DISSERTATION

# INVESTIGATING EXPERIMENTAL AND ENVIRONMENTAL FACTORS TO PROVIDE A MECHANISTIC UNDERSTANDING OF BENTHIC ALGAL BIOMASS ACCUMULATION IN FRESHWATER STREAMS

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# ABSTRACT

## INVESTIGATING EXPERIMENTAL AND ENVIRONMENTAL FACTORS TO PROVIDE A MECHANISTIC UNDERSTANDING OF BENTHIC ALGAL BIOMASS ACCUMULATION IN FRESHWATER STREAMS

Benthic (streambed) algae serve many critical ecological functions in freshwater stream ecosystems, including primary production, nitrogen (N) and phosphorus (P) cycling, and habitat provision for macroinvertebrates. Over the past several decades, N and P concentrations have increased in freshwater systems and estuaries because of human sources like wastewater treatment plant discharges, agricultural fertilizer runoff, urban stormwater runoff, and atmospheric deposition. Additions of these limiting nutrients can dramatically increase algal biomass accrual, causing negative ecosystem-level consequences such as proliferation of nuisance algae and depletion of dissolved oxygen, leading to economic costs conservatively estimated at $2.2 billion per year.

In aquatic ecology studies, algal production and biomass have been shown to respond to nutrient additions in a context-specific manner that depends on experimental methods and environmental conditions. In this dissertation, I investigate these sources of experimental and environmental variability using three approaches: a meta-analysis, methods comparison, and multi-factor experiments. The meta-analysis focuses on experimental, environmental, and geographic factors that influence algal nutrient limitation, whereas the methods comparison identifies experimental design choices that could confound nutrient limitation results. The multi-factor experiments focus on environmental factors including herbivory, temperature, and

background nutrients that mediate algal responses to nutrient additions under field conditions. While uncertainty can never be completely removed from ecological experiments, my research highlights how we can better account for that uncertainty and identify the appropriate scope of inference for individual nutrient addition experiments. Ultimately, this knowledge will contribute to the ability of researchers and managers to more effectively predict and prevent ecological and human health effects of stream eutrophication.

In Chapter 1, I conducted a systematic literature review and meta-analysis of nutrient diffusing substrate (NDS) experiments in freshwater stream and river ecosystems. My objectives were to calculate overall effects of N, P, and N+P additions on algal biomass, while also developing meta-analysis models to determine how experimental (e.g., nutrient concentrations), environmental (e.g., riparian canopy cover, temperature), and geographic factors (e.g., stream order, ecoregion) influence algal responses to nutrients. I extracted results from 649 experiments and found that experimental variables including substrate type, chemical concentration, and experimental length significantly affected P and N+P effect sizes, while NDS chemical compound influenced N, P, and N+P effect sizes. Environmental variables such as in-stream nutrients and riparian canopy cover significantly affected limitation by N, P, and N+P. Temperature, stream discharge, and stream velocity only affected limitation by N+P. Land use, ecoregion, and season showed clear trends in nutrient limitation for all treatments that could generally be tied to environmental factors like in-stream nutrients and riparian canopy cover.

For Chapter 2, I conducted NDS experiments in eight streams along an elevation gradient during two seasons to quantify important spatio-temporal drivers of algal biomass accrual, algal nutrient limitation, and aquatic insect excretion in the Cache la Poudre River watershed, Colorado. In-stream temperature decreased and stream N:P molar ratio increased with elevation

and with time, providing natural gradients for testing hypotheses. Temperature and nutrients had opposite expected effects on some response variables, allowing me to disentangle the relative importance of these drivers. In agreement with prior laboratory experiments, I found that nutrient availability was a stronger driver than temperature on ecosystem processes like algal accrual and aquatic insect N excretion. Additionally, algae were primarily co-limited by N and P, but algal growth responded more strongly to N than to P additions in the watershed. A pilot experiment for Chapter 2 showed strong inhibition of algal growth by P, but this effect was reduced when I lowered P concentrations on the experimental substrates.

Building upon findings from Chapters 1 and 2, I investigated why P additions often inhibit algal growth in NDS experiments. In Chapter 3, I used both meta-analysis models and field experiments to test several potential causes of P-inhibition including direct P toxicity, cation toxicity from the P salt, shifts in pH from the P salt, $H_2O_2$ production in the NDS agar, heterotrophic microbial suppression of algae with P additions, and selective grazing of P-rich algae. I found significant inhibition of algal growth in 12.9% of published P experiments as compared to 4.7% and 3.6% of N and NP experiments. My field experiments showed significantly lower gross primary productivity (GPP) and biomass-specific GPP of benthic algae on monobasic phosphate salts as compared to dibasic phosphate salts, likely because of reduced pH levels. A review of past field experiments and meta-analyses supported the plausibility of direct P toxicity or phosphate form (monobasic vs. dibasic) leading to inhibition of algal growth, particularly when other resources such as N or light are limiting. Given that multiple mechanisms may be acting simultaneously, this chapter recommends practical, cost-effective steps to minimize the potential for P-inhibition of algal growth as an artifact of NDS experimental design.

Finally, although the focus of previous chapters was primarily on abiotic drivers of algal community structure and function, in Chapter 4 I focused on how herbivory interacts with nutrients to structure algal communities. Additionally, I incorporated a seasonal component by completing three separate experiments from early August (summer) to early October (fall) in the South Fork Poudre River, CO. I used an underwater electrical fence to exclude herbivorous insects, and I also deployed NDS within the electrified and control plots. I measured algal biomass, total organic matter, and autotrophy, and I used UPLC-UV-MS pigment analysis to quantify the percent diatoms, green algae, and cyanobacteria in the algal communities. I found that herbivorous insects depleted total organic matter and decreased the proportion of diatoms in the August experiment, but herbivores did not significantly influence algae in the second and third experiments, presumably because many of them had emerged into their terrestrial life stages. Algae were primarily limited by P availability, but the magnitude of nutrient limitation decreased over time as current velocity decreased and temperatures cooled. These results suggest that investigators should proceed with caution when extending findings based on short-term experiments, and they support the need for additional seasonal-scale field research in stream ecology.

In summary, I used laboratory, field, and meta-analysis techniques to show how factors including temperature, herbivory, and streamflow interact with nutrients to influence algal biomass production in stream ecosystems. I also provided recommendations for improved, consistent methodologies for measuring algal nutrient limitation. These studies have led to a better understanding of variation in site-specific algal responses to resources under a range of natural field conditions in small montane streams, as well as the appropriate scope of inference for such studies.

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DEDICATION

*In memory of my amazing father, David Scott Beck (1960-2017), in hopes that I will one day save the Chesapeake Bay.*

TABLE OF CONTENTS

ABSTRACT ..... ii
ACKNOWLEDGEMENTS ..... vi
DEDICATION ..... vii
Chapter 1 – Influence of experimental, environmental, and geographic factors on nutrient-diffusing substrate experiments in running waters ..... 1
Summary ..... 1
Introduction ..... 2
Methods ..... 6
Database search ..... 6
Criteria ..... 6
Data extraction ..... 7
Database composition ..... 9
Effect size and variance metrics ..... 9
Statistical models ..... 10
Results ..... 12
Experimental approach ..... 12
Environmental variables ..... 14
Geographic factors ..... 14
Discussion ..... 16
Overall effect sizes ..... 16
Effects of experimental approach on nutrient limitation ..... 17
NDS and nutrient inhibition ..... 17
NDS substrate type ..... 20
Drivers of spatiotemporal patterns in nutrient limitation ..... 22
Conclusion ..... 26
8 Figures ..... 28
Chapter 2 – Multiple gradients reveal that resource availability is a stronger driver than temperature on mountain stream ecosystem processes ..... 36
Summary ..... 36
Introduction ..... 37
Methods ..... 41
Study sites ..... 41
Nutrient limitation ..... 41
Algal accrual ..... 42
Invertebrate excretion ..... 43
Geographic and environmental variables ..... 43
Statistical analyses ..... 44
Results ..... 45
Patterns of algal responses ..... 45
Drivers of algal responses ..... 46
Drivers of invertebrate excretion ..... 47
Discussion ..... 49

Nutrient limitation across all experiments ....49
Multiple gradients with same directional effects ....50
Multiple gradients with opposing directional effects....51
Algal P-limitation....53
Conclusion ....54
6 Figures....55
1 Table ....61
Chapter 3 – Confounding factors in algal phosphorus limitation experiments ....63
Summary ....63
Introduction....64
Methods....67
Quantitative review ....67
Experiments ....69
Statistical analyses for experiments ....73
Results....75
Quantitative review ....75
Experiments ....76
Discussion ....77
P toxicity ....77
Cation and phosphate form ....80
Heating method....82
Microbial competition....82
Grazer selection ....83
Additional considerations ....84
Conclusion ....85
7 Figures....87
Chapter 4 – Seasonal shifts in the importance of bottom-up and top-down factors on stream periphyton community structure ....93
Summary ....93
Introduction....94
Methods....98
Study site and design ....98
Nutrient addition treatments ....98
Electrical exclusion of grazers ....99
Environmental variables ....100
Response variables and analysis ....101
Statistical analyses ....104
Results....104
Discussion ....106
Conclusion ....111
5 Figures....112
1 Table ....117
REFERENCES ....118
APPENDICES ....129
Appendix 1....129
Appendix 2....129

Appendix 3 ..... 134
6 Figures ..... 134
19 Tables ..... 140

# CHAPTER 1: INFLUENCE OF EXPERIMENTAL, ENVIRONMENTAL, AND GEOGRAPHIC FACTORS ON NUTRIENT-DIFFUSING SUBSTRATE EXPERIMENTS IN RUNNING WATERS[1]


## Summary

Freshwater algal growth is often limited by the availability of nitrogen (N), phosphorus (P), or both nutrients (NP). For over thirty years, investigators have conducted nutrient diffusing substrate (NDS) experiments to quantify algal nutrient limitation or co-limitation in rivers and streams. Previous meta-analyses of NDS have shown that algae are commonly co-limited by N and P and that water column nutrients are weakly predictive of limitation. These analyses have not, however, comprehensively addressed the experimental, environmental, and geographic covariates affecting nutrient limitation results. We surveyed the literature and extracted data for algal biomass effect sizes and a suite of covariates across a total of 649 experiments. We built meta-regression models to identify important controls on NDS results and to gain insights about algal nutrient limitation patterns over space and time. We also reviewed potential mechanisms for the reported result that NDS N and P treatments can inhibit algal growth. Experimental variables including substrate type, chemical concentration, and experimental length significantly affected P and NP effect sizes, while NDS chemical compound influenced N, P, and NP effect sizes. We also found that environmental variables such as in-stream nutrients and riparian canopy cover significantly affected limitation by N, P, and NP. Temperature, stream discharge,


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[1]This chapter is an edited version of: Beck, W. S., Rugenski, A. T., & Poff, N. L. (2017). Influence of experimental, environmental, and geographic factors on nutrient-diffusing substrate experiments in running waters. *Freshwater Biology*, *62*(10), 1667–1680. https://doi.org/10.1111/fwb.12989

and stream velocity only affected limitation by NP. Land use, ecoregion, and season showed clear trends in nutrient limitation for all treatments that could generally be tied to environmental factors like in-stream nutrients and riparian canopy cover. Most experimental and environmental variables that were statistically significant in the meta-regression models produced very low $R^2$ index values, indicating that the models explained little variation in among-site effect sizes. Spatial factors including stream order, ecoregion, and climate classification had the highest $R^2$ index values, but these models still produced a large amount of unexplained variance. In light of these findings, we provide recommendations for improving NDS experimental design and pursuing future research avenues using NDS. We also highlight the need for future experiments to consider algal stressors that may interact with nutrient limitation experiments.

## Introduction

Benthic algae serve many critical ecological functions in freshwater stream ecosystems, including primary production, nutrient cycling of both nitrogen (N) and phosphorus (P), and food and habitat provision for macroinvertebrates and fish (Stevenson 1996). Over the past several decades, N and P concentrations have increased in freshwater systems as a result of human sources such as wastewater treatment plant discharges, agricultural fertilizer runoff, urban stormwater runoff, and atmospheric deposition (Carpenter et al. 1998). Given concerns about stream eutrophication, it is important to understand how algal communities respond to nutrient additions and how a variety of environmental factors can mediate algal responses to increased nutrient loading.

For over 30 years, investigators have used *in situ* nutrient diffusing substrates (NDS) to quantify algal nutrient limitation in freshwater streams and rivers (Fairchild et al. 1985). NDS involve filling replicate vessels (e.g., clay pots, plastic vials) with a medium (agar or water),

which slowly releases nutrient salts to create locally-enriched growth surfaces (Tank et al. 2006). These have become a standard method of assessing nutrient limitation in streams and rivers because they have the advantage of being small, replicable, and relatively low maintenance. After an in-stream deployment period, control and treatment NDS are compared to quantify the effects of nutrient additions on algal growth and accrual. Structural response variables in NDS studies typically include chlorophyll *a* or ash-free dry mass (AFDM), although some experiments have also measured algal species composition (e.g., Biggs et al. 1998), algal biovolume or cell density (e.g., Wellnitz et al. 1996), and fungal biomass (Tank & Dodds 2003). Functional response variables are measured much more infrequently, but NDS studies have addressed the influence of nutrient inputs on gross primary production (GPP; Reisinger et al. 2016), respiration (Hoellein et al. 2010), photosynthetic efficiency (Whorley & Francoeur 2013), and N-fixation (Marcarelli & Wurtsbaugh 2006).

A previous meta-analysis by Keck and Lepori (2012) reported that in-stream dissolved inorganic nitrogen (DIN) and total phosphorus (TP) concentrations were significantly correlated with decreased algal responses to N and P treatments, respectively. However, the statistical models predicting algal responses from in-stream chemistry contained a large amount of unexplained variance, highlighting the potential importance of other factors in regulating algal production and biomass accrual. Another study (Wold & Hershey 1999) found that in-stream $NO_3^-$ and soluble reactive phosphorus (SRP) were poor predictors of nutrient limitation.

Many environmental factors can modify algal responses to nutrient additions by either increasing algal accrual (e.g., light, temperature) or decreasing it (e.g., herbivore grazing, scouring flows). For example, one study found that as light became less limiting, secondary N-limitation became apparent (Taulbee et al. 2005). For environmental factors to affect NDS

results, they must either interact with nutrients in some way or significantly reduce algal biomass across all treatments.

NDS results are also influenced by the methodological approach used to construct and deploy the substrates. For example, investigators may employ clay pots, plastic vials, or periphytometers as the substrate type (Capps et al. 2011), apply different nutrient chemicals (e.g., $KH_2PO_4$ vs. $NaH_2PO_4$), utilize nutrient concentrations which yield different N:P molar ratios (Capps et al. 2011), and deploy NDS substrates for differing periods of time, from less than two weeks (Scrimgeour & Kendall 2002) to greater than two months (Gustina & Hoffmann 2000). Capps et al. (2011) demonstrated that within the same system and over the same time period, the NDS substrate type significantly affected algal nutrient limitation patterns. Moreover, they found potential interactive effects on limitation between the N:P molar ratio of the nutrient addition and the substrate type (Capps et al. 2011).

Limitation of algal growth by N, P, or both nutrients can vary over time within a given system due to shifts in stream physical, chemical, and biological conditions. For instance, a study in New Zealand showed that nutrient limitation was most common in summer and least common in winter. Statistical models showed that these results were due to temperature changes (Francoeur et al. 1999). Wold and Hershey (1999) completed repeated experiments in Michigan streams and showed that within two weeks, a given stream could shift between limitation by N, P, both nutrients, or neither nutrient.

Nutrient limitation clearly varies over space based on regional factors like land use, climate, and nutrient loading. Reisinger et al. (2016) showed regional differences in the nutrient limitation of 15 U.S. streams (spanning the U.S. Midwest, Mountain West, and Arid West) that were associated with in-stream nutrient concentrations and land use. Tank and Dodds (2003)

investigated nutrient limitation of autotrophic and heterotrophic biofilms across eight different biomes, finding that nutrient limitation was also associated with in-stream nutrient concentrations. Furthermore, they showed that autotrophic nutrient limitation was linked to photosynthetically active radiation (PAR; Tank & Dodds 2003).

These highlighted studies reveal that a number of mechanisms may control algal accrual on NDS and that there are context-dependent stream and study characteristics that challenge the replication of results from experiments. Thus, it is not surprising that after 30 years of investigation, no clear consensus has arisen as to how multiple factors (experimental, environmental, geographic) interact to influence algal responses to nutrients from NDS. To address this shortcoming, meta-analyses can be used as they are powerful ecological tools that involve aggregating effect sizes along with associated variances from multiple experiments (Koricheva et al. 2013). Additionally, these analyses can include covariates that may vary across sites, allowing for better insight into their general contributions to algal response variation across systems. Previous meta-analyses have investigated NDS results (Francoeur 2001; Elser et al. 2007; Keck & Lepori 2012); however, none of them included a comprehensive suite of covariates to help inform future experimental design and inference. Such an analysis could ultimately assist in management of benthic algal production and biomass in streams and rivers.

In this review, we use meta-analysis techniques to investigate how experimental approach (e.g., substrate construction, experimental length), environmental conditions (e.g., in-stream nutrients, canopy cover, discharge), and geographic variation (e.g., land use, ecoregion) affect the results of NDS experiments. We also examine the reported result that NDS nutrient treatments can inhibit algal growth (e.g., Sanderson et al. 2009; Bernhardt & Likens 2004) in light of hypothesized mechanisms such as nutrient toxicity (Fairchild et al. 1985) or $H_2O_2$

production when agar and $PO_4^{3-}$ are boiled together in the laboratory (Tanaka et al. 2014). We have four primary objectives: 1) estimate overall effect sizes for N, P, and NP; 2) calculate reporting rates for potentially-important covariates; 3) develop and analyze univariable and multivariable meta-analysis models; 4) provide recommendations for future experiments and novel research avenues.

## Methods

### *Database Search*

We searched for NDS studies using the online databases Web of Science, Science Direct, and Wiley Online Library. The keyword combinations for database searches are reported in Appendix 1 (Supporting Information). Studies were defined as journal articles, dissertations, and reports from the time period January 1, 1986 through December 31, 2015. From the search results, we reviewed titles and abstracts from over 3,000 studies. In addition, we applied the Google Scholar "related searches" tool to a subset of references, and we reviewed citations from previous nutrient limitation meta-analyses.

### *Criteria*

Studies were required to meet several criteria before being used in our meta-analysis. First, they had to include *in situ* NDS experiments in freshwater, lotic systems. Studies were excluded if they contained "unnatural" manipulations to the stream environment, such as complete grazer exclusions (e.g. Lourenco-Amorim et al. 2014). We wanted to directly link environmental variability with effect sizes in this meta-analysis, hence studies with pooled results from multiple streams (e.g. Bechtold et al. 2012) were not considered. We only included studies that measured chlorophyll *a* (biomass per unit area) as the response variable, because this

metric is representative of the algal fraction of periphyton, and it was commonly recorded in NDS studies. Furthermore, we only included studies that reported replicated NDS controls and at least one replicated nutrient treatment (N, P, or NP) that were deployed in natural flowing water for a discrete experimental period.

For the analysis, if NDS were deployed under unique experimental or environmental conditions within the same stream, they were considered to be separate experiments. For instance, some studies tested the effects of different nutrient ratios (e.g., Capps et al. 2011) or light levels (e.g., Elsaholi et al. 2011) within the same stream and the results from these manipulations were considered as independent units. Lack of spatial independence of these experiments was then corrected for in the meta-analysis models (see methods below). If multiple experiments were conducted over time at the same site (e.g., Wold & Hershey 1999), we recorded only one experiment per 30 days to ensure experiments did not overlap.

***Data Extraction***

For controls and treatments in each experiment, we recorded chlorophyll *a* biomass ($ug \cdot cm^{-2}$) mean and variance, and the number of replicates. Some experiments included multiple sampling time points, and in these cases, only the final chlorophyll *a* biomass was recorded. Response variables were extracted from images when necessary, using Web Plot Digitizer version 3.8 (Rohatgi 2015).

Prior to reviewing the studies, we identified a wide variety of experimental, environmental, and geographic factors which could potentially influence effect sizes across experiments (Table S1.1). We recorded values for these factors and determined which ones were commonly reported so that we could use them as predictor variables in meta-analysis models.

Environmental factors were only recorded when they were relevant to the spatial and temporal scale of the NDS experiments. For instance, average channel water depth did not qualify as experimental depth, and watershed forest cover did not qualify as riparian canopy cover. Annually-averaged parameters (e.g., in-stream nutrients, discharge) were not considered to be representative of the experimental period. We computed averages if multiple values were recorded for an environmental variable within the same experimental period. In some cases when environmental data were plotted, the first value from the associated experimental month was extracted using Web Plot Digitizer version 3.8 (Rohatgi 2015). For canopy cover, we recorded categorical and continuous values separately to capture all available information. We recorded the primary watershed land use category stated by the authors. We determined season by comparing solstice and equinox dates with reported experimental dates. If experiments overlapped two different seasons, we recorded the season in which the majority of the experiment took place. All data were assumed to be reliable as reported.

We completed two separate spatial analyses to understand how nutrient effect sizes might change based on ecoregion (North America) and climate classification (global). Ecoregions are areas of similar temperature, precipitation, and vegetation growth potential, and these factors influence background nutrient dynamics and hydrology (Omernik 1987). However, classifications are only available for North America. Köppen-Geiger climate regions are derived from temperature and precipitation regimes (Peel et al. 2007), and classifications are available on a global scale. Layers for North American Level 1 Ecoregions (Fig. 1.1, Commission for Environmental Cooperation 2009) and updated Köppen-Geiger climate classifications (Peel et al. 2007) were downloaded and visualized using Google Earth. We plotted experimental sites using

reported coordinates as well as study site maps and stream names. This allowed us to associate most sites with an ecoregion and/or climate classification.

***Database Composition***

The final database included 649 experiments from 67 studies (Appendix 2 and Fig. S1.1). Of these experiments, 553 recorded effects of N, 534 recorded effects of P, 591 recorded effects of NP, and 487 recorded effects of all three nutrient treatments. Experimental factors were commonly reported, but certain environmental and geographic factors (e.g., pH and stream slope) were not. If factors were reported in more than 25% of experiments, they were used to develop meta-analysis models. We calculated Pearson's correlation coefficients for commonly-reported continuous environmental variables, using pairwise deletion of missing values.

***Effect Size and Variance Metrics***

Meta-analysis models require a measure of effect size and associated variance (Koricheva et al. 2013). We used the log response ratio (LRR, Equation 1) as our effect size because of its demonstrated utility in ecological studies (Hedges et al. 1999). LRRs are easily interpretable and have been used in previous meta-analyses on nutrient limitation (Francoeur 2001; Elser et al. 2007; Keck & Lepori 2012). LRRs were separately calculated in the following way for nitrogen (N-LRR), phosphorus (P-LRR), and nitrogen + phosphorus (NP-LRR) treatments:

$$LRR = \ln (Y_1 / Y_2) = \ln (Y_1) - \ln (Y_2) \quad \text{(Eqn 1.1)}$$

Where $Y_1$ is the mean chlorophyll *a* biomass from the treatment replicates and $Y_2$ is the mean chlorophyll *a* biomass from the control replicates in a given experiment (Koricheva et al. 2013). We also calculated the variance of each LRR using the following equation:

$$LRR\_Var = s_1^2/(n_1Y_1^2) + s_2^2/(n_2Y_2^2) \quad \text{(Eqn 1.2)}$$

Where $s_1$ and $s_2$ are the standard deviations of the treatment and control replicates, and $n_1$ and $n_2$ are the number of treatment and control replicates (Koricheva et al. 2013).

***Statistical Models***

We built meta-analysis models using the metafor package (Viechtbauer 2010) in R version 3.2.2 (R Core Team 2015). Meta-analysis models incorporate experimental effect sizes and variances, and thus can account for variability within and among experiments (Viechtbauer 2010). We chose to use linear mixed models (LMMs) with "site" as a random effect, to account for the correlated effects of experiments within the same stream reach (Gelman & Hill 2006). LMMs were used to estimate the total effect size of each nutrient, and we also built meta-regression models to determine the effect of experimental, environmental, and geographic predictor variables. Predictors were transformed as necessary to meet LMM assumptions. The "rma.mv" function was used for all models.

Each individual model was created using a different subset of the database, depending on the research question and predictor(s) of interest. As a result, models cannot be compared to one another, and inference can only be made for factors within the same model. For models with multiple predictors, we centered the values to put all coefficients on a scale commensurate with the predictor means and standard deviations (Gelman & Hill 2006).

We developed models for commonly reported variables (i.e., > 25% of experiments, Table S1.1). Models with NDS chemical concentration as a predictor did not include experiments that used periphytometers, because aqueous and agar diffusion rates are likely to be

different. Combining multiple predictors reduced the sample size we could consider; therefore, we constructed most models using a single predictor variable.

Investigators use different chemical compounds when constructing NDS. To determine the effect of P substrate chemicals on P-LRR and NP-LRR, we developed univariable models that compared commonly-used compounds ($KH_2PO_4$, $K_2HPO_4$, $NaH_2PO_4$, and $Na_2HPO_4$ ). We then categorized the phosphates into compounds with sodium ($NaH_2PO_4$ and $Na_2HPO_4$) versus potassium ($KH_2PO_4$ and $K_2HPO_4$) cations, and compounds with one hydrogen atom ($K_2HPO_4$ and $Na_2HPO_4$) versus two hydrogen atoms ($KH_2PO_4$ and $NaH_2PO_4$). We used the cation and hydrogen designations as categorical predictors for multivariable models. For N substrate chemicals, we developed univariable models to determine the influence of commonly-used compounds on N-LRR and NP-LRR ($NaNO_3$, $KNO_3$, $NH_4Cl$, and $NH_4NO_3$).

For each model, we evaluated the sign and magnitude of the coefficients, and whether the predictors explained significant variation in the observed effect sizes. For individual estimates, we determined significance using 95% confidence intervals (CIs) that did not overlap with zero. For categorical factors and multivariable models, we determined significance using an omnibus test of parameters. This method uses a test statistic ($Q_M$) and chi-square distribution to test the null hypothesis: $B_1 = B_2 = 0$, where $B$s are model coefficients in the meta-regression models (Viechtbauer 2010). If the null hypothesis is rejected, we can conclude there is a significant effect of the factor(s) being tested.

We calculated an $R^2$ index for each meta-regression model based on the equation from Borenstein et al. (2009):

$$R^2 \text{ index} = \tau^2_{explained} / \tau^2_{total} = 1 - (\tau^2_{unexplained} / \tau^2_{total}) \quad \text{(Eqn 1.3)}$$

where the $\tau^2$ parameter is estimated as part of the LMM procedure, and represents the true variance between studies in the meta-analysis. We used an intercept-only random effects model to calculate the total variance between sites ($\tau^2_{total}$), and meta-regression models with moderators to calculate the unexplained variation between sites ($\tau^2_{unexplained}$). Most index values are expected to range between 0 and 1, but we set any negative values equal to 0 (Borenstein et al. 2009). A high $R^2$ index indicates the moderator explained additional variation between streams, and a low $R^2$ index indicates that the moderator did not explain much more variability than the random effects model alone.

We used models with intercepts to determine model parameters, standard errors, and 95% confidence intervals. Statistical significance was also determined using models with intercepts. We used no-intercept model estimates to graphically represent the meta-analysis model effect sizes and 95% confidence intervals.

**Results**

All nutrient effect sizes were positive and significantly different from zero (Fig. 1.2, Table S1.2). NP had the highest estimate (0.82), followed by N (0.35) and P (0.24).

Many continuous covariates were significantly correlated with other covariates (Table S1.3). Pairs with high correlation coefficients ($r > 0.5$) included stream order and quantitative canopy cover ($r = -0.654$), $NH_4^+$ and TN ($r = -0.622$), $NO_3^-$ and DIN ($r = 0.994$), DIN and TP ($r = 0.886$), TN and DIN ($r = 0.965$), TN and TP ($r = 0.712$), and SRP and TP ($r = 0.989$).

***Experimental Approach***

Substrate had different effects depending on the LRR being considered. Clay pots had the highest N-LRR estimate, followed by vials and periphytometers (Table S1.4, Fig. 1.3), but

results showed no overall effect of substrate on N-limitation ($Q_M$ = 5.31 and p = 0.07). Clay pots had the highest P-LRR estimate, followed by periphytometers and vials (Table S1.4, Fig. 1.3). Periphytometers had the highest NP-LRR estimate, followed by clay pots and vials (Table S1.4, Fig. 1.3). For the P and NP models, substrate type significantly affected nutrient limitation responses (P: $Q_M$ = 36.01 and p < 0.0001; NP: $Q_M$ = 20.47 and p < 0.0001).

$PO_4^{3-}$ compound significantly affected LRRs for both P ($Q_M$ = 16.09 and p = 0.001) and NP ($Q_M$ = 36.14 and p < 0.0001) models. Compounds with potassium ($KH_2PO_4$ and $K_2HPO_4$) produced higher P-LRR and NP-LRR estimates than did $PO_4^{3-}$ compounds with sodium ($NaH_2PO_4$ and $Na_2HPO_4$; Table S1.4, Fig. 1.4). $PO_4^{3-}$ compounds with one hydrogen atom ($K_2HPO_4$ and $Na_2HPO_4$) produced higher P-LRR and NP-LRR estimates than compounds with two hydrogen atoms ($KH_2PO_4$ and $NaH_2PO_4$; Table S1.4, Fig. 1.4). While there were significant main effects of cation and hydrogen in the multivariable models, there was no significant interaction between the two factors (Table S1.4).

Nitrogen compound significantly affected LRRs for both N ($Q_M$ = 201.03 and p < 0.001) and NP ($Q_M$ = 9.07 and p = 0.028). $NH_4NO_3$ had the highest estimates for both LRRs (Fig. 1.5).

The effect of NDS initial concentration and deployment length was not significant for the N models but was significant for the P and NP models (Table S1.4). P concentration and number of days led to decreases in P-LRR and NP-LRR, while N concentration also led to decreases in NP-LRR.

Meta-regression models considering experimental approach generally produced low $R^2$ index values within the range of 0 to 2%. However, P chemical compound explained 4% of the between-stream variability in P- and NP-LRRs, while substrate type explained over 12% of the between-stream variability in P-LRR.

### *Environmental Variables*

Environmental variables significantly affecting all three nutrient responses included quantitative canopy cover, SRP, and season (Tables S1.5-S1.7, Fig. 1.6). Quantitative canopy cover was associated with increases in N-LRR, but decreases in P-LRR and NP-LRR. SRP was associated with decreases in all nutrient responses. N-LRR and NP-LRR exhibited the same seasonal trends whereby fall had the highest estimates, followed by summer, spring, and winter (N: $Q_M = 12.3023$ and $p = 0.0064$; P: $Q_M = 162.7482$ and $p < 0.0001$). P-LRR had the highest estimate during summer, followed by fall, spring, and winter ($Q_M = 97.8791$ and $p < 0.0001$).

Other environmental variables significantly affecting N-LRR included $NO_3^-$ and N:P molar ratio (Table S1.5), which were both associated with decreases in N-limitation. Additional environmental variables significantly affecting NP-LRR included $NH_4^+$, qualitative canopy cover, discharge, $NO_3^-$, temperature, and velocity (Table S1.7). Discharge, $NH_4^+$, $NO_3^-$, and velocity were all associated with decreases in NP-LRR, while temperature was associated with increases in NP-LRR. Open canopy cover had a higher estimate than closed canopy cover ($Q_M = 233.9916$ and $p < 0.0001$).

The $R^2$ indices were also low for the environmental variable models, generally in the range of 0 to 2% (Tables S1.5-S1.7). However, in-stream $NO_3^-$ explained nearly 8% of the variability in among-site N-LRR and 11% of the variability in NP-LRR. In-stream SRP and quantitative canopy cover explained 9% and 4% of the variability in among-site NP-LRR.

### *Geographic Factors*

Land use was significantly related to all three nutrient responses (Fig. 1.7, Table S1.8). Pasture had the highest N-LRR estimate, followed by grassland, forest, urban, and agriculture

($Q_M$ = 13.0620 and p = 0.0110). Agriculture had the highest P-LRR estimate, followed by forest, grassland, pasture, and urban ($Q_M$ = 15.0838 and p = 0.0045). Grassland had the highest NP-LRR estimate, followed by forest, pasture, agriculture, and urban ($Q_M$ = 17.0689 and p = 0.0019). The land use $R^2$ index explained the highest among-stream variability in NP-LRR (12%), followed by P-LRR (10%) and N-LRR (9%). In the P models, stream order was also significantly related to P-LRR, whereby higher stream orders were associated with increases in P-LRR (Table S1.8). The stream order $R^2$ index was 5% for N-LRR and 15% for P-LRR but 0% for NP-LRR.

Response ratios differed by North American ecoregion (Table S1.9, Fig. 1.8), and we found a significant effect of ecoregion in all models (N: $Q_M$ = 22.0441 and p = 0.0025; P: $Q_M$ = 27.9145 and p = 0.0002; NP: $Q_M$ = 49.1990 and p < 0.0001). The Marine Western Forest and Temperate Sierra ecoregions had significantly positive N-LRR estimates. The Marine Western Forests, Northern Forests, and Northwest Forested Mountains had significantly positive P-LRR estimates. Finally, the Eastern Temperate Forests, Great Plains, Northern Forests, Northwest Forested Mountains, and Tundra had significantly positive NP-LRR estimates. The ecoregion $R^2$ index was 8% for N-LRR and P-LRR, and 16% for NP-LRR.

No individual Köppen-Geiger climate classification was significantly related to N- or P-LRR (Table S1.10), but we did find an overall effect of climate classification in those models (N: $Q_M$ = 21.5962 and p = 0.0173; P: $Q_M$ = 23.7271 and p = 0.0048). The Cfa, Dfc, and Dsb classifications were all significantly related to NP-LRR (Table S1.10), and we found a significant effect of climate classification on NP-limitation ($Q_M$ = 59.3328 and p < 0.0001). Climate classification explained 4%, 6%, and 18% of the among-stream variability in N-LRR, P-LRR, and NP-LRR respectively.

## Discussion

By including experimental, environmental, and geographic covariates, our meta-analysis has produced new insights into what drives nutrient limitation in running waters over space and time. Our findings show that algal production on NDS P treatments depends on the experimental approach, including the substrate type, nutrient concentrations, $PO_4^{3-}$ compound chemical composition, and experimental duration. In fact, NDS substrate type explained more variability in P-LRR than any other experimental or environmental variable. This suggests a need to standardize NDS approaches in future experiments, which we discuss below. We also found that spatio-temporal factors (land use, ecoregion, and season) significantly influenced NDS response ratios, with spatial factors having the highest power to explain variation in LRRs. Environmental variables that promote algal growth rates have been well-reported and studied, and our results reaffirm the importance of in-stream nutrients but also identify other factors that may be influencing nutrient limitation. We found that variables that decrease algal growth and accumulation (e.g. turbidity, grazing) are under-studied, but NDS may be a useful method to understand how these factors interact with nutrients to regulate algae.

