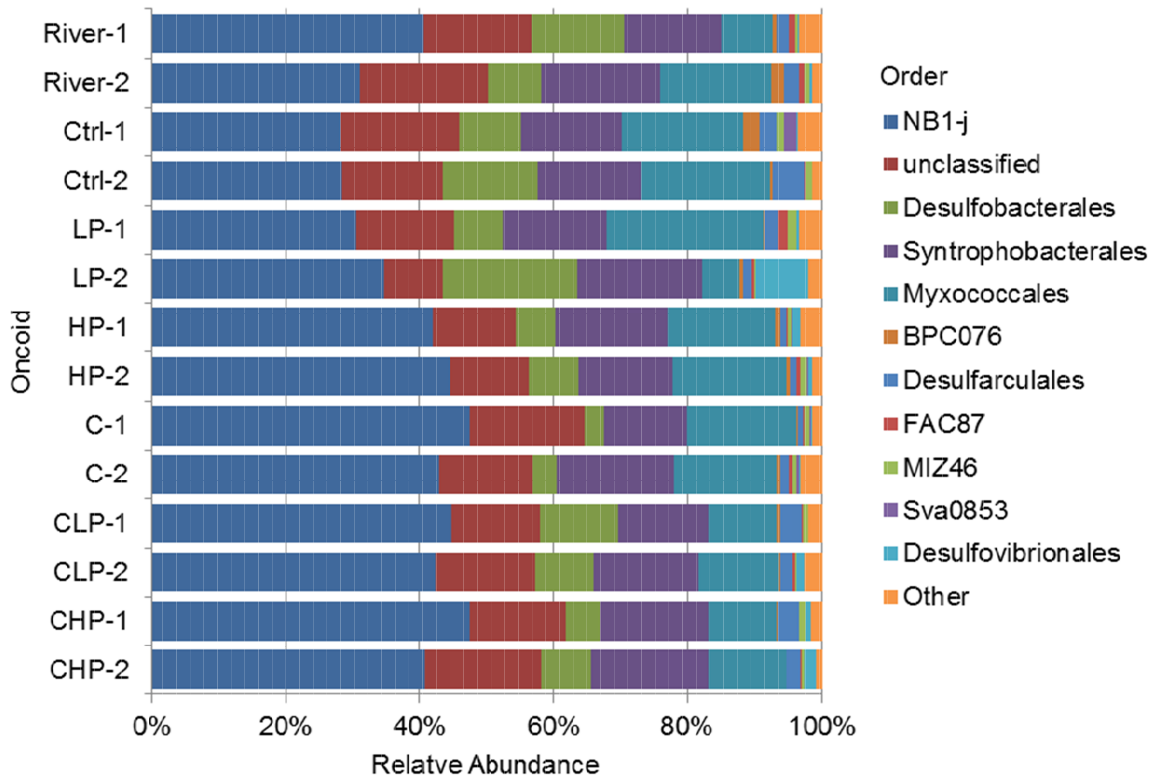
Figure 4. Relative abundance of Deltaproteobacteria. See Figure 3 for description.

DESeq2 was used to determine if the abundance of particular OTUs changed significantly with incubation in mesocosms (river versus Ctrl oncoids) and with nutrient addition (Ctrl versus C, LP, CLP, HP, and CHP oncoids) (Figure 5). The number of OTUs detected as significantly differentially abundant ($p<0.05$) between control and "river" or treatment oncoids ranged from 12 to 30 (a total of 41 across all samples), indicating that a very small proportion of the microbial community changed in response to manipulation. Generally, the significant changes in OTU abundances did not exceed a 4-fold change with standard errors of approximately 0.64.

With incubation of oncoids in mesocosms, 30 OTUs increased in abundance in the control samples relative to the river oncoids (Figure 5). Cyanobacteria accounted for some of the highest fold changes among these OTUs, reflecting the overall increase of this phylum as a proportion of the microbial community in oncoids that did not receive nutrient addition. The remaining OTUs were mostly Proteobacteria, while a few were affiliated with low abundance members of the bacterial community (Actinobacteria and unclassified Bacteria).

Nutrient addition resulted in both decreases and increases in the abundance of particular OTUs (Figure 5). Many OTUs, especially those classified as Cyanobacteria, decreased with nutrient addition in all treatments. However, a few cyanobacterial OTUs and one derived from chloroplast increased in abundance with HP or LP addition. Only two OTUs increased in abundance with organic carbon addition alone. The addition of organic carbon in combination with phosphorus generally resulted in decreases in the abundance of OTUs with the exceptions of an unclassified Bacteroidetes and two OTUs classified as Betaproteobacteria. More OTUs responded favorably to phosphorus addition than organic carbon addition (with and without added P). These included Bacteroidetes, Cyanobacteria, Proteobacteria, including potential nitrogen-fixing alphaproteobacteria and a potentially pathogenic *Legionella*, and OTUs classified as Chloroflexi. Bacteroidetes-affiliated OTUs, particularly those classified as Saprospirae, only exhibited a positive response to nutrient addition (LP, HP, and CLP), also reflecting the apparent increase in the abundance of this phylum in nutrient-amended samples.

Figure 5.

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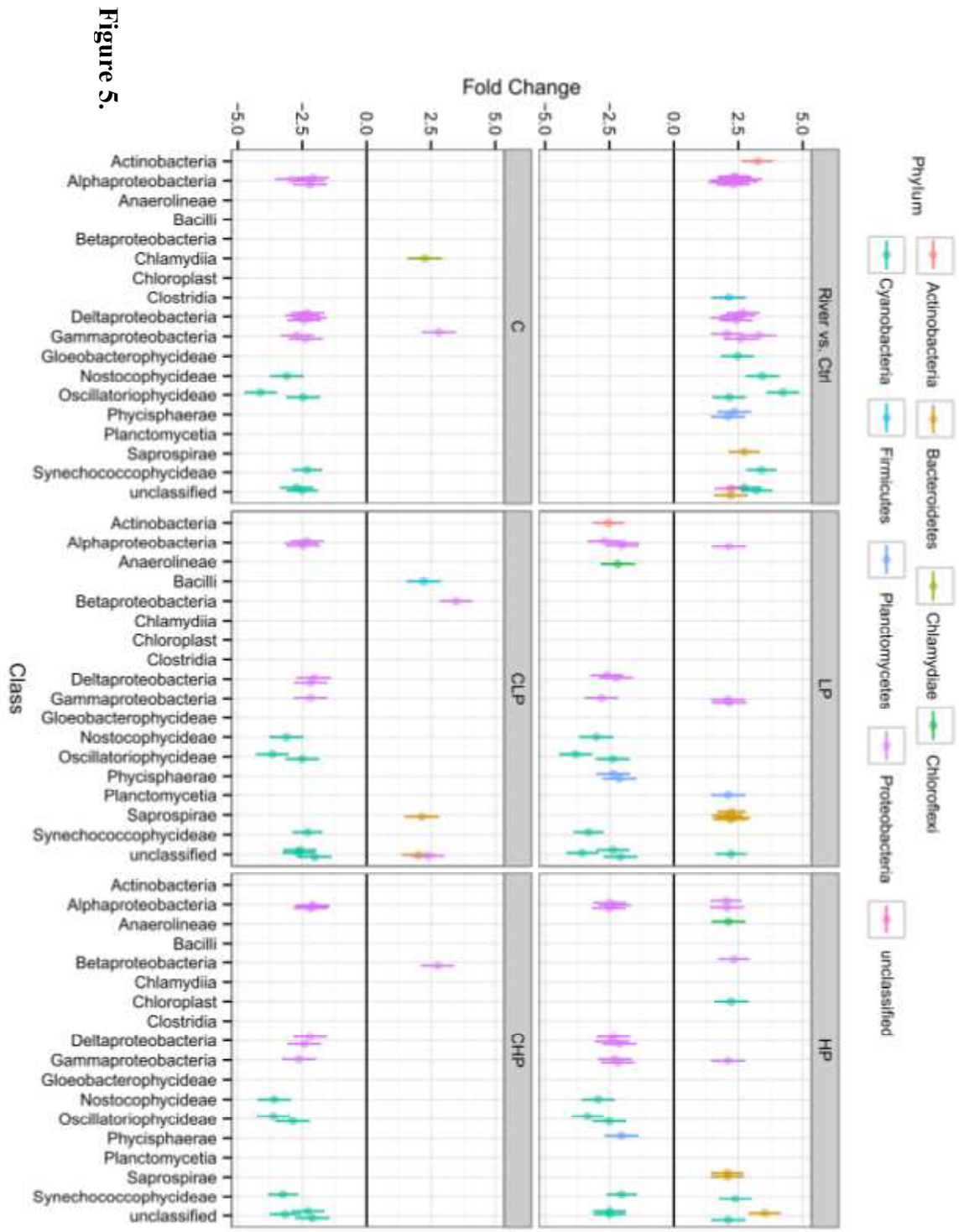
Figure 5*, previous page.* Shifts in OTU abundance as detected by DESeq2. Only OTUs with significant shifts between the pairwise comparison are shown (log2 fold change greater than 2 and false discovery rate <0.05). Pairwise comparisons are either shifts between the river and Ctrl oncoids (panel 1) or the Ctrl and treatment oncoids (panels 2 – 5). Error bars represent the standard error.

Experiment 2. Nutrient limitation of Non-microbialite Associated Photoautotrophic

Microbial Communities

The NDS experiment indicated that nutrient limitation of photoautotrophs was not strong in the non-$CaCO_3$ associated benthic microbial communities. Across all three sites, there was no clear singular N or P effect on Chl *a* accrual, although N and P added in combination nearly led to a significant increase Chl *a* biomass ($F_{1,36}$=3.98, *p*=0.053). Of the three sites in this experiment, only two showed a trend towards nutrient limitation of Chl *a* accrual: N-limitation in Site 2 and NP co-limitation in Site 3 (Figure 6). However, neither Chl *a* responses were statistically significant (Site 2, N, $F_{1,12}$=2.90, *p*=0.11 and Site 3, NP, $F_{1,12}$=4.52, *p*=0.055).
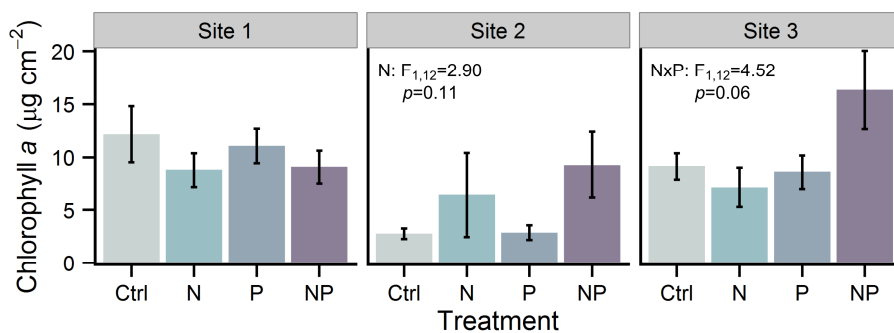
Figure 6. Areal mass of chlorophyll *a* on nutrient-diffusing substrata at the end of the nutrient amendment experiment. Error bars represent ± standard error.

Experiment 3. Influence of Lithification on Nutrient Availability

During the calcification reduction experiment, physicochemical differences across blocks (mesocosms within water baths) were minimal. pH and specific conductivity were constant across blocks at 8.27 ± 0.02 and 3198 ± 36 μS cm^{-1}. Temperature varied significantly between blocks ($F_{3,16}$=7.41, p<0.01), with block A slightly cooler (26.8 ± 0.2 $^{\circ}$C) than B (27.6 ± 0.3 $^{\circ}$C), C (27.3 ± 0.2 $^{\circ}$C), and D (27.4 ± 0.1 $^{\circ}$C). Dissolved oxygen varied significantly between blocks, but only on some days ($F_{39,208} = 3.86$, p<0.001). Across blocks, DO concentration was 6.31 ± 0.28 mg L^{-1}. Differences across blocks were controlled for in the statistical analysis. Calcium removal efficiency of the water softener decreased during the experiment, but was never below 51%.

The Ca^{2+} removal (water softener) treatment provided the greatest stimulation to microbial biomass accumulation, either in terms of phosphorus or organic carbon, compared to the other treatments. While the control and strontium treatments exhibited a similar rate of phosphorus accrual, 0.005 mg P kg oncoid^{-1} week^{-1} (95% Confidence Interval: -0.001, 0.011), the rate of phosphorus accrual was significantly higher in the Ca^{2+} removal treatment ($F_{6,48}$=13.91, p<0.001). Indeed, in the first three weeks, microbial phosphorus contents in the Ca^{2+} removal treatment increased 121% (Figure 7). Organic carbon accumulation in microbial biomass was also slightly higher in the Ca^{2+} removal treatment (Figure 7). While the control and strontium treatments exhibited a similar rate of organic carbon accrual, 5.64 mg C kg oncoid^{-1} week^{-1} (95% CI: 4.11, 7.15), the organic carbon accrual rate was significantly greater ($F_{4,48}$=5.23, p<0.01) in the Ca^{2+} removal treatment, 8.61 C mg kg oncoid^{-1} week^{-1} (95% CI: 5.62, 11.59).