### *Overall Effect Sizes*

Our study supports previous meta-analyses of nutrient amendment experiments that demonstrated high effect sizes of NP treatments in lotic ecosystems (Francoeur 2001; Elser et al. 2007). Furthermore, in agreement with Allgeier et al. (2010), we found synergistic, non-additive effects of NP additions (i.e., the NP-LRR estimate was higher than the sum of the N-LRR and P-LRR estimates). We found a higher N than P effect size, which is in contrast to the slightly higher P effect size reported by Elser et al. (2007) in freshwater streams. However, we restricted

our analysis to NDS experiments, while Elser et al. (2007) included a broader class of nutrient enrichment experiments. Our results show that in many eutrophic systems, reductions of both N and P are likely required to decrease algal biomass and meet management goals.

### *Effects of Experimental Approach on Nutrient Limitation*

#### NDS and Nutrient Inhibition

We tested the effect of multiple experimental factors on NDS study outcomes, with a particular focus on why some studies have found an inhibitory effect of P additions on algal biomass as compared to control treatments (e.g., Sanderson et al. 2009; Bernhardt & Likens 2004). These inhibitory effects introduce questions about whether researchers are actually using appropriate experimental methods to test for P-limitation. Unexpectedly, NDS studies have reported P-inhibition by P treatments but rarely by NP treatments (but see Reisinger et al. 2016), despite the fact that the two treatments generally include the same P concentrations and chemicals. Although we found that P-inhibition was more common than NP-inhibition, we identified a set of experimental factors that led to significant declines in both P-LRR and NP-LRR.

We found that increased NDS P concentrations and longer experimental periods led to decreased P-LRR and NP-LRR (Table S1.4). The experimental lengths ranged from 11-67 days, but depending on how they are constructed, NDS may diffuse nutrients for only 18-20 days (Tank et al. 2006). Higher NDS concentrations lead to more sustained diffusion (Rugenski et al. 2008), but we advise against deploying experiments for more than three weeks unless diffusion rates have been measured. Additionally, benthic algae colonizing bare substrates have been shown to exhibit an accrual phase followed by a loss phase due to autogenic sloughing, grazing,

or the drag of current velocity (Biggs 1996). Researchers are generally most interested in measuring chlorophyll *a* around the time of peak algal biomass, but lengthy experiments might lead to chlorophyll *a* measurements during a biomass loss phase. Algal accrual observations on tiles or scrubbed rocks could be employed to achieve a stream-specific estimate of when peak biomass occurs.

Higher P concentrations may have had a negative effect on P and NP treatment responses by creating an unfavorable N:P molar ratio for algal growth. Many studies use the Redfield ratio (N:P = 16:1; Redfield 1934) to relate stream water chemistry to algal growth, and P additions could induce a ratio below 16:1 in certain streams. However, an unfavorable N:P ratio would not necessarily inhibit algal biomass on P treatments relative to controls. Inhibition could result from direct effects of P toxicity, but this phenomenon has rarely been reported in the literature. P toxicity has sometimes been inferred in NDS experiments when algal biomass on controls exceeds algal biomass on P treatments (Fairchild et al. 1985), but alternative hypotheses should also be considered.

We found higher algal biomass stimulation when experiments used potassium phosphates ($KH_2PO_4$ and $K_2HPO_4$) as compared to sodium phosphates ($NaH_2PO_4$ and $Na_2HPO_4$). Possible explanatory mechanisms could include algal potassium-limitation or sodium-inhibition (Sudhir & Murthy 2004). On the other hand, we found similar effect sizes when experiments used $NaNO_3$ and $KNO_3$, showing that the cation was not important for N-LRR and NP-LRR. This could have been because of the small sample size for $KNO_3$ (n = 33 for N-LRR and n = 28 for NP-LRR), but could also indicate that the cations differentially interact with nitrate and phosphate uptake or assimilation.

The number of hydrogen atoms in the $PO_4^{3-}$ compound may have had an effect on P-LRR and NP-LRR by changing the pH conditions of the growth surface. $KH_2PO_4$ and $NaH_2PO_4$ can raise the pH of the surrounding water, while $K_2HPO_4$ and $Na_2HPO_4$ can lower the pH of the surrounding water (W. Beck, unpublished data). In our analysis, chemicals that lower pH had significantly higher P- and NP-LRRs. Stream pH can affect algal biomass and community composition (Planas 1996) and may be a mechanism by which P chemicals influence LRRs. However, additional field and laboratory experiments are required to explore this mechanism.

Two other ideas about P-inhibition could not be tested using the dataset compiled here. First, Tanaka et al. (2014) reported that autoclaving $PO_4^{3-}$ with agar produces $H_2O_2$ that inhibits microbial growth, and it is plausible that $H_2O_2$ could inhibit algal growth as well. However, it is unclear whether autoclaving is necessary to produce $H_2O_2$, or whether simply boiling the agar solution and $PO_4^{3-}$ can yield the same results. In any case, studies do not commonly report these laboratory methods. In the *Methods in Stream Ecology* textbook, Tank et al. (2006) recommend mixing $PO_4^{3-}$ and agar during the heating process, leading us to speculate that most experiments have used that protocol. If harmful levels of $H_2O_2$ are produced by this method, we might expect P-inhibition of algae to be more common than is currently reported (Reisinger et al. 2016).

Second, it has been proposed that heterotrophic bacteria and fungi could utilize P additions and outcompete algae in epilithic biofilms (Bernhardt & Likens 2004). However, few studies provide enough data to adequately test this idea. Most studies only report broad structural responses to nutrient additions including chlorophyll *a* biomass and (less commonly) AFDM. Bechtold et al. (2012) measured both chlorophyll *a* and AFDM in NDS experiments, finding apparent competitive suppression of chlorophyll *a* when dissolved organic carbon was added as an NDS treatment. It is possible that an analysis of studies reporting both chlorophyll *a* and

AFDM would show the same type of competitive suppression with P additions, but such an analysis was beyond the scope of this study. A few studies have tested functional responses to nutrient additions such as GPP and ER (e.g., Reisinger et al. 2016; Marcarelli et al. 2009) and this approach may provide information about the relative importance of autotrophic versus heterotrophic growth in NDS experiments with different types of $PO_4^{3-}$.

Finally, we did find a significant effect of N chemical compound, but the compound type did not consistently influence N-LRR and NP-LRR. Different algal species may preferentially take up $NO_3^-$ or $NH_4^+$ (Dortch 1990), and we did see that adding both chemicals together elicited the greatest N-LRR and NP-LRR. However, we found that $NH_4Cl$ had the lowest N-LRR while $KNO_3$ had the lowest NP-LRR. Background water chemistry and algal community composition may influence how algae respond when N is added in these different forms.

**NDS Substrate Type**

Our substrate model results contrast a previous experiment that tested the effect of substrate on nutrient limitation. In our analysis, clay pots had the highest reported N-LRR and P-LRR, whereas Capps et al. (2011) found plastic vials to have the highest N-LRR and periphytometers the highest P-LRR. For NP-LRR, however, our findings agree with Capps et al. (2011) that periphytometers have the highest estimate. The difference in these results could be driven by the Capps et al. (2011) experiment being completed in one stream during one season, emphasizing the importance of considering spatial and temporal context when interpreting NDS experimental outcomes.

NDS substrate may affect experimental results via chemical interactions, and if this is the case, in-stream solutes would likely make these interactions very stream-dependent. Brown et al. (2001) concluded that clay pots do not consistently diffuse nutrients because agar clogs the

substrate pores, and pot types vary widely in pore size and diffusion rates. Furthermore, clay pots tend to bind P because of high aluminum and iron oxide content (Brown et al. 2001). In our analysis clay pots had the high coefficient of variation (0.46 to 0.55) across all treatments, which does support Brown et al.'s (2001) conclusions about clay pot porosity and chemistry producing variable results. However, our results showed that rather than completely binding the chemical additions, clay pots had the highest N-LRR and P-LRR estimates. We hypothesize that the unique chemistry of clay pots could negate any inhibitory effects of nutrient additions, such as cation or nutrient toxicity (Fairchild et al. 1985) or $H_2O_2$ toxicity (Tanaka et al. 2014).

Surface texture is another mechanism by which NDS substrate could affect algal accumulation. Rough, heterogeneous surfaces (e.g., clay pots or fritted glass discs in plastic vials) could support higher LRRs by promoting strong algal attachments that are resistant to loss by shear stress (Dudley & D'Antonio 1991). Clay pots supported the highest control, P, and NP chlorophyll *a* biomass means of all the treatments, although the control means were comparable to vials. Periphytometer surfaces generally consisted of smooth glass fiber filters, and these treatments had the lowest control and NP chlorophyll *a* means.

Because NDS substrate type had a higher $R^2$ index for P-LRR than all other experimental and environmental variables, we recommend standardizing this factor across experiments. We caution against using clay pots since they produce highly variable NDS results. We do recognize that in larger rivers clay pots may be easy to anchor and deploy (Scrimgeour & Chambers 1997), but plastic vials have been successfully deployed in large rivers (Marcarelli et al. 2009; Reisinger et al. 2016). Periphytometers are unique because they often employ smooth glass fiber-filter growth surfaces that are not necessarily representative of natural stream substrates. Additionally, they are deployed with growth surfaces perpendicular to water flow (Matlock et al. 1998).

Benefits of periphytometers include a more constant nutrient diffusion rate and the ability to completely recover chlorophyll *a* from the glass fiber filters (Matlock et al. 1998). However, for comparability across experiments and to best emulate natural stream conditions, we concur with Tank et al.'s (2006) recommendation to use vials covered with a rough substrate such as a fritted glass disc whenever possible. Chlorophyll *a* can be completely recovered from these discs when the whole disc is placed in the extraction medium, and vials are easily deployed so that the growth surface is parallel to water flow (Tank et al. 2006).

***Drivers of Spatio-Temporal Patterns in Nutrient Limitation***

Urban and agriculture landscapes, which generally have higher in-stream nutrient loadings compared to forested or otherwise undisturbed land surfaces, produced lower N- and NP-LRRs than forest, grassland, and pasture land uses. This is consistent with the environmental variable models, where stream nutrient concentrations ($NH_4^+$, $NO_3^-$, SRP) were always associated with decreases in LRRs. In a recent study across multiple streams spanning three U.S. regions, Reisinger et al. (2016) also showed a negative relationship between nutrient LRRs and watershed percent developed lands (urban and agriculture), as well as in-stream $NO_3^-$ concentrations.

Contrary to our expectations, P-LRR was highest in agricultural streams. Agriculture can have variable effects on stream N:P molar ratios, but in our dataset, agricultural streams had the highest N:P ratios of any land use ($NO_3^-$ : SRP = 155.7). It is likely that algae in these streams were P-limited, causing the algae to respond to increased P from the NDS.

In-stream SRP was associated with decreases in N-LRR, which could occur if in-stream SRP and N were positively correlated in streams. For instance, we might expect that agricultural and urban land uses produce higher loads of both N and P (Carpenter et al. 1998), leading to a

lower N effect size. In our dataset the correlation between in-stream SRP and $NO_3^-$ was weak but significant (Table S1.3). Because of the weak correlation, a more likely explanation is that SRP lowered N:P molar ratios and decreased the magnitude of N-limitation in streams.

N:P molar ratios have been shown to be good predictors of N-limitation but not P-limitation (Keck & Lepori 2012), which is consistent with our findings. However, neither this analysis nor Keck and Lepori (2012) used TN:TP molar ratios. TN and TP may be important predictors of nutrient limitation because they capture additional information about nutrient resources over the long-term, while DIN and SRP are representative of instantaneous, bioavailable nutrients. In our dataset, TP was highly correlated with SRP and DIN was highly correlated with TN, but this does not necessarily mean the variables are interchangeable. We recommend that future studies should compare the utility of DIN:SRP ratios (commonly reported) and TN:TP ratios (rarely reported) for predicting algal biomass, algal responses to NDS, and nutrient uptake rates.

Canopy cover was an important variable for predicting algal responses to all nutrient treatments, but was not clearly correlated with land use in our study. Even streams in forested watersheds were often described as having "open" riparian canopies. We expected canopy cover to be associated with decreases in all LRRs, as light becomes more limiting than N and P (e.g. Taulbee et al. 2005). However, we found that canopy cover was associated with increases in N-LRR but decreases in P-LRR and NP-LRR. In our dataset, canopy cover was significantly negatively associated with in-stream $NO_3^-$ and $NH_4^+$ but was not associated with in-stream SRP or TP (Table S1.3), which may explain the patterns of higher N-limitation under closed canopies.

While canopy cover can be a useful surrogate for available light, few studies reported turbidity and experimental depth, both of which are confounding factors that would affect light

penetration to substrate surfaces. Continuous PAR measurements would be an ideal method to compare light differences among experiments. However, at a minimum we recommend reporting quantitative canopy cover, experimental depth, and turbidity to calculate a useful estimate of light differences.

In concordance with our land use results, ecoregions with intense urbanization or agriculture (and likely higher nutrient loads) generally had lower responses to all three nutrient treatments, including Mediterranean California, Eastern Temperate Forests, and North American Deserts. Most of the Mediterranean California streams were located in urban areas (Busse et al. 2006). Similarly, Eastern Temperate Forests are characterized by high population density and dominance of urban and agricultural industries (Commission for Environmental Cooperation 1997), and the North American Desert ecoregion is characterized by large-scale irrigated agriculture (Commission for Environmental Cooperation 1997).

In contrast, ecoregions with lower human population densities and more intact forests generally had higher LRRs, including Marine West Coast Forests, Northern Forests, Northwest Forested Mountains, and the Tundra. In the case of Marine West Coast Forests and Northern Forests, soils are known to be relatively nutrient-poor, which could lead to low in-stream nutrients in relatively undisturbed streams (Commission for Environmental Cooperation 1997).

Ecoregions are described by temperature and precipitation regimes, vegetative land cover, topography, and soil nutrient status (Omernik 1987) and may explain some natural variation in algal production. However, human activities greatly modify hydrologic processes and nutrient loading characteristics and thus alter algal dynamics in complex ways. Metrics such as watershed population density or quantitative watershed land use may have helped interpret the ecoregion results, but these were rarely reported in the studies we reviewed.

Köppen-Geiger climate classifications did not produce interpretable geographic patterns in LRRs, but in all models we did find a significant effect of climate classification and a relatively high $R^2$ index as compared to experimental and environmental models. Unfortunately, the climate classification models did not provide clear insight about particular combinations of temperature and precipitation that led to differences in algal responses to nutrient additions. Additional factors that vary over space (e.g., elevation, stream slope) may have helped interpret spatial patterns in response ratios, but this information was rarely reported. Even exact latitude and longitude coordinates of streams were only reported in 25% of experiments. Future studies should provide more detail on geographic setting to facilitate comparisons across experiments.

Stream order was only a significant predictor of P-limitation and explained the highest among-stream variation in P-LRR (15%). Order was not significantly correlated with in-stream P, but was negatively correlated with quantitative canopy cover. It is likely that P-LRR increased with stream order as light became less limiting.

We expected to find temporal differences in nutrient limitation based on season, especially since only nine experiments were conducted in tropical zones with little seasonality. We expected summer to have the highest estimate for all response variables because of higher temperature and insolation. We found that fall had the highest estimates for N-LRR and NP-LRR, while summer had the highest estimate for P-LRR followed by fall. Francoeur et al. (1999) showed that nutrient limitation in 12 New Zealand streams was most common during summer, and that nutrient limitation was significantly associated with temperature. However, temperatures in our dataset (spring: 14°C, summer: 15.4°C, fall: 11.9°C, winter: 6.6°C) varied less by season than did temperatures in the New Zealand dataset (spring: 9.2°C, summer: 14.4°C, fall: 10.5°C, winter: 3.5°C). In our dataset winter had much lower LRRs than did other seasons, indicating

that low temperatures and light levels may have decreased algal growth. Fall may have had high LRRs because of moderate temperatures but low canopy cover. Ultimately, interpreting seasonal variation in algal production requires considering context-specific factors like temperature, light, grazing activity, and in-stream chemical parameters like C, N, and P availability.

Temperature and precipitation regimes can affect algal patterns among streams (Biggs 1996). Temperature does not significantly affect diffusion rates from NDS (Rugenski et al. 2008); however, temperature may increase algal growth rates (Biggs 1996). We did find a significant relationship between temperature and NP-LRR in our dataset. We caution that in NDS studies most temperature measurements are instantaneous, and few experiments reported the full range and variability of temperatures experienced by the algal communities.

**Conclusions**

NDS experiments can be valuable tools to determine stream nutrient limitation when the scope of inference is applied to an appropriate spatio-temporal scale. Our analysis has shown that NDS produce variable results that are not easily explained by single experimental and environmental factors. Broader spatial factors like ecoregion, climate classification, and stream order explained the most variation in nutrient limitation results, but the $R^2$ index was still below 20% for all models. Thus NDS experiments may be most useful for site-specific or regionally-specific research questions. Comparing experiments across regions is likely only informative when methods are standardized (e.g., Tank & Dodds 2003; Reisinger et al. 2016), and such studies should measure environmental gradients that are expected to be important such as light or turbidity.

While many studies have focused on the effects of resources on algal nutrient limitation, there is a clear need for studies that consider how algal stressors interact with nutrients.

Instantaneous discharge and water velocity were sometimes reported, but metrics of flow variability or flood occurrence were lacking in the studies we analyzed (but see Biggs et al. 1998; Francoeur et al. 1999). Similarly, studies rarely reported herbivore grazing metrics, even though grazing is just as important as nutrients for controlling algal accrual (Hillebrand 2002). A few studies (e.g., Biggs et al. 2000; Francoeur et al. 1999) did quantify insect grazers on the NDS growth surfaces at the end of the experimental periods. However, this is an instantaneous measure unlikely to sufficiently quantify grazing activity over the experimental period. A census of grazer densities and complete grazer exclusions would be required to accurately determine the influence of grazers on algal accumulation. Finally, turbidity can affect light penetration into streams as well as scour algal cells from benthic growth surfaces. Turbidity was measured in some studies and not found to be a significant factor (e.g., Atkinson et al. 2013), but we maintain that turbidity may interact with factors like canopy cover, water velocity, water depth, or vertebrate and invertebrate grazing to influence algal growth on NDS.

Determining drivers of nutrient limitation in streams is challenging because of the wide range of experimental methods employed, as well as a lack of reporting for environmental data. Given what has been reported in the literature, our meta-analysis emphasizes the importance of in-stream nutrients, light levels, streamflow, season, and land use, as well as experimental methodologies. We have provided recommendations for standardizing methodologies and reporting environmental variables that may drive and help explain NDS results as they relate to basic research and management of benthic algae. However, understanding the relative contributions of environmental and geographic factors requires future experiments on how methodologies may impact P-inhibition of algal growth. Future studies should also deploy NDS

across a priori-defined gradients of key environmental variables, particularly focusing on algal stressors that are largely understudied.

**Figures**

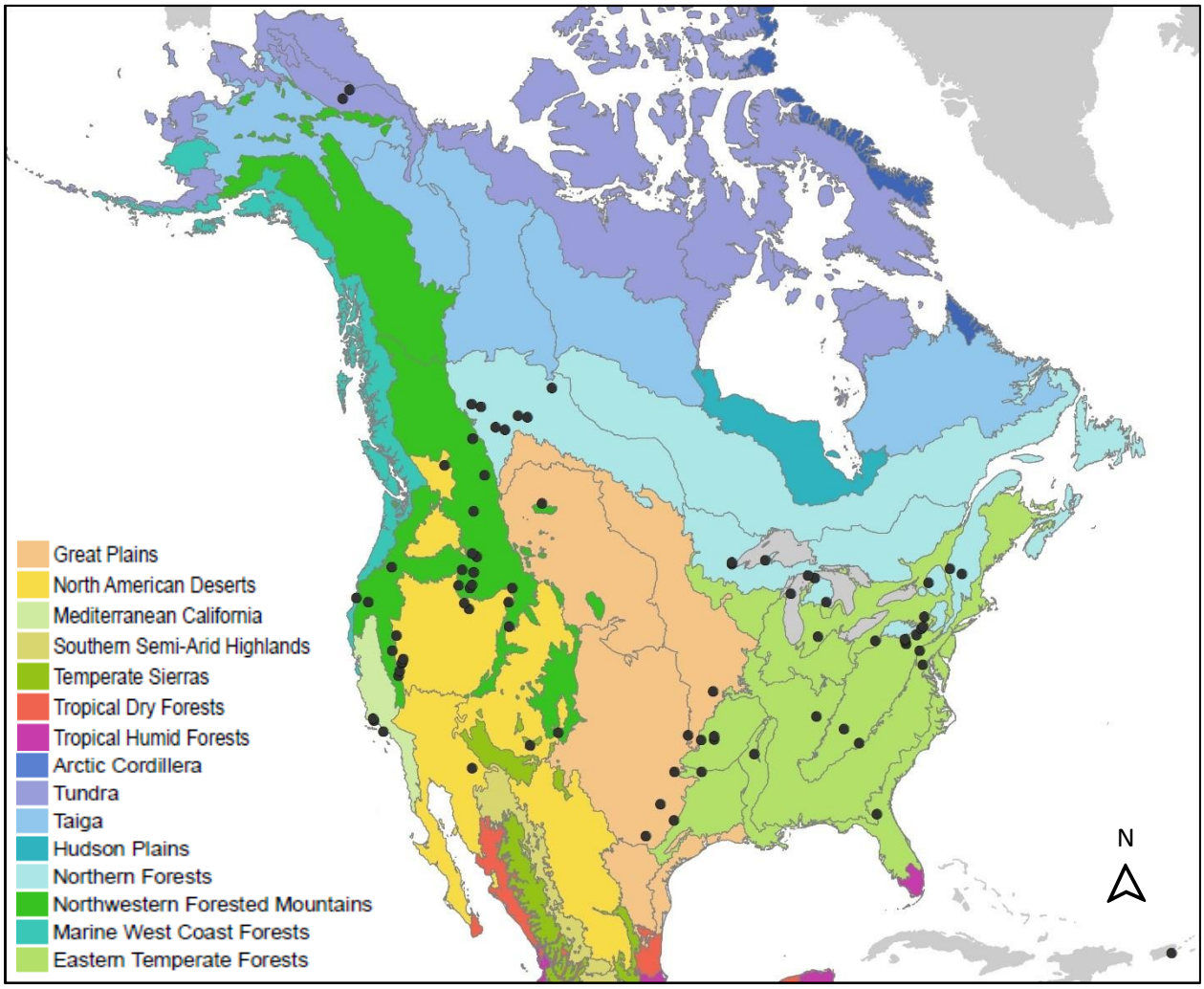


**Figure 1.1: Study sites in North America were mapped based on reported latitudes and longitudes, as well as site maps and stream names. They were classified by Level 1 Ecoregion (Commission for Environmental Cooperation 2009) to facilitate an analysis of geographic patterns in nutrient limitation.**

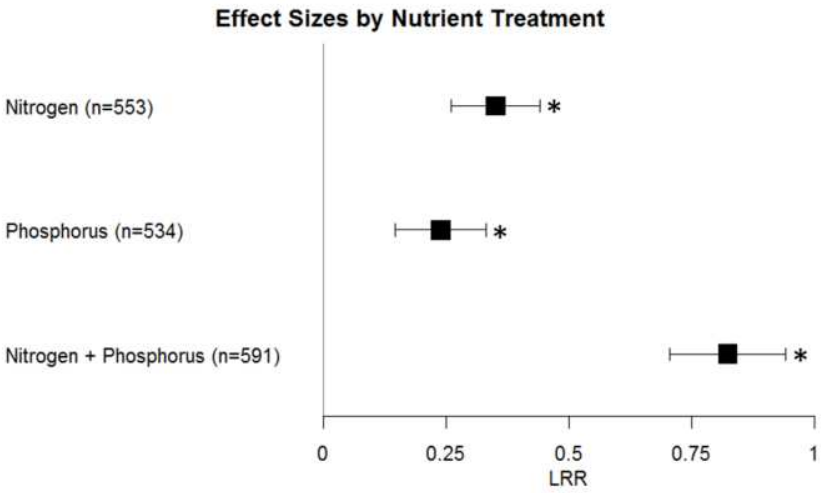


**Figure 1.2: LRR effect size estimates from the no-intercept models by nutrient treatment, with the center square denoting the mean, bars denoting upper and lower 95% confidence intervals (CIs), and "*" indicating a significant effect as determined by CIs that do not overlap with zero in the intercept model (see Table S1.2).**

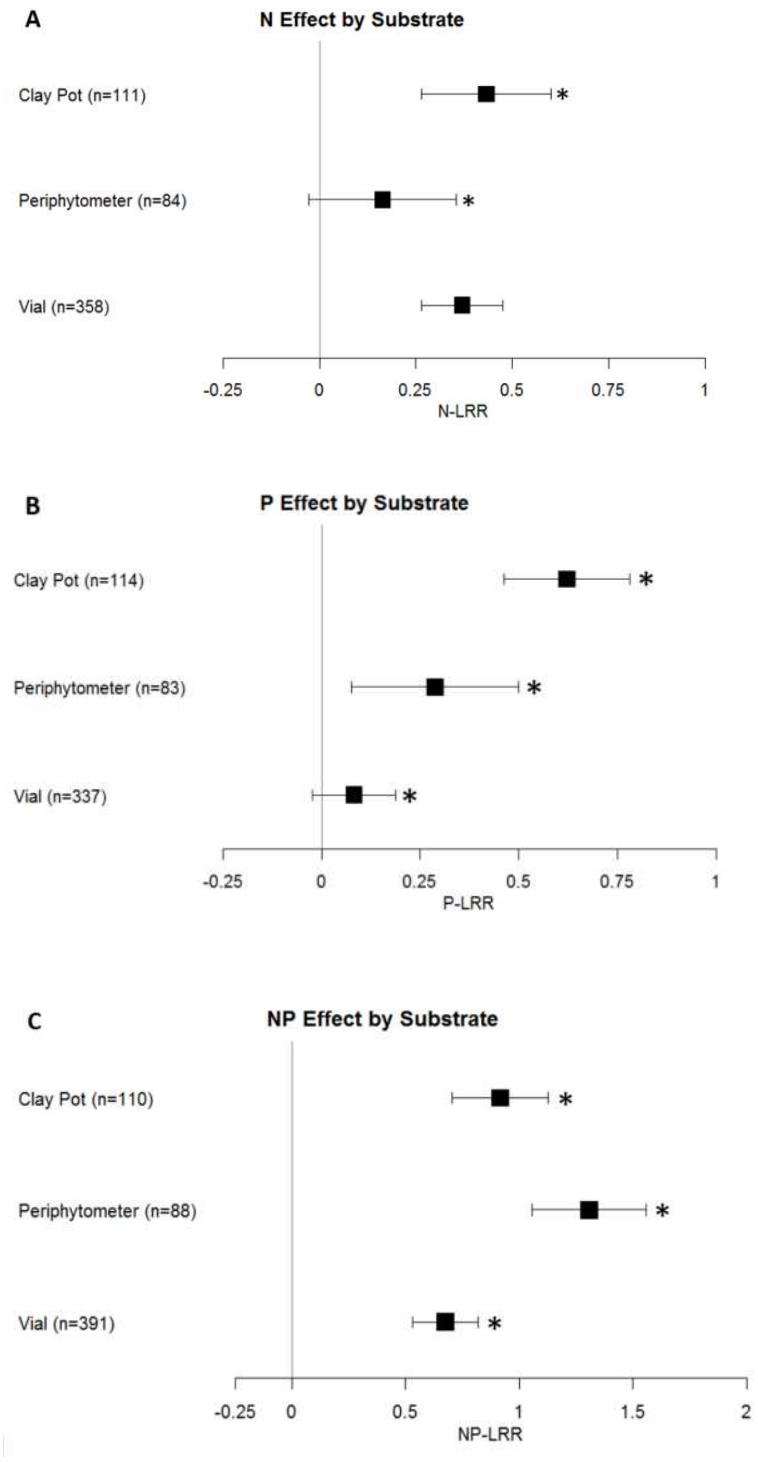


**Figure 1.3: LRR effect size estimates from the no-intercept models by experimental substrate for the N (A), P (B), and NP (C) treatments (see Table S1.4; symbols explained in the Figure 1.2 caption).**

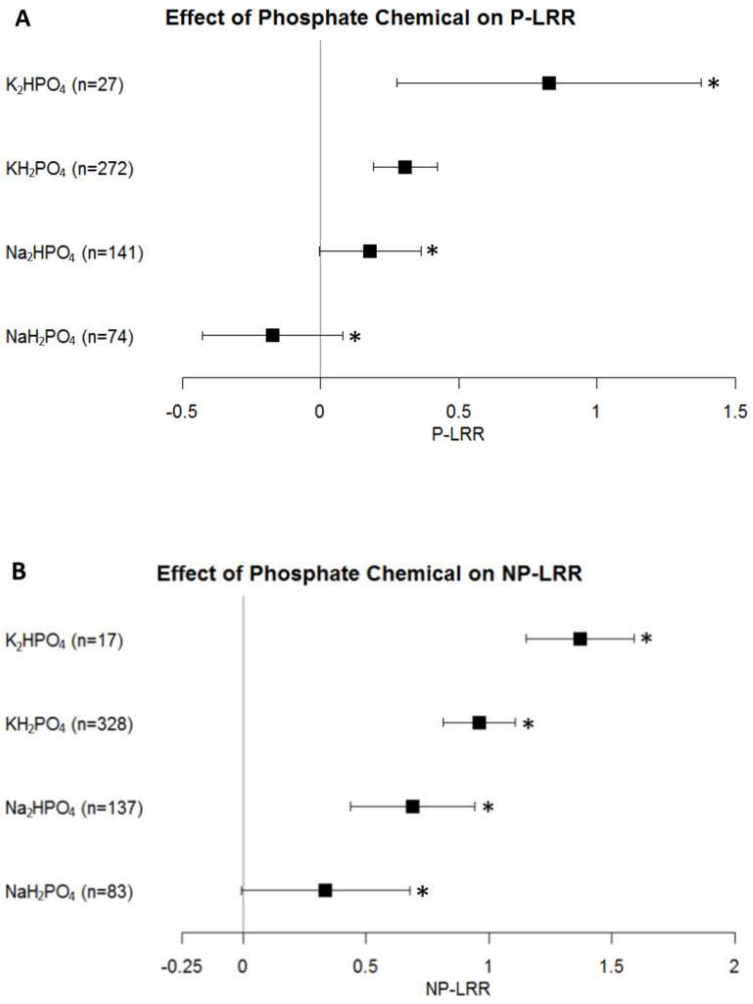


**Figure 1.4: P-LRR (A) and NP-LRR (B) effect size estimates from the no-intercept models by $PO_4^{3-}$ chemical compound composition (see Table S1.4; symbols explained in the Figure 1.2 caption).**

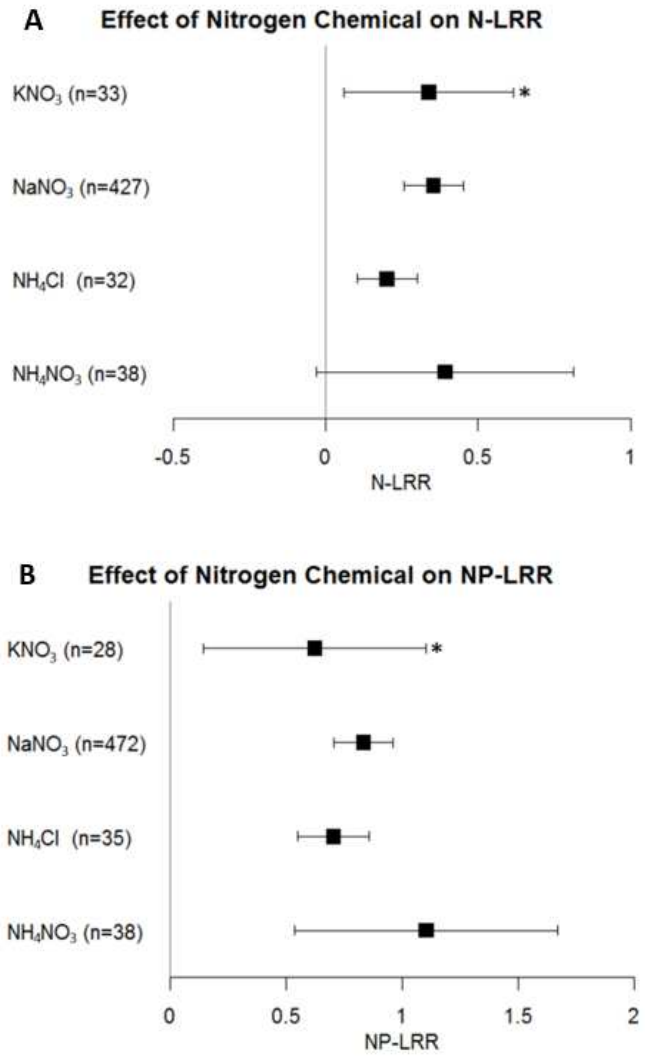


**Figure 1.5: N-LRR (A) and NP-LRR (B) effect sizes estimates from no-intercepts models by N chemical compound composition (see Table S1.4; symbols explained in the Figure 1.2 caption).**

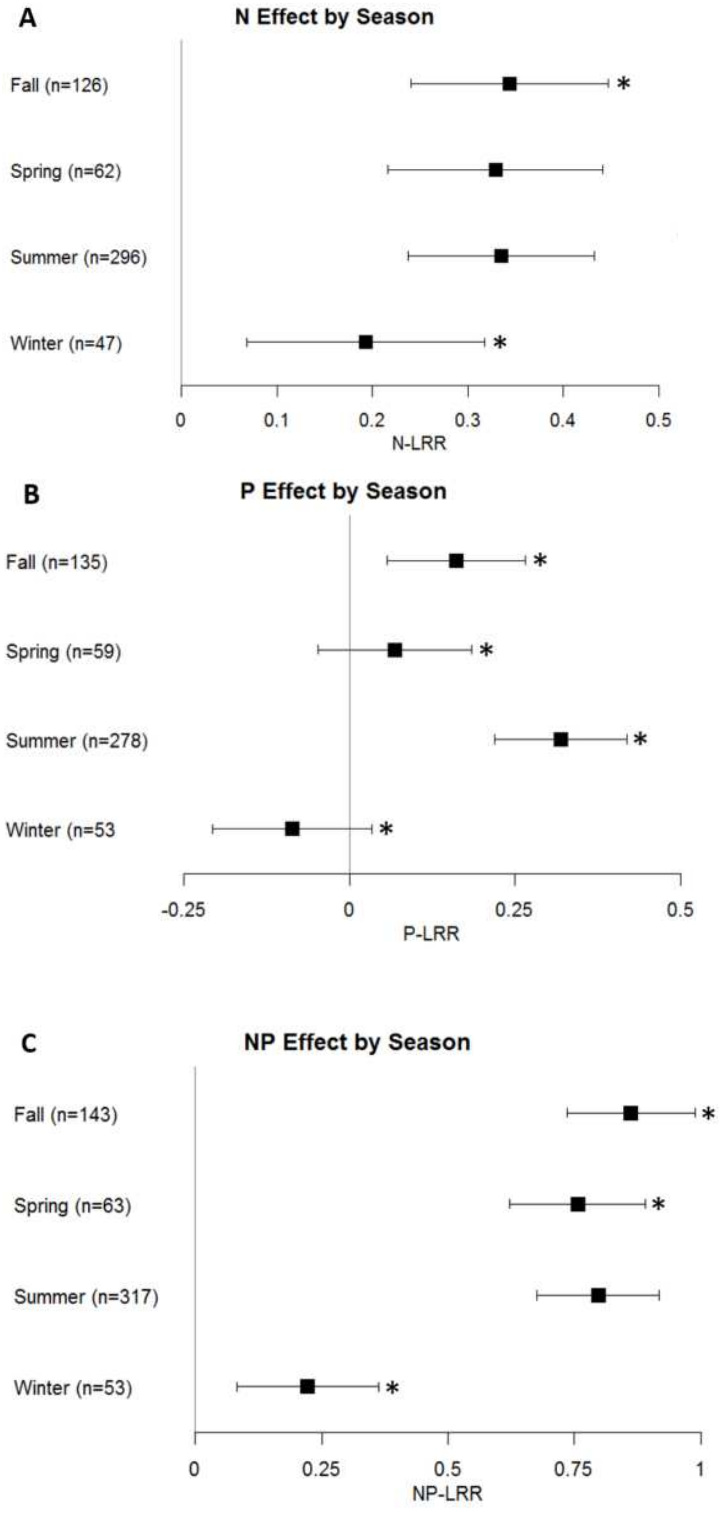


**Figure 1.6: LRR effect size estimates from the no-intercept models by season for the N (A), P (B), and NP (C) treatments (see Tables S1.5, S1.6, and S1.7; symbols explained in the Figure 1.2 caption).**

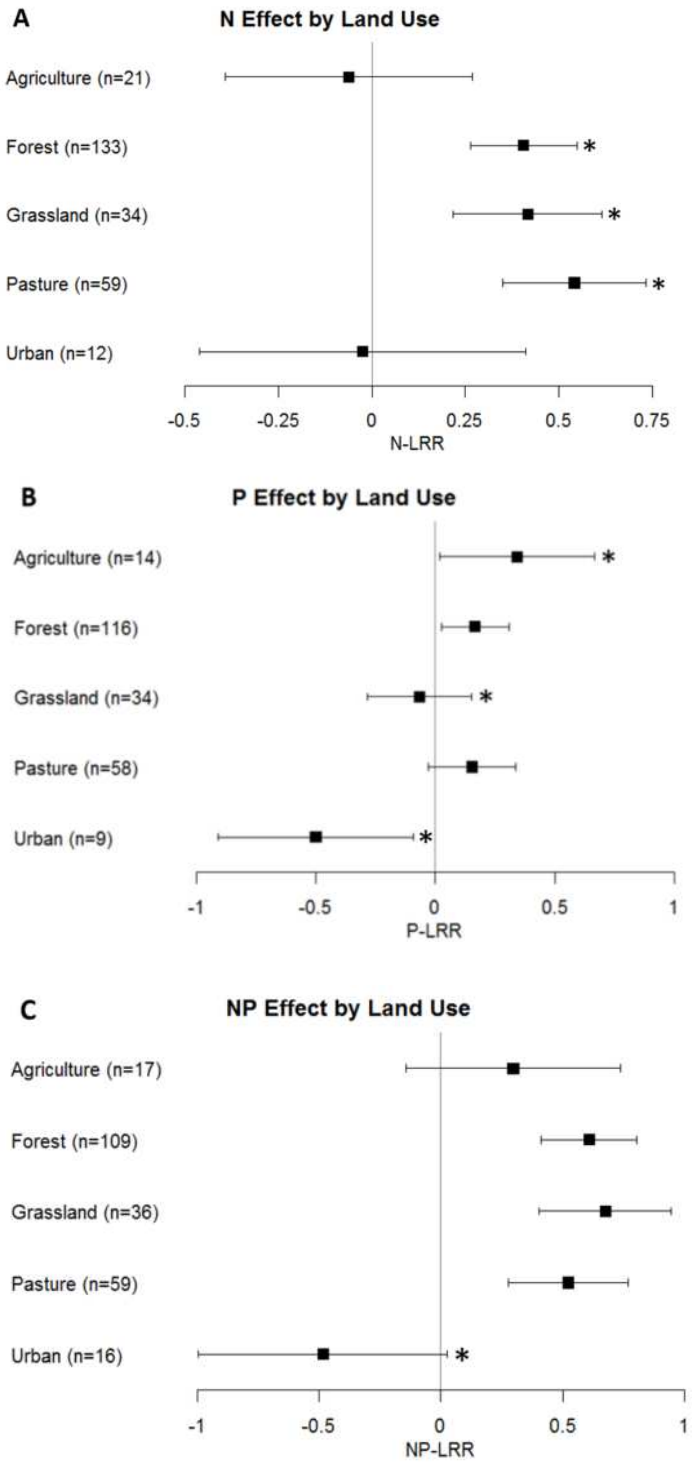


**Figure 1.7: LRR effect size estimates from the no-intercept models by land use for the N (A), P (B), and NP (C) treatments (see Table S1.8; symbols explained in the Figure 1.2 caption).**

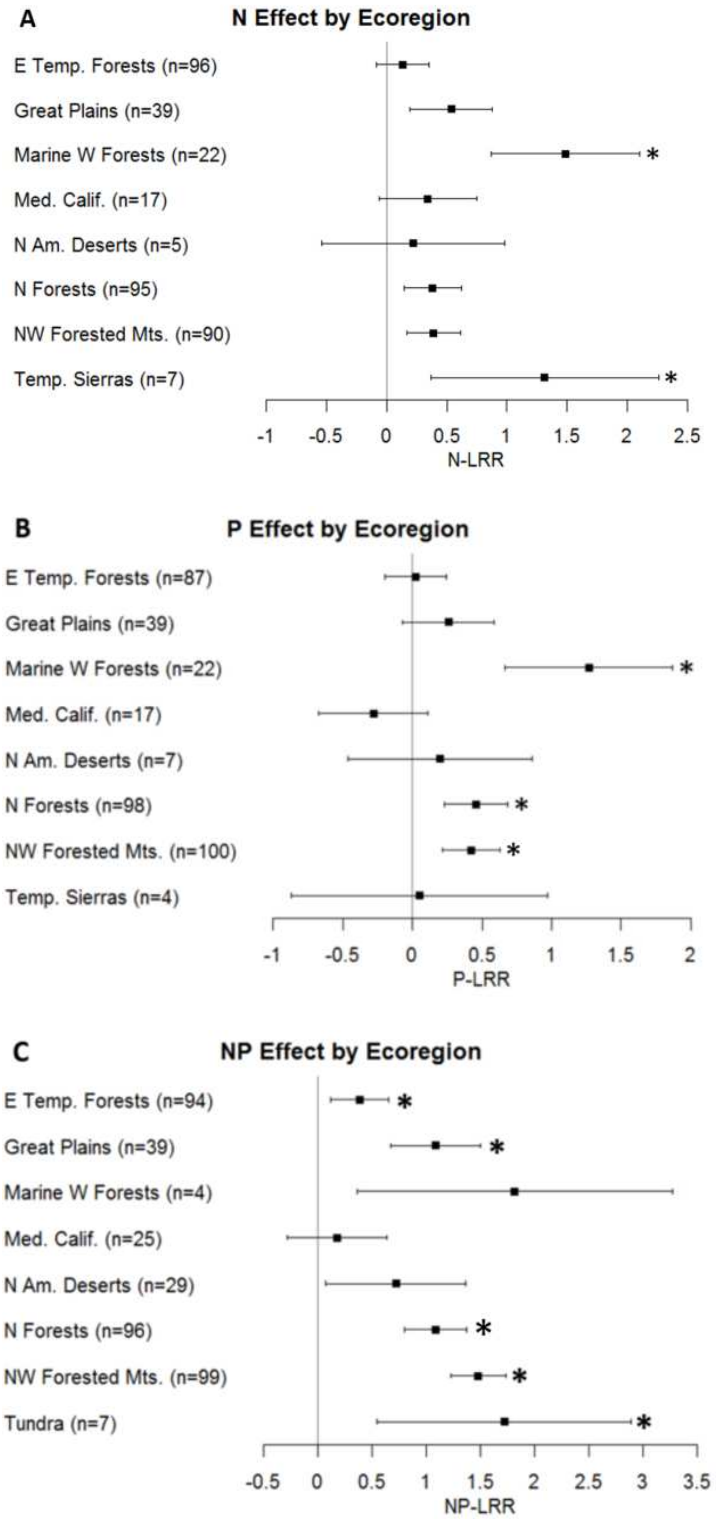


**Figure 1.8: LRR effect size estimates from the no-intercept models by North American Level 1 Ecoregion for the N (A), P (B), and NP (C) treatments (see Table S1.9; symbols explained in Figure 1.2 caption).**

# CHAPTER 2: MULTIPLE GRADIENTS REVEAL THAT RESOURCE AVAILABILITY IS A STRONGER DRIVER THAN TEMPERATURE ON MOUNTAIN STREAM ECOSYSTEM PROCESSES[2]

## Summary

Nitrogen (N) and phosphorus (P) concentrations often increase algal biomass production and algal nutrient content in streams, which in turn stimulate secondary production and regulate consumer nutrient excretion. Studies have often investigated how individual factors like temperature and background nutrients influence these algal and animal-driven dynamics, yet few have explicitly tested hypotheses along multiple gradients of covarying environmental factors. We conducted experiments in eight streams to quantify how algal accrual rates, algal nutrient limitation, and invertebrate excretion rates changed along an elevation gradient (2000-3200 masl) with increasing in-stream N and decreasing temperature. We also investigated how algal parameters changed over time (summer to fall) with decreasing discharge and temperature. First, we confirmed previous findings that algal biomass was NP co-limited but responded more strongly to N additions than P additions. As expected, algal N-limitation magnitude decreased with elevation and invertebrate excretion N:P molar ratios increased with elevation. For some response variables, the multiple gradients comprised environmental drivers with opposing directional effects. As elevation increased, the positive effect of in-stream N concentrations outweighed the negative effect of cooling water temperature on algal accrual and invertebrate N

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[2]This chapter is an edited version of: Beck, W. S., Rugenski, A. T., & Poff, N. L. (2019). Multiple gradients reveal that resource availability is a stronger driver than temperature on mountain stream ecosystem processes. In Review, *Limnology and Oceanography*.

excretion rates. Algal accrual increased from summer to fall in association with increased resources (N and light), despite decreasing temperatures. These results demonstrated that resource availability was a stronger driver than temperature on ecosystem processes in the watershed. This and other studies on multiple gradients can help advance our ability to predict ecosystem responses of streams to future changes in temperature, hydrology, and nutrient loading.

## Introduction

Benthic algae are commonly the most important primary producers in streams (Minshall 1978), transforming solar energy into biomass that fuels secondary production of macroinvertebrates and fish (Benke 1993). Algae play a primary role in nitrogen (N) and phosphorus (P) cycling through the uptake, mineralization, and trophic transfer of nutrients (Borchardt 1996). Algal growth and biomass are often limited by the availability of N, P, or co-limited by both nutrients in freshwater systems (Francoeur 2001; Elser et al. 2007; Beck et al. 2017). Nutrient limitation leads not only to reduced algal production but may also reduce the nutritional quality of algae (i.e., higher C:N and C:P ratios), which can thus limit secondary production of consumers (Atkinson et al. 2017). Further, algal nutrient limitation can also decrease rates of invertebrate nutrient recycling, which feeds back to reduce water column nutrient concentrations and further exacerbate algal nutrient limitation (Cross et al. 2005; Spooner & Vaughn 2006) and its trophic consequences. Not surprisingly, understanding the multiple consequences of nutrient limitation on ecosystem productivity in streams has received considerable effort in recent years (Francoeur 2001; Beck et al. 2017).

Algal nutrient limitation is commonly measured using nutrient diffusing substrates (NDS, Fairchild et al. 1985), which are constructed by filling vials with agar that contain control, N, P, and N+P treatments. After a deployment period, the identity and magnitude of the limiting nutrient(s) are determined by comparing the response variable on nutrient treatments versus the control. Nutrient diffusing substrates have frequently been deployed along single gradients of environmental variables like light (Ambrose et al. 2004; Taulbee et al. 2005; Von Schiller et al. 2007) and nutrients (Snyder et al. 2002; Bowman et al. 2005) to identify important drivers of algal nutrient limitation. However, studies have rarely tested explicit hypotheses about how algal production is limited by nutrients in combination with other important environmental factors that commonly covary over space and time.

Multiple gradients of covarying environmental factors are common in nature and decomposing the influence of individual factors along such gradients can provide insight into ecosystem responses to environmental change (Keddy 1991). Along multiple gradients, variation in individual environmental factors could positively, negatively, or neutrally influence the response variable(s) of interest, resulting in complex dynamics that can be difficult to disentangle. Where correlated environmental factors have neutral or the same directional effects on a response variable, experiments cannot always determine the relative influence of interacting environmental drivers (e.g., Hoellein et al. 2010). However, where correlated environmental factors have opposing directional effects on a response variable, experiments can be designed to disentangle the relative influence of environmental drivers. In this study we test hypotheses about algal accrual and nutrient limitation as well as invertebrate nutrient excretion along multiple gradients primarily comprised of resource availability and temperature. Depending on the response variable being considered, these gradients have the same or opposing directional

effects. Disentangling the importance of individual environmental variables across multiple streams at different times can advance ecological understanding and improve prediction of stream ecosystem responses to anticipated future alterations in temperature, hydrology, and nutrient loading (Kundzewicz et al. 2008; Whitehead et al. 2009).

Environmental factors such as light, current velocity, and temperature may interact with nutrients to influence algal growth and nutrient limitation. For instance, light can both increase maximum algal growth rates and increase nutrient uptake rates (Hill 1996). Faster current velocities can increase nutrient availability (Hiatt et al. 2018) by decreasing the boundary layer of low mixing between the water column and algal cells (Borchardt et al. 1994; Larned et al. 2004). High current velocities can also decrease algal growth rates via physiological stress (Biggs et al. 1998), particularly when algae are nutrient-limited (Horner & Welch 1981; Borchardt 1996). Finally, a recent review (Cross et al. 2015) suggests that optimal temperatures increase maximum algal growth rates and nutrient uptake rates.

Environmental factors and their interactions with nutrients strongly influence algal primary production and nutrient limitation, conferring impacts on the trophic transfer of nutrients from algae to aquatic consumers. Algal nutrient content (Cross et al. 2015) and temperature (Hall et al. 2007) generally increase invertebrate grazer nutrient excretion rates. Algal nutrient content also influences invertebrate nutrient excretion ratios, with higher N leading to higher N:P ratios and higher P leading to lower N:P ratios (Atkinson et al. 2017). However, the effect of temperature on N:P ratios is less clear. Experiments have rarely used multiple gradients or factorial manipulations to determine the relative importance of temperature and nutrients on invertebrate nutrient excretion rates and ratios. Furthermore, many studies have examined how invertebrates affect nutrient concentrations and algae in streams (Atkinson et al. 2017) but none

have determined whether algal nutrient limitation could serve as a useful indicator for invertebrate nutrient excretion rates and ratios.

We used eight mountain streams at different elevations in the Cache la Poudre River watershed (Colorado, USA) to test hypotheses about multiple environmental controls on algal accrual, algal nutrient limitation, and invertebrate nutrient recycling. Pilot NDS experiments demonstrated NP co-limitation in the watershed, with stronger algal biomass responses to N additions than P additions (W. S. Beck unpubl.). These streams comprise multiple spatial gradients because water temperature decreases and in-stream N increases with elevation (Kohler 2013). Additionally, these snowmelt-driven streams experience multiple temporal gradients as high discharge in early summer declines to baseflow by early fall, leading to decreases in depth and velocity and increases in water column light penetration. Stream water temperatures also decrease over this time period (Shah et al. 2017). We conducted experiments to quantify how algal accrual rates, algal nutrient limitation, and invertebrate excretion rates changed along the elevation gradient (2000-3200 masl), and how algal response metrics changed seasonally (July-October 2016).

For some response variables, the multiple gradients included environmental drivers with the same or neutral expected effects (Fig. 2.1). We hypothesized that (1a) N-limitation magnitude would decrease with elevation because of increased in-stream N (negative effect) and decreased temperature (negative effect). We also hypothesized that (1b) invertebrate excretion N:P molar ratios would increase with elevation because of increased in-stream N (positive effect) and decreased temperature (neutral effect). For some response variables, the multiple gradients included environmental drivers with opposing directional effects, and we expected to gain insight on the relative importance of individual drivers when results deviated from the null

hypotheses (Fig. 2.1). We hypothesized that (2a) algal accrual and (2b) mass-specific N excretion would not change with elevation because of the competing influence of increased in-stream N (positive effect) and decreased temperatures (negative effect) with elevation. Furthermore, we hypothesized that (3a) algal accrual and (3b) N-limitation magnitude would not change from summer to fall because of reduced depth and current velocity (positive effect) along with decreased temperature (negative effect). Lastly, we hypothesized that (4) algal P-limitation magnitude would not change with elevation or season because P would be less limiting to algal growth than N (Fig. 2.1).

## Methods

### *Study sites*

We completed experiments in eight low order streams (2000-3200 masl) of the Cache la Poudre River watershed, located in the Roosevelt National Forest, CO, along the Front Range of the Rocky Mountains (Fig. 2.2). Nutrient diffusing substrates were deployed at all sites in July 2016 ("summer") and September 2016 ("fall"), algal accrual was measured from late June to late July ("summer") and mid-August to mid- September 2016 ("fall"), and invertebrate nutrient excretion was measured in July 2016.

### *Nutrient limitation*

Nutrient diffusing substrates were created by filling 6 replicate 30 mL plastic vials (Item #66159, U.S. Plastic Corps, Lima, OH) with 2% agar (control), agar + 0.5 $NaNO_3$ (N), agar + 0.05 $KH_2PO_4$ (P), or agar + 0.5 $NaNO_3$ + 0.05 $KH_2PO_4$ (NP, Tank et al. 2017). Vials were capped with fritted glass discs (5.7 $cm^2$, Item #C4505, EA Consumables, Pennsauken, NJ) and attached randomly to plastic L-bars (Item #45031, U.S. Plastic Corp, Lima, OH), which were

staked directly into the streambeds. NDS were collected after 2-3 weeks, depending on the season and accessibility of the streams. We stored discs at -20°C until chlorophyll *a* analysis, within 30 days. We calculated log response ratios (LRRs) for each nutrient treatment using the following equation:

$$\mathrm{LRR_X} = \ln \frac{\text{x chlorophyll } a \text{ mean}}{\text{control chlorophyll } a \text{ mean}} \qquad \text{(Eqn 2.1)}$$

where x refers to the N, P, or NP treatments (Tank & Dodds 2003).

***Algal accrual***

We measured algal accrual rates during summer and fall by attaching fritted glass discs (5.7 $cm^2$, Item #C4505, EA Consumables, Pennsauken, NJ ) to bricks with silicon glue. At each site, we deployed three bricks with three discs in riffles, and measured chlorophyll *a* on one disc from each brick on each of three dates, spaced in 7-14 day intervals. We calculated separate net algal accrual rates for the two experiments (Stevenson 1996), using only the data from the last day of the experiments:

$$\text{Accrual } (\mu g \cdot day^{-1}) = \frac{\text{average chlorophyll } a \text{ (µg)}}{\text{number of days}} \qquad \text{(Eqn 2.2)}$$

We extracted and quantified chlorophyll *a* biomass directly from NDS and accrual discs using 90% buffered ethanol and a handheld Aquafluor® fluorometer (Turner Designs, San Jose, CA) with an acidification correction (U.S. EPA 1997). In fall 2016, we measured NDS chlorophyll *a* using a spectrophotometer (Genesys 20™, Thermo Fisher Scientific, Waltham, MA) because of a fluorometer technical issue. To avoid any confounding influence of methodology, we used NDS chlorophyll *a* effect sizes (LRRs) rather than raw values to make inferences about seasonal changes.

### *Invertebrate excretion*

We also sought to quantify whether stream environmental conditions and algal nutrient limitation could be related to mass-specific excretion of aquatic insect grazers. We measured N excretion of grazing macroinvertebrates from two mayfly families: Baetidae (*Baetis bicaudatus* and *Baetis tricaudatus*) and Heptageniidae (*Cynigmula spp.* and *Epeorus spp.*). Individual nutrient excretion rates were measured using methods described by Whiles et al. (2009). Briefly, we collected macroinvertebrates of similar size classes at all eight streams using a 250 μm mesh kick net. One individual was placed in an incubation vial with 20 mL of filtered stream water (0.7 μm) for 60 minutes. Vials with only filtered stream water were used as control blanks, and all samples were incubated at ambient stream temperature. At the end of incubations, individuals were removed and total body length was measured to the nearest mm. We measured $NH_4^+$ in the field with a Turner Designs 10-AU fluorometer following methods described by Holmes et al. (1999) with modifications by Taylor et al. (2007). Water samples for total dissolved P analysis were frozen in the field and analyzed using the acid-molybdate method after persulfate digestion (APHA 1998). We determined mass-specific excretion rates (μmol $mg^{-1}$ $hr^{-1}$) of each taxon as the quotient of macroinvertebrate biomass (mg dry mass) and the per capita excretion rate corrected for blank controls.

### *Geographic and environmental variables*

We used field measurements and online databases to characterize geographic characteristics at the sites (Table S2.1). United States Geological Survey Streamstats version 4 was used to calculate watershed area and watershed percent forest cover for each site. Elevation was determined using Google Earth, and we measured slope using a clinometer.

We measured environmental variables upon experimental deployment, collection, or both (Table S2.2). We measured discharge using a Marsh McBirney flow meter (Hach, Loveland, CO), pH and conductivity using a multimeter and probes (Thermo Fisher Scientific, Waltham, MA), and canopy cover using a densiometer (Forestry Suppliers, Jackson, MS). Temperature loggers (miniDOT®, PME, Vista, CA) collected data every 10 minutes throughout the duration of the summer experiments, but during the fall experiments the loggers were removed from streams after 10-14 days. We calculated temperature means and coefficients of variation for dates that overlapped with each experiment. We filtered stream water through Type A/E glass fiber filters (0.45 µm, Pall Corporation, Port Washington, NY) into duplicate 60 mL nalgene® bottles and used an ALPKEM® Flow Solution IV autoanalyzer (O.I. Analytical, College Station, TX) to measure $NO_3^-$ with the Cd reduction method (U.S. EPA Method 353.2 1993) and orthophosphate with the ascorbic acid method (Murphy & Riley 1962). We also analyzed stream water samples for $NH_4^+$ using the methods described above for excretion analysis (Holmes et al. 1999; Taylor et al. 2007).

***Statistical analyses***

All statistical analyses were performed in R version 3.5.0 (R Core Team 2017). We used ANCOVAs to quantify the effects of elevation, season, and their interaction on algal response variables, including accrual and NDS effect sizes for each nutrient (N-LRR, P-LRR, and NP-LRR). We used contrasts of least-squared means with Tukey-adjusted p-values ($\alpha$=0.05) to determine significant differences between seasons.

We developed a series of models to determine the influence of environmental and geographic factors on algal response variables. Because of high multicollinearity in our predictor

dataset (Table S2.3), we standardized predictors by their means and standard deviations and used principal components analysis (PCA) to reduce their dimensionality. We used the first four principal components as predictors in multiple regression models for each algal response metric to quantify the influence of multiple environmental and geographic variables in each model. We also developed simple linear regression models to examine the effects of single geographic and environmental factors on each response variable.

We used multiple regression models to determine the effect of environmental characteristics (in-stream temperature and DIN:SRP molar ratio) on mass-specific insect N excretion and insect excretion N:P molar ratios. The models included insect dry mass and insect family (Baetidae or Heptageniidae) as additional covariates, and backward selection based on p-values was used for model selection. Because algal N-limitation magnitude was highly correlated with both temperature and in-stream DIN:SRP molar ratio, we used separate regression models to determine the influence of N-limitation magnitude, insect dry mass, and insect family on insect N excretion and excretion N:P molar ratios.

## Results

### *Patterns of algal responses*

Algal biomass was NP co-limited in 10 of the 16 experiments, with primary N-limitation in five experiments across four streams and primary P-limitation in one experiment (Fig. 2.3, Table S2.4). N-LRR decreased with elevation ($F_{1,12}$=4.85, p=0.048) but was not influenced by season (p>0.05, Table 2.1). By contrast, algal accrual was not influenced by elevation (p>0.05) but increased from summer to fall ($F_{1,10}$=24.985, p<0.001, Table 2.1). Elevation and season had no significant effect on P-LRR or NP-LRR (p>0.05, Table 2.1).

***Drivers of algal responses***

The first four principal components explained 79.3% of the variation in the environmental variables, with the first component explaining 33.2% of the variation and the second component explaining 20.6% of the variation (Fig. 2.4). The first component included high loadings for variables that varied along the elevation gradient (Fig. S2.1), including elevation (0.407) and DIN:SRP molar ratio (0.287), along with average temperature (-0.410), watershed area (-0.361), and conductivity (-0.350). The second component included high loadings for seasonal and light variables (Fig. S2.1) including canopy cover (0.431), season (0.326), velocity (-0.418), and depth (-0.352). The third component comprised a variety of factors (Fig. S2.1) including season (-0.463), watershed area (-0.300), $NH_4^+$ (0.404), SRP (0.367), canopy cover (0.291), and slope (0.264). Finally, the fourth component included high loadings (Fig. S2.1) for temperature CV (0.531), $NH_4^+$ (0.300), DIN:SRP molar ratio (-0.465), velocity (-0.366), and canopy cover (-0.306).

Overall, N-limitation magnitude was primarily associated with elevation-related factors. The four PCA axes explained 55.1% of the variability in algal N-limitation, and N-LRR was significantly negatively related to PCA axis 1 ($p=0.018$). The simple linear regression models (Tables S2.5-S2.6) showed that N-limitation was significantly positively related to watershed area ($R^2=0.378$, $p=0.011$); marginally positively related to average temperature ($R^2=0.229$, $p=0.061$) and depth ($R^2=0.220$, $p=0.067$); and significantly negatively related to slope ($R^2=0.263$, $p=0.043$), DIN:SRP molar ratio ($R^2=0.372$, $p=0.012$), and $NO_3^-$ ($R^2=0.704$, $p<0.001$).

We found that P-LRRs were related to a mixture of chemical, hydrologic, and light variables. The four PCA axes explained a moderate amount of variability in algal P-limitation

magnitude ($R^2$=0.455), and we found a significant negative effect of PCA axis 2 on P-LRRs (p=0.013). Simple linear regression models (Tables S2.5-S2.6) showed that P-limitation was marginally positively related to velocity ($R^2$=0.229, p=0.061); significantly positively related to discharge ($R^2$=0.348, p=0.016); marginally negatively related to forest cover ($R^2$=0.235, p=0.057); and significantly negatively related to canopy cover ($R^2$=0.309, p=0.025), conductivity ($R^2$=0.221, p=0.066), and pH ($R^2$=0.290, p=0.038).

NP-limitation was associated with similar factors as N-limitation, although some unique relationships were found. The four PCA axes explained a high amount of variation in NP-LRRs ($R^2$=0.672), which had a significant negative relationship with axis 1 (p=0.024) and axis 3 (p=0.007). The simple linear regression models (Tables S2.5-S2.6) showed that NP-limitation magnitude was positively related to watershed area ($R^2$=0.438, p=0.005) and depth ($R^2$=0.329, p=0.020), but marginally negatively related to DIN:SRP molar ratio ($R^2$=0.210, p=0.074) and negatively related to slope ($R^2$=0.349, p=0.016), canopy cover ($R^2$=0.286, p=0.033), and $NO_3^-$ ($R^2$=0.477, p=0.003).

The four PCA axes explained a moderate amount of variability in algal accrual ($R^2$=0.421), which was significantly positively related to PCA axis 2 (p=0.049). Simple linear regression results (Tables S2.5-S2.6) showed that accrual was positively related to DIN:SRP molar ratio ($R^2$=0.372, p=0.020), negatively related to average temperature ($R^2$=0.371, p=0.021), and marginally negatively related to velocity ($R^2$=0.241, p=0.075).

***Drivers of invertebrate excretion***

In the first N excretion multiple regression model, environmental and biological predictor variables explained 63.2% of the variability in mass-specific invertebrate N excretion (Fig. 2.5).

Insect family was highly significant ($p<0.001$), with Baetidae having higher N excretion rates than Heptageniidae ($p<0.001$). Mass-specific N excretion significantly decreased with increasing insect dry mass ($p=0.001$) and stream temperature ($p<0.001$) but increased with increasing stream DIN:SRP molar ratio ($p<0.001$). Finally, we found a negative interaction between stream temperature and stream DIN:SRP molar ratio ($p<0.001$), demonstrating that DIN:SRP molar ratio had more of an effect on mass-specific N excretion at lower temperatures. In the second model, algal N-LRR and biological predictor variables explained 52.9% of the variability in mass-specific invertebrate N excretion (Fig. 2.5). Insect family and dry mass followed the same trends as the previously-described model (both $p<0.001$), and algal N-LRR had a significant negative relationship with invertebrate N excretion ($p<0.001$).

In the first N:P molar ratio multiple regression model, environmental and biological predictor variables explained only 20.7% of the variability in invertebrate excretion N:P molar ratios (Fig. 2.6). Temperature was not a significant predictor and therefore was dropped from the final model. We found that stream DIN:SRP molar ratio ($p<0.001$) and insect dry mass ($p=0.014$) led to significant increases in excretion N:P molar ratios, and that Heptageniidae had lower excretion N:P molar ratios compared to Baetidae ($p=0.017$). In the second model, algal N-LRR and biological predictor variables explained 16.5% of the variability in invertebrate excretion N:P molar ratios. Insect family and dry mass followed the same trends as the previously-described model ($p=0.019$ and $p=0.013$), while algal N-LRR had a significant negative relationship with excretion N:P molar ratios ($p<0.001$).

## Discussion

We found that when multiple gradients had the same or neutral directional effects on the response variable of interest, we correctly predicted the direction of the response variable, but we could not disentangle the relative influence of driving factors. However, when multiple gradients had opposing directional effects on the response variable of interest, we could often determine the relative importance of environmental drivers and gain new insight about the ecosystem process of interest. We summarize our findings in light of the original hypotheses and provide recommendations for how future experiments can utilize multiple gradients in natural systems.

### *Nutrient limitation across all experiments*

We found strong NP co-limitation in the Poudre watershed across the spatial and temporal scales tested. Co-limitation has commonly been demonstrated in previous studies (Francoeur 2001; Beck et al. 2017), because algae are heterogenous communities where individual species may be limited by different nutrients (Francoeur 2001). Additionally, the addition of the primary limiting nutrient may cause another nutrient to become secondarily limiting (Francoeur 2001). We also found that four streams were primarily N-limited during at least one season, and algal accrual was significantly positively related to the water column DIN:SRP molar ratio. Algal biomass frequently responded positively to N additions, but algal biomass responded negatively to P additions in half of the experiments. Other studies have shown strong algal biomass responses to N additions (Grimm & Fisher 1986; Sanderson et al. 2009), especially in streams with low DIN:SRP molar ratios that are comparable to ratios in our study streams. Algal growth was primarily P-limited in one stream from our study because of a

high DIN:SRP molar ratio, likely driven by limited development and a small wastewater treatment plant in the watershed (W. S. Beck, unpubl.).

***Multiple gradients with same directional effects***

We found strong support for our hypothesis (1a) that algal N-limitation magnitude would decrease with elevation because of increases in N and decreases in temperature. However, given the nature of how these factors varied along the elevation gradient, we could not disentangle their relative influences on nutrient limitation. Multiple meta-analyses have found that stream DIN significantly decreases N-limitation (Keck & Lepori; Beck et al. 2017), and individual studies have also shown spatial variation in N-limitation based on in-stream DIN (Reisinger et al. 2016) or DIN:SRP molar ratio (Tank & Dodds 2003). However, we are not aware of studies that have examined spatial variation in N-limitation magnitude based on temperature.

We also found support for our hypothesis (1b) that invertebrate excretion N:P molar ratios would increase with elevation because of increased in-stream N. However, while excretion N:P molar ratios did increase with in-stream DIN:SRP molar ratio and decrease with algal N-limitation magnitude, the variance explained by the models was relatively low. We tested excretion N:P molar ratios at only five sites, and three of the sites had similar in-stream DIN:SRP molar ratios and nutrient limitation effect sizes. While it is reasonable to expect that low stream N and high algal N-limitation magnitudes would lead to lower relative invertebrate excretion of that nutrient, a wider range of stream DIN:SRP molar ratios would be needed to more clearly elucidate the influence of stream nutrient content on invertebrate nutrient recycling.

***Multiple gradients with opposing directional effects***

Multiple gradients with opposing directional effects on response variables provided a valuable opportunity to disentangle the relative importance of environmental drivers. First, we found no evidence that algal accrual changed with elevation (supporting hypothesis 2a). However, we did find that across all experiments, the influence of N availability was stronger than the influence of temperature on accrual. In fact, we found a negative regression relationship between algal accrual and temperature which likely only occurred because the coldest streams had the highest N availability. The Cache la Poudre watershed streams were relatively oligotrophic, with in-stream $NO_3$-N concentrations below 100 μmol $L^{-1}$ at all sites. Average individual stream temperatures were between 7° and 16° C in summer (9° C range) and between 4° to 11° C in fall (7° C range). We did not find a significant positive relationship between algal accrual and temperature, although this has been shown elsewhere with relatively small changes in temperature. For instance, an Icelandic stream was experimentally warmed from 5.8° C to 9.1° C, which tripled net primary productivity and increased nutrient mineralization, $N_2$ fixation, and overall nutrient use efficiency (Cross et al. 2015). However, a recent phytoplankton laboratory study found that temperature-dependence of algal growth rates is weak under nutrient-poor conditions (Marañón et al. 2018), which reflects our field-scale results.