Conversely, the dark treatment dampened the rate of biomass accumulation in the

oncoids. As with the Ca^{2+} removal treatment, the effect of light removal on phosphorus

accrual in microbial biomass was apparent within the first three weeks of the experiment

(Figure 7). Indeed, phosphorus contents had decreased 42% in the dark compared to

initial phosphorus contents. In addition, similar to the Ca^{2+} removal treatment, the effect

of light removal on phosphorus content stabilized for the remainder of the experiment.

The dark treatment also led to no or slightly negative organic carbon accumulation during

the initial three weeks of the experiment. However, after this initial period, organic

carbon accrual was similar to that in the remaining treatments. This led to an overall

organic carbon accumulation rate that was lower than the other treatments, 2.89 mg C kg^{-1} oncoid week^{-1} (-1.73, 7.50), but this difference was only marginally significant

(p=0.069).
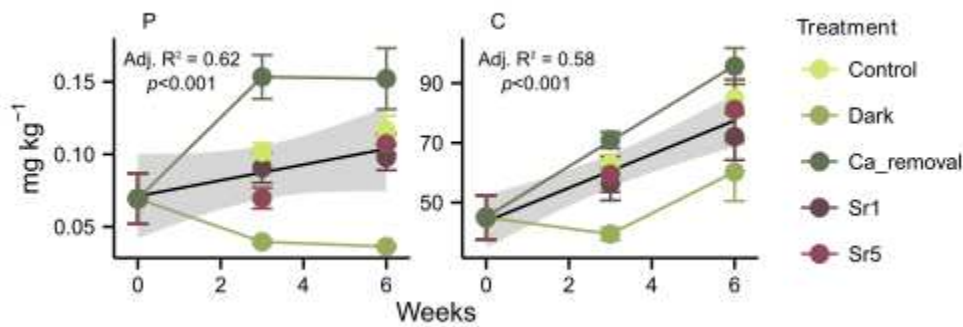
Figure 7. Changes in microbial biomass phosphorus (P) and organic carbon (C) contents
during the calcification manipulation experiment for the control ("Control"), light
removal ("Dark"), calcium removal ("Ca_removal"), and strontium (at 1 mM Sr, "Sr1",
and 5 mM Sr, "Sr5") treatments. The P or C accumulation rate is shown as a black line;
shading represents the 95% confidence interval for the Control, Sr1, and Sr5 treatments.
Accumulation rates for the Ca_removal and Dark treatments are depicted as colored
lines. Accumulation rates for Ca_removal and Dark were not linear (based on a time x
treatment interaction in the model); therefore, the relationship is drawn with a connecting
line.

At the end of the experiment, the dark and Ca^{2+} removal treatments led to the greatest changes in microbial biomass nutrient contents (Figure 8). Both treatments led to significant changes in microbial biomass phosphorus contents ($F_{4,12}=14.596$, $P<0.001$); Ca^{2+} removal led to a 30% increase in phosphorus content compared to the control. Only the dark treatment affected nitrogen contents in microbial biomass ($F_{4,12}=21.97$, $P<0.0001$). Despite some differences in rates of accrual of microbial organic carbon, final concentration of microbial organic carbon was not significantly influenced by the treatments (Figure 8). Changes in the biomass nutrient contents are reflected in changes in the molar ratios of C:P and N:P in the microbial biomass, which differed significantly between treatments ($F_{4,12}=8.97$, $p<0.01$ and $F_{4,12}=6.68$, $p<0.01$, respectively) (Figure 8).
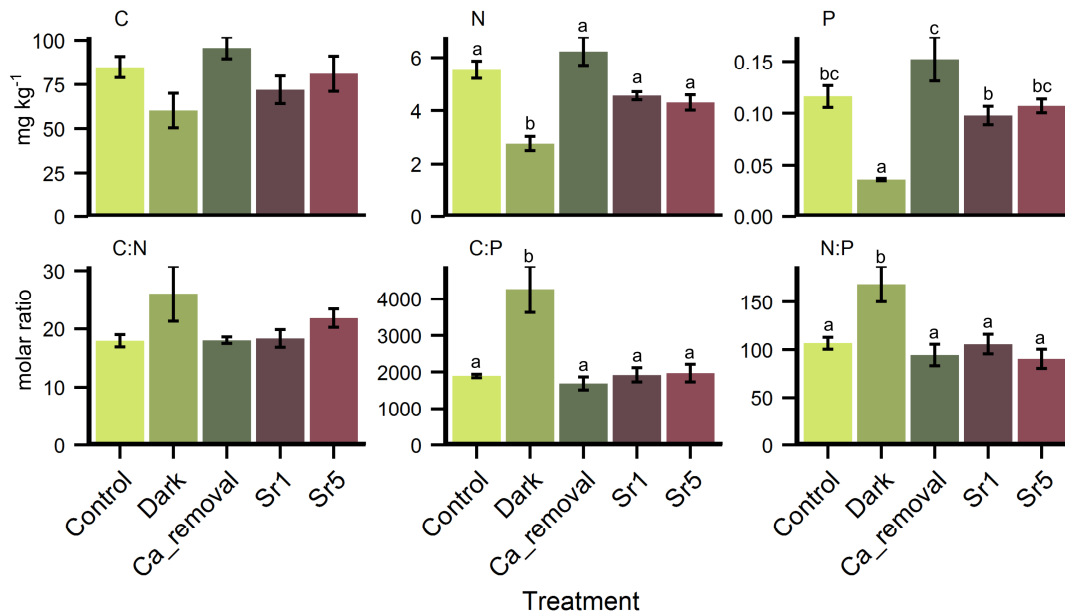
Figure 8. Microbial biomass carbon (C), nitrogen (N), and phosphorus (P) content and ratios following a 5 weeks of $CaCO_3$ reduction by chemical inhibition (Sr1, Sr2), light reduction (Dark), and calcium removal (Ca_removal). Letters indication significant differences ($p<0.05$) between treatments based on Tukey's post hoc comparison.

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DISCUSSION

Nutrient Limitation in Río Mesquites

Chemical analyses of Río Mesquites water samples from this (Table 1) and earlier studies (Elser et al 2005; Elser et al 2006) indicate strong potential for phosphorus limitation given low concentrations of phosphorus in stream water and high N:P ratios. This study supports and builds upon earlier work (Elser et al. 2005, Elser et al. 2006) in demonstrating that phosphorus limits both autotrophic and heterotrophic metabolism of the microbes living in oncoids. Surprisingly, organic carbon enrichment did not strongly alter aerobic respiration rates when added in conjunction with phosphorus (Experiment 1; Figure 1). Indeed, stimulation of aerobic respiration by phosphorus alone (Figure 1) supports several other studies that have also shown that inorganic nutrient availability, e.g., N or P, can directly increase bacterial production (Cotner et al. 1997, Sala et al. 2002, Hitchcock and Mitrovic 2013, Liu et al. 2014), suggesting that nutrients, and not just organic carbon substrate availability, should be considered when studying bacterial growth in lithifying, as well as non-lithifying, microbial communities.

While phosphorus and organic carbon enrichment led to shifts in ecosystem processes (e.g., GPP, NPP, and calcification), major shifts in bacterial biodiversity were not detected. Elser et al. (2005) found increases in diatoms in oncoids in response to phosphorus enrichment; I did not assess diatom responses in these experiments. However, I did observe a lower relative abundance of Cyanobacteria in nutrient-enriched mesocosms (Figure 3) suggesting that eukaryotic photoautotrophs must have been responsible for the observed NPP and GPP increases. Additionally, I did detect small-

scale shifts in the heterotrophic microbial community (Figure 3), suggesting that some species were responsive to experimental treatments. For example, the Saprospirae-classified OTUs that increased in abundance with nutrient addition (Figure 5) tend to be found in association with photoautotrophs and may actually prey on other bacteria and, specifically, cyanobacteria (Saw et al. 2012; Shi et al. 2006). Given the decrease in abundance of Cyanobacteria, I suggest that phosphorus additions led to increased predation by Saprospirae.

As I did not observe large-scale changes in bacterial community composition, it is likely that increasing resource availability resulted in changes in metabolic activity in the microbial community already present in the oncoids (versus potential colonizers from the twice daily stream water changes). More research is needed to determine whether the shifts in respiration or calcification were due to increases in metabolic activity across the entire microbial community or solely to those bacterial species that responded favorably to increases in resource availability.

While nutrient limitation of the photoautotrophic communities differed between those associated with oncoids and those that were not, it cannot be determined if the non-microbialite associated heterotrophic microbial communities were nutrient-limited. By looking at Chl a as a response variable, I excluded the response of heterotrophic microbes. However, the differential responses between the photoautotrophic communities to resource additions supports the role of lithification in controlling nutrient cycling and, therefore, nutrient limitation of microbial growth. Indeed, as nutrient cycling is thought to be more tightly coupled in mature microbial biofilms (Pringle 1990, Frossard et al. 2013), one would not necessarily expect nutrient limitation to be strong in the microbialites. Yet,

that is what I found – stronger nutrient limitation in oncoid microbial communities than on the freshly colonized surfaces of the nutrient-diffusing substrata.

Calcification and Phosphorus Availability in Río Mesquites Oncoids

When I tested if calcification accentuated phosphorus limitation, I expected each treatment to lower $CaCO_3$ deposition rates and, therefore, increase phosphorus bioavailability to the microbes. Indeed, the predicted increase in the rate of biomass accrual and an increase in biomass phosphorus concentration occurred in the Ca^{2+} removal treatment. This result suggests that phosphorus limitation of microbial communities found in this and other $CaCO_3$ microbialites (Rosen et al. 1996, Elser et al. 2005, Elser et al. 2006, Valdespino-Castillo et al. 2014) is due to $CaCO_3$ coprecipitation of phosphate. However, phosphorus accrual in microbial biomass did not change consistently across treatments. Nonetheless, these differences do not necessarily lessen support for my original hypothesis. Instead, I suggest that the loss of microbial biomass in the dark treatment and the lack of an effect of the strontium treatment give some new insights into the ecological processes within lithifying microbial communities.

The light reduction treatment resulted in a visible loss of the phototrophic microbial community – while this may have reduced calcification rates, it also removed an important component of the oncoid microbial community (Garcia-Pichel et al. 2004, Breitbart et al. 2009). Without photoautotrophs fixing carbon, biomass was not accruing in the oncoids in the dark treatment in the first three weeks (Figure 5). Furthermore, the significant decrease in biomass nitrogen and phosphorus contents (Figure 6) suggests decomposition of microbial biomass led to the loss of nutrients from the oncoid

ecosystem. Therefore, while a positive balance between autotrophic and heterotrophic processes can support lithification (Dupraz and Visscher 2005), it may also be important in retaining nutrients within an oncoid.

The dark treatment was also the only treatment in which I detected a shift in microbial biomass accumulation rates over the six-week experiment. Chemoautotrophs may have functionally replaced the photoautotrophs after 2.5 weeks of incubation, hence the apparent recovery in biomass organic carbon concentrations (Figure 7). Furthermore, stability of phosphorus concentrations suggests that, as there was no other source of phosphorus, once the chemoautotrophic community was established, phosphorus was able to be recycled internally, potentially reestablishing the tight biogeochemical patterns present in the oncoid consortia with photoautotrophs. This hypothesis cannot be tested with the current data, but deserves further consideration as it could shed light into resilience of microbialites to disturbance.

Unexpectedly, the strontium treatments had no effect (or a slightly negative effect) on nutrient content in the microbial communities. This lack of impact may be because strontium likely inhibits $CaCO_3$ deposition only at the surface of the biofilm. However, deposition may actually be occurring within or below the microbial biofilm, as occurs with sulfate reduction (Visscher et al. 1998). Thus, the strontium treatments may have been ineffective in reducing lithification because lithification was not occurring at the surface of the oncoid. At high concentrations (>5 mM), strontium can have toxic biological effects (Mei et al. 2006). While the treatment was well below this threshold, the slightly lower accrual of nitrogen and phosphorus in the biomass in the strontium treatment (Figure 8) may nevertheless have been a consequence of acute biological stress.

Evidence of Sulfate Reduction as a Mechanism of CaCO₃ Deposition

Calcification in the oncoid microbialites of Río Mesquites is thought to be photosynthetically driven (Garcia-Pichel et al. 2004, Elser et al. 2005), but results here, as well as from other work, suggest another process may also be driving lithification. If photosynthesis is the main driver of calcification, environmental conditions that increase the rate of photosynthesis would be expected to increase the rate of calcification, as shown in Elser et al. (2005) and Garcia-Pichel et al. (2004). Furthermore, the relationship between O_2 produced and Ca^{2+} taken up should be in a 1:1 molar ratio (Garcia-Pichel et al. 2004):

$$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_{3(s)} + CH_2O + O_2$$
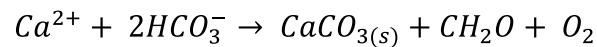
However, in experiments manipulating the rates of photosynthesis in oncoids from Río Mesquites, the increases of calcification were not stoichiometrically constrained to rates of photosynthesis. In my experiment, there was no relationship between phosphorus additions (which stimulated photosynthesis) and rates of calcification. In Garcia-Pichel et al. (2004), the ratio of Ca^{2+} drawdown to O_2 uptake was greater than 1. In Elser et al. (2005), phosphorus enrichment of the oncoids increased GPP by ~50%, but calcification by ~100%. Furthermore, photosynthetically induced calcification would be expected to occur at the surface of the microbes (Bissett et al. 2008). However, my experimental manipulation of calcification reliant on this assumption, strontium-inhibition, did not seem to lower calcification rates.

My results, along with the insight from other studies, suggest that other metabolic pathways may be contributing to calcification in the oncoids of Río Mesquites. Visscher et al. (2000) reported that sulfur metabolism, and more specifically, sulfate reduction, can produce lithified micritic laminae in marine stromatolites. More recently, sulfate reduction has been found in stromatolites in hypersaline lakes in Argentina (Gómez et al. 2014). Sulfate reduction, a process performed by heterotrophic bacteria during the oxidation of organic carbon compounds, leads to the reduction of sulfate to sulfide (Visscher and Stolz 2005, Plugge et al. 2011). Indeed, when coupled with aerobic photosynthesis, sulfate reduction can lead to a net of 4 mol of $CaCO_3$ produced per 3 mol of CO_2 fixed (Visscher and Stolz 2005), which would partially account for the stoichiometrically imbalanced relationship between photosynthesis and $CaCO_3$ deposition detected in this study and in the experiments of Garcia-Pichel et al. (2004) and Elser et al. (2005). Since sulfate reduction causes lithification in anaerobic, inner layers of a stromatolite (Visscher et al. 2000), it would also be the reason that the strontium treatments did not have the expected results. Finally, if $CaCO_3$ deposition in the oncoids is supported by heterotrophic metabolisms linked to sulfate reduction, it would also explain why addition of organic carbon induced $CaCO_3$ deposition.

Both the genetic evidence from this work and that of previous work (Breitbart et al. 2009) support a potential role for sulfate reduction in $CaCO_3$ deposition in the Río Mesquites oncoids. In this study, the Deltaproteobacteria represented between 4.5 to 12.8% of the microbial community (Figure 3); within the Deltaproteobacteria, Desulfarculaceae, Syntrophaceae, and Syntrophobacteraceae are families that include sulfate reducers (Kuever et al. 2005, Miletto et al. 2011, Kuever 2014).

Deltaproteobacteria represent the greatest number of sulfate reducers, but this phenotypic group is also found among Firmicutes, Nitrospirae, Crenarchaeota, and Euryarchaeota (Liu et al. 2003, Muyzer and Stams 2008, Plugge et al. 2011). Therefore, while the decrease in the relative abundance of potential sulfate reducers with resource addition (Figure 4) might suggest that sulfate reduction may not be responsible for positive response of $CaCO_3$ to organic carbon addition (Figure 2), it could also suggest that sulfate reducers from another taxonomic group are responding more strongly to the resource additions. In a metagenomic analysis of a Río Mesquites oncoid, Breitbart et al. (2009) found that the microbialite community indeed contained genes for sulfate reduction (Breitbart et al. 2009). The authors suggested that anaerobic or aerobic sulfate reducers might be present in the oncoids as genes for protection of oxidative stress, which would otherwise limit a sulfate-reducers ability to tolerate aerobic conditions, were also found in the oncoids.

Garcia-Pichel et al. (2004) acknowledged the likelihood that sulfate reduction may account for the imbalance between photosynthesis and calcification in their study, but dismissed it as being important "in the absence of exogenous carbon sources." However, I did not find evidence of limitation of aerobic heterotrophic metabolism by organic carbon availability, suggesting a portion of the heterotrophic microbial community is able to access enough carbon for growth. Furthermore, DOC concentrations, while low in Río Mesquites, were detectable (Table 1). Carbon demand in oncoids may also be supported by macrophytic plant production within the riparian zone of Río Mesquites. In an earlier study, Winsborough et al. (1994) observed the importance of *Phragmites* marshes, and the egested byproduct of this plant material by snails, as

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being abundant enough in Río Mesquites to partially fill oncoid interstitial areas.
Therefore, I suggest that dissolved and particulate organic carbon sources are readily
available in Río Mesquites to support heterotrophic microbial growth and, potentially,
sulfate reduction. Indeed, the combined experimental findings give strong
biogeochemical and genetic evidence suggesting that sulfate reduction is important to
$CaCO_3$ deposition in these oncoids.

CONCLUSIONS

This study supports the role of lithification as a mechanism driving phosphorus
limitation in microbialites primarily composed of $CaCO_3$, suggesting that, despite the
physicochemical conditions of the surrounding aquatic ecosystem, lithification imposes
unique nutrient conditions on microbes living in $CaCO_3$-depositing environments relative
to their counterparts in non-$CaCO_3$-depositing habitats. Whether or not extant microbial
communities have unique adaptations to access phosphate co-precipitated with $CaCO_3$
deserves further investigation. These results also support the role of biological processes
in causing lithification. While much previous work has focused on photosynthetically
induced $CaCO_3$ deposition, these data are some of the first from freshwater ecosystems
showing that sulfate reduction may contribute to $CaCO_3$ deposition. Further research is
needed to elucidate the role of anaerobic heterotrophic metabolism in lithification of
other freshwater microbialites.

ACKNOWLEDGEMENTS

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CHAPTER 5

CALCIUM CARBONATE DEPOSITION DRIVES NUTRIENT CYCLING IN

MONTANE, HEADWATER STREAMS

ABSTRACT

Calcium carbonate ($CaCO_3$) deposition is common in aquatic ecosystems and may reduce

phosphorus availability via coprecipitation. To determine if photosynthetically driven

$CaCO_3$ coprecipitation reduces phosphorus (P) availability in streams, I studied paired

streams (with and without active $CaCO_3$ deposition) and subjected a reach within each to

experimental shading, monitoring changes in ecosystem attributes (e.g., periphyton

biomass content, nutrient spiraling, periphyton nutrient limitation, and leaf litter

decomposition). In the stream with active $CaCO_3$ deposition, shading reduced rates of

$CaCO_3$ deposition by over 50%, suggesting a substantial proportion of $CaCO_3$ deposition

is supported by photosynthetically induced changes in alkalinity. Shading-induced

reductions in $CaCO_3$ deposition resulted in increases in periphyton growth ($F_{2,12}=5.79$,

$p<0.05$) and epilithon biomass P content ($p<0.05$). Reductions in $CaCO_3$ deposition also

eliminated nutrient limitation of periphyton growth by P ($F_{3,16}=59.32$, $p<0.001$),

increased P uptake lengths by at least an order of magnitude, and decreased areal P

uptake rates by 82% ($F_{2,3}=13.19$, $p<0.05$). Finally, while shading caused reductions in

leaf litter decomposition in the non-$CaCO_3$ depositing stream ($F_{5,7}=22.45$, $p<0.001$),

shading had no effect of leaf litter decomposition in the stream with active $CaCO_3$

deposition. These results indicate that $CaCO_3$ deposition is an important process regulating P bioavailability, as has been suggested in lake and wetland ecosystems.

INTRODUCTION

Calcium carbonate (CaCO$_3$) deposits are found in aquatic ecosystems across the world. They are widespread in oceanic systems, e.g., coral reefs and the tests of various organisms, but also occur in inland waters. Indeed, CaCO$_3$ formations are prominent in aquatic ecosystems fed by aquifers rich in carbonate minerals, such as the hardwater lakes and wetlands in temperate regions of North America and Europe (e.g., Neal 2002, Robertson et al. 2007, Hamilton et al. 2009) or in the wetlands of tropical or subtropical regions, such as in Florida (Noe et al. 2001) or the Yucatán Peninsula (Cole 1979). Calcium carbonate deposits can take a variety of forms: loose, unconsolidated material (e.g., periphyton mats of Everglades, FL, USA, Hagerthey et al. 2011), distinct, bulbous structures (e.g., oncoids of River Alz, Germany, Hägele et al. 2006; Río Mesquites, México, Garcia-Pichel et al. 2004), or cement-like layers and dams of travertine (e.g., Fossil Creek, AZ, USA, Malusa et al. 2003; Falling Springs Creek, VA, USA, Herman and Lorah 1987; Barkly karst, Queensland, AUS, Carthew et al. 2006). Indeed, the depositional form of CaCO$_3$ seems to influence how CaCO$_3$ deposition interacts with ecological processes.

Calcium carbonate deposits are associated with a variety of ecological processes and functions in streams. Flow regimes can be transformed by CaCO$_3$ deposits that form dams and create step-pools (Chafetz and Folk 1984, Fuller et al. 2011). Biodiversity and algal biomass tend to be higher in CaCO$_3$-depositing versus non-CaCO$_3$-depositing reaches of a stream, possibly due to the novel habitat created by the CaCO$_3$ structures (Marks et al. 2006, Carter and Marks 2007). Decomposition rate can also be influenced

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by CaCO$_3$ deposition. For example, increased turbulence created by water flowing over CaCO$_3$ terraces has been associated with elevated rates of leaf litter decomposition in Fossil Creek, AZ (Carter and Marks 2007), and in the fluvial lakes of the Plitvice region of Croatia (Milisa et al. 2010). Conversely, the physical shielding of leaf litter by CaCO$_3$ deposits has been suggested as a cause of reduced rate of leaf litter decomposition in calcareous streams of the Nervión River catchment in Spain (Martínez et al. 2014).

Beyond these physical processes, CaCO$_3$ deposition may also have a strong chemical influence on ecosystems due to its impacts on phosphorus cycling. Deposition of CaCO$_3$ coprecipitates phosphorus, through either the adsorption of phosphate onto the CaCO$_3$ mineral grains or incorporation into the CaCO$_3$ mineral matrix, thereby lowering phosphorus concentrations in solution (Kitano et al. 1978, Ishikawa and Ichikuni 1981, Rodriguez et al. 2008, Hu et al. 2014). Phosphorus coprecipitation with CaCO$_3$ occurs in a variety of ecosystems: terrestrial soils (Lajtha and Schlesinger 1988), agricultural soils (Tunesi et al. 1999), benthic photosynthetic mats (Noe et al. 2001 and references therein, Borovec et al. 2010), wetland soils (Boyer and Wheeler 1989), streams (House and Donaldson 1986, Jarvie et al. 2006), sea ice (Hu et al. 2014), and lakes (Lawrence Lake, Michigan, USA, Otsuki and Wetzel 1972; Lake Wallersee, Austria, Jäger and Röhrs 1990; Lake Constance, Europe, Kleiner 1988; Gull Lake, Michigan, USA, Hamilton et al. 2009). When phosphorus coprecipitation with CaCO$_3$ occurs in the water column of lakes and the CaCO$_3$ subsequently sinks from the photic zone into the aphotic sediments, it is thought to reduce phosphorus concentrations that might otherwise fuel primary production (Koschel et al. 1983, Robertson et al. 2007, Hamilton et al. 2009). In periphyton mats in Florida (Noe et al. 2001, Hagerthey et al. 2011) and in benthic

cyanobacterial mats in Belize (Rejmánková and Komárková 2005, Borovec et al. 2010), $CaCO_3$ deposits form diurnally due to photosynthesis. In this scenario, coprecipitation leads to a temporary, daytime sink of phosphorus that is regenerated when respiration rates lead to nighttime dissolution of $CaCO_3$ and subsequent phosphorus release. Therefore, the role of $CaCO_3$ as an ecosystem sink of phosphorus may be mediated both by the relative proximity of the deposits to metabolically active organisms and by the temporal stability of $CaCO_3$ deposits. In calcareous streams, $CaCO_3$ deposition generally occurs on the stream bottom where its formation may be mediated by periphyton activities, but also where it might influence phosphorus bioavailability to the periphyton. Hence, understanding how $CaCO_3$ deposition influences phosphorus cycling in streams may provide insight into how a geochemical process influences and interacts with nutrient limitation and ecosystem dynamics.