As we observed with algal accrual, positive effects of in-stream nutrients appeared to override the negative effects of temperature on invertebrate N excretion. Invertebrate metabolism and excretion are temperature-dependent processes, and N and P excretion rates have been shown to increase with water temperature (Devine & Vanni 2002; Allen & Gillooly 2009). However, we found that mass-specific invertebrate N excretion increased with elevation (i.e. negative relationship with temperature), leading us to reject our hypothesis that excretion would

not change with elevation (2b). Concentrations of in-stream N were positively related to streambed algal biomass and may have increased algal N content at the highest elevation streams (Stelzer & Lamberti 2001; Murdock et al. 2011), which could stimulate invertebrate N excretion. Future studies should analyze periphyton and invertebrate tissue stoichiometry to confirm this potential mechanism for the relationship between in-stream N and invertebrate N excretion. More broadly, the strength of causal linkages among temperature, stream nutrients, algal growth, algal elemental content, algal nutrient limitation, and consumer excretion are still unknown but could be quantified using an approach like structural equation modeling (Grace 2006) coupled with path analysis (e.g., Atkinson et al. 2013). Because consumer growth and excretion rates can be more sensitive to changes in nutrients under warmer temperatures (Cross et al. 2015), future research measuring the effects of nutrient and temperature levels in a factorial field or mesocosm design could reveal generalities about the interactions among resources, temperature, and excretion.

Our models generally demonstrated stronger effects of environmental factors on baetid mayfly mass-specific N excretion and excretion N:P molar ratios as compared to heptageniid mayflies. Other studies have found differences in body stoichiometry within and among aquatic invertebrate species (e.g., Liess & Hillebrand 2005), and variability in nutrient requirements could influence nutrient recycling rates. Furthermore, Baetidae generally have shorter life cycles and higher growth rates than Heptageniidae, which is likely to influence nutrient requirements (Breitenmoser-Würsten & Sartori 1995).

We found increased algal accrual from summer to fall, leading us to reject our hypothesis that accrual would not change over time (3a). In addition to decreased current velocity and increased light penetration in shallower waters over time, unexpected increases in DIN:SRP

molar ratios may have driven increases in algal accrual despite decreasing temperatures. Results from an adjacent montane watershed suggest that streambed algal biomass decreases dramatically during spring snowmelt and associated scour but increases throughout the summer and particularly during the fall to reach a winter maximum (Lewis & McCutchan 2010), which supports the generality of our seasonal algal accrual results.

Season had no effect on algal N-LRRs which supported our hypothesis 3b. Certain factors varied with season that could decrease the magnitude of N-limitation, including reduced temperatures (Francoeur et al. 1999) and higher in-stream N:P molar ratios (Keck & Lepori 2012). However, shallower water levels should have increased light penetration in these non-turbid streams, and that could increase N-limitation if light was a primary or secondary limiting resource (Taulbee et al. 2005). A recent meta-analysis showed a significant effect of season on N-limitation, with winter producing the lowest algal responses to N additions (Beck et al. 2017). Many streams in our study develop an ice layer during winter and also become inaccessible because of snowpack. However, if we had been able to complete NDS studies later in the year, we expect lower temperatures and insolation would have ultimately decreased N-limitation.

### *Algal P-limitation*

Algal P-limitation magnitude was not influenced by elevation or season (supporting hypothesis 4), and P was rarely the primary limiting nutrient. In fact, we observed slight inhibitory effects of P on algal biomass in some experiments. Phosphorus-limitation tended to decrease with increasing canopy cover, which supports findings from a recent meta-analysis of 534 NDS experiments (Beck et al. 2017). Phosphorus toxicity may be more likely to occur when other resources like N and light are limiting, because algal cells can take up P that is not

immediately used ("luxury" P, Stevenson & Stoermer 1982b), and the concentrations can reach toxic intracellular levels (Jensen et al. 1976). Furthermore, we found that P-limitation decreased as in-stream pH increased. We used a monobasic form of phosphate, which acidifies agar compared to control treatments (Beck & Hall 2018) and may lead to lower algal growth responses in some streams (Beck et al. 2017). This effect could be stronger in more alkaline streams if algal communities are not adapted to the low pH conditions. In addition to environmental factors, NDS P concentrations may also affect P-limitation or inhibition magnitudes. During pilot experiments in summer 2015, we used 0.2 M P in the NDS treatments and found significant P-inhibition of algae at some sites (W. S. Beck unpubl.); however, using 0.05 M P in 2016 resulted in reduced P-inhibition.

**Conclusion**

In this study, we used multiple gradients in environmental factors like in-stream resources and temperature to test hypotheses about controls on ecosystem processes in a natural field setting. The multiple gradient design provided important insights, particularly when environmental factors had opposing directional effects on the response variables of interest. We showed that in-stream N concentrations rather than temperature drive algal accrual and grazer N excretion in the mountainous Cache la Poudre watershed. Our results add to the limited number of field studies investigating how temperature and resources interactively influence stream ecosystems, and our multiple gradient design can serve as a template for future studies to better predict stream responses to global climatic changes (Friberg et al. 2009).

**Figures**

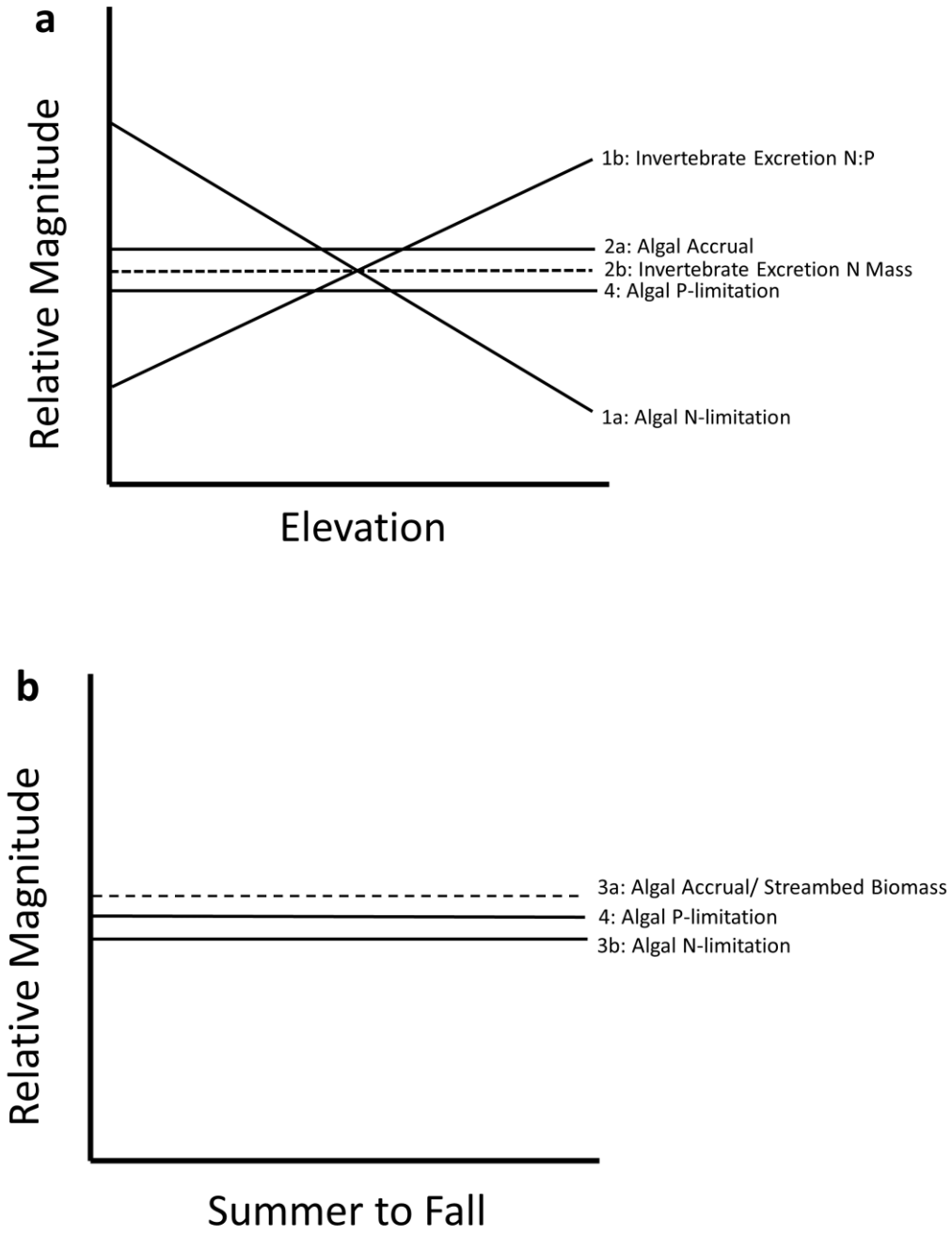


**Figure 2.1: Hypotheses about changes in algal accrual, algal nutrient limitation, and invertebrate excretion across (A) an elevation gradient of 2000-3200 masl and (B) a seasonal shift of summer to fall. Numbers correspond to hypotheses in the text. Solid lines represent hypotheses supported by our experiments, and dashed lines represent hypotheses that were rejected based on our experiments. Relative response magnitudes (y-axis) should only be compared for a given response variable, not among response variables.**

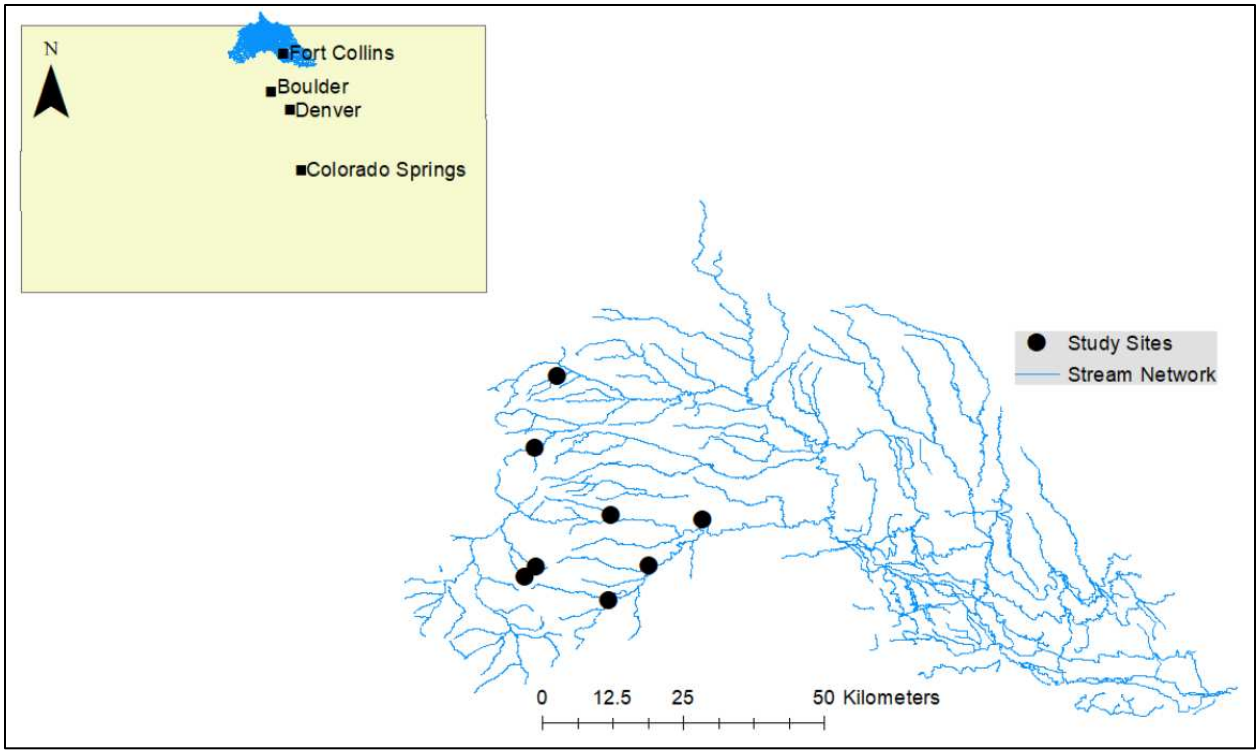


**Figure 2.2: Experiments were completed in eight streams of the Cache la Poudre watershed, located in northern Colorado.**

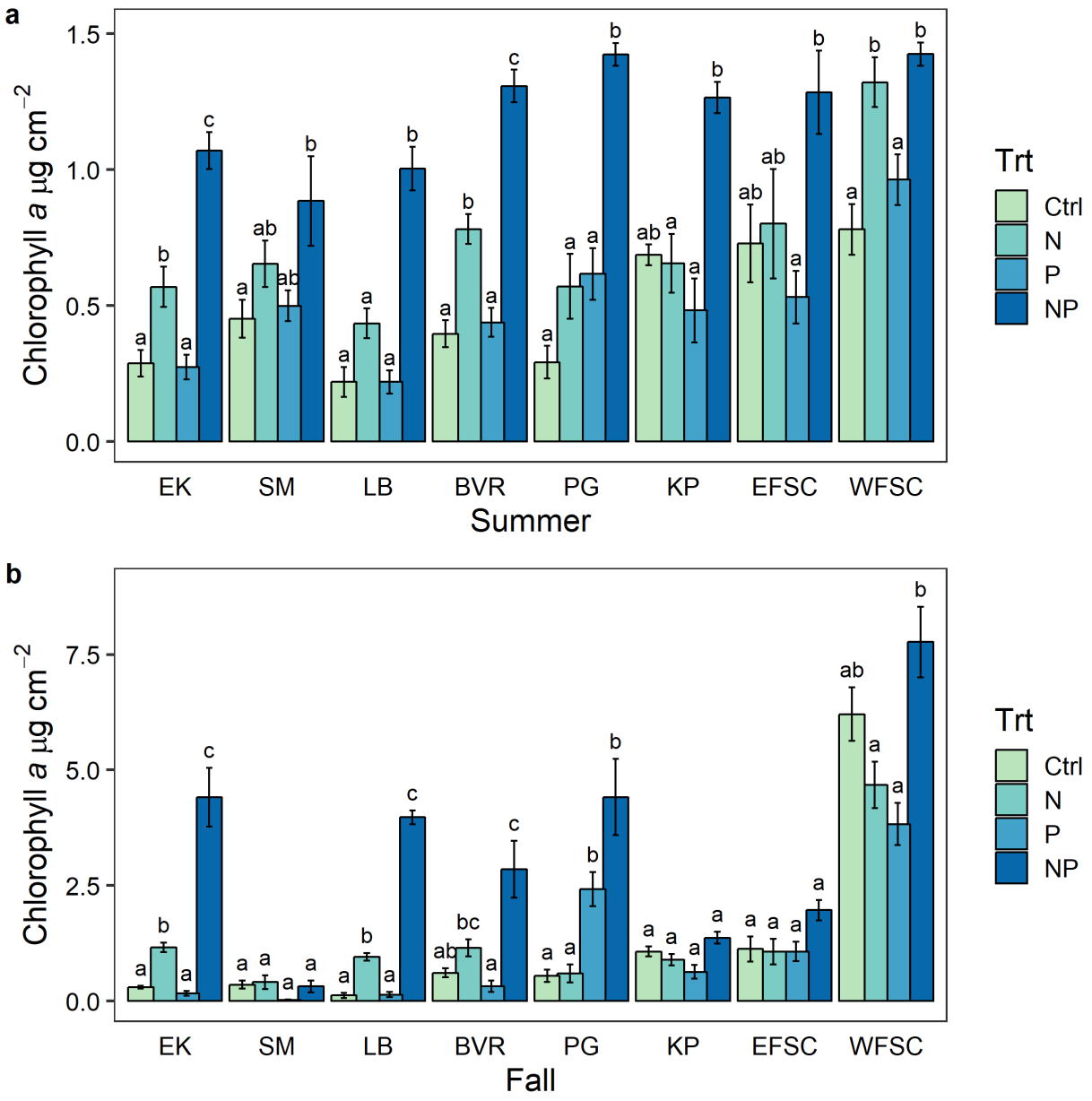


**Figure 2.3: Chlorophyll *a* results for NDS experiments completed at eight streams in (A) July 2016 and (B) September 2016, where treatments are indicated as Ctrl=control, N=nitrogen, P=phosphorus, NP=nitrogen and phosphorus. Treatment comparisons are based on one-way ANOVAs within each site, but not across sites. Sites are arranged from low (2000 masl) to high (3200 masl) elevation on the x-axis, and note the different y-axis scales.**

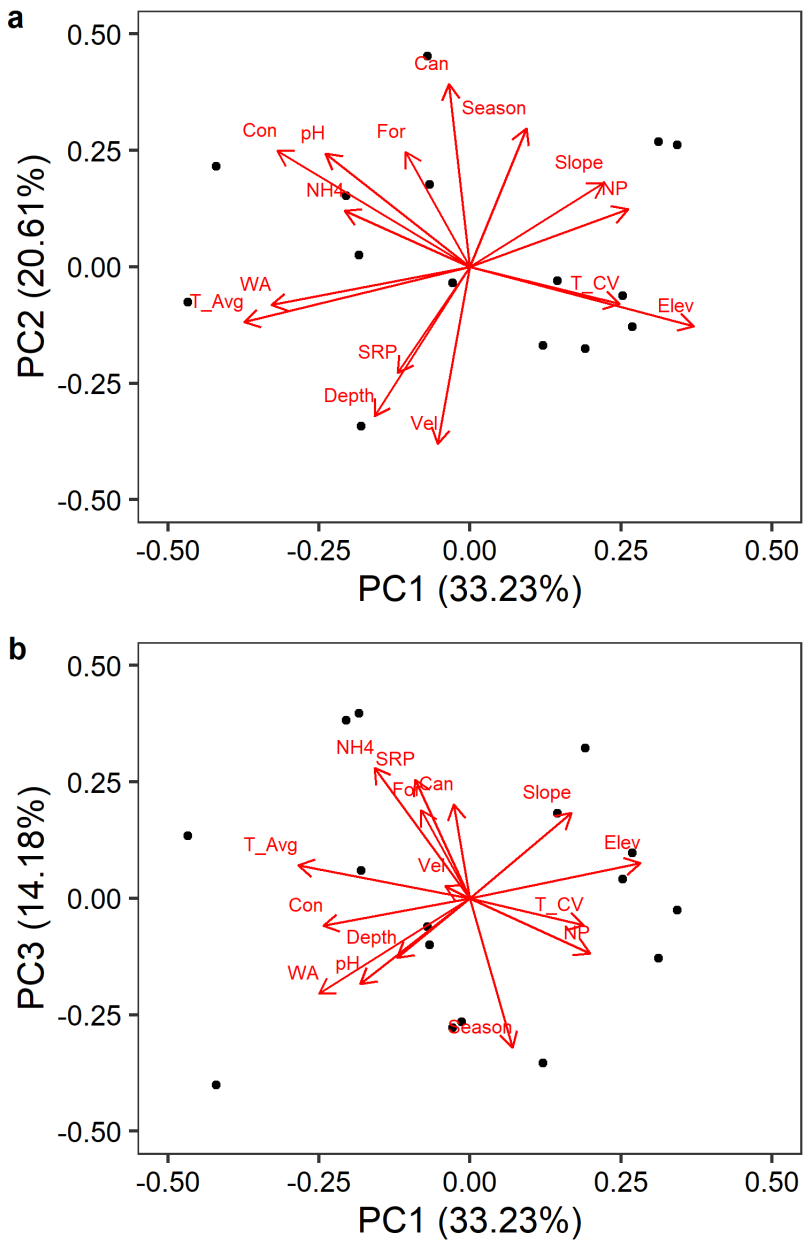


**Figure 2.4: Principal components analysis biplot results comparing (A) PC1 and PC2, and (B) PC1 and PC3 for an analysis of geographic and environmental variables collected in Cache la Poudre River watershed streams in 2016. Together, the first four PCs explained 79.3% of the variability in these variables, but principal component 4 was not a significant factor in any of the regression models. Can = percent canopy cover, Con = conductivity, Elev = elevation, For = percent watershed forest cover, NP = in-stream DIN:SRP molar ratio, T_Avg = average temperature, T_CV = temperature coefficient of variation, Vel = velocity, WA = watershed area. Summer and fall values for the eight stream sites are shown as points on the graph.**

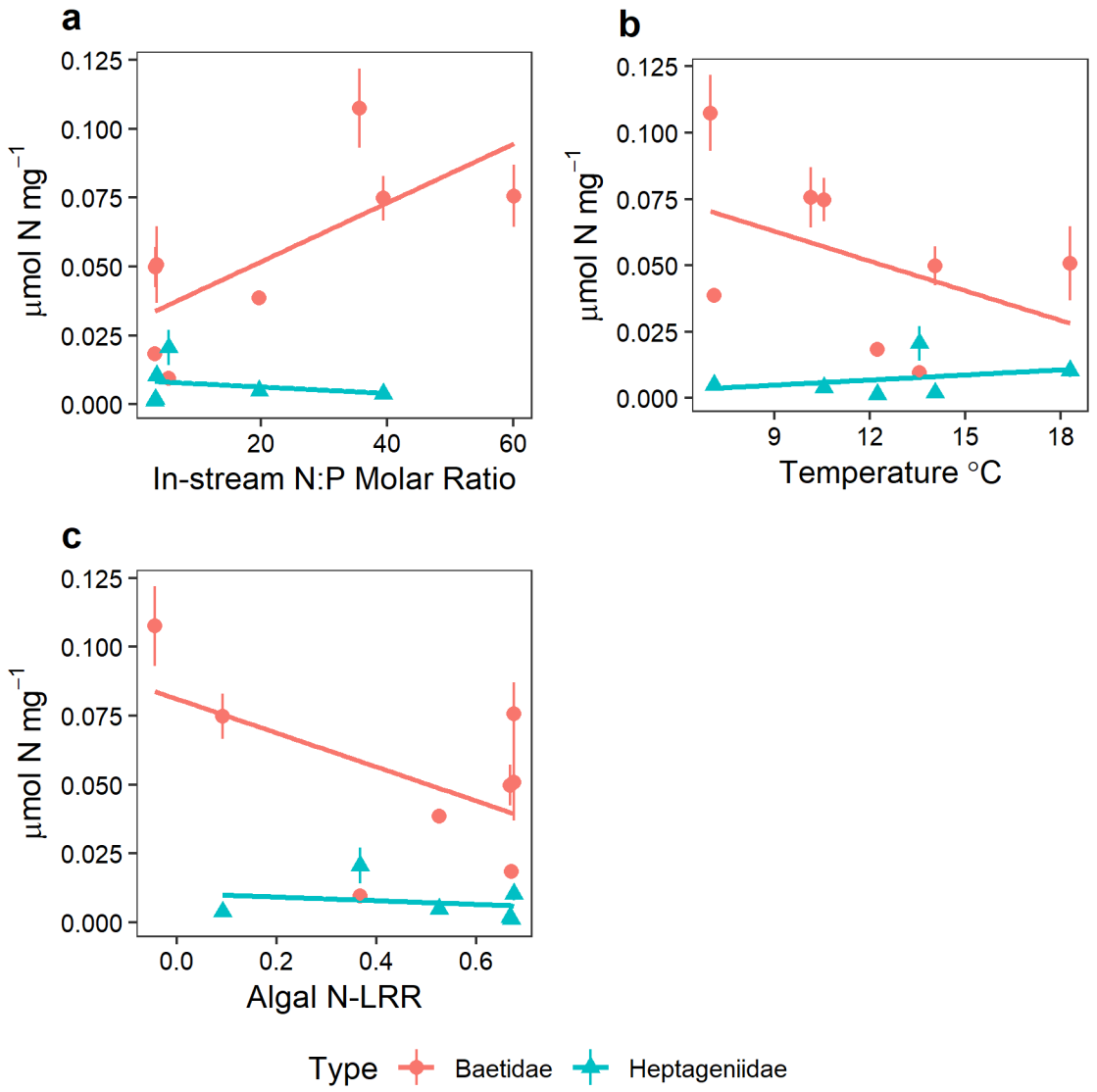


**Figure 2.5: Mass-specific N excretion means ± 1 SE for baetid (total n=133, with n=8-20 per site at 8 sites) and heptageniid (total n=56, with n=8-10 per site at 6 sites) mayflies collected from streams in the Cache la Poudre watershed, Colorado. Excretion was positively related to (A) streamwater DIN:SRP molar ratio but negatively related to (B) in-stream temperature and (C) algal N-limitation magnitude.**

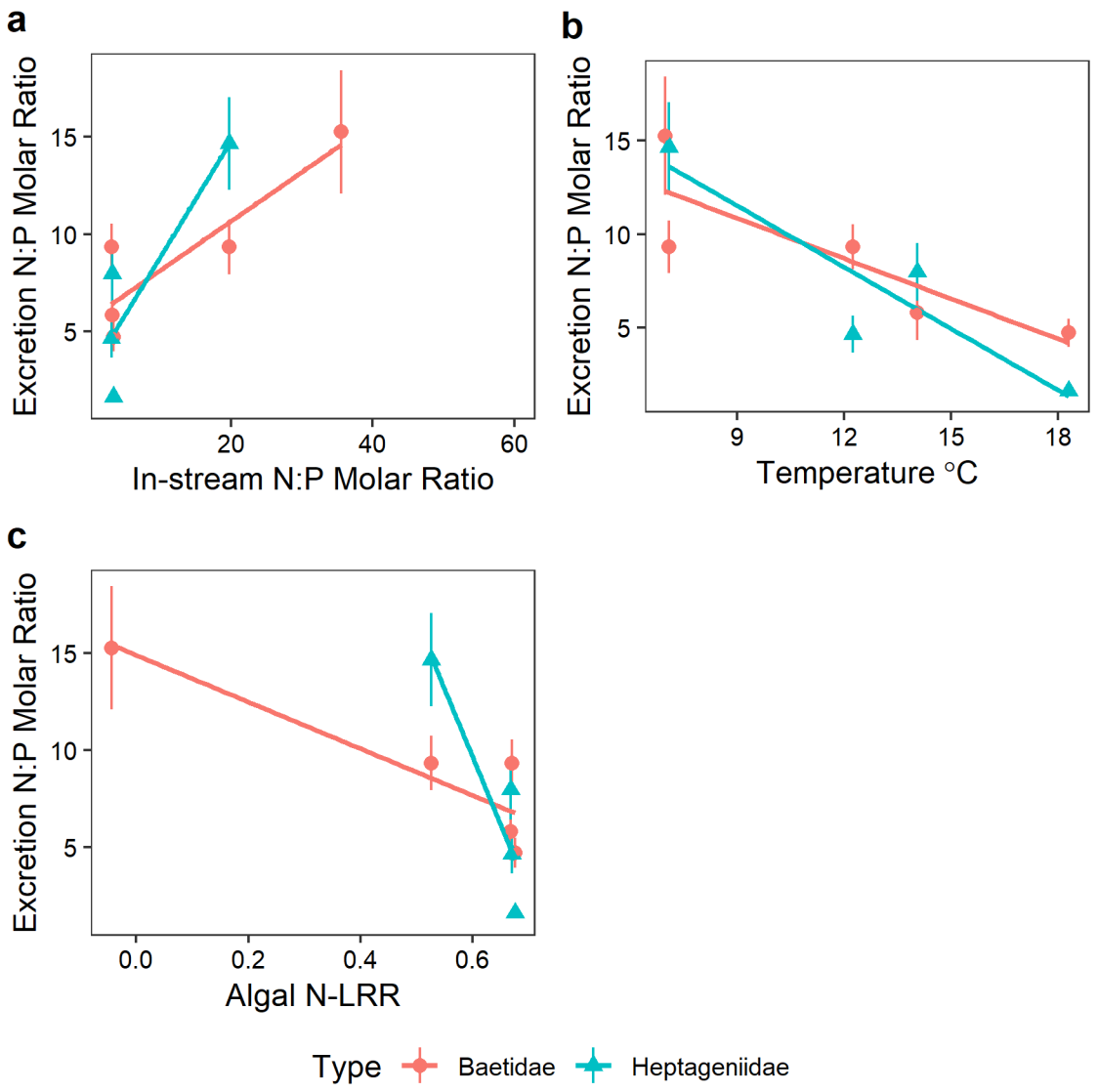


**Figure 2.6: Excretion N:P molar ratio means ± 1 SE for baetid (total n=86, with n=15-19 per site at 5 sites) and heptageniid (total n=30, with n=4-9 per site at 4 sites) mayflies collected from streams in the Poudre watershed, Colorado. Excretion N:P molar ratios were positively related to (A) in-stream DIN:SRP molar ratio but negatively related to (B) in-stream temperature and (C) algal N-limitation magnitude.**

**Tables**

**Table 2.1: Results from ANCOVAs testing the effects of elevation, season, and their interaction on NDS effect sizes (N-LRR, P-LRR, NP-LRR) and algal accrual rates. $p<0.05$ is denoted by "*".**

| Response | Parameter | Df | Sum Sq | F-value | P-value |
|---|---|---|---|---|---|
| N-LRR | *Elevation | 1 | 1.385 | 4.846 | 0.048 |
| | Season | 1 | 0.005 | 0.018 | 0.895 |
| | Elevation:Season | 1 | 0.513 | 1.795 | 0.205 |
| | Residuals | 12 | 3.429 | | |
| P-LRR | Elevation | 1 | 0.800 | 0.995 | 0.338 |
| | Season | 1 | 0.892 | 1.109 | 0.313 |
| | Elevation:Season | 1 | 0.887 | 1.104 | 0.314 |
| | Residuals | 12 | 9.647 | | |
| NP-LRR | Elevation | 1 | 2.291 | 2.567 | 0.135 |
| | Season | 1 | 0.460 | 0.515 | 0.487 |

| | | | | | |
|---|---|---|---|---|---|
| | Elevation:Season | 1 | 0.544 | 0.609 | 0.450 |
| | Residuals | 12 | 10.708 | | |
| Accrual | Elevation | 1 | 0.000 | 1.365 | 0.270 |
| | *Season | 1 | 0.001 | 24.985 | <0.001 |
| | Elevation:Season | 1 | 0.000 | 1.414 | 0.262 |
| | Residuals | 10 | 0.001 | | |

# CHAPTER 3: CONFOUNDING FACTORS IN ALGAL PHOSPHORUS LIMITATION EXPERIMENTS[3]


## Summary

Assessing algal nutrient limitation is critical for understanding the interaction of primary production and nutrient cycling in streams, and nutrient diffusing substrate (NDS) experiments are often used to determine limiting nutrients such as nitrogen (N) and phosphorus (P). Unexpectedly, many experiments have also shown decreased algal biomass on NDS P treatments compared to controls. To address whether inhibition of algal growth results from direct P toxicity, NDS preparation artifacts, or environmental covariates, we first quantified the frequency of nutrient inhibition in published experiments. We also conducted a meta-analysis to determine whether heterotrophic microbial competition or selective grazing could explain decreases in algal biomass with P additions. We then deployed field experiments to determine whether P-inhibition of algal growth could be explained by P toxicity, differences in phosphate cation (K vs. Na), differences in phosphate form (monobasic vs. dibasic), or production of $H_2O_2$ during NDS preparation. We found significant inhibition of algal growth in 12.9% of published NDS P experiments as compared to 4.7% and 3.6% of N and NP experiments. The meta-analysis linear models did not show enhanced heterotrophy on NDS P treatments or selective grazing of P-rich algae. Our field experiments did not show inhibition of autotrophic growth with P additions, but we found significantly lower gross primary productivity (GPP) and biomass-specific GPP of benthic algae on monobasic phosphate salts as compared to dibasic phosphate


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[3]This chapter is an edited version of: Beck, W. S. and Hall, E. K. (2018). Confounding factors in algal phosphorus limitation experiments. *PLoS One, 13*, e0205684.

salts, likely because of reduced pH levels. Additionally, we note that past field experiments and meta-analyses support the plausibility of direct P toxicity or phosphate form (monobasic vs. dibasic) leading to inhibition of algal growth, particularly when other resources such as N or light are limiting. Given that multiple mechanisms may be acting simultaneously, we recommend practical, cost-effective steps to minimize the potential for P- inhibition of algal growth as an artifact of NDS experimental design.

**Introduction**

Benthic algal production provides an important energy source to higher trophic levels (Lamberti 1996), and in low productivity streams, growth of macroinvertebrate and fish grazers may be limited by the availability of algal food resources (Lewis & McCutchan 2010). Freshwater algal growth is often limited by the availability of nitrogen (N), phosphorus (P), or both nutrients (Francoeur 2001; Elser et al. 2007), but human activities are increasing N and P inputs to streams via sources such as wastewater treatment effluent, agricultural runoff, and atmospheric N deposition (Carpenter et al. 1998). These excess nutrients may result in harmful levels of algal biomass that degrade ecological habitat (Carpenter et al. 1998), stream aesthetics (Suplee et al. 2009), and drinking water quality (Carpenter et al. 1998). Identifying nutrients that limit algal productivity in individual stream reaches can inform stream management plans that promote human and ecosystem health.

For over thirty years, nutrient diffusing substrate (NDS) experiments have been used to determine nutrient limitation of benthic algal communities (Fairchild et al. 1985; Pringle 1987). Nutrient diffusing substrate experiments are constructed by filling a small vessel (e.g., a plastic vial or clay pot) with agar and a nutrient solute of choice (e.g., $KH_2PO_4$), and comparing growth

with a paired control vessel containing only agar (Tank et al. 2017). Differences in algal responses can then be compared across the NDS treatments. Contrary to the long-held paradigm that P frequently limits algal productivity in freshwater ecosystems (Schindler 1977), one of the first published NDS experiments observed that treatments with 0.5 M P had lower algal biomass than treatments with 0.05 M P (Fairchild et al. 1985). Many studies have since found that algal biomass can be inhibited by P as compared to controls (e.g., Bernhardt & Likens 2004; Sanderson et al. 2009), and it is unclear how often or why this phenomenon occurs. Whereas P may not always enhance growth due to limitation by N or other resources (light, Fe, etc.), it is surprising that increasing levels of P in NDS can result in decreased algal biomass relative to the control treatment. These results suggest that artifacts associated with NDS experiments may be leading to the underreporting or misrepresentation of P-limitation in freshwater ecosystems.