In streams, much of what is known about the effects of $CaCO_3$ deposition comes from laboratory studies or *in situ* observation (e.g., House et al. 1986, House and Denison 2000, Machesky et al. 2010). Indeed, the author's own work in the streams in the Huachuca Mountains has allowed the opportunity to use a natural gradient of $CaCO_3$ deposition rates (see Ch. 2) to assess the consequences of $CaCO_3$ deposition on ecological processes. Three years of stream monitoring suggest that $CaCO_3$ coprecipitation of phosphorus reduces downstream phosphorus availability (see Ch. 2) and can lead to phosphorus limitation of periphyton growth (see Ch. 3). In this study, I build on these previous findings using an *in situ*, manipulative experiment to determine if $CaCO_3$ deposition influences phosphorus availability and ecosystem processes.

In the Huachuca Mtns, $CaCO_3$ deposition is likely influenced by periphyton metabolic activities. I suggest this because (1) stream benthic surveys have found that $CaCO_3$ cover is tightly linked to periphyton growth and (2) the $CaCO_3$ saturation index (in terms of calcite, $SI_{calcite}$) is around 0.79 and 0.73 in the two streams with active $CaCO_3$ deposition (Garden Canyon and Huachuca Canyon, respectively) (*unpublished data*). The $SI_{calcite}$, while still suggesting supersaturation, is below or on the lower end of values from streams with little to no biological influence on $CaCO_3$ deposition: Fossil Creek, AZ, $(1.28 - 1.49)$ (Malusa et al. 2003) and Falling Springs, VA $(0.6 - 1.3)$ (Lorah and Herman 1988). Therefore, based on the assumption that $CaCO_3$ is promoted by photosynthetic activity in the Huachuca Mtn streams, I placed a shade structure over 30-m reaches in two streams differing in $CaCO_3$ deposition activity to lower *in situ* rates of $CaCO_3$ deposition. By using two streams, one with active $CaCO_3$ deposition and one without, I was able to distinguish ecological effects caused by reductions in light from those specific to reductions in $CaCO_3$ deposition. I monitored both ecosystem components (water chemistry and periphyton chemistry) and ecosystem processes (nutrient cycling, nutrient limitation of periphyton growth, and decomposition). Based on the hypothesis that co-precipitation with $CaCO_3$ deposition reduces phosphorus availability, I predict that reductions in $CaCO_3$ deposition will lead to increases in phosphorus concentrations in the stream water and reductions in phosphorus retention. Furthermore, I expect processes that are potentially controlled by phosphorus availability, e.g., periphyton growth and decomposition, will increase in response to shade-induced reduction in $CaCO_3$ deposition. I also monitor for responses of ecosystem components or ecosystem processes both under the shaded reach and downstream of the shaded reach to

determine if the effects of reduced $CaCO_3$ deposition extend beyond the location of $CaCO_3$ deposition reduction.

METHODS

Study Sites and Experimental Design

This study was carried out in the Huachuca Mountain Range within the Upper San Pedro River basin, southeastern Arizona. The Huachuca Mountains are part of the Madrean Sky Island Region, so named for the region's distinct flora and fauna inhabiting the isolated mountain peaks, which rise to nearly 2800 m above sea level. The climate is semi-arid: mean annual precipitation is ~80 cm (WRRC 2014). Oak (*Quercus*) and pine (*Pinus*) forests dominate the higher altitudes while grasslands and mesquite (*Prosopis*) desert scrub dominate the lower alluvial fans and river valleys.

Two streams along the northeastern side of the Huachuca Mountains were used in this experiment: Garden Canyon and Ramsey Canyon (see Ch. 2, Figure 1). These streams are generally spring-fed and perennial above ~1500 m elevation (Jaeger and Olden 2012). Stream channel morphology is characterized by cascade and bedrock reaches in the upper canyons and turns into step-pool, plane bed, and pool-riffle reaches downstream. Substrata are limestone bedrock or cobbles and boulders. Active $CaCO_3$ deposition is found in Garden Canyon but not in Ramsey Canyon (see Ch. 2). Both streams are alkaline and have low discharge (median discharge from 2011 – 2014 was 0.003 m^3 s^{-1} in Ramsey and 0.006 m^3 s^{-1} in Garden) and low concentrations of dissolved

inorganic nitrogen (DIN; <0.1 mg L^{-1}) and soluble reactive phosphorus (SRP; <20 µg L^{-1}) (see Ch. 2).

I established artificially shaded reaches ("S") with paired upstream ("U") and downstream ("D") reaches ("experimental units") in each stream. Artificial shades were created using dark greenhouse shade cloth (10% light transmittance) secured 2 m above the stream with rebar poles and ropes. Shaded regions were ~30 m long x ~6 m wide, covering the width of the stream. Experimental units were 150 m long to allow buffer zones between sampling locations upstream and downstream of the shaded reach. Daytime photosynthetically active radiation (PAR) reaching the water surface was measured over both non-shaded and shaded stream reaches at 3 – 5 locations around noon on 24, 25, and 29 Jun 2013 and hourly for 6 – 10 h on 15 and 16 Jun 2013 (LiCor 1400 Photometer, LI-COR, Lincoln, NE, USA). Shaded reaches were at 1700 m and 1800 m elevation in Garden Canyon and Ramsey Canyon, respectively.

Sampling Procedure

In each stream, I established sampling sites within each reach for stream water and epilithon samples. Stream water sampling occurred weekly. Using handheld probes, I measured water temperature and specific conductivity (YSI 85, Yellow Springs, OH, USA) and pH (Beckman-Coulter 255 pH/mV, Beckman Coulter Inc., Brea, CA, USA) directly in stream. I collected stream water from the deepest portion of the stream, or from the center if stream depth was uniform, using acid-cleaned HDPE bottles for chemical analysis. Epilithic periphyton was sampled only at the end of the experiment as intensive sampling of epilithic periphyton involves destructive techniques. To sample

epilithon from substrata, I used a Loeb sampler (Loeb 1981), which is a cylinder with a

brush-fitted plunger. To create an "epilithon slurry," I collected 6-7 epilithon samples at

each sampling location with the Loeb sampler. Sampling locations were located at 5 m

intervals within each reach. In Garden, I collected five replicate slurries within each

reach; in Ramsey, due to threatening field conditions on sample collection day, I

collected only four replicate slurries within each reach. Epilithon slurries were kept in a

cooler on ice until they could be processed that same evening. At each site, I also

measured stream water temperature continuously with HOBO temperature loggers (Onset

Computer Corp., Bourne, MA, USA).

Assessment of Stream Ecosystem Processes

<u>Stream nutrient uptake rates</u>

I used short-term tracer additions to determine nutrient cycling parameters (uptake

length and areal uptake rate) at each site in the experimental unit. Stream tracer tests were

performed initially across the entire experimental unit and at week 3 and at week 5 in

each reach. The methods generally followed those of Webster and Valett (2006). Briefly,

reactive tracers or nutrients (nitrogen as KNO_3 and phosphorus as KH_2PO_4) were

combined with inert tracers (chloride as NaCl and bromide as NaBr), dissolved in stream

water, and pumped upstream of the respective reach at a constant rate. Once plateau

concentrations of the inert tracer were detected at the furthest downstream site (by a

change in conductivity), water samples were collected for chemical analysis. Water

samples were collected at five or more locations per reach. The enriched uptake lengths

were estimated by regressing the downstream plateau flux of the nutrient relative to the

inert tracer against distance. The uptake length (S_w, m) of the nutrient is the inverse of this regression slope (Stream Solute Workshop 1990). For each nutrient, S_w was used with stream velocity, mean depth, and background nutrient concentration to calculate the areal uptake rate (U, $\mu g\ m^{-2}\ min^{-1}$). Stream tracer tests were always performed at the downstream reach first.

Periphyton nutrient limitation

Nutrient limitation of periphyton growth was monitored in each stream at each site using nutrient diffusing substrata (NDS). I constructed NDS based on the methods of Tank et al. (2006). Holes (diameter = 2.5 cm) were drilled into the lids of plastic cups (Poly-Cons, Madan Plastics, Crawford, NJ, USA); plastic cups were then filled with nutrient-enriched 2% agar solution (or 3% agar solution, in the case of the dual nutrient treatment), covered with a fritted-glass disk, and sealed with the lid of the cup. Treatments consisted of nutrient enrichments of nitrogen ("N", 0.5 M NH_4Cl), phosphorus ("P", 0.5M KH_2PO_4), or nitrogen and phosphorus ("NP", 0.5 M NH_4Cl + 0.5M KH_2PO_4), or a control ("C", no amendment). I affixed 5 replicates of each treatment to stainless steel L-bars. One L-bar was placed in each reach for 21 days, beginning the first day of the experiment. L-bars were placed parallel to flow to limit sedimentation on the NDS. On the last day of the incubation, I collected and froze the glass crucible covers for chlorophyll *a* (Chl *a*) analysis. Chlorophyll *a* concentrations were determined as described below for epilithon Chl *a* concentrations.

Leaf litter decomposition and calcium carbonate deposition

Decomposition rates during the experiment were estimated in each reach by calculating breakdown rates of maple leaves. Maple leaves were chosen for the

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experiment because maple is a common deciduous taxon in the region and its leaves are less recalcitrant than leaves of other taxa found in the region (Webster and Benfield 1986), and, thus, were more likely to show detectable differences in decomposition rate within the short time-span of the experiment. Senescent leaves of bigtooth maple (*Acer grandidentatum*) from trees in the riparian zone of Ramsey Canyon were collected on 4 December 2010 and transported immediately to the laboratory where they were dried at 50°C. Leaves were stored in a climate-controlled storage facility until use. In May 2013, leaves were re-dried and placed into mesh pecan bags (Gulf Coast Bag and Bagging Company, Houston, TX, USA) containing about 5 g of dried leaves each. Litter bags were deployed in each reach on 12 June 2013; one bag was retrieved per reach each week over the duration of the experiment. Bags retrieved on the final date (10 July) were partially buried under sediments from a recent flood and were not processed. Four control bags were brought to the field and processed without deployment in the stream to measure the amount of material lost in transport; this value was subtracted from all final dry mass totals. I processed litter bags by rinsing leaves to collect all macroinvertebrates that had colonized them, then drying the remaining leaf material at 50°C, homogenizing the leaf material, and combusting a subsample at 500°C to measure ash-free dry mass (AFDM). I calculated leaf litter breakdown rate (k) using the AFDM data following the methods of Benfield (2006).

Leaves from the leaf litter breakdown experiment were also used to determine $CaCO_3$ deposition rates, as in Ch. 2. Briefly, combusted leaf material was dissolved in 2% nitric acid and analyzed for Ca^{2+} (description below). The change in the amount of Ca^{2+} per dry leaf mass over time was used to determine the $CaCO_3$ accumulation rate (mg

CaCO$_3$ g^{-1} dry leaf day^{-1}). As the accumulation rate was based on the deposition of Ca^{2+} on leaf material and leaf material was likely lost during the incubation due to decomposition or physical breakdown, these rates serve as conservative estimates of CaCO$_3$ deposition.

Laboratory Analyses

Stream water chemical analysis

Stream water samples were prepared in the field whenever possible. Whole (unfiltered) samples were collected for determinations of total nitrogen (TN), total phosphorus (TP), and alkalinity. Stream water was also collected and filtered (0.45-μm Supor membrane, Acrodisc) for determinations of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), dissolved inorganic nitrogen (DIN), total dissolved phosphorus (TDP), soluble reactive phosphorus (SRP), other major anions and cations, and trace elements. Samples for cation and trace elements analysis (calcium, Ca^{2+}, magnesium, Mg^{2+}, sodium, Na$^+$, silica, Si, and potassium, K$^+$, and vanadium, V, manganese, Mn, iron, Fe, copper, Cu, zinc, Zn, arsenic, As, molybdenum, Mo, and cadmium, Cd) and for DOC and TDN analysis were acidified immediately to pH <2 with nitric acid or hydrochloric acid, respectively. The rest of the samples were stored on ice in the field. The following samples were processed within 6 h of collection at the field station (method in parentheses): alkalinity (Gran titration; APHA 2005), ammonium (NH$_4^+$; OPA-fluorometric technique of Taylor et al. 2007), and SRP (molybdate colorimetric analysis on a spectrophotometer; APHA 2005). The remaining samples, except for the acidified ones, were frozen. All samples not processed at the field station

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were analyzed at Arizona State University (Tempe, AZ, USA). Cation samples were analyzed with ion coupled plasma optical emission spectrometry (ICP-OES, Thermo Fisher Scientific iCAP6300, Waltham, MA, USA), trace elements with ICP mass spectrometry (ICP-MS, Thermo Fisher Scientific X Series 2, Waltham, MA, USA), and DOC and TDN with a Shimadzu analyzer (Shimadzu Corporation, Kyoto, Japan). TP and TDP were measured following persulfate oxidation by the colorimetric method used for SRP (Solorzano and Sharp 1980). I analyzed nitrate (NO_3^-) and nitrite (NO_2^-) and other anions (sulfate, $SO_4^=$, bromide, Br^-, and chloride, Cl^-) directly and TN following persulfate oxidation (to convert all forms of nitrogen to NO_3^-) with ion chromatography (Dionex ICS-2000 IC System, Sunnyvale, CA, USA). NO_2^- was always below detection limit, so DIN was calculated as the sum of NH_4^+ and NO_3^-. Dissolved organic nitrogen (DON) was estimated from the difference between TDN and DIN; dissolved organic phosphorus (DOP) was estimated from the difference between TDP and SRP.