Several hypotheses have been introduced to explain why addition of P in NDS would result in a decrease of algal biomass. As a macronutrient, P is required for algal growth and maintenance (Borchardt 1996), but high P concentrations may result in direct physiological toxicity and this has been hypothesized as a reason for observed P-inhibition (Fairchild et al. 1985). While mechanisms for this toxicity in algae are unclear, excessive P concentrations in growth media of terrestrial plants can negatively affect the availability, uptake, and metabolic processing (Jones Jr. 1998) of Fe (Christie & Moorby 1975), K (Christie & Moorby 1975), and Zn (Loneragan et al. 1982), leading to deficiency of these essential nutrients and slowing or inhibiting plant growth. Other hypothesized mechanisms for P-inhibition of algal growth have focused on artifacts related to preparation of the NDS, including: 1) phosphate cation type (K vs. Na) and toxicity, 2) phosphate form (monobasic vs. dibasic), and 3) $H_2O_2$ production from phosphate reacting with agar during autoclaving. A limited number of laboratory studies have

tested whether high concentrations of the phosphate salt cations ($K^+$, $Na^+$, $H^+$) may inhibit algal growth. These studies have shown that K phosphates and KCl are toxic to algae at lower concentrations than Na phosphates and NaCl (Chu 1943; Lehman 1976). The phosphate form may also influence algae, as monobasic forms ($KH_2PO_4$ and $NaH_2PO_4$) tend to have lower NDS effect sizes than dibasic forms ($K_2HPO_4$ and $Na_2HPO_4$, Beck et al. 2017). This suggests algae are either inhibited by acidic pH levels (induced by monobasic forms) or are experiencing cation limitation (alleviated by dibasic forms). Lower pH may influence algae directly by changing concentrations of $H^+$ around the cell or indirectly through the effects of pH on metal toxicity or nutrient availability (e.g., via slowed nitrification rates or binding of P by Al, Planas 1996). Finally, the preparation of the NDS media may affect how the P treatments influence algal growth. Autoclaving phosphate and agar together produces $H_2O_2$, a toxin that may inhibit microbial growth (Tanaka et al. 2014). It has been suggested that the common NDS construction method of combining the two compounds on a hotplate could also produce the same result (Tank et al. 2017) thus leading to inhibition of algal growth in treatments that contain P; however, to our knowledge this has never been directly tested.

Beyond these direct artifacts of NDS preparation, there may also be a series of indirect effects of adding phosphate to NDS that could inhibit algal growth. For example, P could disproportionately stimulate heterotrophic microbes (Bechtold et al. 2012) and increase competition between autotrophs and heterotrophs for other limiting nutrients, ultimately suppressing algal growth. It is also possible that P amendments may induce additional top-down pressure if insect grazers selectively graze P-rich algal biofilms (Hood et al. 2014), resulting in lower algal biomass on NDS P treatments as compared to controls (Bernhardt & Likens 2004). Selective foraging has been supported by a theoretical analysis (Neeson et al. 2013), and

laboratory experiments provide some additional support that grazers can engage in P-specific foraging (Hood et al. 2014; Mooney et al. 2016).

Determining why P-inhibition of algal growth has been observed in NDS experiments is not only an important methodological question but one with important implications for an ecological understanding of lotic ecosystems. Each of the previously-described mechanisms may potentially affect the response of algae to P treatments in NDS experiments, but there is no single study that simultaneously evaluates each mechanism. We used quantitative analyses of published data and our own field experiments to investigate how frequently and why NDS P treatments inhibit algal growth. First, we surveyed the literature to determine how frequently significant inhibition of algal growth was reported for P, N, and NP treatments. We also used meta-analysis random effects models and linear mixed models to determine if there was consistent evidence for heterotrophic microbial competition or top-down grazing control that could be leading to P-inhibition of algal growth across multiple study systems. Finally, we completed field experiments to directly address the effects of NDS preparation on several common response variables used to evaluate algal growth: chlorophyll *a* (a measure of algal biomass), ash-free dry mass (AFDM, a measure of total biofilm organic matter), a calculated index of autotrophy (AI), gross primary productivity (GPP), and biomass-specific GPP (GPP/chlorophyll *a*).

## Methods

### *Quantitative Review*

To explicitly quantify how often nutrient treatments inhibit algal growth in NDS experiments, we used the database assembled by Beck et al. (2017). Briefly, this database includes 649 NDS experiments from 1985-2015 that used algal biomass (chlorophyll *a*) as a

response variable. The database was previously used to determine overall effect sizes of P, N , and NP additions and to quantify the influence of over thirty experimental, environmental, and geographic covariates. However, in this study our goal was to determine how many individual experiments detected significantly lower algal biomass levels on P treatments (n=534), N treatments (n=553), and NP treatments (n=591) as compared to controls ($\alpha$=0.05). Although we could have used a meta-analysis approach, we used separate two-tailed t-tests for each NDS experiment to better represented how investigators analyzed data in individual studies.

To determine whether P treatments significantly influenced the proportion of autotrophy in microbial communities and whether grazers selected for algal biomass on NDS P treatments, we used meta-analysis models (see below). To assess whether P additions changed algal-heterotrophic interactions, we identified any experiments from the previously compiled database (Beck et al. 2017) that also reported AFDM as a response variable, as a proxy for total biofilm organic matter. For this study, we extracted AFDM data using Webplot Digitizer version 3.12 (Rohatgi 2015), and calculated an autotrophic index (AI) for each experiment and treatment as follows:

$$\mathrm{AI} = \frac{\mathrm{AFDM}}{\text{chlorophyll } a} \qquad \text{(Eqn. 3.1)}$$

We interpret lower values of the AI as a higher proportion of autotrophy in the microbial community (Bechtold et al. 2012). For NDS P treatments we calculated AI log response ratios (LRRs, a measure of effect size) as:

$$\mathrm{LRR} = \ln\frac{\mathrm{Y_1}}{\mathrm{Y_2}} = \ln(\mathrm{Y_1}) - \ln(\mathrm{Y_2}) \qquad \text{(Eqn. 3.2)}$$

Where $Y_1$ is the mean AI from the P treatments and $Y_2$ is the mean AI from the control treatments in a given experiment (Koricheva et al. 2013). LRRs greater than zero indicate there is a positive P treatment effect, but LRRs less than zero indicate an inhibitory effect of the P treatment as compared to controls. We also calculated the variance of effect sizes using:

$$\text{LRR\_Var} = \frac{s_1^2}{n_1 Y_1^2} + \frac{s_2^2}{n_2 Y_2^2} \quad \text{(Eqn. 3.3)}$$

Where $s_1$ and $s_2$ are the standard deviations of the P and control treatments, and $n_1$ and $n_2$ are the number of P and control replicates (Koricheva et al. 2013). To determine whether P additions influence grazer selection of algal biofilms, we identified NDS experiments that also incorporated grazer exclusion treatments. We calculated LRR and LRR_Var metrics for P treatments compared to controls in grazed and ungrazed plots (Equations 2-3).

We used the metafor package in R to build meta-analysis models based on experimental effect sizes and variances (Viechtbauer 2010). Meta-analysis models account for variability within and among experiments by weighting effect sizes by their variances (Viechtbauer 2010). In all models, we used "site" as a random effect, to account for the correlated effects of experiments deployed in the same stream reach (Gelman & Hill 2007). Models were used to determine how P additions influenced AI effect size, and to determine how grazers influenced P treatment effects on algal biomass. For the grazer models, we included grazer treatment as a covariate in the meta-analysis models (Viechtbauer 2010).

***Experiments***

To investigate how NDS preparation methods influence P-inhibition of algal growth, we prepared and deployed a series of complementary NDS experiments in a sub-alpine stream. To

address the influence of cation type, monobasic vs. dibasic phosphate form, and the potential for $H_2O_2$ formation, the first two experiments involved crossing four different phosphate chemicals ($KH_2PO_4$, $K_2HPO_4$, $NaH_2PO_4$, or $Na_2HPO_4$ all at 0.1 M concentrations) with two laboratory heating methods (boiling agar and phosphate together vs. separately) for a total of eight preparation treatments (Fig 3.1). In a third experiment, we again crossed the four phosphate compounds with two laboratory heating methods but to assess direct toxicity of excess P, we also used two different concentrations of P (0.05 M and 0.5 M) for a total of 16 preparation treatments. Concentrations were chosen based on the most commonly used NDS concentrations. Furthermore, Fairchild et al. (1985) previously observed a difference in algal biomass responses to 0.05 M and 0.5 M of P.

We constructed NDS according to standard methods in the literature (Tank et al. 2017). Briefly, we boiled 2% agar with deionized water, poured the solutions into 30 mL vials (Item #66159, U.S. Plastic Corps, Lima OH), and capped the cooled agar with fritted glass discs (5.7 $cm^2$, Item #C4505, EA Consumables, Pennsauken, NJ). A plain agar solution was used for the control, and the specified phosphate salt was added to the agar according to the experimental design described above (Fig 3.1). For the "heated together" treatment, we boiled the agar and the phosphate salt together. For the "heated separately" treatment, we added the phosphate salt once the agar had cooled to handling temperature (55-65° C) and mixed it thoroughly with a magnetic stir plate before solidification.

While preparing the NDS treatments we also measured pH, a putative mechanism for how phosphate form mediates P-inhibition of algal growth. We tested the effects of phosphate compound and heat treatment on agar and water pH in the lab. To do this we used litmus pH test strips to measure the pH of cooling agar for four vials from each phosphate treatment and the

control. To measure effects of phosphate compound and heat treatment on water pH, we constructed two replicate NDS representing the four phosphate compounds crossed with two heat treatments, as well as a control. These NDS were placed in separate plastic bags with 1 L of distilled water. We used a multimeter with a pH probe (Thermo Fisher Scientific A329, Waltham, MA) to measure the initial pH and remeasured the pH after 24 hours.

We deployed all NDS experiments at Little Beaver Creek, a low-order, open-canopy stream in the mountains of the Roosevelt National Forest in Colorado (40.625° N, -105.527° W). Our research was conducted in accordance with a U.S. Forest Service research permit. Experiments 1 and 2 were deployed in summer 2016, while experiment 3 was deployed in summer 2017 (Table S3.1). Previous NDS experiments in summer 2015 showed that Little Beaver was co-limited by N and P, but primarily N-limited, with P treatments causing inhibition of algal biomass relative to the control (Beck, unpublished). We randomized 6 replicates of each treatment and attached 6-8 individual NDS vials to plastic L-shaped bars (Item #45031, U.S. Plastic Corp, Lima, OH) that were anchored into the streambed using metal stakes. Upon deployment, collection, or both, we measured in-stream pH, conductivity, and temperature using a multimeter and probes (Thermo Fisher Scientific Orion Star A329, Waltham, MA); collected duplicate filtered (0.45 µm Type A/E filters, Pall Corporation, Port Washington, NY) water samples in 60 mL Nalgene bottles for nutrient analysis; measured canopy cover using a densiometer (Forestry Suppliers, Jackson, MS); and measured flow using a Marsh McBirney meter (Hach, Loveland, CO). We used a flow meter (Schiltknecht, Switzerland) to measure 2.5 cm-scale current velocity above three evenly-spaced vials on each L-bar. We measured $NO_3^-$ using the Cd reduction method (EPA 1993) and orthophosphate using the ascorbic acid method

(Murphy and Riley 1962) on an Alpkem Flow Solution IV autoanalyzer (O.I. Analytical, College Station, TX).

We analyzed primary production rates on NDS discs after each in-stream experiment (Tank et al. 2017). Briefly, upon collecting the discs, we immediately placed them in 50 mL centrifuge tubes with unfiltered river water, capping the tubes underwater to exclude any air bubbles. Tubes were stored on ice during transport back to the laboratory (less than two hours), where we used filtered (0.45 µm Type A/E filters, Pall Corporation, Port Washington, NY) river water to run light and dark incubations with the NDS discs. We measured the initial temperature and dissolved oxygen (DO) values of the water before light and dark incubations using a ProDO meter (YSI, Yellow Springs, OH). We also included four "blank" tubes with water only, to control for background changes in DO because of temperature changes or exposure to atmospheric oxygen during the measurement process. During light treatments, tubes were incubated in sunlight for two hours in the afternoon, then we measured the ending DO concentration in each tube and ending temperature in several representative tubes. For dark incubations, we replaced the filtered water and incubated tubes in the dark for two hours, after which we measured the final DO concentrations in each tube and ending temperature in several representative tubes.

We calculated net primary productivity (NPP) as the increase in oxygen during the light incubations, correcting for the change in the blank stream water tubes. We calculated respiration as the decrease in oxygen during the dark incubations, correcting for the change in the blank stream water tubes. Gross primary productivity was calculated as follows, then all variables were standardized by disc area and time:

$$\text{GPP} = \text{NPP} + |\text{Respiration}| \quad (4)$$

After the incubations, we immediately placed the discs in black film canisters and extracted chlorophyll *a* for 12-24 hours using buffered 90% ethanol. We measured chlorophyll *a* with an acidification correction (EPA 1997) using an Aquafluor fluorometer (Turner Designs, San Jose, CA). We calculated biomass-specific GPP (GPP/ chlorophyll *a*) as an additional response metric for each disc.

For experiments 1 and 2, we saved all liquid slurry from the chlorophyll *a* extractions and filtered the liquid through a pre-combusted filter (500° C for one hour, 0.45 µm Type A/E, Pall Corporation, Port Washington, NY). We then used both the filter and glass disc associated with each NDS to measure AFDM (Bechtold et al. 2012). For experiment 3, we allowed the chlorophyll *a* extraction slurry to evaporate in a weigh boat under a fume hood and used all remaining material and the glass disc to measure AFDM. We dried the samples for 48 hours at 50° C, pre-weighed their masses, and combusted them at 500° C for one hour in a muffle furnace. We then rehydrated the discs with deionized water, dried them for another 48 hours at 50° C, and weighed the final masses. This procedure was to account for any water that might have been lost from clay particles in the muffle furnace. The difference in weights was calculated as AFDM. We standardized both chlorophyll *a* and AFDM by disc area as is common in NDS experiments (Tank et al. 2017). We also used the chlorophyll *a* and AFDM measurements to calculate AI as described in Equation 1.

***Statistical Analyses for Experiments***

We completed all statistical analyses in R version 3.5.0 (R Core Team 2018). To test the effect of treatment classes on the pH of the NDS agar and water incubated with the NDS, we

used two-way ANOVAs with phosphate form (monobasic vs. dibasic) and heating method as factors. We compared means using post-hoc Tukey's HSD tests ($\alpha$=0.05).

Because the field experiments comprised an incomplete factorial design (i.e., crossed treatments and a separate control, Fig 3.1), we used three separate approaches to analyze the results. First, to determine whether P preparation treatments significantly stimulated or inhibited response variables relative to controls, we used one-way ANOVAs with post-hoc Dunnett tests to compare each of the eight P preparation treatments (Fig 3.1) with the controls. The Dunnett test is a multiple comparison procedure that compares individual treatment means to control means while maintaining a family-wise error rate that is below $\alpha$ ($\alpha$ = 0.05 in this study). We used only data from experiments 1 and 2 and included experiment as a fixed effect block to control for experiment-specific artifacts. Second, to determine whether the treatment classes (cation type, phosphate form, and heat, Fig 3.1) influenced NDS P effect sizes, we used data from experiments 1 and 2 to run separate one-way ANOVAs for each treatment class and response variable (algal biomass, AFDM, AI, GPP, and biomass-specific GPP). We included experiment as a fixed effect block, and the model response variables were P treatment values of each response variable divided by their respective experimental control means (Tank et al. 2017). Third, to determine whether treatment classes interacted with P concentration to influence NDS P effect sizes, we used data from experiment 3 to run separate two-way ANOVAs crossing each treatment class with concentration.

## Results

### *Quantitative Review*

In our analysis of 649 of experiments from the literature we found that algal biomass was more commonly inhibited by NDS P treatments than either N or NP treatments. Phosphorus additions produced a significant negative effect in 12.9% of experiments. However, N and NP additions produced a significant negative effect in only 4.7% and 3.6% of experiments, respectively (both within the commonly assumed type I error rate of 5%).

We next looked for published evidence of hypothesized biological mechanisms that would explain inhibition of algal growth by P. To address the potential for heterotrophic suppression of autotrophs in microbial communities on NDS P treatments, we identified 45 experiments from 11 studies where an AI effect size could be calculated (Ambrose 2003; Ambrose et al. 2004; Corkum 1996; Eckert & Carrick 2014; Elsaholi et al. 2011; Grimm & Fisher 1986; Gustina & Hoffmann 2000; Lang et al. 2012; Mosisch et al. 1999; Ribot et al. 2015; Rier et al. 2014; Snyder et al. 2002). However, the meta-analysis of AI effect sizes showed neither a positive nor negative response to P treatments (Fig 3.2). We found even fewer examples of studies that could be used to examine nutrient additions in conjunction with grazer exclusions. Only five experiments from three studies reported the effects of grazer exclusions on P treatments in NDS experiments (Flecker et al. 2002; Lourenco-Amorim et al. 2014; Winterbourn & Fegley 1989). In these studies, algal biomass effect sizes on the P treatments were not significantly influenced by the presence or absence of grazers (Fig 3.2, $Q_M = 2.594$, $p=0.107$).

***Experiments***

To address the remaining hypothesized mechanisms for inhibition of algal growth by P amendments we conducted a series of field experiments in a small sub-alpine stream. We found no significant differences between any of the eight P preparation treatments (Fig 3.1) and control treatments (all $p > 0.05$) for any of the algal response variable raw values, indicating neither inhibition nor stimulation of algal growth was induced by the addition of P relative to the control in our experiments.

When we grouped the eight P preparation treatments by three different treatment classes (cation type, phosphate form, and heating method, Fig 3.1) and tested treatment class effects on response variable effect sizes (Table S3.2), we found a significant effect of phosphate form (monobasic or dibasic) on GPP ($F_{1,88}=5.057$, $p=0.027$) and biomass-specific GPP ($F_{1,87}=5.578$, $p=0.020$). Dibasic treatments produced higher rates for both measures of primary production (Figs 3.3 and 3.4). Furthermore, phosphate form significantly altered the pH of the agar ($F_{1,28}=1408.333$, $p<0.001$) and the water ($F_{1,12}=503.244$, $p<0.001$) in the laboratory experiments. Monobasic chemicals significantly lowered pH means (agar pH=4.81, water pH=5.45) and dibasic chemicals significantly raised pH means (agar pH=8.88, water pH=8.28) relative to controls (agar pH=7.25, water pH=6.35). We did not find an effect of the phosphate cation salt or heating treatment on any algal response variable effect sizes ($p>0.05$) in the field experiments (Figs 3.3-3.7, Table S3.2). We also did not find any significant main effects of phosphate concentration or interactions between concentration and other treatment classes on response variable effect sizes in the field experiments (Table S3.3).

## Discussion

Our experiments and quantitative literature analyses did not identify a clear mechanism to explain why 12.9% of past NDS experiments reported a significant negative effect of P treatments on algal growth, a number more than twice as high as the type I error rate (5%) and higher compared to what was observed on N and NP treatments (both <5%). In our experiments we observed slight but non-significant P stimulation rather than inhibition, consistent with the evidence for P-limitation in many freshwater streams (Elser et al. 2007). However, past laboratory experiments (Chu 1943; Lehman 1976) and a previous meta-analysis (Beck et al. 2017) support the plausibility of direct P toxicity, cation toxicity, or phosphate form as mechanisms that may inhibit algal growth. Given that multiple mechanisms may be acting simultaneously, and some mechanisms are most likely ecosystem dependent, we discuss the implications of each of our findings and recommend a series of practical steps for future NDS experiments to reduce the likelihood of P-inhibition of algal growth resulting from artifacts of experimental design or experimental preparation.

### *P toxicity*

While our experiments did not show evidence of P toxicity as a mechanism for P-inhibition of algal growth, previous research supports the possibility of P toxicity occurring across a range of stream ecosystems. In contrast with previous research (Fairchild et al. 1985), in our experiments we saw no significant difference in algal biomass between the low (0.05 M) P concentrations and high (0.5 M) P concentrations. Phosphorus toxicity is dependent upon biological and environmental context (see below), and the P concentrations we used in this experiment may have been too low to induce toxicity. There is evidence that NDS experiments in

other systems may commonly exceed the concentrations required to detect toxicity, as a previous meta-analysis of 534 NDS experiments showed that higher P concentrations in NDS treatments significantly decreased P effect sizes (Beck et al. 2017). The physiological mechanisms that cause P toxicity in algae are not well defined, but some insight can be gained from the terrestrial plant literature (Christie & Moorby 1975; Loneragan et al. 1982).

Terrestrial plant studies have demonstrated that excess P within a cell can induce Fe (Christie & Moorby 1975), K (Christie & Moorby 1975), or Zn (Loneragan et al. 1982) deficiencies. This leads to leaf necrosis and discoloration (Musick 1978), reduced growth rates (Christie & Moorby 1975), and plant death (Heddle & Specht 1972; Groves & Keraitis 1976). These plant symptoms have occurred even when studies maintained optimal levels of other nutrients and pH (Christie & Moorby 1975), and when multiple phosphate forms have been tested in the same study (Rossiter 1952). Algae (Stevenson & Stoermer 1982a), like terrestrial plants (Chapin et al. 1986), can take up excess P for storage (i.e. "luxury P uptake"), a strategy to deal with heterogenous nutrient supplies common in stream ecosystems. The mechanisms and potential consequences of luxury P consumption for algae are less clear, but it is plausible that P could accumulate to toxic levels within cells. For instance, laboratory experiments on the freshwater cyanobacterium *Plectonema boryanum* have shown that excess P in the culture medium leads to high levels of intracellular polyphosphates as well as increased cell lysis and cell death (Jensen & Sicko-Goad 1976). Measuring additional algal response variables in NDS experiments such as nutrient content and enzyme activity could provide valuable information on how P concentrations mechanistically influence algal production. However, toxicity from luxury P consumption would only occur if NDS nutrients diffuse at high enough rates that excess P could accumulate in the water-cell boundary layer or biofilm.

Previous studies have measured NDS nutrient diffusion rates in beakers of distilled water to estimate stream water diffusion rates. For instance, a study showed that plastic vial NDS (5.1 $cm^2$ area) constructed with 50 $mmol \cdot L^{-1}$ $KH_2PO_4$ can release 0.321 $mmol\ P \cdot L^{-1} \cdot hr^{-1}$ at day 0, but that diffusion rate declines to 0.001 $mmol\ P \cdot L^{-1} \cdot hr^{-1}$ by day 14 (Capps et al. 2011). Clay pot NDS (86.8 $cm^2$ area) constructed with 50 $mmol \cdot L^{-1}$ $KH_2PO_4$ can release 0.113 $mmol\ P \cdot L^{-1} \cdot hr^{-1}$ at day 0, with a diffusion rate that declines to 0.011 $mmol\ P \cdot L^{-1} \cdot hr^{-1}$ by day 14 (Capps et al. 2011). Because diffusion rates decline in a log-linear fashion over time, algal populations may initially experience concentrations of P from NDS that are sufficiently high to induce toxicity and inhibition of growth (e.g., 0.019 $mmol\ P \cdot L^{-1}$, Lehman 1976). To optimize NDS experiments, future studies could complete pilot experiments that empirically measure diffusion rates to determine appropriate P starting concentrations and experimental lengths (described in detail by Costello et al. 2016). Ultimately, studies should be long enough to surpass the initial high pulses of P released from NDS when direct toxicity may occur, but short enough to maintain a measurable nutrient flux that is significantly enriched from that of the control treatment.

There is also evidence that the relative availability of P and other resources can influence P toxicity. Because P toxicity of algal growth is concentration dependent, it is important to consider the bioavailability of diffused NDS P and water column P, which may adsorb to sediments, form complexes with Al- and Fe-oxides, or precipitate from metal complexes depending on stream hydrochemistry (Wetzel 2001). Rapid P-cycling clearly regulates both P limitation and P toxicity, and the one-time dissolved inorganic P measurements taken in most studies do not fully capture these dynamics. Furthermore, studies generally use colorimetric methods to measure dissolved inorganic P from the water column, which can underestimate

bioavailable P (e.g., organic forms, Van Moorleghem et al. 2013) and challenge our ability to connect NDS study results with P that is available to algal communities.

Additionally, studies suggest that nitrogen- or light-limitation could potentially induce toxicity at lower concentrations of P, if luxury P (Stevenson & Stoermer 1982a) accumulates without being used for growth due to limitation by other resources. Terrestrial plant studies have shown a positive relationship between P toxicity concentrations and N:P and K:P resource ratios (Grundon 1972), likely because high growth rates supported by N and K availability can reduce tissue P concentrations (Rossiter 1952). While these results may not translate directly to algae because of physiological differences between algae and terrestrial plants, P-inhibition of algal growth does tend to be stronger in shaded areas (Beck et al. 2017) where light may be limiting and where $NO_3^-$ reduction could be limited by the availability of NADPH from photosynthesis (Grant & Turner 1969). Thus, thresholds for P toxicity appear to be closely linked with the probability of secondary limitation by another resource, which would in part explain the inconsistency of reported P-inhibition in NDS studies as environmental conditions change among different ecosystems.

***Cation and Phosphate Form***

Few NDS studies have controlled for the effects of phosphate cation or phosphate form (monobasic vs. dibasic). However, experimental evidence suggests that toxicity thresholds differ based on the cation in the phosphate salt (Lehman 1976). In general, it appears K leads to toxicity at lower phosphate salt concentrations than Na. Growth of the freshwater chrysophyte *Dinobryon sociale* was maintained in the laboratory at P concentrations of 0.032 mmol $P \cdot L^{-1}$ for $NaH_2PO_4$ but declined when P concentrations were raised from 0.005 mmol $P \cdot L^{-1}$ to 0.019 mmol $P \cdot L^{-1}$ for

$KH_2PO_4$ (Lehman 1976). Similarly, a laboratory study on a cyanobacterium, *Microcystis* spp., showed lower toxicity thresholds for KCl as compared to NaCl (Parker et al. 1997), further supporting the potential for cation toxicity with the same cation but for different salts. However, across hundreds of published field studies, P effect sizes for algal biomass were higher for K phosphates as compared to Na phosphates (Beck et al. 2017). Taken together these results suggest that K-toxicity of algal biomass can be induced under laboratory conditions, but K concentrations in NDS phosphate salts may not be high enough to induce toxicity in field experiments.

Although the cation in the phosphate salt does not appear to strongly influence the effect of NDS P treatments on algal growth, it is likely that the phosphate form (monobasic vs. dibasic) used influences experimental outcomes by modifying pH at the surface of the NDS. In our experimental stream, the pH varied between 7.79 and 8.10, which differed substantially from the agar amended with monobasic phosphates (pH 4.81 ± 0.09) but was more comparable to the agar amended with dibasic phosphates (pH 8.88 ± 0.06). These differences in pH between the P treatments may have contributed to the difference in GPP and biomass-specific GPP we saw between these treatments, i.e., increased productivity on dibasic treatments as compared to the monobasic treatments. These experimental results are consistent with a previous meta-analysis which showed P and NP effect sizes for algal biomass were significantly higher on dibasic NDS treatments relative to those effects on monobasic treatments (Beck et al. 2017). Our meta-analysis in this study showed that P-inhibition was reported more commonly than N-inhibition of algal growth, which could be driven by P compounds containing easily disassociated $H^+$ ions while N compounds often do not. To avoid the artifact of pH on NDS P treatments we recommend that NDS experiments mix monobasic and dibasic phosphates to reflect the

background pH of study streams as best as possible. For many years, microbial cultivation studies have involved buffering nutrient-enriched media to prevent pH changes (Vacin & Went 1949), and this principle should be applied to NDS field studies as well. This simple step would avoid the confounding influence of alteration of pH in the P treatment of NDS experiments.

### *Heating Method*

We also investigated the potential for $H_2O_2$ production during the preparation of NDS P treatments to create an artifact in NDS experimental results, as has been hypothesized (Reisinger et al. 2016). We found that heating phosphate and agar together vs. separately on a hotplate did not produce significant differences in algal response metrics. We did not directly test whether $H_2O_2$ was produced in our experiments as was found by a laboratory study that autoclaved phosphate and agar together (Tanaka et al. 2014), but either $H_2O_2$ production requires the combination of heat and pressure (from autoclaves) and was not produced in this experiment, or the concentrations of $H_2O_2$ in our experiments were not high enough to inhibit algal growth. A simple solution to avoid the potential inhibiting effect of $H_2O_2$ would be to heat P and agar separately (described by Tank et al. 2017) to avoid the possibility of $H_2O_2$ interference with algal growth. This approach would require little extra effort in NDS experiment preparation and completely remove this potentially confounding artifact.

### *Microbial Competition*

Indirect mechanisms have also been proposed to explain why P additions commonly inhibit algal growth. Heterotrophic and autotrophic microbial communities interact in complex ways that may change along nutrient gradients. At low concentrations of P, heterotrophic microbes are expected to be competitively dominant because of their strong affinity for P and

high surface area relative to volume (Brown et al. 1981). However, heterotrophs may also regenerate nutrients that can stimulate autotrophic production and autotrophs produce organic C that fuels heterotrophy, leading to a coupling of the two communities (Scott et al. 2008; Hoagland et al. 1993). When nutrients are added to the system these community dynamics are altered, affecting the biomass and diversity of both heterotrophs and autotrophs within the biofilm (Pepe-Ranney & Hall 2015). A study of Idaho streams found a strongly stimulated AI (i.e., a higher proportion of heterotrophs) when C was added, and a weakly stimulated AI when P was added (Bechtold et al. 2012). Furthermore, a study of Texas streams showed a decoupling of autotrophic and heterotrophic production when nutrients were added (Scott et al. 2008). Our experiments and meta-analysis produced no evidence that heterotrophic-autotrophic interactions were influenced by P additions (i.e., no change in AI), but it is clear that environmental variability of C, N, P, and other nutrients may influence microbial interactions within and among streams. Background nutrients were not considered in the meta-analysis model, but incorporating nutrient pools and dynamics in future studies may lead to a more predictive understanding of autotroph-heterotrophic interactions over space and time. We also recommend that future studies consider alternative response metrics for measuring heterotrophic microbial biomass if heterotrophic estimates are required to answer study-specific research questions.

### *Grazer Selection*

In addition to heterotrophic microbes affecting algal growth through ecological interactions, we hypothesized that P effect sizes on grazer exclusion treatments may be larger than on grazed treatments because some grazers have been shown to selectively consume P-enriched resources (Hood et al. 2014; Mooney et al. 2016). Primary consumers exhibit preferences for different types of resources (e.g., detritus vs. periphyton) based on their

nutritional content (Hood et al. 2014). For instance, a study of forested stream segments showed that despite low algal productivity, over 50% of invertebrate biomass depended at least partially on algal food resources (McNeeley et al. 2007). However, within periphyton mats, it has been challenging to determine whether N- or P-specific foraging occurs. A recent study found that periphyton C:P and N:P increased in the presence of *Glossosoma intermedium* (Mooney et al. 2016) . However, this result could suggest either of two effects that are challenging to disentangle: selective feeding of *G. intermedium* on P-rich periphyton, or higher P-retention by *G. intermedium.* Our meta-analysis did not produce evidence to suggest that P effect sizes differed between grazed and ungrazed plots in NDS experiments. However, very few studies to date have investigated resource selectivity in grazers under field conditions (n=5 experiments in the meta-analysis; Flecker et al. 2002; Lourenco-Amorim et al. 2014; Winterbourn & Fegley 1989). Theoretical models show that nutrient-specific foraging could have important ecological consequences in streams (Neeson et al. 2013), and we recommend that future experiments consider the interactions between nutrient-specific foraging and algal nutrient limitation to determine whether grazing leads to apparent P-inhibition of algal growth. One option is to construct electrical exclusion treatments which can prevent grazing from macroinvertebrates and vertebrates across a wide range of body sizes (Lourenco-Amorim et al. 2014; Pringle & Blake 1994; Moulton et al. 2004).

### *Additional Considerations*

There are several potential reasons why our experiments did not show significant P treatment effects that have previously been described in the literature. First, deploying only six replicates per treatment produced low statistical power, given the large number of treatments and the small effect sizes of P additions in our NDS experiment. Furthermore, phosphate and cation

inhibition of algae have been demonstrated using controlled laboratory experiments that can achieve a wider and more precise concentration gradient as compared to field experiments. We previously observed P-inhibition of algal biomass at the same stream reach used in this study. However, NDS diffusion rates (Corkum 1996), environmental characteristics, and algal community composition in the field are clearly variable over space and time, which may have obscured our ability to connect field-scale results with proposed mechanisms that are largely based on laboratory studies and meta-analyses of field studies.

**Conclusion**

Phosphorus additions have significantly inhibited algal biomass in 12.9% of past NDS studies, and investigators have hypothesized that this may be an artifact of NDS preparation or an indirect effect of increased heterotrophic microbial competition or top-down grazer control. We found that phosphate form (monobasic vs. dibasic) likely influences algal growth on NDS P treatments by mediating biofilm pH levels, and acidic monobasic treatments may inhibit algal growth. Furthermore, the literature supports direct P toxicity as a mechanism for P-inhibition of algal growth (Chu 1943; Lehman 1976; Beck et al. 2017), particularly when other resources such as light or N are limiting (Jensen & Sicko-Goad 1976). We did not find support for phosphate salt cation toxicity occurring under field conditions, nor did we find evidence that laboratory heating method influenced algal responses to P. Based on our analyses, it is also unlikely that P stimulates heterotrophic microbes relative to autotrophic microbes or that P stimulates grazing rates.

Considering that multiple mechanisms may be operating simultaneously to inhibit algal growth on NDS P treatments, we recommend several low effort, cost-effective steps for the NDS

preparation process that could reduce the potential for P-inhibition in future experiments. First, future experiments could measure background stream nutrient concentrations to determine the most appropriate P treatment concentrations for NDS construction (Keck & Lepori 2012), with the goal of avoiding the potential for levels of P that are directly toxic to algae. In addition, measuring NDS diffusion rates (Capps et al. 2011; Costello et al. 2016; Rugenski et al. 2008) under conditions that mimic natural systems would allow investigators to further determine appropriate concentrations and experimental lengths for NDS studies. Experiments should be long enough to surpass the potential for initial P toxicity when diffusion concentrations are at their highest but short enough to maintain stimulatory P diffusion from NDS. Because monobasic and dibasic phosphates influence NDS pH levels, we encourage investigators to mix the two phosphate forms to reflect the background pH in experimental streams to the extent possible. Finally, while we did not find evidence that laboratory construction methods inhibited algal growth, it seems prudent (and logistically simple) to use the separate agar and phosphate heating methods outlined by Tank et al. (2017) to avoid the potential for $H_2O_2$ production that inhibits microbial growth (Tanaka et al. 2014). Avoiding confounding factors in NDS experiments will ensure that studies are not underestimating P-limitation of primary producers in aquatic ecosystems, improving our understanding of how resources and environmental conditions interact to affect algal growth and stream ecosystems as a whole.