Epilithon analysis

From each homogenized epilithon slurry, I took quantitative sub-samples for Chl *a*, dry mass (DM) and ash-free dry mass (AFDM), carbon:nitrogen:phosphorus (C:N:P) stoichiometry, and community composition. Chl *a* samples were collected on GF/C filters (Whatman) and frozen for >24 h prior to extraction in 90% buffered acetone for 16 h in the dark (Lorenzen 1967, Arar and Collins 1997). The extractant was analyzed fluorometrically (TD-700, Turner Designs, Sunnyvale, CA, USA) to estimate concentrations of Chl *a* and phaeophytin. All Chl *a* values are reported as phaeophytin-corrected concentrations. I filtered DM and AFDM subsamples on preweighed, precombusted GF/C filters (Whatman). Filters were dried overnight at 60°C to calculate

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DW. Then, filters were combusted in a muffle furnace at 550°C and reweighed to calculate AFDM. To determine an "autotrophic index" (AI), an indicator of trophic state (Flotemersch et al. 2006), Chl a values were divided by AFDM values. Subsamples of the slurry for C and N analysis were dried in an oven at 60°C and ground to a fine powder. Particulate C and N contents were measured directly with a Perkin Elmer CHN analyzer (Perkin Elmer, Waltham, MA, USA). Organic carbon values were determined by acid fumigation to remove carbonates prior to CHN analysis (Harris et al. 2001). Particulate P was analyzed colorimetrically on filters used for AFDM, as described for TDP and TP.

Statistical Analyses

For each water physicochemical variable, a linear regression model with a factor for each reach (Ramsey Upstream, Ramsey Shaded, Ramsey Downstream, Garden Upstream, Garden Shaded, and Garden Downstream) was used to model the value of the physicochemical variable. Wald tests were then performed to determine if values were significantly different between reaches. Upstream reach comparison were used to determine differences between streams while within-stream comparisons were made for Upstream-Shade, Shade-Downstream, and Upstream-Downstream pairings to determine experimental effects. Weekly water chemistry samples were used as replicates within each group (n=5) as models that incorporated repeated measures or time series analysis were not significantly different than this simpler model that assumed weekly samples as independent. This procedure was repeated for the periphyton variables. To facilitate visual comparisons, parameters were normalized based on the mean concentration of the

upstream reach prior to graphing water or periphyton chemistry variables that showed significant differences between reaches.

To examine the nutrient spiraling metrics, I used a one-way ANOVA to test for differences in nutrient uptake rates between reaches within a stream. To examine algal colonization rates, concentrations of Chl a on control NDS were compared between reaches within a stream using a one-way ANOVA. To examine nutrient limitation of periphyton, I performed a one-way ANOVA on changes in Chl a biomass in response to nutrient amendments within each reach of each stream. When there was a significant increase in Chl a biomass in a nutrient treatment compared to the control, I determined the identity of the limiting nutrient based on the definitions of Harpole et al. (2011). Briefly, "single nutrient limitation" was defined as a positive response that was equal to or greater than the combined ("NP") nutrient treatment. "Serial limitation" was defined as a positive response in a single nutrient treatment combined with a response for the NP treatment that was greater than the single nutrient treatment. "Co-limitation" was defined as a positive response for the NP treatment but not for either single nutrient treatment. To examine leaf litter decomposition, the effects of shading on the percentage loss of AFDM were tested separately by stream using a linear mixed model.

Values are reported as either means ± standard error or means [with 95% confidence intervals in brackets]. Prior to analysis, normality of residuals and heterogeneity of variance were checked. All statistics were performed in R (ver. 3.0.2) (R Core Team 2014).

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RESULTS

Light and CaCO$_3$ deposition rate

  Greenhouse shade cloth led to reductions in PAR and CaCO$_3$ deposition. Average

reductions in PAR were 80.3 ± 8.8% leading to average irradiance levels of 40.9 μmol

m^{-2} s^{-1} ± 5.1 and 19.5 μmol m^{-2} s^{-1} ± 4.9 in the shaded reaches of Garden and Ramsey

Canyons, respectively. Reductions were apparent on both cloudy and sunny days (Figure

1). Shading corresponded to an approximately 57% decrease in CaCO$_3$ deposition in

Garden Canyon, decreasing from 1.01 g CaCO$_3$ g^{-1} leaf day^{-1} [0.67, 1.35] to 0.43 g

CaCO$_3$ g^{-1} leaf day^{-1} [0.27, 0.59].
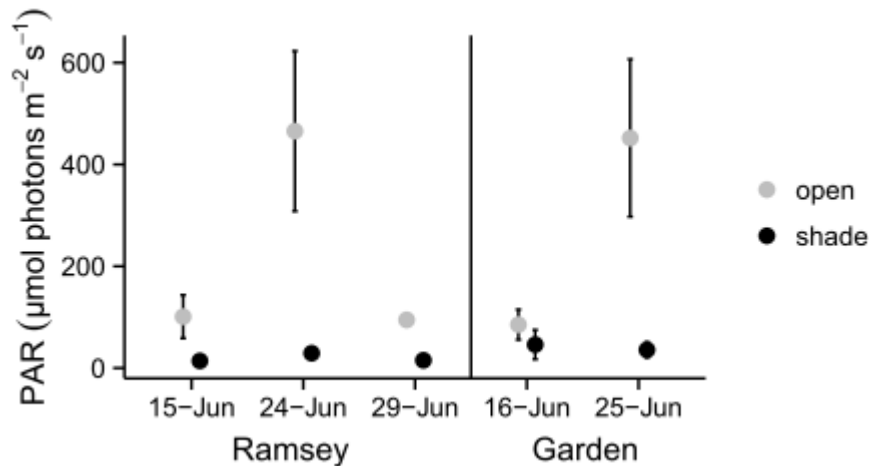
Figure 1. Photosynthetically active radiation (PAR) in the experimental units in Ramsey
and Garden Canyon on different dates. Error bars represent the standard error of multiple
measurements on that day. Points are offset within dates to alleviate overplotting.

Stream Water Physicochemistry

Based on the data loggers, stream water temperatures were higher in Garden Canyon (17.9 ± 0.1 $^{\circ}$C) than Ramsey Canyon (16.4 ± 0.1 $^{\circ}$C) (Figure 2). Shading tended to reduce daily maximum temperatures in Ramsey Canyon, but did not influence mean or minimum daily temperatures (Figure 2). Unfortunately, the data logger was lost from the shaded reach in Garden Canyon, so this comparison cannot be made. Based on the weekly temperature readings from the handheld probe, shading did not influence temperatures between reaches in either stream ($p > 0.05$) (Table 1).
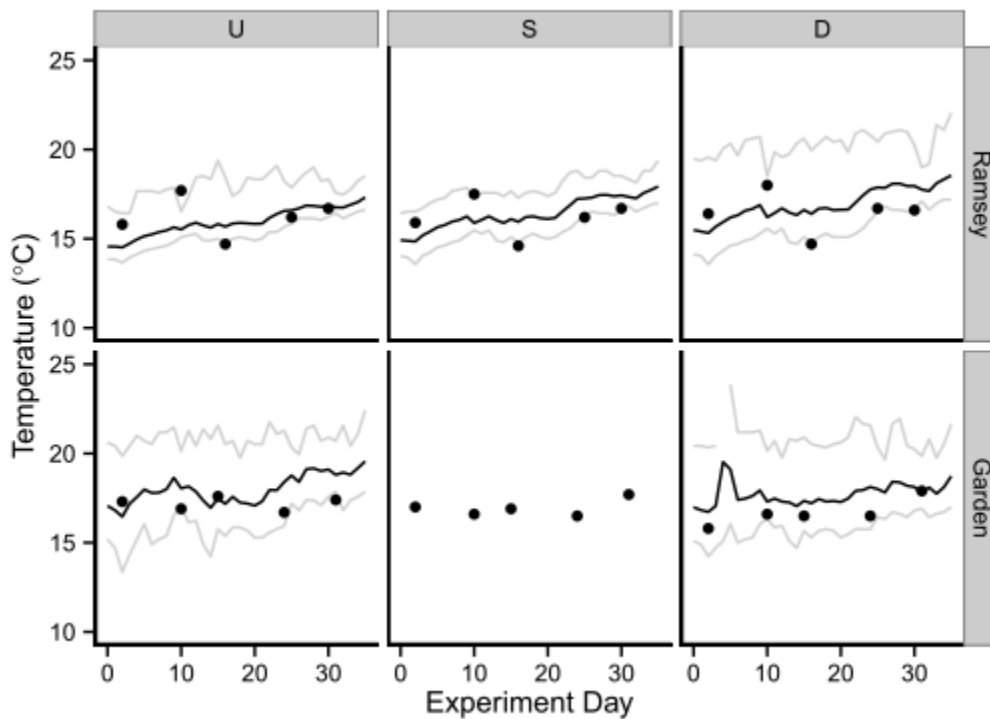
Figure 2. Stream water temperature in the upstream (U), shaded (S), and downstream (D) reaches in Ramsey and Garden Canyon, AZ, during the experiment. Lines represent daily mean, maximum, and minimum temperatures from continuous *in situ* data loggers while points indicate weekly readings from a hand-held probe. Continuous data is not shown for Garden S as the data logger was lost in the stream.

Table 1. *p*-values for upstream reach comparisons of water chemistry parameters. All parameters are based on weekly field samples. Bolded *p*-values indicate significant differences (*p*<0.05) and italicized *p*-values indicate marginally significant differences (*p*>0.05 and *p*<0.10).

Parameter	Mean (SE)		
	Garden	Ramsey	*p*-value
Basic			
Alkalinity (meq L^{-1})	5.72 (0.28)	4.98 (0.17)	**0.02**
pH	8.08 (0.03)	8.12 (0.01)	0.29
Specific Conductivity ($\mu S\ cm^{-1}$)	534 (5)	465 (8)	**0.01**
Temperature (^{o}C)	17.2 (0.2)	16.2 (0.5)	*0.10*
Major Ions (mg L^{-1})			
Ca^{2+}	80 (<1)	65 (<1)	**<0.01**
Mg^{2+}	14.5 (0.1)	13.6 (0.1)	**<0.01**
Na^+	3.02 (0.04)	4.44 (0.04)	**<0.01**
K^+	0.29 (0.03)	0.63 (0.03)	**<0.01**
Si	4.18 (0.03)	6.35 (0.06)	**<0.01**
SO_4^{2-}	10.6 (1.3)	13.1 (1.4)	0.16
Cl^-	1.91 (0.29)	2.24 (0.34)	0.41
Br^-	0.001 (0.001)	0.001 (0.001)	0.90
Organic Carbon and Nutrients (mg L^{-1})			
DOC	0.74 (0.09)	0.55 (0.04)	0.38
NH_4^+-N	3.21 (0.69)	2.47 (0.5)	*0.08*
NO_3^--N	0.07 (0.02)	0.008 (0.006)	**<0.01**
DON	0.029 (0.027)	0.033 (0.023)	*0.08*
TDN	0.1 (0.04)	0.04 (0.03)	0.21
TN	0.092 (0.031)	0.045 (0.009)	0.23
SRP ($\mu g\ L^{-1}$)	3.72 (1.24)	7.74 (1.55)	0.15
DOP ($\mu g\ L^{-1}$)	4.34 (0.31)	7.74 (2.17)	0.91
TDP ($\mu g\ L^{-1}$)	8.05 (1.24)	15.49 (3.72)	*0.06*
TP ($\mu g\ L^{-1}$)	4.96 (0.93)	10.22 (1.24)	*0.10*
Ratio of Total Nitrogen:Total Phosphorus	47 (13)	10 (2)	**0.02**
Trace Elements ($\mu g\ L^{-1}$)			
As	0.21 (0.02)	0.19 (0.011)	0.40
Cd	0.003 (0.001)	0.016 (0.002)	**<0.01**
Cu	0.3 (0.22)	0.36 (0.08)	0.89
Fe	23.4 (2.3)	3.2 (0.6)	**<0.01**
Mn	17 (2)	5.6 (0.7)	**<0.01**
Mo	0.091 (0.021)	0.425 (0.018)	**<0.01**
V	0.3 (0.09)	0.2 (0.05)	0.26
Zn	7.8 (6.3)	8.7 (4.9)	0.88

Specific conductivity differed between the streams ($p<0.05$), consistent with differences in major ion concentrations (Table 1). In Garden Canyon, the specific conductivity was 15% higher than in Ramsey Canyon. Indeed, concentrations of Ca^{2+} and Mg^{2+} are 24% and 7% greater in Garden Canyon than Ramsey Canyon, respectively. In both streams, cations and anions had similar relative concentrations with cation concentrations ranked as $Ca > Mg > Si > Na > K$ and anion concentrations as $SO_4^= > Cl^-$ $>> Br^-$. Alkalinity also differed between streams ($p<0.05$), with higher values in Garden Canyon than Ramsey Canyon (Table 1).