**Figures**

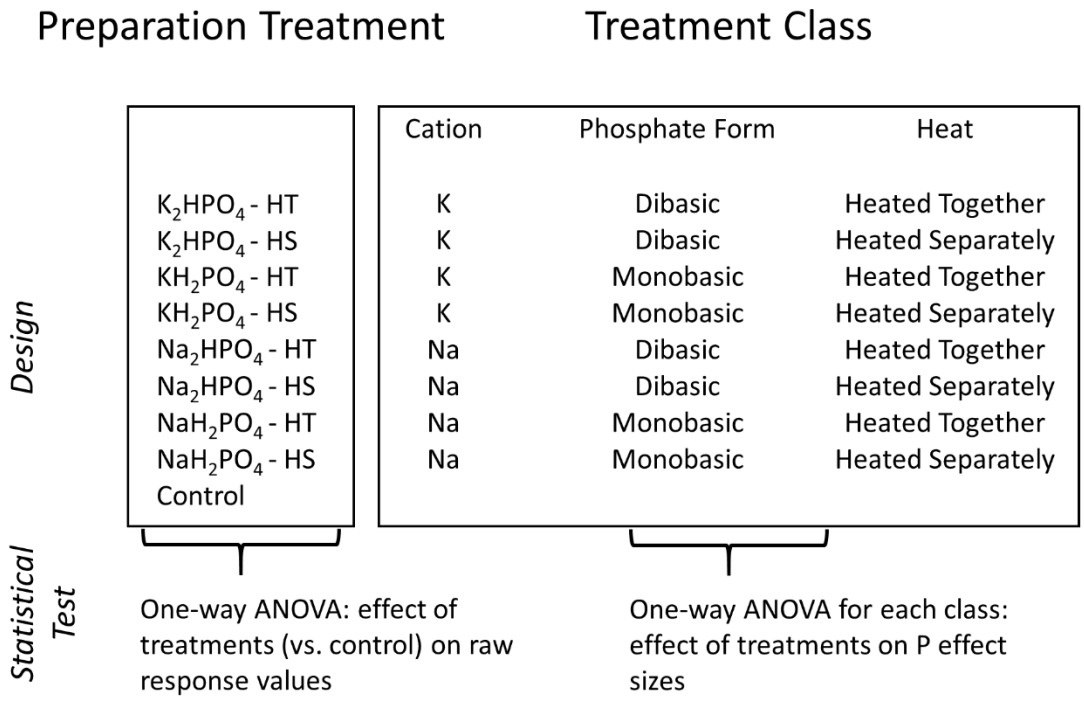


**Figure 3.1: Field experiment preparation methods and treatment classes. Four different phosphate chemicals crossed with laboratory heating methodology were deployed in two NDS experiments in 2016, for a total of eight preparation treatments. The eight treatments were grouped by three treatment classes including cation, phosphate form, and heating method. The same preparation treatments were crossed with two different phosphate concentrations in a 2017 experiment, for a total of sixteen preparation treatments. The sixteen treatments were grouped by four treatment classes including cation, phosphate form, heating method, and concentration. Heated Together = phosphate and agar boiled together, Heated Separately = agar boiled and phosphate added at pouring temperature of 55-65° C.**

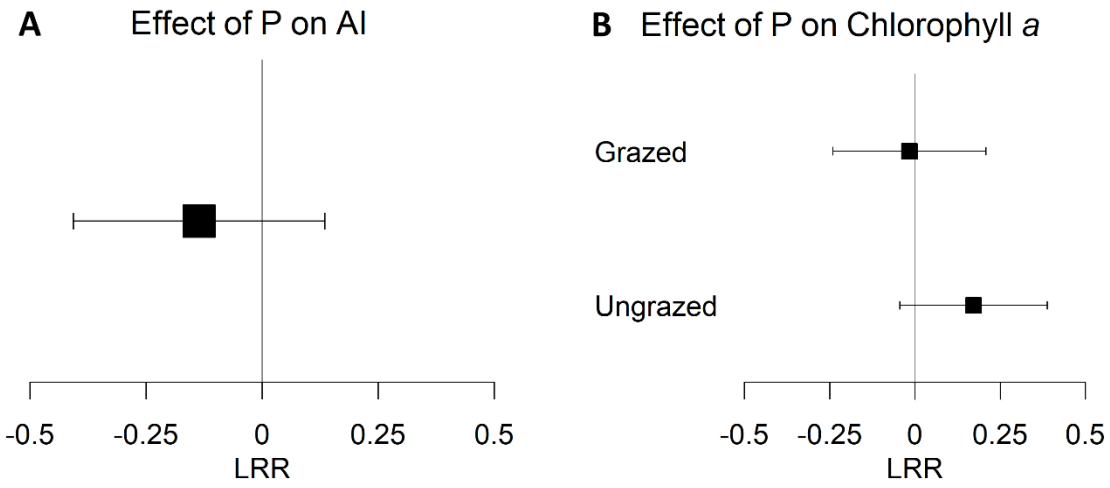


**Figure 3.2: Meta-analysis model results. (A) Results of a meta-analysis model testing the significance of NDS P treatment autotrophic index (AI) effect sizes (n=45 experiments), where lower values indicated a higher proportion of autotrophy in microbial communities. (B) Results of a meta-analysis model testing the effect of grazing on NDS P treatment algal biomass (chlorophyll *a*) effect sizes (n=5 experiments per grazing treatment). Squares are log response ratio (LRR, see equation 2) mean estimates surrounded by 95% confidence interval bars.**

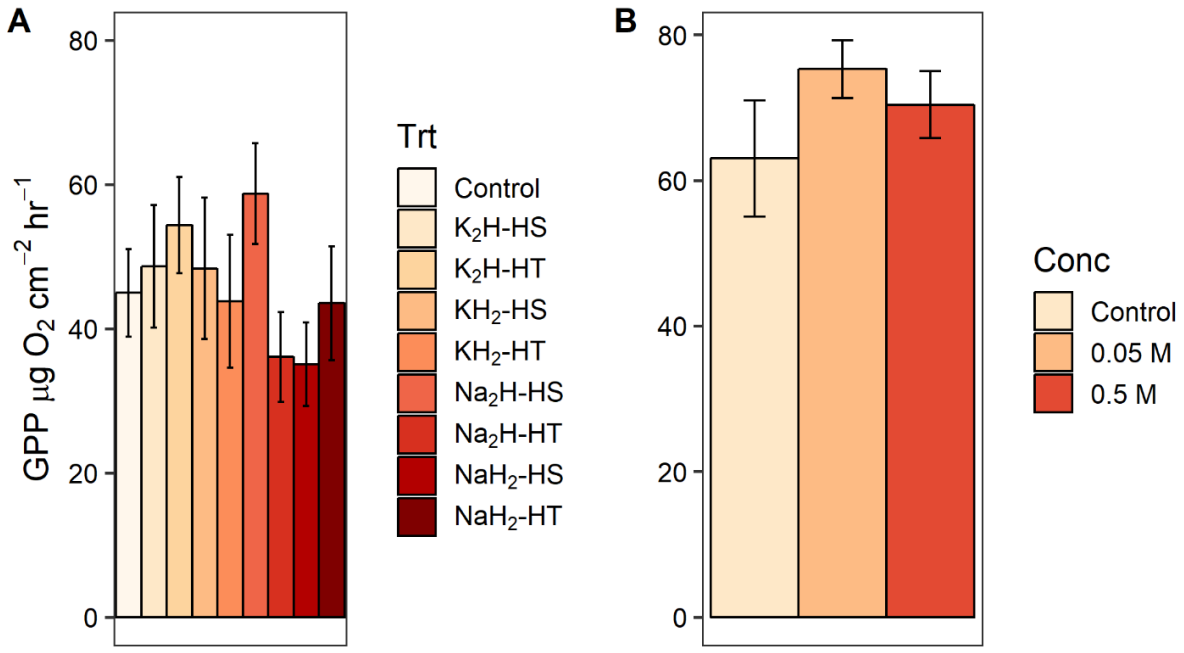


**Figure 3.3: Effect of experimental preparation treatments on gross primary productivity (GPP). Gross primary productivity means ± 1 standard error from nutrient diffusing substrate (NDS) field experiments. (A) Results from experiments 1-2 (total n=103), where treatments consisted of crossing four chemicals ($K_2HPO_4$, $KH_2PO_4$, $Na_2HPO_4$, and $NaH_2PO_4$) and two heat treatments (agar and phosphate heated together vs. heated separately, denoted as "HT" and "HS"). An agar-only control treatment was included in each experiment. While no individual treatments significantly differed from the control, we found that monobasic phosphate forms significantly inhibited GPP compared to dibasic forms. (B) Results from experiment 3 (total n=104), where treatments included the same factors as experiments 1-2, except low (0.05 M) and high (0.5 M) phosphate concentrations were included as an additional factor. Gross primary productivity values by concentration are presented by averaging over all other factors.**

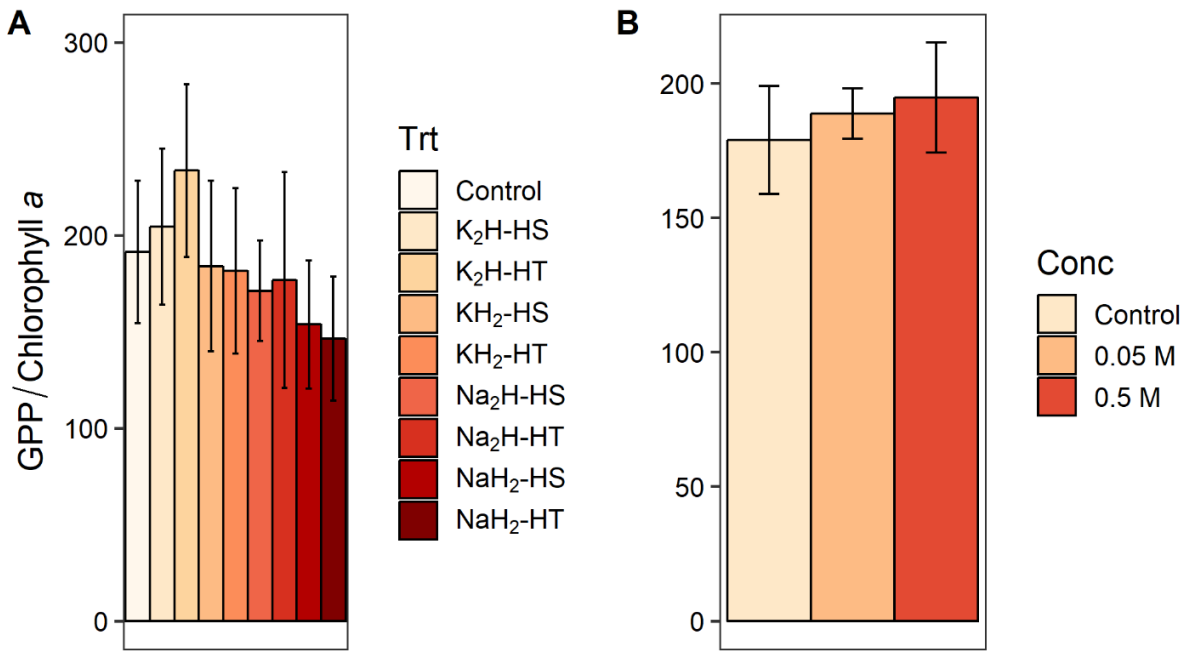


**Figure 3.4: Effect of experimental preparation treatments on biomass-specific gross primary productivity (GPP). Biomass-specific GPP means ± 1 standard error from NDS field experiments (see Fig 2 caption). (A) For experiments 1-2, total n=102. While no individual treatments significantly differed from the control, we found that monobasic phosphate forms significantly inhibited biomass-specific GPP compared to dibasic phosphate forms. (B) For experiment 3, total n=103.**

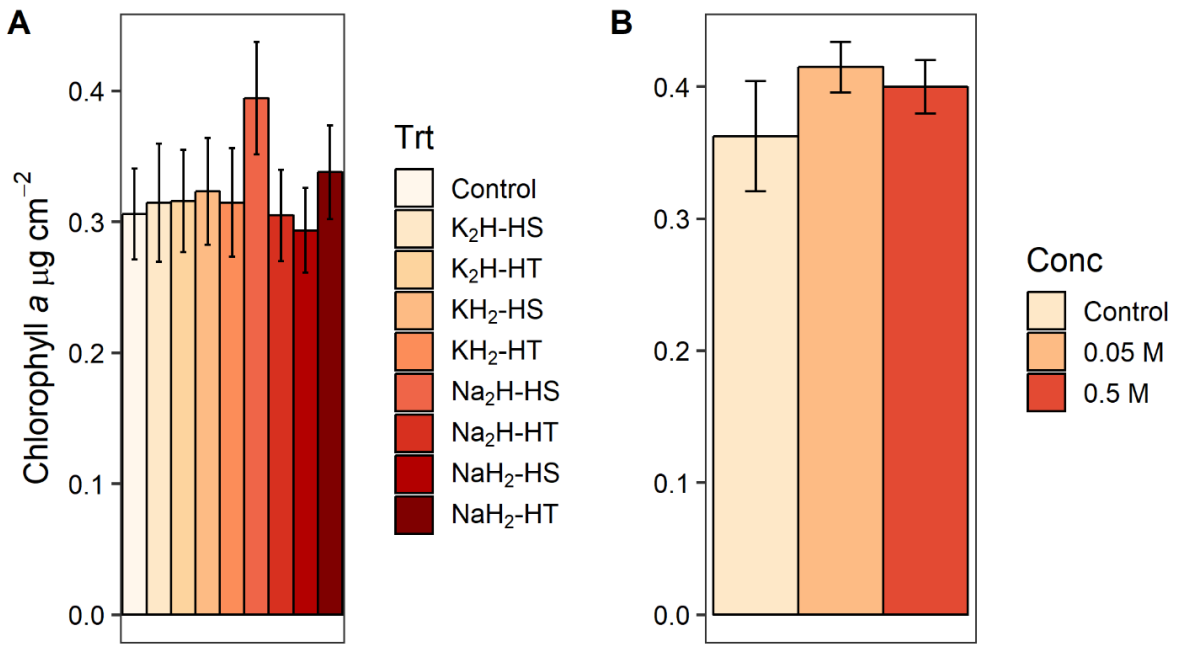


**Figure 3.5: Effect of experimental preparation treatments on chlorophyll *a*. Chlorophyll *a* means ± 1 standard error from NDS field experiments (see Fig 2 caption). (A) For experiments 1-2, total n=107. (B) For experiment 3, total n=105.**

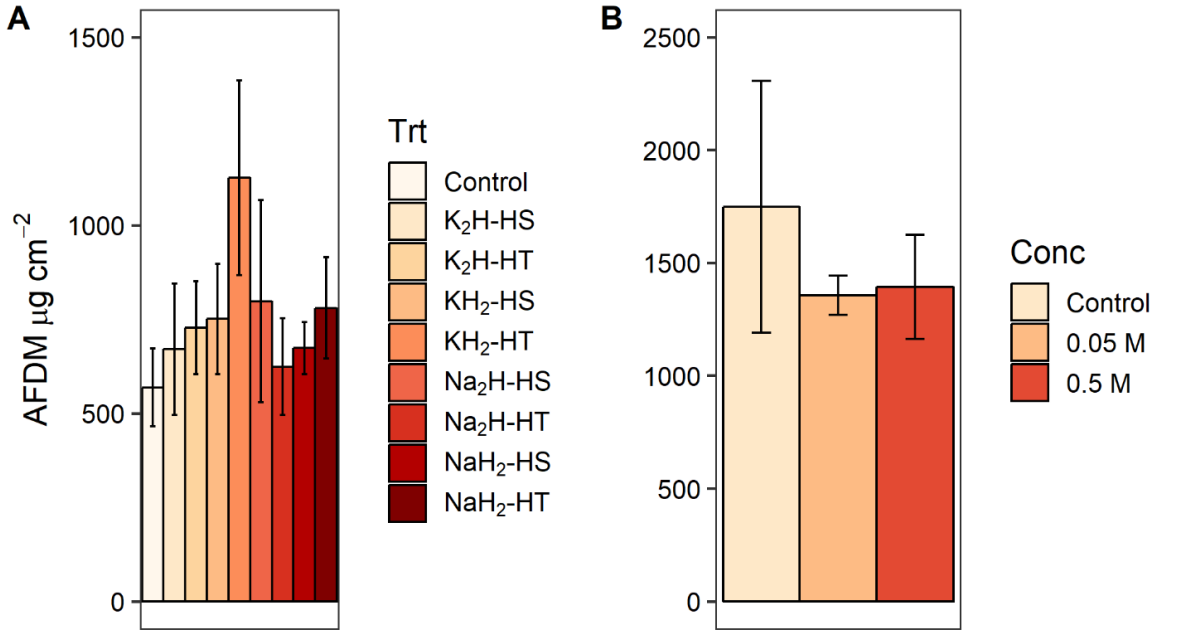


**Figure 3.6: Effect of experimental preparation treatments on ash-free dry mass (AFDM). Ash-free dry mass means ± 1 standard error from NDS field experiments (see Fig 2 caption). (A) For experiments 1-2, total n=93. (B) For experiment 3, total n=104.**

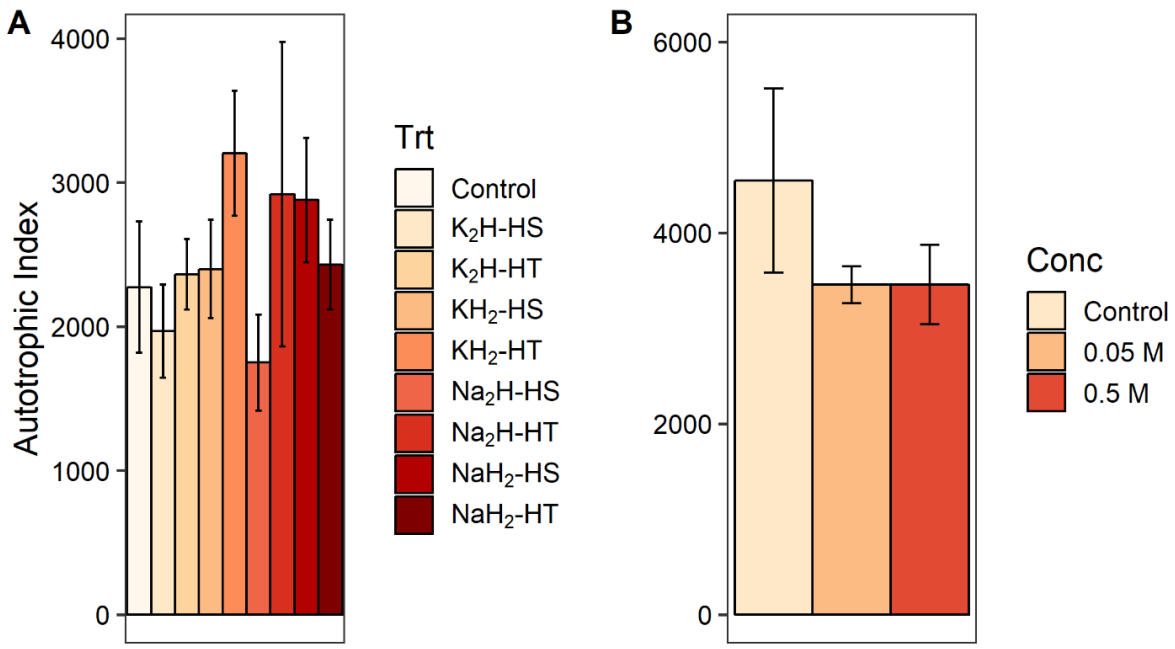


**Figure 3.7: Effect of experimental preparation treatments on an autotrophic index (AI). Autotrophic index means ± 1 standard error from NDS field experiments (see Fig 2 caption). (A) For experiments 1-2, total n=92. (B) For experiment 3, total n=104.**

# CHAPTER 4: SEASONAL SHIFTS IN THE IMPORTANCE OF BOTTOM-UP AND TOP-DOWN FACTORS ON STREAM PERIPHYTON COMMUNITY STRUCTURE[4]


## Summary

We examined the importance of temporal variability in top-down and bottom-up effects on the accumulation of stream periphyton, which are complex associations of autotrophic and heterotrophic microorganisms. Periphyton contributes to primary production and nutrient cycling and serves as a food resource for herbivores (grazers). Periphyton growth is often limited by the availability of nitrogen and phosphorus, and biomass can be controlled by grazers. In this study we experimentally manipulated nutrients and grazers simultaneously to determine the relative contribution of bottom-up and top-down controls on periphyton over time. We used nutrient diffusing substrates to regulate nutrient concentrations and an underwater electric field to exclude grazing insects in three sequential 16-17 day experiments from August to October in montane Colorado, USA. We measured algal biomass, periphyton organic mass, and algal community composition in each experiment and determined densities of streambed insect species, including grazers. Phosphorus was the primary limiting nutrient for algal biomass, but it did not influence periphyton organic mass across all experiments. Effects of nutrient additions on algal biomass and community composition decreased between August and October. Grazed substrates supported reduced periphyton biomass only in the first experiment, corresponding to high benthic abundances of a dominant mayfly grazer (*Rhithrogena spp.*). Grazed substrates in


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[4]This chapter is an edited version of: Beck, W. S., Markman, D. W., Oleksy, I. A., Lafferty, M. H., and Poff, N. L. (2018). Seasonal shifts in the importance of bottom-up and top-down factors on stream periphyton community structure. *Oikos*, *in press*. https://doi.org/10.1111/oik.05844

the first experiment also showed altered algal community composition with reduced diatom relative abundances, presumably in response to selective grazing. We showed that top-down grazing effects were strongest in late summer when grazers were abundant. The effects of phosphorus additions on algal biomass likely decreased over time because temperature became more limiting to growth than nutrients, and because reduced current velocity decreased nutrient uptake rates. These results suggest that investigators should proceed with caution when extending findings based on short-term experiments. Furthermore, these results support the need for additional seasonal-scale field research in stream ecology.

## Introduction

Streambed periphyton is a complex association of autotrophic and heterotrophic microbes that can facilitate stream nutrient cycling (Battin et al. 2003) and provide a food source for primary consumers (Feminella & Hawkins 1995). It is critical to understand factors that regulate the biomass and composition of periphyton because of its important role in stream food webs and ecosystem functioning. Furthermore, stream periphyton serves as a water quality indicator because these species-rich communities respond rapidly to environmental changes (Stevenson 2014), and nuisance periphyton blooms may impose significant burdens on human health and ecosystems which requires management intervention to reduce biomass levels (Carpenter et al. 1998). A wealth of research has shown that periphyton can be regulated by both bottom-up and top-down factors (Francoeur 2001; Hillebrand 2002; Elser et al. 2007; Hillebrand 2009), although the strength of periphyton responses to resource additions and herbivory is influenced by stream context. Factors that change over time such as disturbance, nutrients, temperature, light, and predator and prey densities may all influence the strength of bottom-up and top-down forces in natural ecosystems (Power 1992), thereby limiting the temporal scope of inference for

many studies. Experiments and conceptual models from terrestrial (Hunter & Price 1992; Boyer et al. 2003; Gratton & Denno 2003) coastal marine (Thompson et al. 2008; Whalen 2013), and lentic systems (Weisse 1991) have incorporated temporal heterogeneity when determining the strength of bottom-up and top-down factors, but experiments from stream systems have rarely considered temporal variation in these factors.

Studies focusing on bottom-up effects on periphyton have added nutrients to streams, streamside channels, or mesocosms and measured responses of algal biomass (Fairchild et al. 1985), fungal biomass (Tank & Dodds 2003), bacterial abundance (Hoch 2008), and periphyton organic mass (Bechtold et al. 2012). Regardless of the microbial group being considered, periphyton growth is commonly limited by nitrogen (N) and phosphorus (P) availability in stream ecosystems (Francoeur 2001; Elser et al. 2007; Beck et al. 2017). Furthermore, nutrient availability alters periphyton community composition by mediating microbial competitive (Brown et al. 1981) and facilitative (Lang et al. 2012) interactions.

Periphyton responses to nutrients may also be highly dependent on variability in stream environmental conditions such as light, temperature, and current velocity (Beck et al. 2017). Nutrient diffusing substrates (NDS) are often used to experimentally test algal responses to N and P additions by providing artificial colonization surfaces for periphyton (Fairchild et al. 1985; Francoeur 2001), and NDS have been used to demonstrate the importance of stream context. For instance, NDS experiments showed enhanced algal responses to a limiting nutrient (N) as stream light levels increased (Taulbee et al. 2005). Furthermore, seasonal manipulations of NDS have demonstrated higher levels of algal nutrient limitation in summer, most likely because of the warmer temperatures (Francoeur et al. 1999). These studies show that under some conditions, light or temperature can be more limiting to algal growth than nutrients. Finally, NDS have

shown that stream current velocity can increase algal biomass responses to limiting nutrients (Hoch 2008) because of increased nutrient uptake with faster current (Borchardt et al. 1994).

Top-down control of periphyton by grazers such as aquatic insects and snails has also been demonstrated in many laboratory and field studies (Feminella & Hawkins 1995; Lamberti et al. 1995). Indeed, a meta-analysis found that grazer removal had a stronger positive effect on periphyton biomass than nutrient additions, although both effect sizes were significant (Hillebrand 2002). Grazers consume periphyton but may also cause non-consumptive biomass losses through physical disruption of periphyton communities (Eichenberger & Schlatter 1978; Lamberti et al. 1995). Furthermore, grazers have been shown to change algal community composition by selectively removing palatable diatoms (Rosemond et al. 1993) or overstory taxa (Feminella & Hawkins 1995), depending on the morphological traits of the grazers and growth forms of periphyton communities being studied (Steinman 1996).

Stream biological and environmental conditions such as grazer densities, predator densities, temperature, and current velocity all vary over time and can substantially influence top-down control of periphyton by grazers. In field experiments, higher grazer abundances are linked to higher periphyton consumption rates (Hillebrand 2009), but grazer abundances change over time based on species' phenologies and grazing rates may decrease with predator abundances (e.g., Lourenço-Amorim et al. 2014). Temperature increases metabolic rates of grazers and may lead to higher periphyton consumption rates, as has been demonstrated across a wide range of laboratory and field experiments in lentic, lotic, and marine systems (Hillebrand 2009). Finally, consumption rates may depend on how the grazer of interest responds to variation in stream current velocity (Poff et al. 2003).

A number of studies have also quantified periphyton responses to interactions between bottom-up and top-down factors (reviewed by Hillebrand 2002), but rarely have these interactions been investigated over time. Seasonal changes incorporate largely predictable shifts in environmental conditions that are likely to affect bottom-up and top-down influences on periphyton, but only one previous field study has examined the seasonal changes in resource and grazer regulation of periphyton. Rosemond et al. (2000) held grazing snail densities constant in experimental streamside channels and found that snails significantly reduced periphyton biomass and altered periphyton community composition across three seasons. Furthermore, resource additions generally only influenced periphyton structure when grazers were removed. The interaction between nutrients and grazers in regulating periphyton biomass and community composition has not previously been examined with in-stream experiments that account for seasonal changes in grazer densities. Yet these seasonal changes are likely to be important, as studies from lake (Weisse 1991) and tidal systems (Thompson et al. 2008) have shown seasonally-variable top-down pressure on phytoplankton because of changes in grazer abundances. Additionally, research has shown that seasonal changes in resource quality and predator abundances influence the strength of bottom-up and top-down control on herbivores in grassland systems (Boyer et al. 2003; Gratton & Denno 2003).

In this study, we sought to understand how seasonal shifts in abiotic limiting factors and insect grazer abundance affect the relative importance of top-down and bottom-up factors on periphyton community structure in a temperate mountain stream. We completed a series of in-stream experiments from summer to early fall, using NDS to add nutrients (Fairchild et al. 1985, Tank et al. 2017) and underwater electric fields to exclude grazers (Pringle & Blake 1994; Opsahl et al. 2003; Moulton et al. 2004; Lourenço-Amorim et al. 2014). We measured multiple

periphyton responses to these treatments including algal biomass, periphyton organic mass, an autotrophic index (AI), and algal community abundances of chlorophytes (green algae), bacillariophytes (diatoms), and cyanobacteria.

## Methods

### *Study Site and Design*

We completed experiments in the South Fork Poudre River at the Colorado State University Mountain Campus (40.57° N, -105.59° W), a low-order stream with an elevation of 2740 meters. We selected an open-canopy study reach with sand, gravel, and cobble substrate. Three experiments were deployed sequentially in the same reach: August 12-28 (Exp 1), September 1-17 (Exp 2), and September 17-October 4 (Exp 3) of 2017. During each experiment, we employed a split-plot design and designated two replicate grazer exclusion plots and two replicate control plots (the whole plot factor) with light, velocity, and depth conditions that were as homogeneous as possible. Each plot contained six replicate vials of four nutrient treatments (the sub-plot factor).

### *Nutrient Addition Treatments*

We constructed NDS (Tank et al. 2017) by filling 30 mL plastic vials (Item #66159, U.S. Plastic Corp, Lima, OH) with either 2% agar (control treatment), agar + 0.5 M $NaNO_3$ (N treatment), agar + 0.05 M $KH_2PO_4$ + 0.05 M $K_2HPO_4$ (P treatment), or agar + all three nutrient chemicals (NP treatment). Two types of phosphate were used to create an agar pH that was close to neutral, to avoid any confounding influences of pH alterations on periphyton (Beck and Hall 2018). Individual vials were capped with fritted glass discs (5.7 $cm^2$, Item #C4505, EA Consumables, Pennsauken, NJ) and randomly attached to three plastic L-bars (Item #45031, U.S.

Plastic Corp, Lima, OH) so that each plot contained three parallel L-bars holding six replicates of each of the four nutrient treatment types (Fig. 4.1). The L-bars were submerged and anchored to paving stones (15 cm x 22 cm) using zip-ties.

***Electrical Exclusion of Grazers***

To measure the effect of aquatic insect grazers on periphyton communities, we constructed a solar-powered, battery-operated electrical exclusion system (Fig. 4.1) modified from Lourenço-Amorim et al. (2014) and Moulton et al. (2004). A 100-watt solar panel (Acopower, Walnut, CA) was wired in parallel to a 10 amp rated charge regulator (Sunforce, Montreal West, QC) using insulated cables. The charge regulator supplied and regulated the charge of two 12-volt, 35 amp-hour, deep-cycle sealed lead acid batteries (Mighty Max, Edison, NJ) which were wired in parallel using 8 gauge insulated wire repurposed from automotive jumper cables. Batteries supplied power to a Speedrite™ 6000 electric fence energizer (Tru-test Ltd., New Zealand), which provided 6700 volts at 500 ohms with a maximum output of 6 Joules. In accordance with Ohm's Law, a high-voltage energizer was necessary to account for low stream water conductivity (Utz et al. 2017) and the small body size of the stream grazers to be excluded (Lourenço-Amorim et al. 2014). Two 12-meter lengths of insulated 12.5-gauge steel wire were connected to the "active" (positive) terminal of the fence energizer and two identical lengths of wire were connected to the "ground" (negative/relative ground) terminal. The opposite end of each wire was spliced to 12-gauge uninsulated copper wire using specialized wirenuts (Ideal Twister Al/Cu, Ideal Industries, Ontario, Canada) to avoid galvanic corrosion between dissimilar metals. Splice connections were filled with waterproof dielectric grease prior to sealing with heat shrink tubing to ensure water resistance.

Rectangular exclosures were created on the streambed immediately surrounding the treatment vials using the uninsulated copper wires originating from the positive terminal of the fence energizer. Plastic tent stakes were used to maintain the rectangular configuration of the exclosure. The uninsulated copper wires originating from the ground terminal of the fence energizer were anchored ~1 cm above the center L-bar in each electrified plot to generate an electrical gradient covering the entire treatment plot (Fig. 4.1). The electrical exclosures measured 45 cm by 30 cm and electrified and control plots were 1-2 meters apart to prevent interaction. Previous research has shown that electrical exclosures do not significantly influence periphyton growth rates (Brown et al. 2000). To ensure the fence was excluding a broad range of insect size classes, we observed the behavior of Ephemeroptera, Plecoptera, and Coleoptera individuals when exposed to the electrified exclosures. Twitching and contractions were observed across all tested insect orders during electric pulses. Insects were unaffected if they were greater than ~5 cm outside the exclosure. The fence energizer delivered an electric pulse every 2.5 seconds during the day and every 1.5 seconds at night, with more frequent pulses at night to account for increased insect drifting (Waters 1972).

***Environmental Variables***

At the beginning and end of each experiment, we measured fine-scale flow velocity immediately above NDS discs at three points on each L-bar using a 2.5 cm-scale MiniWater® 20 flow meter (Schiltknecht, Switzerland). At the beginning of each experiment, we also measured canopy cover at each plot using a densiometer (Forestry Suppliers, Jackson, MS) and collected duplicate filtered and unfiltered water samples from one point upstream. We filtered stream water through Type A/E glass fiber filters (0.45 µm retention, Pall Corporation, Port Washington, NY) into 60 mL Nalgene® bottles and analyzed samples for $NO_3^-$ using the Cd

reduction method (U.S. EPA Method 353.2 1993) and orthophosphate using the ascorbic acid method (Murphy & Riley 1962) on an ALPKEM® Flow Solution IV autoanalyzer (O.I. Analytical, College Station, TX). We analyzed unfiltered stream water for total N using a Shimadzu TOC/TN analyzer (Shimadzu Scientific Instruments, Inc.). During each experiment, we took at least six underwater photographs per plot on three different dates to investigate algal disc colonization by invertebrates. At the end of each experiment, we measured streamflow using a Marsh McBirney meter (Hach, Loveland, CO) and measured pH, conductivity, and temperature using a multimeter and probe (Thermo Fisher Scientific, Waltham, MA). Finally, at the end of each experiment we collected two Hess samples of aquatic invertebrates from the streambed surrounding the experimental plots to characterize community composition. In the laboratory, we used a dissecting microscope to separate macroinvertebrates from the substrate. The invertebrates were then identified to the lowest taxonomic unit needed to assign functional traits (Poff et al. 2006), which was genus for most individuals. Over 1000 individuals were identified from 31 taxa groups.

***Response Variables and Analysis***

At the end of each experiment, we collected NDS discs and stored them at -20° C until chlorophyll *a* analysis within 30 days. We extracted chlorophyll *a* (a measure of algal biomass) directly from four replicate discs using 90% buffered ethanol, and we quantified the pigment mass using a handheld Aquafluor® fluorometer (Turner Designs, San Jose, CA) with an acidification correction (U.S. EPA 1997).

After chlorophyll *a* analysis, we allowed the liquid from the extraction slurry and NDS discs to evaporate in weigh boats under a fume hood then measured ash-free dry mass (AFDM,

APHA 2005) using all remaining material (Bechtold et al. 2012). Ash-free dry mass incorporates not just algal biomass, but also the biomass of heterotrophic microbes and detritus from periphyton. We dried the weigh boat contents (including the NDS discs and particulates from the slurry) for 48 hours at 50° C, measured their initial masses, and combusted them at 500° C for one hour. We then rehydrated the weigh boat contents with deionized water, dried them for another 48 hours at 50° C, and measured their final masses. The rehydration step allowed us to account for water that was lost from clay particles during combustion. The difference in initial and final masses was calculated as AFDM, and we calculated an autotrophic index (AI) as the ratio of chlorophyll *a* to AFDM (APHA 2005). Lower values of the index indicated a higher proportion of autotrophy in the microbial community (Bechtold et al. 2012). We also used chlorophyll *a* values and periphyton AFDM values to calculate separate log response ratios (LRR, a measure of effect size) for electrical exclusions and nutrient additions:

$$LRR_E = \ln \frac{\text{electric mean}}{\text{control mean}} \quad \text{(Eqn. 4.1)}$$

$$LRR_N = \ln \frac{\text{nutrient mean}}{\text{control mean}} \quad \text{(Eqn. 4.2)}$$

Treatments had a positive effect on the response variable of interest when the LRR was greater than zero and a negative effect when the LRR was less than zero (Tank & Dodds 2003).

We used the remaining two replicate discs to determine algal community composition using a UPLC-UV-MS system, modifying the procedure of Fu et al. (2012). Previous studies have shown strong agreement between LC-based measurements and microscopic determinations of algal community composition (Wright et al. 1996; Schlüter et al. 2006). Variation in environmental factors like light levels can obscure relationships between the two measurement methods (Havens et al. 1999), but environmental conditions among our plots were highly

standardized compared to studies of lake and ocean phytoplankton communities. We measured three target pigments to capture variability in algal community composition: chlorophyll *b* for chlorophytes (green algae), fucoxanthin for bacillariophytes (diatoms), and myxoxanthophyll for cyanobacteria (Leavitt & Hodgson 2002). Hereafter, we use the algal group name rather than the pigment name. We also measured total chlorophyll *a* as the sum of chlorophyll *a* and three primary breakdown products in our samples, including chlorophyll *a '*, pheophytin *a*, and pheophytin *a '* (Sartory 1985). We extracted algal pigments directly from discs using an 85:10:5 by volume acetone:methanol:water solution (Steinman et al. 2017). We filtered the extractant through 0.22 µm nylon syringe filters (Argos Technologies Inc., Vernon Hills, IL) and dried the solution under $N_2$ gas until no liquid remained (Steinman et al. 2017). We then resuspended the pigments with a 1:1 by volume acetonitrile:MTBE solution (Fu et al. 2012). We used a maXis™plus Q-TOF mass spectrometer (Bruker Corporation, Billerica, MA) to identify pigments based on their known masses, which were confirmed with pigment standards (DHI Lab Products, Denmark). During 6-minute runs for each sample, we used an Acquity ultra performance liquid chromatography (UPLC®) system with a tunable UV detector (Waters Corporation, Milford, MA) to separate pigment compounds from sample mixtures and measure their intensities (Fu et al. 2012). We integrated UV curve areas using Compass Hystar data software (Bruker Corporation, Billerica, MA). We used calibration curves from pigment standards to convert UV areas to masses (Leavitt & Hodgson 2002), then standardized community composition masses by total biomass as follows:

$$\text{Chlorophytes} = \frac{\text{chlorophyte mass}}{\text{total chlorophyll } a \text{ mass}} \quad \text{(Eqn. 4.3)}$$

$$\text{Bacillariophytes} = \frac{\text{bacillariophyte mass}}{\text{total chlorophyll } a \text{ mass}} \quad \text{(Eqn. 4.4)}$$

$$\text{Cyanobacteria} = \frac{\text{cyanobacteria mass}}{\text{total chlorophyll } a \text{ mass}} \quad \text{(Eqn. 4.5)}$$

***Statistical Analyses***

We performed statistical analyses in R version 3.5.0 (R Core Team 2018). To analyze data from the split plot design, we separated the analyses by experiment and used ANOVAs with electricity and nutrients as factors along with plot as a random block to quantify treatment effects on algal biomass (chlorophyll *a*), periphyton AFDM, and AI. We used ANOVAs with electricity and nutrients as factors but no random block for the algal community response metrics, because we sampled fewer replicates and therefore had lower statistical power in those models. Finally, we used one-way ANOVAs with experiment as a predictor and aquatic insect order abundances as response variables to test for changes in streambed insects. When there was a particularly abundant family within an order, we used that family as a separate response variable. For all models, we used contrasts of least-squared means with Tukey-adjusted p-values to determine significant differences among factors ($\alpha = 0.05$).

**Results**

Both nutrients and insect grazing regulated periphyton biomass metrics and community composition, but the independent and interactive effects depended on the response variable and time period being considered. Nutrients had a significant effect on algal biomass in experiment 1 ($F_{3,55}$=127.503, p<0.001) and experiment 2 ($F_{3,56}$=53.534, p<0.001), with higher values on P and NP treatments than no-nutrient controls (p<0.001). Nutrients also had a significant effect on algal biomass in experiment 3 ($F_{3,53}$=5.877, p=0.002), with NP treatments being marginally significantly higher than controls (p=0.068). Nutrients influenced AI in experiment 2 ($F_{3,53}$=5.068, p=0.003), with reduced values and therefore higher autotrophy on P and NP

treatments (p=0.011 and p=0.018). Periphyton AFDM did not respond to nutrients in any of the experiments (p>0.05).

Algal community composition was also modified by nutrients, with strongest effects occurring earlier in the study (Fig. 4.3). In the first experiment, bacillariophytes ($F_{3,23}$=8.915, p<0.001) and cyanobacteria ($F_{3,21}$=8.389, p<0.001) were higher on the NP treatments (p<0.001 for both). In the second experiment, nutrient treatment only influenced cyanobacteria ($F_{3,21}$=14.260, p<0.001), which was higher on NP treatments compared to no-nutrient controls (p<0.001). No algal groups responded to nutrient treatments in the third experiment (p>0.05).

The effects of grazing on periphyton biomass metrics and algal community composition were determined using electrical exclusion and were strongest in the first experiment (Figs. 4.2, 4.4). Periphyton AFDM and AI were significantly higher in electrical exclusion plots (i.e., reduced grazers) as compared to grazed controls ($F_{1,51}$=22.325, p=0.0470 and $F_{1,51}$=43.302, p<0.001), but algal biomass was not influenced by electricity (p>0.05). Bacillariophytes were more abundant ($F_{1,23}$=6.396, p=0.019) on electricity treatments as compared to grazed controls. However, this pattern was not observed on N treatments, as we found a significant electricity*nutrient interaction for bacillariophytes ($F_{3,23}$=4.589, p=0.012). We found no main effect of electricity on periphyton biomass metrics or algal community composition in the second or third experiments (p>0.05). However, we did find an interactive effect of electricity and nutrients on bacillariophytes in the third experiment ($F_{3,20}$=3.720, p=0.028), with higher relative abundances on grazed controls for all nutrient treatments except P.

We observed seasonal trends in the electricity and nutrient addition effect sizes as well as streambed grazer abundances. Algal biomass LRRs for both electricity and limiting nutrient (P)

additions tended to decrease over time (Fig. 4.2), and periphyton AFDM LRRs for electricity also decreased over time. We found a marginally significant effect of experiment on heptageniid mayflies ($F_{2,3}$=7.775, p=0.065), with the first experiment having higher abundances compared to the second (p=0.074) and third (p=0.095) experiments. We found no effect of experiment on other aquatic insect groups.

**Discussion**

In this study, the strength of bottom-up and top-down factors on periphyton structure changed over time in a temperate mountain stream. This is the first demonstration of seasonal changes in these factors by an in-stream study (but see Rosemond et al. 2000 for a streamside mesocosm experiment), but it is likely a common phenomenon in seasonally-varying streams with substantial implications for generalizations about top-down and bottom-up drivers of periphyton dynamics. Our results add to a growing body of literature across a wide variety of ecosystems demonstrating temporal heterogeneity in the strength of bottom-up and top-down drivers (Weisse 1991; Boyer et al. 2003; Whalen et al. 2013). We also found that periphyton responded differently to electricity and nutrient treatments depending on the response variable being considered, which highlights the importance of treating periphyton as a heterogeneous microbial community in experimental studies. We outline the likely drivers of periphyton changes in our study, but also discuss how experimental design can influence both outcomes and appropriate scales of inference for stream field studies.

In the South Fork Poudre River, P was a primary driver of algal biomass accrual, and the effect of P additions changed over time. Pigments indicative of algal biomass and community composition responded to P and NP additions, but periphyton AFDM did not. It is likely that the

heterotrophic component of the community was limited by another factor such as carbon (Bechtold et al. 2012), which we did not measure during this study. As a result, autotrophs generally comprised a higher proportion of the periphyton community as compared to heterotrophs on NDS P treatments. This pattern has been demonstrated in other studies as well, whereby limiting nutrient additions lead to a decoupling of heterotrophic and autotrophic components of periphyton (Scott et al. 2008). Specifically, it has commonly been hypothesized that heterotrophs are better competitors for P under limiting conditions (Brown et al. 1981), but autotrophs are expected to increase with P additions as we found in this experiment. In terms of algal community composition, we found that bacillariophytes and cyanobacteria increased on NP nutrient treatments during the first experiment, and cyanobacteria also increased on NP treatments in the second experiment. Bacillariophytes and cyanobacteria likely depended more on NP substrate additions as compared to chlorophytes. The chlorophytes in the South Fork Poudre River were dominated by filamentous taxa such as *Chaeotophera spp.*, *Cladophora spp.*, and *Spirogyra spp.* (personal observation, Author 1), and these taxa had more access to water column nutrients as compared to the adnate (low stature) bacillariophytes and cyanobacteria (largely *Oscillatoria spp.*). Bacillariophytes in particular tend to be early successional colonizers (Peterson and Stevenson 1990) and may have thrived on the limiting nutrient substrates.

We observed temporal changes in both periphyton nutrient limitation and algal community composition which were likely related to changing environmental conditions. Particularly in high elevation and high latitude streams, algal growth may be limited by factors like temperature (Cross et al. 2015) and light levels (Gustina & Hoffmann 2000) in addition to N and P availability (Toetz et al. 1999; Cardinale et al. 2009; Bowman et al. 2011). Temperatures cooled throughout the summer, as point temperature measurements decreased from 11.5°C in

August to 6-7°C in September and October (Table 4.1). A study in the same watershed used water temperature logger data to show substantial cooling from August to October (Shah et al. 2017), which can decrease algal responses to nutrient additions (Francoeur et al. 1999). Decreased insolation has also been shown to reduce nutrient limitation in past experiments (Rosemond et al. 2000; Taulbee et al. 2005), but in our study seasonal decreases in insolation may have been balanced by decreases in water depth over time (Table 4.1) that likely increased light availability in the water column. Current velocity also decreased over time, which may have decreased nutrient uptake rates (Horner & Welch 1981; Borchardt et al. 1994) and led to weaker nutrient limitation. Additionally, the algal communities changed in response to these environmental conditions, specifically with chlorophytes decreasing and bacillariophytes increasing throughout the season, probably reflecting the higher tolerance of bacillariophytes to cooler temperatures (DeNicola 1996). Cyanobacteria also decreased over time and always comprised a low proportion of the community because these taxa are intolerant of cool temperatures (DeNicola 1996). These results highlight how factors change seasonally and indicate that investigators should proceed with caution when extending nutrient limitation findings based on short-term experiments.

We also found that grazing treatments influenced most periphyton response metrics, and as the streambed grazer densities declined over time, so did the inferred strength of top-down control. In the first experiment, periphyton AFDM and the proportion of bacillariophytes were significantly reduced (Figs. 4.2, 4.4), and this corresponded to the highest abundances of mayfly grazers (*Heptageniidae,* primarily *Rhithrogena spp.*) on the streambed (Fig. 4.5). Although we did not observe aquatic insects on NDS surfaces during the day, many aquatic insects in our system (including heptageniid mayflies) commonly drift through the water column at night (Poff

et al. 2006), and they could easily access the artificial substrates used to grow algae (see Opsahl et al. 2003). Grazers can selectively feed on bacillariophytes (Rosemond et al. 1993; Rosemond et al. 2000), and grazers as well as non-grazers can cause non-consumptive losses of periphyton through physical disruption as they move over the substrate (Eichenberger & Schlatter 1978; Lamberti et al. 1995).

None of our response metrics were significantly influenced by insect grazers in the second and third experiments. Positive or neutral effects of grazers on periphyton biomass have been reported in other studies and likely occur because of indirect effects related to insect competition, nutrient cycling, or sediment and detritus removal (Hillebrand 2009). However, periphyton removal likely decreased over time because we found a marginal decrease in *Rhithrogena* spp. after the first experiment (despite low statistical power from n=2 samples), which almost certainly emerged from the stream as adults (B.C. Kondratieff, Colorado State University, personal communication). Indeed, in an energetics study in a neighboring watershed, Carlisle (2002) found *Rhithrogena* spp. streambed abundances decreased from summer to fall, leading to an increased standing stock of algal biomass and decreased consumption of bacillariophytes. Studies from lentic and tidal systems have also demonstrated seasonal changes in grazer abundances that significantly decreased top-down pressure on phytoplankton (Weisse 1991; Thompson et al. 2008). For those insects remaining in the stream during our study, overall aquatic insect activity and metabolic rates may have decreased with cooling temperatures over time, contributing to reduced periphyton removal as has been shown across a wide variety of stream, lake, and marine experiments (Hillebrand 2009). These results indicate that top-down control may be variable and is dependent on grazer community composition and environmental conditions that shift over time. However, it is important to consider that grazers may have

influenced algal community composition at a finer scale than what was measured during this study, as grazers have been shown to select for palatable and accessible taxa even within bacillariophyte, chlorophyte, or cyanobacteria communities (Steinman et al. 1992).

Past meta-analyses have found that experimental duration was an important factor influencing the relative strength of bottom-up and top-down effects on periphyton (Feminella & Hawkins 1995; Hillebrand 2002; Hillebrand 2009). The positive effect of nutrients on periphyton decreases with experimental duration, while the negative effect of grazing on periphyton increases with experimental duration (Hillebrand 2002; Hillebrand 2009). This is likely because more developed periphyton communities can internally recycle nutrients and rely less on external sources, but grazing rates increase with available periphyton biomass (Hillebrand 2002; Hillebrand 2009). In our study, nutrient additions had a stronger influence on periphyton biomass metrics than did grazer exclusions, and we saw no interaction between grazer removal and nutrient additions on periphyton biomass metrics. We completed relatively short periphyton colonization experiments because nutrient diffusion rates from NDS decline logarithmically over time (Rugenski et al. 2008). Nutrients would be expected to have strong control on periphyton biomass in the early successional communities that developed. Only one other experiment has used NDS in conjunction with electrical exclusions, finding that nutrients had a much larger effect than grazer exclusions on algal biomass in a similarly short, 15-day experiment (Lourenço-Amorim et al. 2014). However, it is important to consider that a longer experiment would require replenishing nutrients in the NDS agar. In addition, periphyton may surpass peak biomass in a longer experiment and begin to slough from growth surfaces (Biggs 1996), making it difficult to distinguish autogenic sloughing from treatment effects.

While any experimental design includes compromises, field experiments employing underwater electric fences allowed for the observation of complex dynamics between periphyton communities and temporally variable drivers like shifts in insect communities and background environmental conditions. Laboratory and streamside enclosure experiments are often not representative of in-stream conditions and tend to estimate higher grazing rates than field experiments (Feminella & Hawkins 1995; Hillebrand 2009). In contrast, in-stream experiments maintain natural insect densities and processes like drifting, emergence, predation, and abiotic variability which significantly influence grazing. While field experiments on herbivory may be challenging, electric fences have the advantage of excluding aquatic insects with small body sizes (Moulton et al. 2004) while also avoiding the effects of sedimentation or altered current velocity that may accompany other in-stream exclosure devices.

**Conclusion**

We found that the strength of bottom-up and top-down controls on periphyton decreased over time from summer to early fall in the South Fork Poudre River, which adds to the growing number of studies demonstrating temporal heterogeneity of top-down and bottom-up importance across terrestrial (Hunter & Price 1992; Boyer 2003; Gratton & Denno 2003), marine (Thompson et al. 2008, Whalen et al. 2013), and lentic ecosystems (Weisse 1991). Nutrient additions had a substantial effect on algal biomass and community composition, while grazer exclusions affected periphyton AFDM and algal community composition early in the season because of consumptive and non-consumptive biomass losses. We recommend that future experiments consider temporal variability when investigating bottom-up and top-down regulation of stream periphyton in natural stream ecosystems to better account for physical and biological controls on nutrient limitation and herbivory. In addition, management decisions based on stream periphyton

experiments should carefully consider the limited temporal scope provided by individual experiments.

## Figures

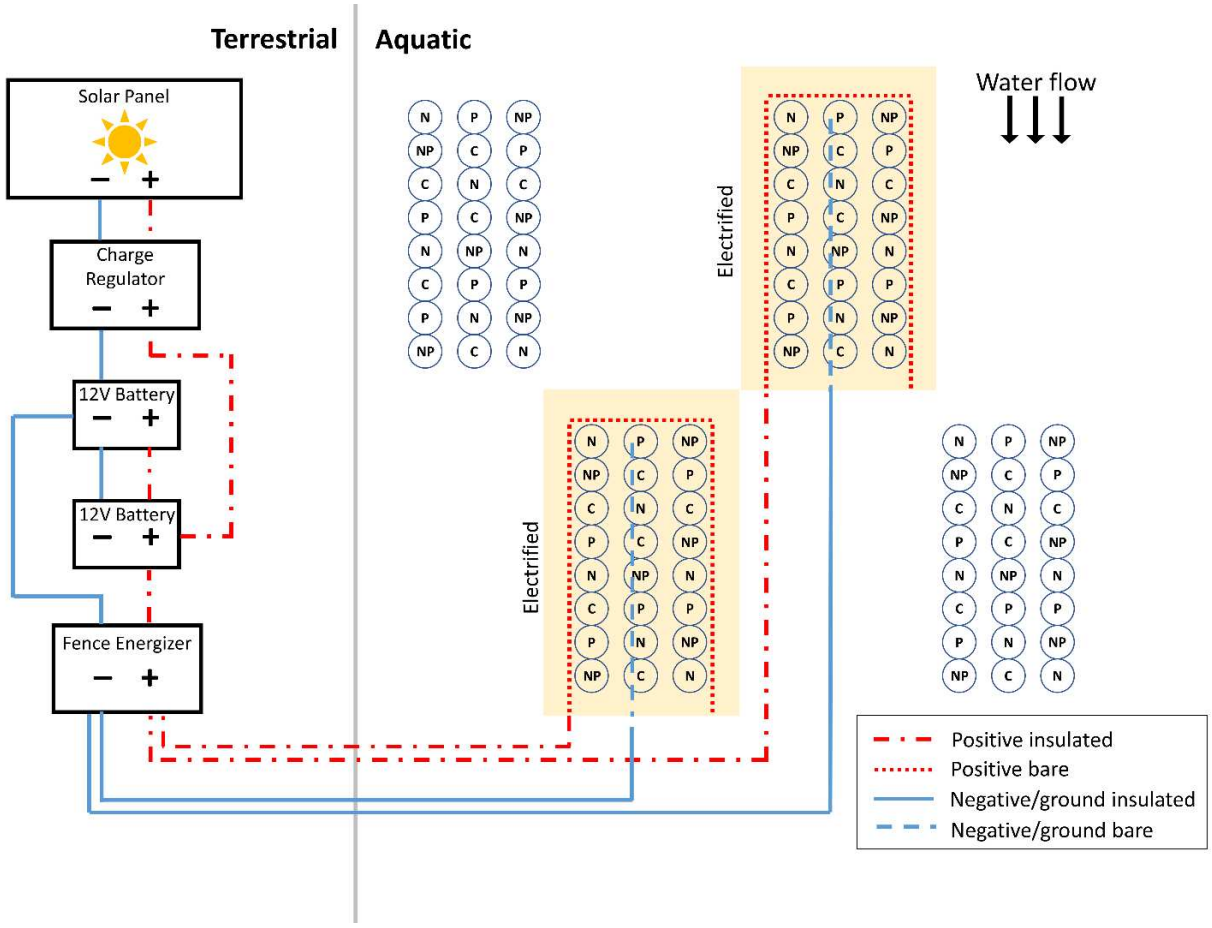


**Figure 4.1: Conceptual schematic for electrical exclusion of stream grazers. An exclusionary zone (box surrounding two center experimental plots) was created by electrical current moving from bare wires carrying positive charge to bare wires designated as negative/ground. Insulated wires were connected to the positive or negative/ground terminals of each device as indicated in the figure key. Plots were staggered to avoid electrical or nutrient interference between plots. Diagram not shown to scale.**

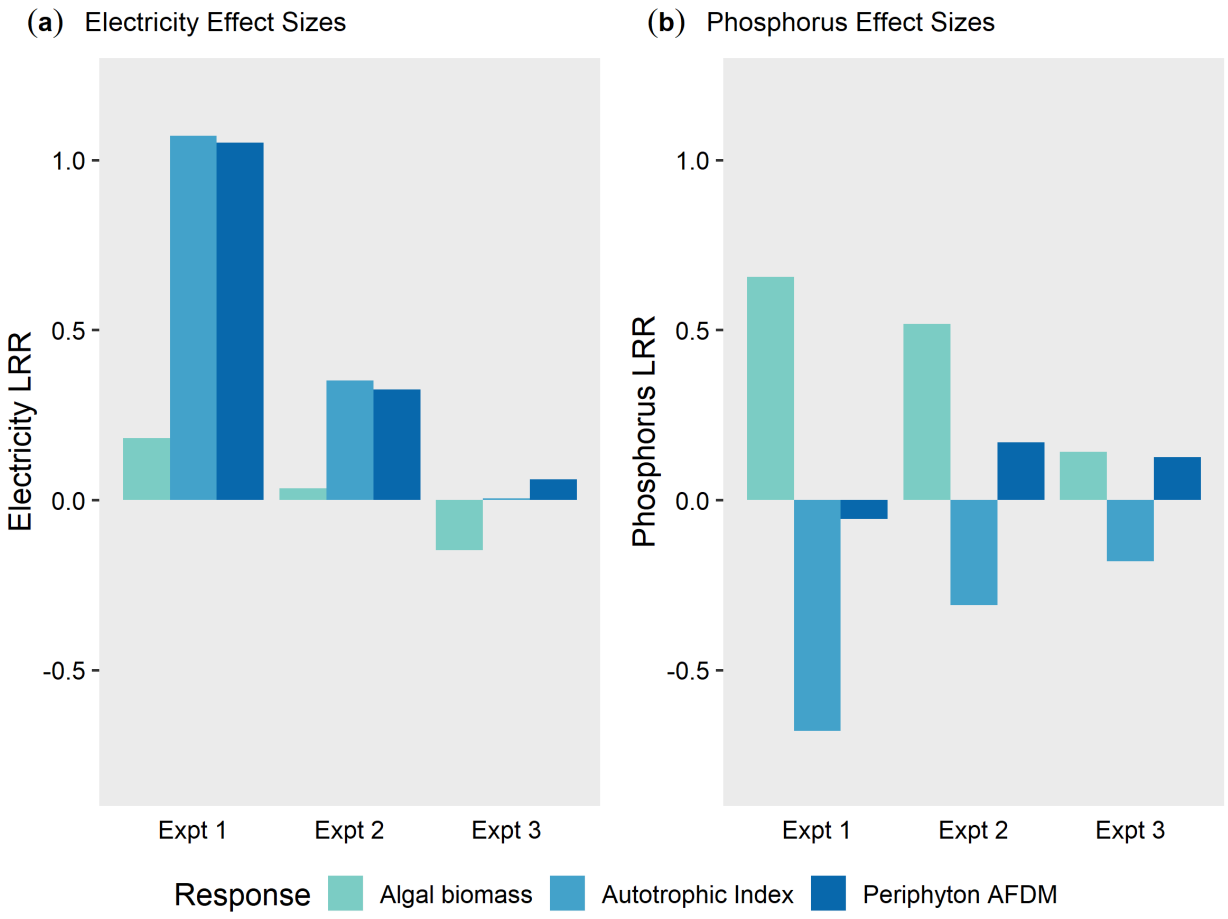


**Figure 4.2: Experimental effect sizes (measured as log response ratios, LRRs, equations 1-2) of electric and primary limiting nutrient (phosphorus) treatments calculated separately for algal biomass, periphyton ash-free dry mass (AFDM), and an autotrophic index (AI). See supplemental figures S4.1 and S4.3 for statistical test results from untransformed values, showing a significant effect of electricity on AFDM and AI in the first experiment and a significant effect of P treatments on algal biomass in the first and second experiments. See table 4.1 for experimental time periods.**

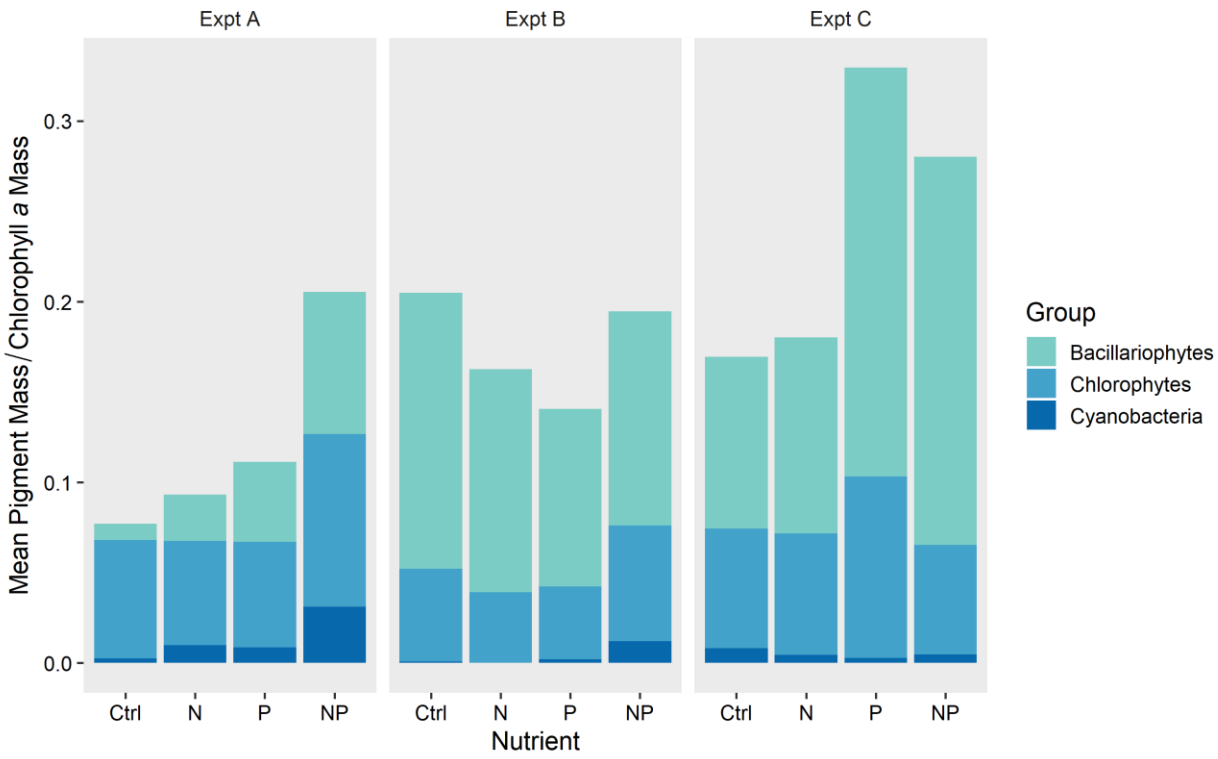


**Figure 4.3: Mean bacillariophyte, chlorophyte, and cyanobacteria responses to nutrient treatments (Ctrl= control, N= nitrogen, P= phosphorus, NP= nitrogen and phosphorus) during three sequential experiments at the South Fork Poudre River. See Supplemental Figure S4.2 for variances and a summary of statistical tests.**

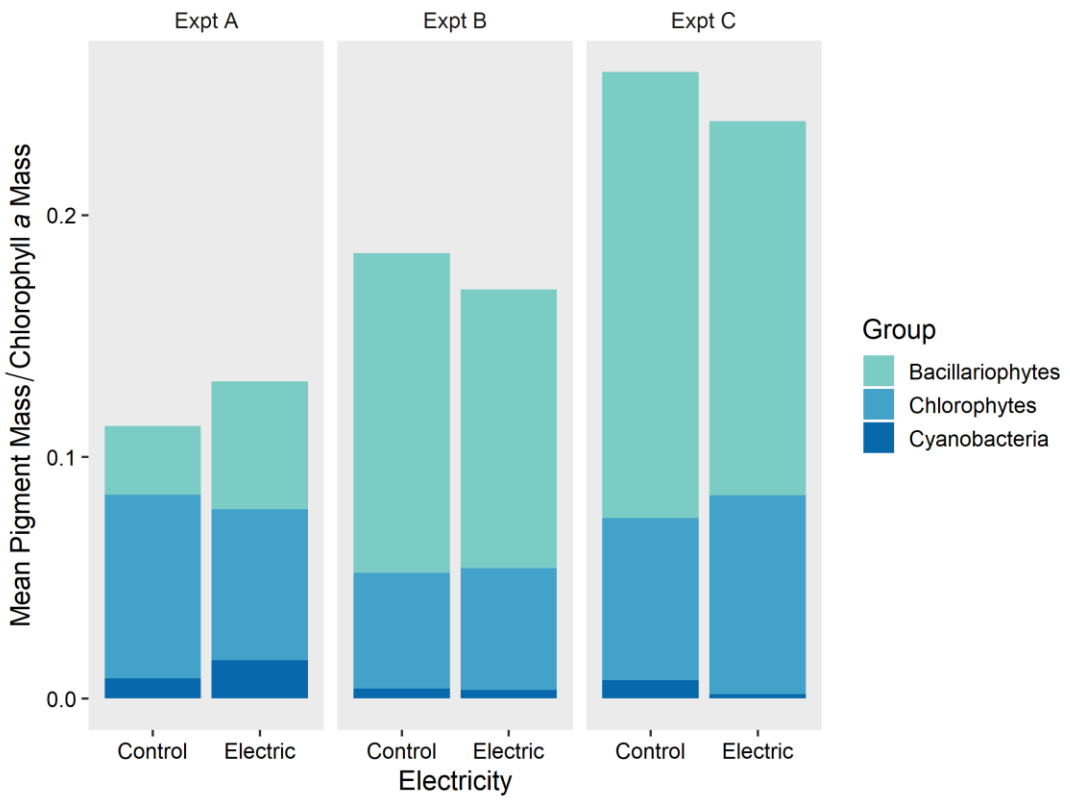


**Figure 4.4: Mean bacillariophyte, chlorophyte, and cyanobacteria responses to electrical grazer exclusion treatments during three sequential experiments at the South Fork Poudre River. See Supplemental Figure S4.4 for variances and a summary of statistical tests.**

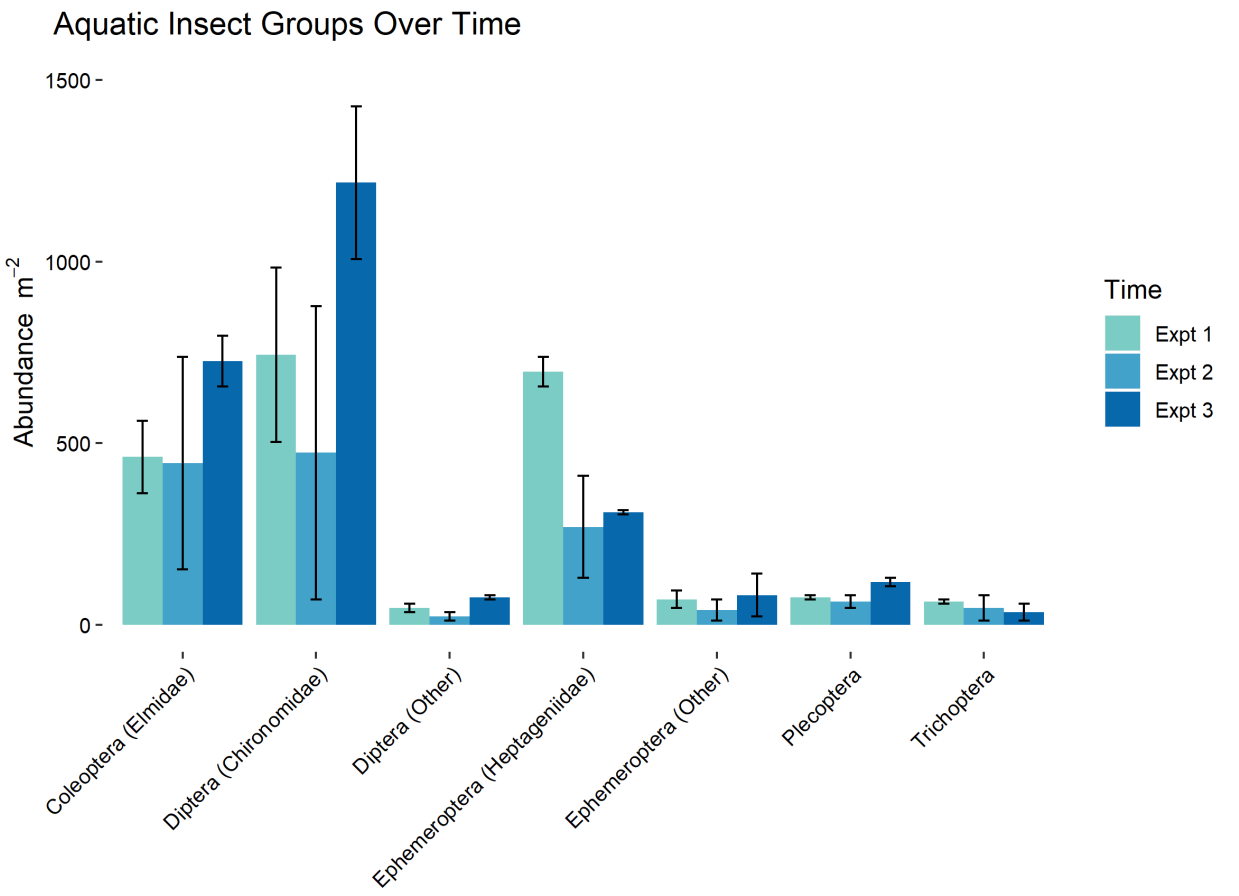


**Figure 4.5: Streambed aquatic insect groups (mean ± SE) measured with two replicate Hess samples at the end of each of three sequential experiments in the South Fork Poudre River.**

## Tables

**Table 4.1: Environmental conditions at the South Fork Poudre River, Colorado during three experiments from August 2017 to October 2017. Canopy cover and nutrient measurements were made at the beginning of each experiment, velocity and depth measurements were made at the beginning and end of each experiment, and all other measurements were made at the end of each experiment. Data with multiple measurements are reported as mean ± SE.**

| Exp | Dates | Temp (°C) | Cond (µS cm$^{-1}$) | Discharge (m$^3$ sec$^{-1}$) | Canopy % | Velocity (cm sec$^{-1}$) | Depth (cm) | Nitrate-N (µg L$^{-1}$) | Phosphate-P (µg L$^{-1}$) | TN (µg L$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 8/12/17-8/28/17 | 11.5 | 20.92 | 2.32 | 7.28 | 20.42 ± 1.56 | 21.13 ± 1.87 | 85.9 ± 2.10 | <detection | 117.9 ± 1.9 |
| 2 | 9/1/17-9/17/17 | 5.8 | 27.3 | 0.42 | 4.75 | 16.25 ± 0.68 | 13.54 ± 1.29 | 58.10 ± 0.80 | 0.79 ± 0.29 | 103.71 ± 7.09 |
| 3 | 9/17/17-10/04/17 | 6.7 | 27.85 | 0.26 | 1.13 | 11.81 ± 0.97 | 17.08± 0.54 | 61.05± 0.65 | <detection | 107.15 ± 5.85 |

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# APPENDICES

## Appendix 1: Meta-analysis Keyword Search Terms

nutrient AND (*diff* OR *substrat* OR artificial OR amend* OR enrich*) AND (freshwater OR stream OR lotic OR creek OR river) AND (algae OR periphyton OR biofilm OR chlorophyll)

"nutrient diff*" AND (freshwater OR stream OR lotic OR creek OR river)

nutrient AND (clay OR periphytometer) AND (freshwater OR stream OR lotic OR creek OR river) AND (algae OR periphyton OR chlorophyll)

## Appendix 2: List of Studies Included in the Meta-analysis


Allen, N. S., & Hershey, A. E. (1996). Seasonal changes in chlorophyll a response to nutrient amendments in a North Shore tributary of Lake Superior. *Journal of the North American Benthological Society*, **15**, 170–178.