Nutrient concentrations did not differ greatly between streams, although nitrate concentrations were significantly higher in Garden Canyon than Ramsey Canyon (Table 1). Phosphorus concentrations tended to be lower in Garden Canyon than Ramsey Canyon. The slightly higher values of N species and lower values of P species in Garden compared to Ramsey led to significantly higher ratios of TN:TP in Garden Canyon than in Ramsey Canyon ($p<0.05$).

As with the major cations, some trace elements differed significantly in concentration between streams (Table 2). Cadmium and molybdenum were markedly higher in Ramsey Canyon than Garden Canyon while iron and manganese were higher in Garden Canyon than Ramsey Canyon.

Within streams, shading influenced several chemical parameters (Figures 3 and 4). In Garden Canyon, shading slightly, but significantly, decreased pH from 8.08 ± 0.03 to 7.99 ± 0.04 (Figure 3). In Ramsey Canyon, specific conductivity declined 14% between the upstream and downstream reaches (Figure 3), although this was not associated with any shifts in the major cations tested. In Garden Canyon, but not Ramsey

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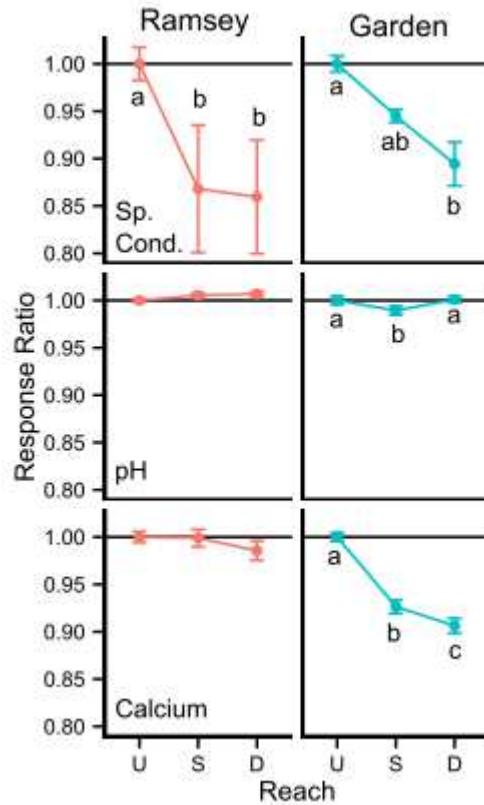
Figure 3. Response of specific conductivity, pH, and calcium concentration to experimental shading in the shaded ("S"), and downstream ("D") reaches normalized to the upstream ("U") reach in Ramsey and Garden Canyon streams. Responses ratios are in terms of average upstream values. The horizontal line represents the distinction between a negative (<1) or positive (>1) response in the reach compared to the upstream value. Letters indicate significant differences based on Wald tests between groups ($p<0.05$).
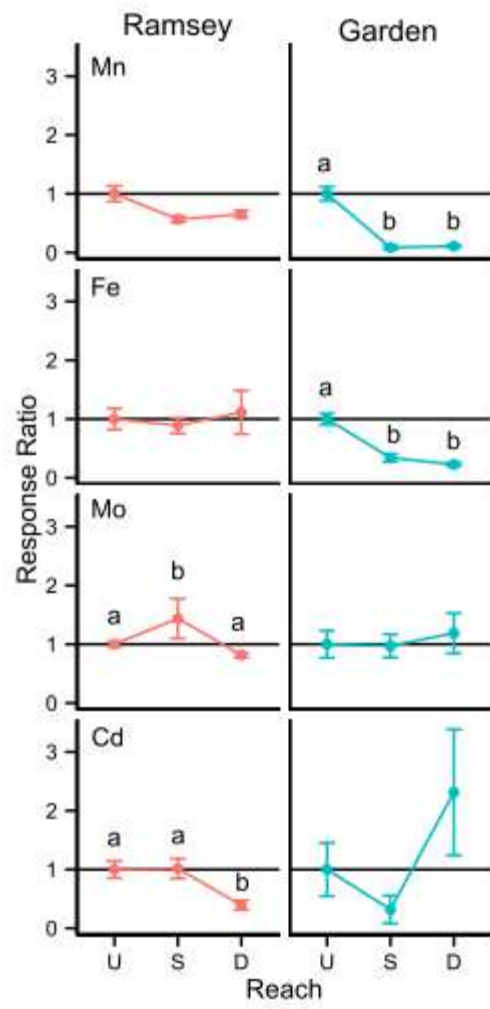
Figure 4. Response of trace elements (manganese, Mn, iron, Fe, molybdenum, Mo, and cadmium, Cd) to experimental shading. See Figure 3 for description.

Canyon, shading influenced Ca^{2+} concentrations (Figure 3), consistent with shifts in $CaCO_3$ deposition rates. Shading was also associated with some shifts in trace element concentrations (Figure 4). In Garden Canyon, shading was associated with 66% and 91% decreases in iron and manganese concentrations, respectively (Figure 4). In Ramsey Canyon, shading was associated with a temporary, 44%, increase in molybdenum concentrations and a 61% decline in downstream concentrations of cadmium (Figure 4). Shading did not strongly influence dissolved organic carbon or nutrient concentrations.

Epilithon Chemistry

The epilithon differed markedly between the two streams (Table 2). Epilithon biomass was higher in Ramsey Canyon, both in terms of AFDM ($p<0.01$) and Chl *a* ($p=0.055$). The organic C, nitrogen, and phosphorus contents of the epilithon biomass were also higher in Ramsey Canyon, although the lower molar ratios of C:N and C:P in Garden Canyon suggest that the epilithon biomass was of higher stoichiometric quality to consumers in the $CaCO_3$-depositing stream.

Shading influenced Chl *a* biomass and the absolute and relative concentrations of nutrients in the epilithon, although the effect differed by stream (Figure 5). In Ramsey Canyon, Chl *a* increased significantly between the shaded and downstream reach (Figure 5). However, in Garden Canyon, there was no effect of shading on Chl *a* concentrations. Similarly, concentrations of organic C and N shifted in response to shading in Ramsey Canyon but not in Garden Canyon (Figure 5). In Ramsey Canyon, concentrations of both organic C and N declined 41% and 35%, respectively. In Garden Canyon (the $CaCO_3$-depositing stream), epilithon P concentrations and N:P ratio were significantly influenced

by shading (Figure 5). Epilithon P concentrations were higher in the shaded reach

compared to the downstream reach ($p<0.05$), although the difference between the

upstream and shaded reach was only marginally significant ($p=0.08$). Epilithon N:P

downstream of shade more than doubled to 23. Importantly, shading did not affect

epilithon P concentrations nor N:P ratios in Ramsey, the non-$CaCO_3$-depositing stream.

Table 2. *p*-values for upstream reach comparisons of epilithon chemical parameters
including ash-free dry mass (AFDM), chlorophyll *a* (Chl *a*), autotrophic index (AI), and
percent by mass of carbon (C), nitrogen (N), and phosphorus (P). The ratios of carbon to
nitrogen (C:N), carbon to phosphorus (C:P), and nitrogen to phosphorus (N:P) are
reported as molar ratios. All parameters are based off samples collected in Week 5.

Parameter	Mean (SE)		*p*-value
	Ramsey	Garden	
AFDM (mg cm^{-2})	4.71 (3.2)	0.32 (0.07)	**<0.01**
Chl a (µg cm^{-2})	3.19 (0.37)	0.95 (0.29)	*0.06*
AI	1293 (828)	404 (108)	0.25
% Organic C	14.7 (1)	2.5 (0.2)	**<0.01**
% N	0.82 (0.09)	0.18 (0.01)	**<0.01**
% P	0.068 (0.002)	0.024 (0.003)	**<0.01**
C:N	21 (0.9)	14.3 (1)	**0.01**
C:P	553 (101)	306 (71)	**0.03**
N:P	26 (6)	13 (0)	**0.02**
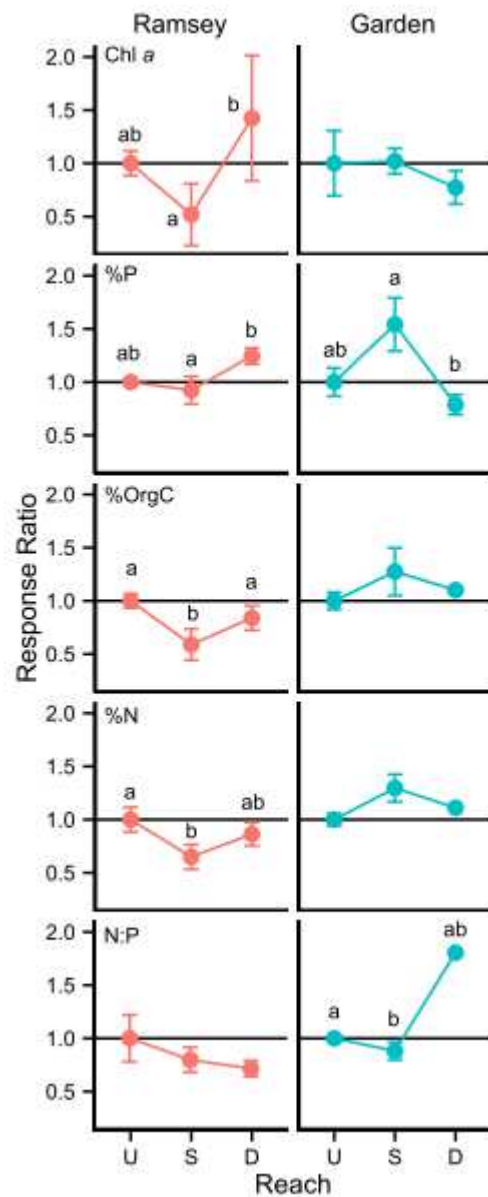
Figure 5. Relative response of epilithon biofilm characteristics (chlorophyll *a*, Chl *a*, biomass phosphorus concentration, %P, biomass organic carbon concentration, %C, biomass nitrogen concentration, %N, and the molar ratio of N:P in the biomass) to experimental shading. See Figure 3 for description.

Ecosystem Processes

Nutrient uptake

      Nutrient spiraling assays compared uptake responses at the start of the experiment

and after three and five weeks of shading. Initially, spiraling lengths (S_w) across the

experimental units were similar between streams for NO_3^- but differed for SRP (Table 3).

Throughout the experiment, SRP uptake lengths were often too long to be detected in

Ramsey Canyon, but this only occurred once in Garden Canyon (Table 3). Overall, areal

uptake rates of NO_3^- were higher in Garden Canyon than in Ramsey Canyon, while areal

uptake rates of SRP were more similar (Figure 6). In Garden Canyon, shading was

expected to increase the S_w of P and decrease areal P uptake lengths, as these are both

Table 3. Uptake lengths in Garden Canyon and Ramsey Canyon from nutrient
enrichment experiments prior to shading (Week 0) and after 3 and 5 weeks of shading.
After shading began, uptake lengths were determined separately in the upstream ("U"),
shaded ("S"), and downstream ("D") reaches.

Stream	Week	Reach	Uptake Length, S_w (m)	
			NO_3	SRP
Garden	0	U	38	12
	3	U	43	25
		S	52	45
		D	13	50
	5	U	49	29
		S	77	44
		D	35	>150
Ramsey	0	U	52	>150
	3	U	57	>150
		S	>150	124
		D	49	86
	5	U	147	>150
		S	39	>150
		D	51	>150

indicative of decreased P coprecipitation and less P limitation. As expected, shading noticeably increased SRP uptake lengths in Garden Canyon (Table 3) and significantly decreased areal uptake rates of SRP in Garden Canyon ($F_{2,3}=13.19$, $p<0.05$) (Figure 6). There were no consistent effects of shading on spiraling metrics in Ramsey Canyon.
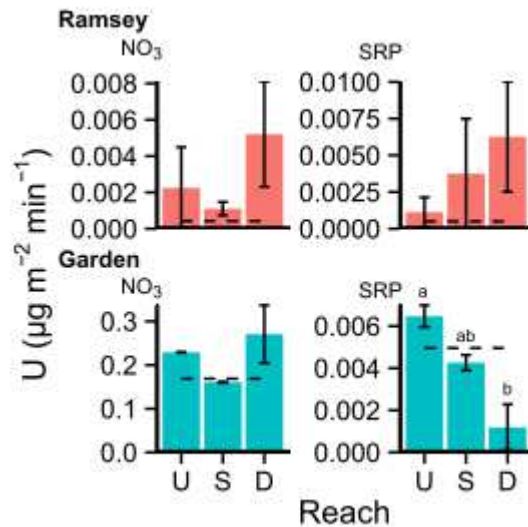
Figure 6. Mean uptake rates in Ramsey Canyon and Garden Canyon from nutrient enrichment experiments. Letters indicate significant differences between reaches (upstream, "U", shaded, "S", and downstream, "D") based on Tukey's post hoc comparisons ($p<0.05$). Error bars represent the standard error. The dotted, horizontal line indicates the pre-experimental manipulation uptake rate.

Periphyton nutrient limitation and colonization rate

Chlorophyll *a* biomass on the artificial substrates responded positively to nutrient amendments in both streams. In Ramsey Canyon, shading reduced nutrient limitation, but did not change the identity of the limiting nutrient (Figure 7). Chlorophyll *a* biomass increased significantly with N amendments in both the upstream ($F_{3,16}=5.64$, $p<0.01$) and the downstream ($F_{3,16}=108.18$, $p<0.001$) reaches of Ramsey Canyon. However, in Garden

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Canyon, shading led to a shift in nutrient limitation, reducing the role of P between the

upstream and downstream reaches. In the upstream and shaded reach of Garden Canyon,

I found serial limitation, with N and NP leading to successive increases in Chl a biomass

(upstream: $F_{3,16}=10.26$, $p<0.001$, shade: $F_{3,16}=74.55$, $p<0.001$). However, downstream of

the shade, Chl a biomass increased with N, but serial limitation was no longer apparent

($F_{3,16}=59.32$, $p<0.001$).
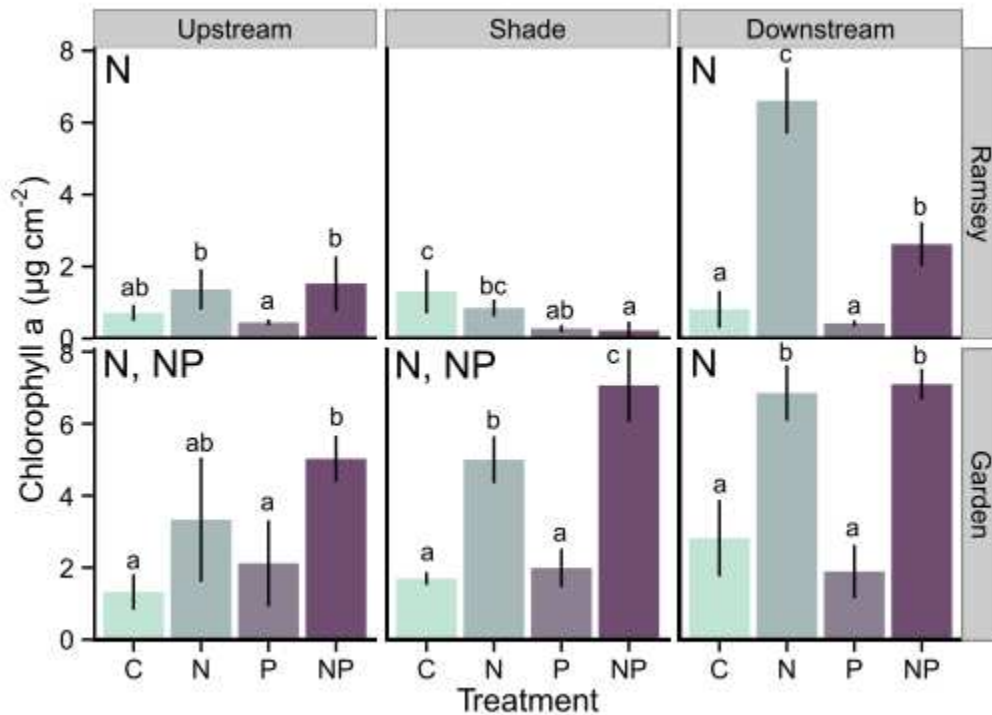
Figure 7. Responses of chlorophyll a biomass on artificial substrata in response to nutrient treatments (unamended, C, nitrogen, N, phosphorus, P, or nitrogen and phosphorus, NP) in each reach in Ramsey (top row) and Garden (bottom row) Canyon following a three-week incubation. Where applicable, the identity of the limiting nutrient (or, multiple nutrients in the case of serial limitation) is listed in the upper left corner of the panel. Letters indicate significant differences between nutrient treatments based on Tukey's Post Hoc comparisons ($p<0.05$). Error bars represent the standard error.

The amount of Chl *a* on the control NDS did not differ between reaches in Ramsey Canyon (*p*=0.13), but did in Garden Canyon ($F_{2,12}$=5.79, *p*<0.05). In Garden Canyon, Chl *a* concentrations were lowest in the upstream reach and increased in the shaded and downstream reaches. Indeed, downstream Chl *a* concentrations were 113% higher in the downstream reach compared to the upstream reach.

Leaf litter Decomposition

Overall, breakdown rates of leaf litter were low, but detectable, in both streams (Figure 8). Rates were higher in Garden Canyon, 0.0156 day^{-1} [0.008, 0.023], than in Ramsey Canyon, 0.003 day^{-1} [0.001, 0.004]. However, an effect of shading on leaf litter decomposition was only found in Ramsey Canyon. In Ramsey Canyon, shading decreased leaf litter decomposition significantly ($F_{5,7}$=22.45, *p*<0.001), lowering it 32% in the shaded reach and 75% in the downstream reach.
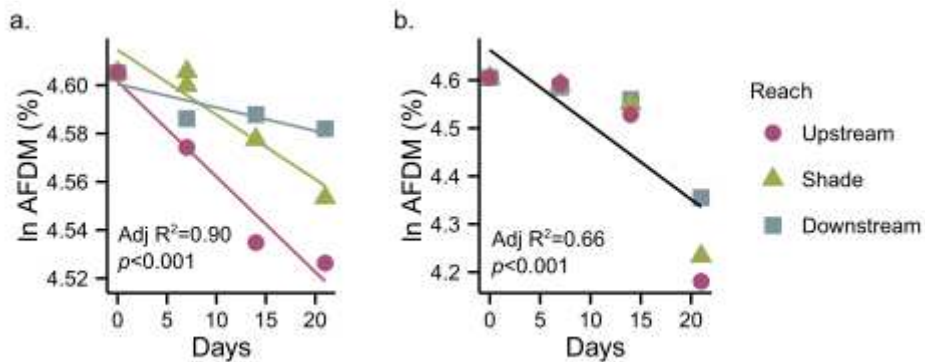
Figure 8. Decrease of ash-free dry mass (AFDM) of leaf litter from bags in (a) Ramsey Canyon and (b) Garden Canyon stream.

DISCUSSION

In Garden Canyon, the CaCO$_3$-depositing stream, shading-induced decreases of CaCO$_3$ deposition led to several strong responses of ecosystem processes related to phosphorus availability. Lowering CaCO$_3$ deposition increased phosphorus bioavailability, alleviating signs of phosphorus limitation of periphyton and lowering benthic areal P removal rates. These findings support the role of CaCO$_3$ co-precipitation of P as an important process regulating P cycling in streams, as has been suggested in lakes (Koschel et al. 1983, Robertson et al. 2007, Hamilton et al. 2009) and wetlands (Noe et al. 2001, Rejmánková and Komárková 2005, Borovec et al. 2010, Hagerthey et al. 2011). More importantly, by manipulating *in situ* CaCO$_3$ rates in stream reaches, I provide evidence that this process is important at the ecosystem scale.

Methodological Considerations

The shading manipulation led to substantial reductions in PAR in both streams (Figure 1) and CaCO$_3$ deposition rates in Garden Canyon. Therefore, I expected to find increases in stream nutrient concentrations due to decreased biological activity (Hill 1996, Rier et al. 2006, Rier et al. 2014) or, in the case of phosphate, decreased coprecipitation rates. However, I was unable to detect significant changes in nutrient concentrations in either stream in response to the manipulation. Nonetheless, this lack of response is not unprecedented. In other studies in which shade manipulations were performed and stream water nutrient concentrations were monitored, researchers also did not find significant changes in stream water nutrient concentrations (Steinman et al. 1991,

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Hepinstall and Fuller 1994). Hepinstall and Fuller (1994) did not offer an explanation for this observation; Steinman et al. (1991) suggested it was because reduced metabolic rates in periphyton in the shaded treatment were balanced by nutrient regeneration in the non-shaded treatment. I suggest that, because other shifts in P cycling patterns were detected (e.g., Figure 6), a longer experimentally shaded reach may be needed in these streams to perceive detectable changes in stream water nutrient concentrations.

Given the known relationship between photosynthesis and $CaCO_3$ deposition (Garcia-Pichel et al. 2004, Visscher and Stolz 2005), the observed increase in periphyton growth under the shaded reach in Garden Canyon (Figure 7) might have been expected to counter-act the depression of $CaCO_3$ deposition in the shaded reach. However, periphyton growth and rates of net primary production (NPP), a key determinant of $CaCO_3$ deposition rate (Dupraz et al. 2009), are not always linearly related (Falkowski and Raven 2007). Indeed, in an investigation of light effects on diatom physiology, Hill and Knight (1988) found that shading reduced NPP, but not growth, of the algae. In this system, any stimulation of photosynthetic activities related to increased periphyton growth appeared to have been outweighed by concomitant increases in aerobic respiration, therefore maintaining a net decrease in $CaCO_3$ deposition.

While shading led to decreased rates of $CaCO_3$ deposition in Garden Canyon, it did not reduce $CaCO_3$ deposition entirely. This is evident in the positive rate of $CaCO_3$ deposition on leaves in the shaded reach and the response of stream water Ca^{2+} concentrations to shading (Figure 3): Ca^{2+} concentrations decreased from the upstream to shaded and downstream reaches, indicating net deposition of $CaCO_3$. This "background rate" of $CaCO_3$ deposition is also likely the cause for lowered Mn and Fe concentrations

in the shaded and downstream reaches of Garden Canyon. Both of these elements are known to adsorb to $CaCO_3$ in aquatic ecosystems (Cave and Talens-Alesson 2005, Pentecost 2005, Wang et al. 2013). Mn and Fe are important enzyme cofactors (Neilands 1995, Jakubovics and Jenkinson 2001, Kehres and Maguire 2003, Moore and Helmann 2005); research on the $CaCO_3$-trace element interaction from an ecological perspective is needed to determine if $CaCO_3$ may be affecting the bioavailability of these metals, as shown with phosphorus in this study.

The experimental manipulation was necessarily limited in space (two streams, only one with active $CaCO_3$ deposition) and time (lasting five weeks). Replication of $CaCO_3$-manipulating experiments like this one in other streams will aid in determining the ecological impacts of this geochemical process. However, the results of this experiment are consistent with longer-term and widespread observations of lowered phosphorus concentrations in $CaCO_3$-depositing streams and rivers (see Ch. 2, House and Denison 1997, Elser et al. 2005, Withers and Jarvie 2008), suggesting that conclusions from this study have broader application. Additionally, the temporal scale of this experiment was necessarily limited due to impending monsoon storms and related flooding, events common in this region in July (Jaeger and Olden 2012). However, this timescale was still sufficient for assessing the ecological impacts of shade-induced reductions of $CaCO_3$ deposition in a stream. Conversely, longer-term experiments may allow for community successional shifts and therefore would provide the opportunity to study how $CaCO_3$ deposition impacts community species composition, food web dynamics, and other ecological processes that occur on greater timescales (Dodds et al. 2012).

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CaCO₃ and Phosphorus Cycling

Despite the fact that the experimental design did not completely eliminate $CaCO_3$ deposition from Garden Canyon nor was the duration long-term, decreases in $CaCO_3$ deposition still led to substantial changes in the stream ecosystem. As expected, effects of shading in Garden Canyon differed from those in Ramsey Canyon. This could be because the PAR that penetrated shading was greater in Garden Canyon (40 μmol m^{-2} s^{-1} vs 20 μmol m^{-2} s^{-1}). Therefore, larger decreases in Chl *a* concentrations and lower rates of leaf litter breakdown found in Ramsey Canyon in the shade may be due to the fact that less PAR was able to penetrate the shade cloth in this stream. However, this explanation is unlikely because shading in Garden Canyon also lead to increased P concentrations in epilithon biomass (Figure 5) and a decrease in stream water pH (Figure 3), suggesting that the negative effect of light reductions in Garden Canyon were countered by the shifts in $CaCO_3$ dynamics in the shaded reach that increased P availability. In the shaded reach, shifts in $CaCO_3$ dynamics may have occurred in two ways: reduction of $CaCO_3$ deposition rates (as evidenced by lower $CaCO_3$ deposition on the leaf litter) or by dissolution of $CaCO_3$ already present in the stream prior to the start of the experiment. Dissolution of $CaCO_3$ may have been promoted by the decreases in stream water pH, as pH can be lowered due to increased aerobic respiration. If dissolution occurred, it would have released phosphorus co-precipitated with the mineral, as in the periphyton mats of Florida (Hagerthey et al. 2011). Therefore, either reductions in $CaCO_3$ deposition or dissolution of $CaCO_3$ would lead to increased P availability. My results do not let me distinguish between these alternatives. However, regardless of the way(s) in which $CaCO_3$ dynamics shifted in the shaded reach, the greatest influence of the increased

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phosphorus availability seemed to be downstream, where increased phosphorus availability appeared to be able to meet periphyton P demand (Figures 6 and 7).

The stream nutrient tracer experiments also supported my hypothesis about the impacts of $CaCO_3$ coprecipitation of phosphorus on stream ecosystem-level dynamics. Even with only a 57% reduction in $CaCO_3$ deposition, phosphorus S_w increased to >150 m, similar to values in the non-$CaCO_3$ depositing stream. However, because S_w values were much greater than the length of experimental reach, the lengths cannot be quantified (Stream Solute Workshop 1990). Interestingly, only moderate increases in phosphorus S_w were seen in the shaded reach and it was not until further downstream that the substantial increase in phosphorus S_w was observed. I suggest that this is because the stream water is continually moving downstream and moving the nutrients along with it. At weeks 3 and 5, phosphorus S_w values were roughly equivalent to the length of the shaded reach (Table 3). Therefore, much of the phosphorus that otherwise would have been co-precipitated with $CaCO_3$ would "spiral" to the downstream reach before interacting with the substrate ($CaCO_3$ or periphyton) again (Stream Solute Workshop 1990).

Effects of Shading on Streams

In this experiment, a 90% shade cloth corresponded to an 80% reduction in PAR, a light reduction within the range of other experiments designed specifically to test the effects of light exclusion in streams: 70% shade cloth was used in Hepinstall and Fuller (1994), 80% light reduction was achieved in Proia et al. (2012), 81 – 88% reduction in PAR in Lagrue et al. (2011), and 99% reduction in PAR in Rier et al. (2014). Therefore, while the main purpose of this experiment was to examine how $CaCO_3$ deposition

influences stream ecosystems, the experimental approach also allows insight into how light influences stream ecosystems more generally.

Results from this experiment support the findings of Johnson (2004) that solar irradiance can influence stream water temperatures. In both this experimental stream shading and the experimental stream shading by Johnson (2004), shading reduced maximum stream temperatures, but did not strongly affect minimum and mean temperatures. Indeed, these studies support earlier work on riparian canopy removal that found increases in maximum stream temperatures with greater solar irradiance (e.g., Swift and Messer 1971).

As expected based on previous shading experiments (Steinman et al. 1991, Rier et al. 2014), shading reduced ambient levels of Chl *a* in periphyton absent interactions with $CaCO_3$ deposition. Experimental shading is also expected to reduce periphyton colonization rates on artificial substrata (Hepinstall and Fuller 1994, Rier et al. 2014). In Ramsey Canyon, this is not what I found; instead, my results from the control NDS suggest periphyton colonization rates were not affected by shading (Figure 7). However, periphyton colonization rates were inferred by estimates of the standing stock of periphyton (i.e., Chl *a* concentrations) after a three-week *in situ* incubation. While this is a generally accepted method of determining periphyton growth rates (Tank and Dodds 2003), it does not quantify grazing rates or other scouring processes that may influence the amount of biomass on the substrate. Therefore, if periphyton removal processes differ between reaches, comparisons of chlorophyll *a* standing stock are not a valid indicator of colonization rates (Hill 1996); unfortunately, the data to test this hypothesis is not currently available from this experiment.

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Results from this experiment also support previous observations on the effects of shading on leaf litter decomposition. Rates of leaf litter breakdown have been found to decrease under shaded conditions (Lagrue et al. 2011), as was found in Ramsey Canyon. This intriguing result may be due to the loss of algal exudates and algae-derived nutrients from the leaf biofilm that would otherwise stimulate heterotrophic activity (Hepinstall and Fuller 1994, Albarino et al. 2008).

CONCLUSION

Understanding nutrient cycling patterns in streams is important both for understanding *in situ* ecological processes (Cross et al. 2005) and in determining downstream nutrient transport (Alexander et al. 2007). My data indicate that $CaCO_3$ deposition increases phosphorus retention in streams and limits primary production. Similar patterns of phosphorus concentrations and $CaCO_3$ deposition rates in other ecosystems (e.g., Rosen et al. 1996, Noe et al. 2001, Elser et al. 2005) suggest that such responses could be common in calcareous freshwater ecosystems. Many streams are facing extensive threats from nutrient pollution (Gilinsky et al. 2009, Smith and Schindler 2009). Therefore, managing calcareous streams to maintain $CaCO_3$ deposition rates (e.g., by manipulating flow regimes, as in Malusa et al. 2003) may help attenuate nutrient flows to receiving waters and minimize eutrophication.

ACKNOWLEDGEMENTS

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CHAPTER 6

CONCLUDING REMARKS

Synthesis of Major Findings

<u>Chapter 2</u>

Analyzing physicochemical conditions across three streams with differing rates of calcium carbonate ($CaCO_3$) deposition in the Huachuca Mountains, I found strong correlations between $CaCO_3$ deposition and nutrient concentrations and ratios. As expected, $CaCO_3$ deposition was negatively correlated with phosphorus concentrations. Surprisingly, $CaCO_3$ deposition was also positively correlated with nitrogen concentrations, suggesting that the stoichiometric effect of $CaCO_3$ deposition on aquatic ecosystems is due not only to coprecipitation of phosphate, but also to phosphorus-related constraints on nitrogen uptake. In addition, I showed the importance of using multiple metrics of $CaCO_3$ deposition to determine spatial and temporal variation in $CaCO_3$ deposition rates.

<u>Chapter 3</u>

Growth of periphyton in streams is often co-limited by multiple nutrients (Dodds and Welch 2000, Francoeur 2001, Elser et al. 2007). In contrast, I found phosphorus limitation of growth of photoautotrophs was unique to the streams of the Huachuca Mountains with active $CaCO_3$ deposition. This finding supports the predictions of nutrient limitation based on nutrient availability patterns (Chapter 2). However, phosphorus limitation of growth was seasonally dependent, suggesting that nutrient demand, as well as supply, is important in determining nutrient limitation of periphyton.

Chapter 4

In Río Mesquites, primary production and respiration are limited by phosphorus in microbial communities associated with $CaCO_3$ deposits. Conversely, based on chlorophyll *a* accumulation, photoautotrophs in microbial communities not associated with $CaCO_3$ deposits do not respond to phosphorus additions. When I reduced $CaCO_3$ deposition rates in the microbialites, phosphorus concentrations of microbial biomass increased. I also found that photoautotrophs play an important role in nutrient acquisition of the microbial communities. Finally, I present evidence that sulfate reduction may support lithification in these microbial communities, a process found in stromatolites of oceanic and hypersaline origin (Visscher et al. 2000, Gómez et al. 2014) but not yet described in freshwater habitats.

Chapter 5

This study is the first study to experimentally manipulate $CaCO_3$ deposition rates in a stream. My results demonstrate that partial light exclusion can lower rates of $CaCO_3$ deposition and lead to increases in phosphorus availability in the stream. My findings show that $CaCO_3$ deposition has many ecosystem effects, including moderating periphyton growth via phosphorus limitation and decreasing downstream transport of phosphorus (supporting the findings from long-term nutrient data from Chapter 2). Leaf litter decomposition may also be dampened due to decreased phosphorus availability from $CaCO_3$ coprecipitation of phosphate. Additionally, these results suggest that changes in canopy cover over streams may have important consequences for patterns of $CaCO_3$ deposition and, therefore, phosphorus cycling.

Overarching Themes: Cross-Site Comparisons

Calcium carbonate deposition occurs in a wide number of forms. In this dissertation, I studied two types of $CaCO_3$ deposits: travertine (in the Huachuca Mountains) and microbialite (in Río Mesquites). The environment in which these deposits accrue differs, too. The Huachuca Mountain streams are characterized by steep topography, a primarily deciduous riparian canopy, and low discharge while Río Mesquites has little relief, an open canopy, and higher discharge (Chapter 2 and Chapter 4). Despite these differences, my results suggest $CaCO_3$ deposition influences phosphorus cycling similarly.

In both systems, $CaCO_3$ deposition lowered phosphorus concentrations in the water column. This led to phosphorus limitation of microbial growth, a phenomenon that is apparently confined to the microbes associated with $CaCO_3$ deposits in these ecosystems. These results suggest the effects of $CaCO_3$ deposition on phosphorus cycling are widespread.

Experimental manipulation of $CaCO_3$ deposition revealed novel insights into each system as well. In contrast to other streams where $CaCO_3$ is an abiotic process (Herman and Lorah 1987, Malusa et al. 2003), shading in the Huachuca Mountains reduced rates of $CaCO_3$ deposition, highlighting the importance of photoautotrophic metabolism to travertine development. In Río Mesquites, my experimental results, combined with observations from earlier studies (Garcia-Pichel et al. 2004, Elser et al. 2005, Breitbart et al. 2009), suggest sulfate reduction may be an important process in lithification in the microbialites of Cuatro Ciénegas.

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Implications for Management and Future Research Directions

Aquatic systems are vital for human well-being. Yet, most surficial aquatic ecosystems are facing extensive threats from nutrient pollution (Gilinsky et al. 2009, Smith and Schindler 2009). Humans have increased nutrient bioavailability through increased fertilizer use, land-use change, and untreated wastewater discharge, leading to eutrophication in many aquatic systems. While the consequences of eutrophication are well studied, there are still many challenges to mitigating its occurrence (Conley et al. 2009). My results suggest the importance of $CaCO_3$ deposition in regulating downstream flows of phosphorus. Streams with active $CaCO_3$ deposition are geographically widespread. In a comprehensive assessment of travertine, Pentecost (2005) describes over 100 streams with actively depositing $CaCO_3$ distributed across six continents. Therefore, because $CaCO_3$ deposition is widespread and may be a sink for P, land managers should take advantage of this geochemical characteristic when developing nutrient management policies.

However, it should be noted that the ability of $CaCO_3$ coprecipitation of phosphate (PO_4^{3-}) to lower phosphorus concentrations in a stream likely loses effectiveness at high PO_4^{3-} concentrations (e.g., > 100 mg P L^{-1}), as PO_4^{3-} can then inhibit $CaCO_3$ formation (House 1990, Neal 1999). Thus, there is a threshold stream water phosphorus concentration at which $CaCO_3$ will no longer act as a sink for phosphorus (House and Denison 2000). Furthermore, stream beds are not pure $CaCO_3$ substrates, and the presence of other minerals, organic matter, or biofilms may moderate expected relationships between $CaCO_3$ deposition rates and PO_4^{3-} coprecipitation (Jarvie et al. 2002). Kinetic experiments with sediment sorption capacity may be useful in determining

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phosphorus uptake potentials (Jarvie et al. 2005, Demars 2008). Finally, it should be emphasized that, while my results highlight the potential for managing $CaCO_3$ to minimize downstream phosphorus flows, they also suggest that $CaCO_3$ deposition may have the opposite effect on nitrogen flows.

My findings have also established that $CaCO_3$ deposition can cause or enhance phosphorus limitation of microbial communities. However, it remains unknown if microbes receive some benefit from associating with $CaCO_3$ deposits. Microbes may grow on $CaCO_3$ deposits simply because it is novel habitat, as is found with the microbes of Mammoth Springs, WY, USA (Fouke et al. 2000). However, I suggest that microbes may grow on $CaCO_3$ deposits because of the interactions between $CaCO_3$ and phosphorus. Biofilm formation has been linked to phosphorus limitation in axenic cultures (Danhorn et al. 2004). In the Florida Everglades, formation of calcareous periphyton mats is also linked to phosphorus limitation – when phosphorus is replete, these $CaCO_3$ structures no longer form (Noe et al. 2001, Gaiser et al. 2012). One potential explanation for this phenomenon is that the microbes associated with $CaCO_3$ structures interact with the $CaCO_3$ because they are able to access some of the coprecipitated phosphate. Indeed, some microbes are able to access geologic phosphorus, such as the phosphorus in apatite minerals in basalt (Wu et al. 2007). Also, in a recent study of microbes living in subsurface environments, it was found that neutrophilic sulfur-oxidizing bacteria grew to higher biomass on carbonate rocks with phosphorus (limestone and dolomite) than carbonate rocks without phosphorus (pure calcite) (Jones and Bennett 2014). These results suggest that microbes in my study systems might in fact exploit coprecipitated phosphate. I recommend future research consider the interactions

between microbial growth and $CaCO_3$ formation and, more specifically, the role of

phosphorus limitation in the formation of biogenic carbonate structures.

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