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Wold, A. P., & Hershey, A. E. (1999). Spatial and temporal variability of nutrient limitation in 6 North Shore tributaries to Lake Superior. *Journal of the North American Benthological Society*, **18**, 2–14.

**Appendix 3: Supplementary Figures and Tables**


**Figure S1.1: Global study sites were mapped based on reported latitudes and longitudes, as well as site maps and stream names.**

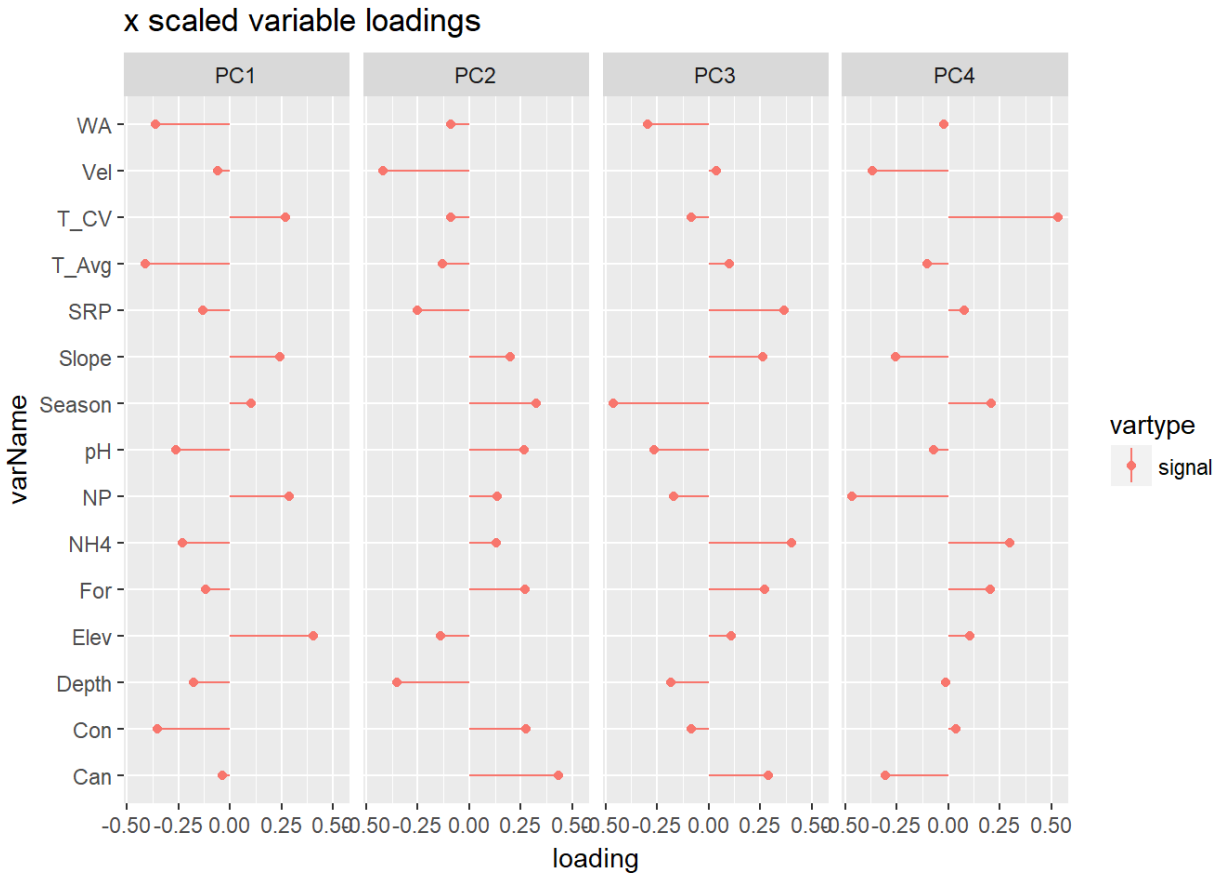


**Figure S2.1: Standardized loadings for predictors from the primary four principal components in the principal components analysis. Predictors are standardized relative to their means and standard deviations. Nitrate was not included in the analysis because it was highly correlated with DIN:SRP molar ratio and slope; discharge was not included because it was highly correlated with velocity. Can = percent canopy cover, Con = conductivity, Elev = elevation, For = percent watershed forest cover, NP = in-stream DIN:SRP molar ratio, T_Avg = average temperature, T_CV = temperature coefficient of variation, Vel = velocity, WA = watershed area.**

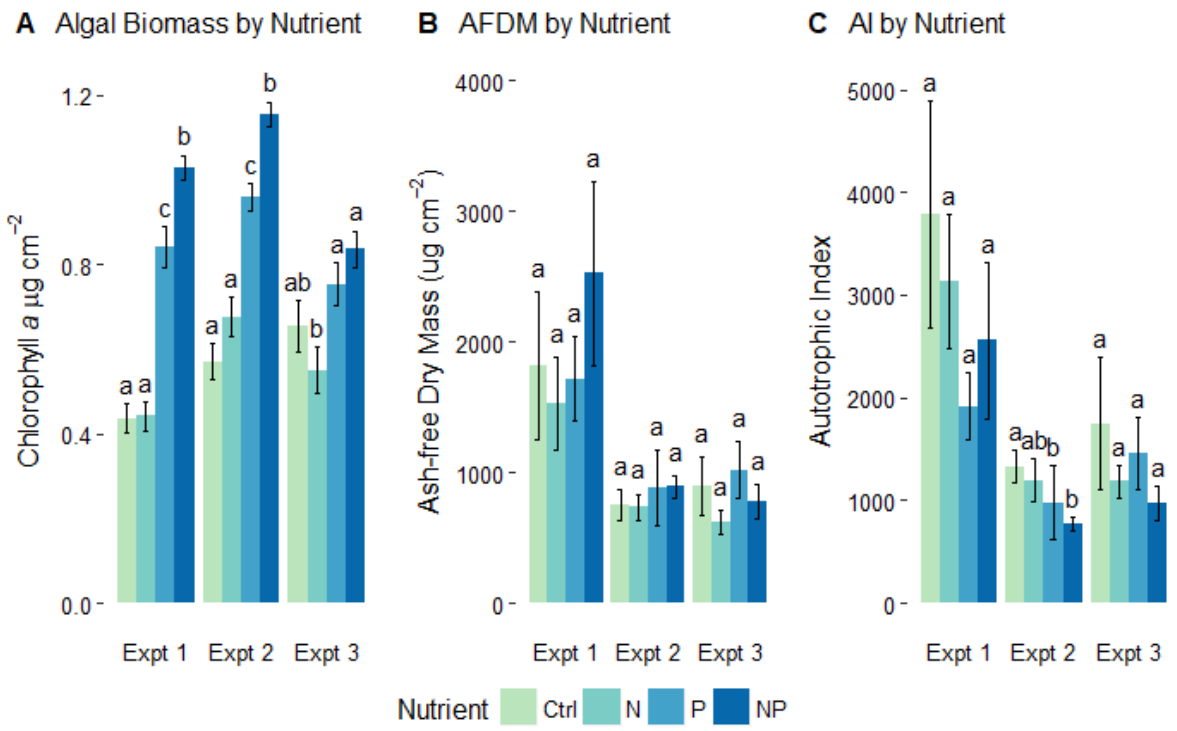


**Figure S4.1: Algal biomass (a), periphyton ash-free dry mass (b), and autotrophic index (c) responses to nutrient treatments (mean ± SE) during three sequential experiments at the South Fork Poudre River. ANOVA contrasts were calculated within each experiment but not across experiments. See table 1 for experimental time periods and note the differing y-axis scales.**

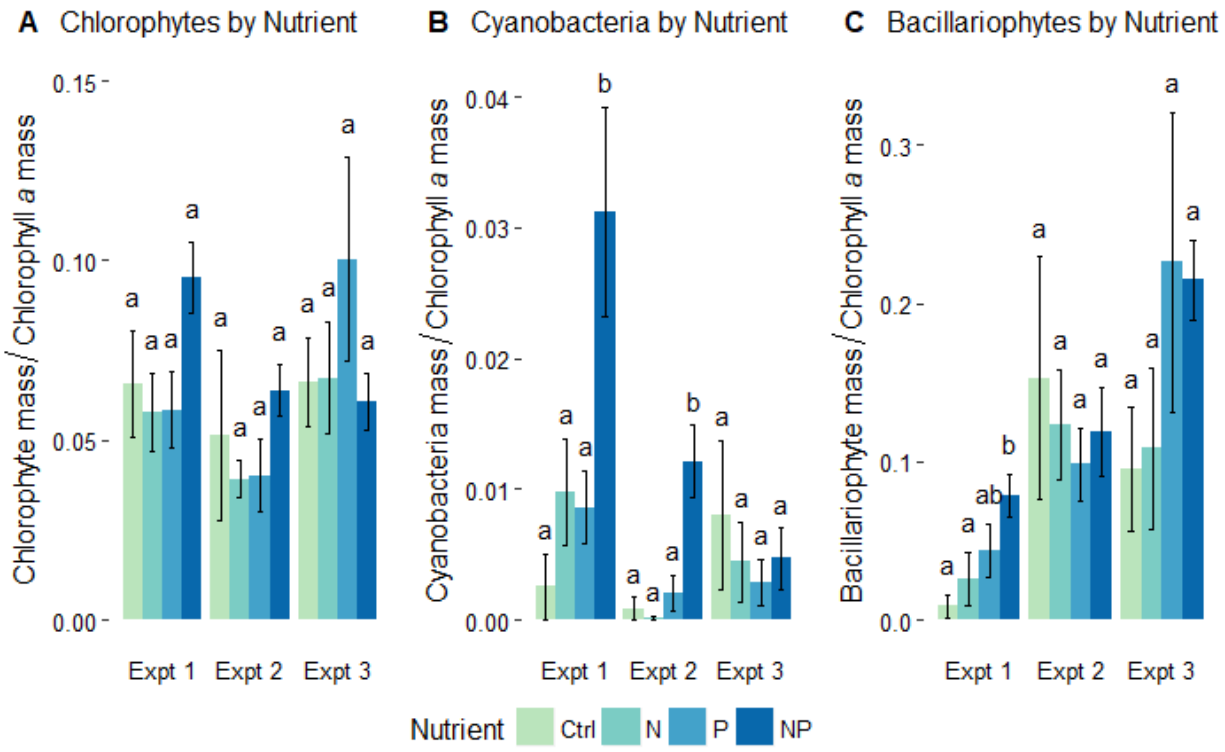


**Figure S4.2: Chlorophyte (a), cyanobacteria (b), and bacillariophyte (c) responses to nutrient treatments (mean ± SE) during three sequential experiments at the South Fork Poudre River. ANOVA contrasts were calculated within each experiment but not across experiments. See table 1 for experimental time periods and note the differing y-axis scales.**

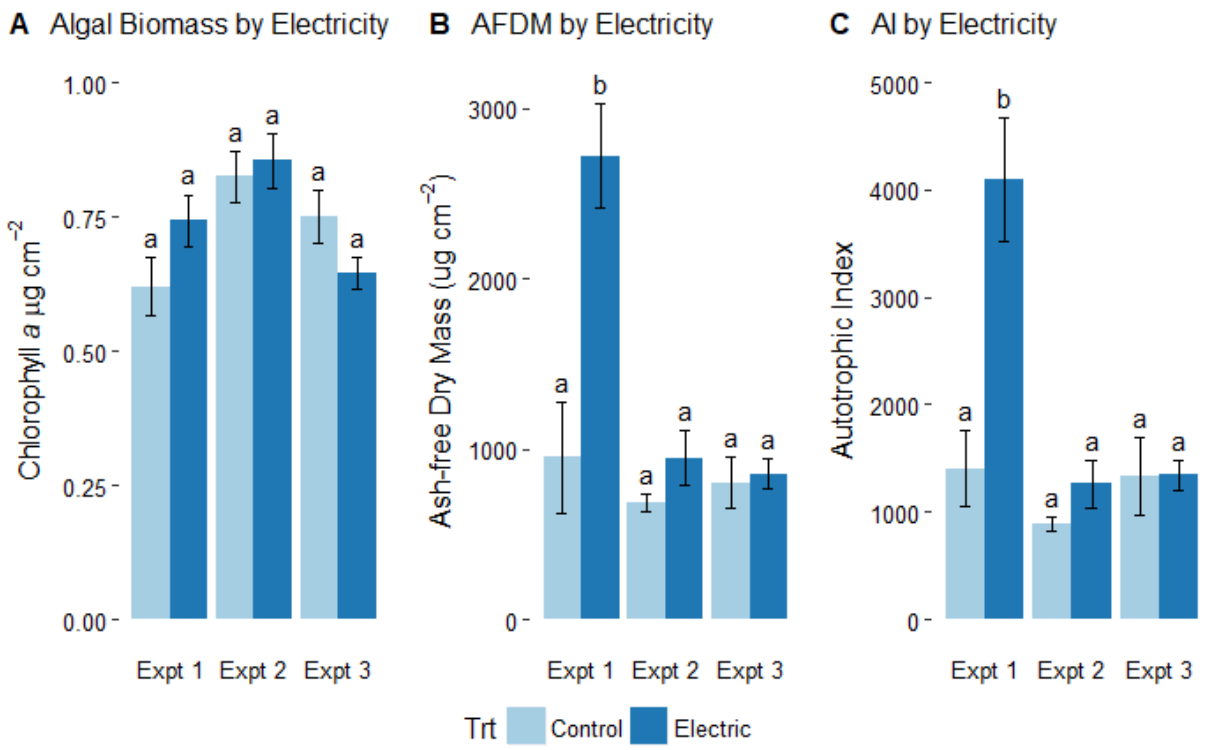


**Figure S4.3: Algal biomass (a), ash-free dry mass (b), and autotrophic index (c) responses to electrical grazer exclusion treatments (mean ± SE) during three sequential experiments at the South Fork Poudre River. ANOVA contrasts were calculated within each experiment but not across experiments. See table 1 for experimental time periods and note the differing y-axis scales.**

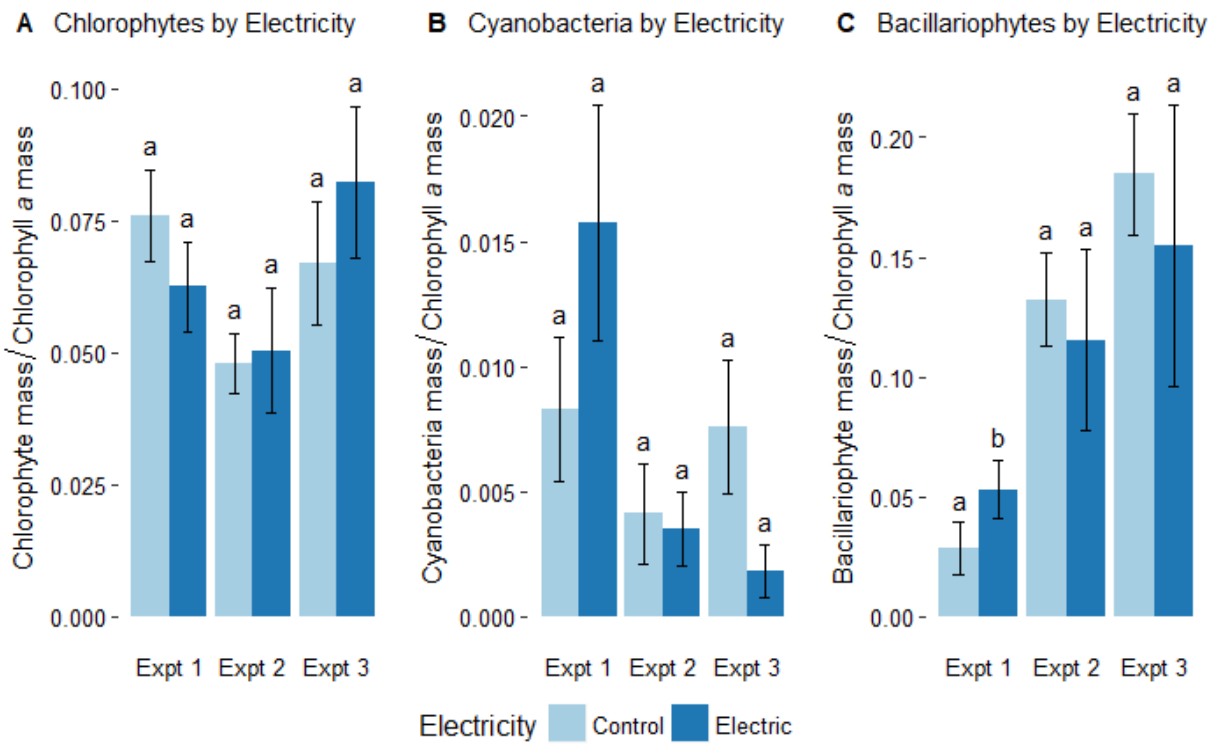


**Figure S4.4: Chlorophyte (a), cyanobacteria (b), and bacillariophyte (c) responses to electrical grazer exclusion treatments (mean ± SE) during three sequential experiments at the South Fork Poudre River. ANOVA contrasts were calculated within each experiment but not across experiments. See table 1 for experimental time periods and note the**

**Table S1.1: Predictor variables extracted from 649 published NDS experiments, along with the percentage of experiments that reported each predictor. *N_Conc, P_Conc, and Nutrient_Trt reporting rates were calculated separately for each nutrient response variable: N (n=553), P (n=534), NP (n=591).**

| Predictor | Description | Reporting Rate |
|---|---|---|
| Experimental Approach | | |
| N_Conc, P_Conc | Molarity of NDS nutrient | *N: 98%<br>*P: 98%<br>*NP: 97% |
| Days | Number of days of NDS deployment | 100% |
| NDS_Sub | NDS substrate category: clay pot, periphytometer, vial | 100% |
| Nutrient_Trt | Chemical compound of NDS treatment nutrient | *N: 97%<br>*P: 98%<br>*NP: 98% |
| Environmental Factors | | |
| Ammonium | In-stream ammonium ($ug \cdot L^{-1}$) | 42% |
| Canopy_Qual | Riparian canopy qualitative: closed, open, slightly shaded | 47% |
| Canopy_Quant | Riparian canopy quantitative: percent cover | 29% |
| Conductivity | In-stream conductivity ($uS \cdot cm^{-1}$) | 21% |
| Depth | NDS depth (m) | 23% |
| Discharge | Stream discharge ($m^3 \cdot sec^{-1}$) | 29% |
| DO | In-stream dissolved oxygen ($mg \cdot L^{-1}$) | 9% |
| Nitrate | In-stream nitrate ($ug \cdot L^{-1}$) | 65% |
| N:P | Molar ratio of in-stream nitrate to in-stream SRP | 54% |

| | | |
|---|---|---|
| pH | In-stream pH | 19% |
| Season | Experiment season category: fall, spring, summer, winter | 97% |
| SRP | In-stream soluble reactive phosphorus ($ug \cdot L^{-1}$) | 67% |
| Temp | In-stream temperature (Celsius) | 55% |
| TN | In-stream total nitrogen ($mg \cdot L^{-1}$) | 17% |
| TP | In-stream total phosphorus ($ug \cdot L^{-1}$) | 24% |
| Turbidity | In-stream turbidity (NTU) | 8% |
| Vel | Stream velocity ($m \cdot sec^{-1}$) | 28% |
| Geographic Factors | | |
| Eco | Level 1 North American Ecoregions category (Fig. 1.1) | N/A |
| Elevation | Elevation (m) | 21% |
| KG | Köppen-Geiger climate classification category (Table S1.10) | N/A |
| Latitude | Decimal degrees | 25% |
| LU | Land use category: agriculture, forest, grassland, pasture, urban | 49% |
| Order | Strahler stream order | 39% |
| Slope | Stream slope (%) | 12% |
| WA | Watershed area ($km^2$) | 31% |

**Table S1.2: Modeled LRR results for each nutrient treatment, where SE= standard error, CI L.B.= 95% confidence interval lower bound, and CI L.B.= 95% confidence interval upper bound.**

| Treatment | Sample Size | Estimate | SE | CI L.B. | CI U.B. |
|---|---|---|---|---|---|
| Nitrogen | 553 | 0.351 | 0.046 | 0.260 | 0.442 |
| Phosphorus | 534 | 0.239 | 0.047 | 0.148 | 0.331 |
| Nitrogen + Phosphorus | 591 | 0.824 | 0.060 | 0.707 | 0.942 |

**Table S1.3: Pearson's correlation coefficients for continuous environmental and geographic variables in the dataset, where significance is shown as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Sample sizes differ because coefficients are based on pairwise deletions of missing values.**

| | Order | Discharge | Vel | Canopy_ Quant | Ammon-ium | Nitrate | DIN | TN | SRP | TP |
|---|---|---|---|---|---|---|---|---|---|---|
| **Temp** | 0.255** | -0.049 | -0.138 | -0.273** | 0.303*** | 0.064 | 0.365* | -0.248 | 0.038 | 0.034 |
| **TP** | -0.131 | -0.229 | -0.205 | 0.184 | 0.431*** | 0.296*** | 0.886*** | 0.712*** | 0.989*** | |
| **SRP** | 0.139 | -0.217** | -0.062 | 0.092 | 0.250*** | 0.366*** | 0.434*** | 0.006 | | |
| **TN** | 0.540 | 0.131 | -0.320* | 0.090 | -0.622* | -0.007 | 0.965*** | | | |
| **DIN** | NA | -0.406 | -0.115 | 0.182 | 0.334 | 0.994*** | | | | |
| **Nitrate** | 0.084 | -0.351*** | -0.074 | -0.332*** | -0.002 | | | | | |
| **Ammon-ium** | 0.238** | -0.272** | -0.144 | -0.485*** | | | | | | |
| **Canopy_ Quant** | -0.654*** | -0.194 | 0.023 | | | | | | | |
| **Vel** | 0.014 | 0.080 | | | | | | | | |
| **Discharge** | 0.499*** | | | | | | | | | |

**Table S1.4: LRR estimates by nutrient treatment, from univariable and multivariable models with experimental predictors (see Table S1.1). Lines delineate separate models, where SE=standard error, CI L.B. = 95% confidence interval lower bound, and CI U.B. = 95% confidence interval upper bound.**

| Coefficient | Sample Size | Estimate | SE | CI L.B. | CI U.B. | $R^2$ Index |
|---|---|---|---|---|---|---|
| Nitrogen | | | | | | |
| Intercept | 553 | 0.336 | 0.077 | 0.185 | 0.487 | 0.000 |
| Days | | 0.001 | 0.003 | -0.005 | 0.006 | |
| Intercept | 457 | 0.372 | 0.055 | 0.265 | 0.479 | 0.000 |
| LogN_Conc | | 0.0001 | 0.006 | -0.012 | 0.012 | |
| Intercept(Clay Pot) | 553 | 0.433 | 0.086 | 0.264 | 0.601 | 0.010 |
| NDS_Sub(Periphytometer) | | -0.269 | 0.122 | -0.508 | -0.030 | |
| NDS_Sub(Vial) | | -0.063 | 0.094 | -0.247 | 0.122 | |
| Intercept($KNO_3$) | 530 | 0.338 | 0.142 | 0.060 | 0.616 | 0.000 |
| Nutrient_Trt($NaNO_3$) | | 0.015 | 0.145 | -0.269 | 0.299 | |
| Nutrient_Trt($NH_4Cl$) | | -0.137 | 0.145 | -0.421 | 0.147 | |
| Nutrient_Trt($NH_4NO_3$) | | 0.055 | 0.258 | -0.451 | 0.560 | |
| Phosphorus | | | | | | |
| Intercept | 535 | 1.217 | 0.242 | 0.742 | 1.692 | 0.000 |
| LogDays | | -0.320 | 0.078 | -0.473 | -0.168 | |
| Intercept | 439 | 0.113 | 0.056 | 0.004 | 0.222 | 0.000 |
| LogP_Conc | | -0.080 | 0.007 | -0.093 | -0.067 | |
| Intercept(Clay Pot) | 534 | 0.623 | 0.081 | 0.463 | 0.782 | 0.123 |
| NDS_Sub(Periphytometer) | | -0.335 | 0.135 | -0.599 | -0.070 | |
| NDS_Sub(Vial) | | -0.540 | 0.090 | -0.718 | -0.363 | |
| Intercept($K_2HPO_4$) | 513 | 0.827 | 0.280 | 0.277 | 1.377 | 0.041 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Nutrient_Trt($KH_2PO_4$) | | -0.521 | 0.287 | -1.083 | 0.041 | |
| Nutrient_Trt($Na_2HPO_4$) | | -0.648 | 0.296 | -1.228 | -0.068 | |
| Nutrient_Trt($NaH_2PO_4$) | | -1.001 | 0.309 | -1.607 | -0.394 | |
| Intercept(Cation(Potassium)) | 513 | 0.252 | 0.070 | 0.115 | 0.388 | 0.041 |
| Nutrient_Trt(Cation(Sodium)) | | -0.528 | 0.135 | -0.792 | -0.264 | |
| Nutrient_Trt(Hydrogens) | | 0.422 | 0.169 | 0.092 | 0.753 | |
| Nutrient_Trt(Cation) * Nutrient_Trt(Hydrogens) | | -0.156 | 0.305 | -0.753 | 0.441 | |
| Nitrogen + Phosphorus | | | | | | |
| Intercept | 592 | 1.111 | 0.092 | 0.931 | 1.290 | 0.000 |
| Days | | -0.012 | 0.003 | -0.018 | -0.006 | |
| Intercept | 488 | 0.991 | 0.073 | 0.847 | 1.134 | 0.002 |
| SqrtP_Conc | | -0.254 | 0.057 | -0.365 | -0.143 | |
| Intercept | 488 | 1.135 | 0.084 | 0.970 | 1.300 | 0.000 |
| SqrtN_Conc | | -0.378 | 0.069 | -0.513 | -0.244 | |
| Intercept(Clay Pot) | 589 | 0.915 | 0.109 | 0.702 | 1.128 | 0.000 |
| NDS_Sub(Periphytometer) | | 0.392 | 0.147 | 0.104 | 0.680 | |
| NDS_Sub(Vial) | | -0.240 | 0.122 | -0.479 | -0.002 | |
| Intercept($KNO_3$) | 573 | 0.623 | 0.245 | 0.143 | 1.103 | 0.000 |
| Nutrient_Trt($NaNO_3$) | | 0.211 | 0.253 | -0.286 | 0.707 | |
| Nutrient_Trt($NH_4Cl$) | | 0.081 | 0.257 | -0.423 | 0.585 | |
| Nutrient_Trt($NH_4NO_3$) | | 0.481 | 0.379 | -0.262 | 1.225 | |
| Intercept($KH_2PO_4$) | 565 | 1.371 | 0.112 | 1.151 | 1.591 | 0.037 |
| Nutrient_Trt($KH_2PO_4$) | | -0.410 | 0.085 | -0.576 | -0.244 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Nutrient_Trt($Na_2HPO_4$) | | -0.683 | 0.171 | -1.018 | -0.347 | |
| Nutrient_Trt($NaH_2PO_4$) | | -1.036 | 0.207 | -1.441 | -0.630 | |
| Intercept(Cation(Potassium)) | 565 | 0.823 | 0.070 | 0.686 | 0.960 | 0.037 |
| Nutrient_Trt(Cation(Sodium)) | | -0.625 | 0.150 | -0.918 | -0.333 | |
| Nutrient_Trt(Hydrogens) | | -0.346 | 0.088 | -0.519 | -0.173 | |
| Nutrient_Trt(Cation) * Nutrient_Trt(Hydrogens) | | 0.050 | 0.203 | -0.348 | 0.447 | |

**Table S1.5: N-LRR results from univariable models with environmental predictors (see Table S1.1). Lines delineate separate models, where SE=standard error, CI L.B. = 95% confidence interval lower bound, and CI U.B. = 95% confidence interval upper bound.**

| **Coefficient** | **Sample Size** | **Estimate** | **SE** | **CI L.B.** | **CI U.B.** | **$R^2$ Index** |
|---|---|---|---|---|---|---|
| Intercept | 208 | 0.269 | 0.074 | 0.125 | 0.413 | 0.000 |
| LogAmmonium | | 0.007 | 0.019 | -0.031 | 0.044 | |
| Intercept(Closed) | 272 | 0.376 | 0.058 | 0.262 | 0.491 | 0.000 |
| Canopy_Qual(SlightShade) | | 0.314 | 0.401 | -0.471 | 1.010 | |
| Canopy_Qual(Open) | | -0.050 | 0.029 | -0.107 | 0.006 | |
| Intercept | 148 | 0.213 | 0.064 | 0.087 | 0.340 | 0.000 |
| Canopy_Quant | | 0.003 | 0.001 | 0.0000 | 0.005 | |
| Intercept | 179 | 0.276 | 0.066 | 0.147 | 0.405 | 0.000 |
| LogDischarge | | -0.043 | 0.063 | -0.168 | 0.081 | |
| Intercept | 347 | 0.606 | 0.072 | 0.465 | 0.747 | 0.077 |
| LogNitrate | | -0.077 | 0.016 | -0.107 | -0.047 | |
| Intercept | 305 | 0.447 | 0.065 | 0.320 | 0.574 | 0.022 |
| LogN:P | | -0.051 | 0.017 | -0.085 | -0.017 | |
| Intercept(Fall) | 531 | 0.344 | 0.053 | 0.241 | 0.447 | 0.001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Season(Spring) | | -0.015 | 0.037 | -0.088 | 0.058 | |
| Season(Summer) | | -0.009 | 0.032 | -0.071 | 0.054 | |
| Season(Winter) | | -0.151 | 0.045 | -0.240 | -0.063 | |
| Intercept | 357 | 0.493 | 0.094 | 0.308 | 0.678 | 0.000 |
| LogSRP | | -0.065 | 0.026 | -0.116 | -0.014 | |
| Intercept | 314 | 0.214 | 0.065 | 0.086 | 0.341 | 0.000 |
| Temp | | 0.002 | 0.002 | -0.002 | 0.006 | |
| Intercept | 118 | 0.288 | 0.077 | 0.137 | 0.438 | 0.000 |
| LogVel | | 0.150 | 0.240 | -0.312 | 0.620 | |

**Table S1.6: P-LRR results from univariable models with environmental predictors (see Table S1.1). Lines delineate separate models, where SE=standard error, CI L.B. = 95% confidence interval lower bound, and CI U.B. = 95% confidence interval upper bound.**

| **Coefficient** | **Sample Size** | **Estimate** | **SE** | **CI L.B.** | **CI U.B.** | **$R^2$ Index** |
|---|---|---|---|---|---|---|
| Intercept | 187 | 0.097 | 0.075 | -0.049 | 0.243 | 0.000 |
| LogAmmonium | | -0.010 | 0.016 | -0.042 | 0.022 | |
| Intercept(Closed) | 246 | 0.121 | 0.066 | -0.008 | 0.251 | 0.000 |
| Canopy_Qual(Slight Shade) | | 0.215 | 0.429 | -0.627 | 1.056 | |
| Canopy_Qual(Open) | | -0.050 | 0.032 | -0.114 | 0.013 | |
| Intercept | 140 | 0.331 | 0.096 | 0.143 | 0.520 | 0.002 |
| Canopy_Quant | | -0.001 | 0.002 | -0.009 | -0.002 | |
| Intercept | 160 | 0.230 | 0.075 | 0.083 | 0.378 | 0.016 |
| LogDischarge | | 0.061 | 0.055 | -0.048 | 0.169 | |
| Intercept | 322 | 0.254 | 0.084 | 0.090 | 0.417 | 0.000 |
| LogNitrate | | -0.029 | 0.017 | -0.063 | 0.006 | |
| Intercept | 279 | -0.053 | 0.068 | -0.187 | 0.080 | 0.013 |

| | | | | | | |
|---|---|---|---|---|---|---|
| LogN:P | | 0.032 | 0.018 | -0.004 | 0.067 | |
| Intercept(Fall) | 514 | 0.161 | 0.054 | 0.056 | 0.266 | 0.000 |
| Season(Spring) | | -0.092 | 0.039 | -0.169 | -0.015 | |
| Season(Summer) | | 0.158 | 0.032 | 0.094 | 0.221 | |
| Season(Winter) | | -0.248 | 0.040 | -0.327 | -0.170 | |
| Intercept | 331 | 0.383 | 0.080 | 0.226 | 0.540 | 0.018 |
| LogSRP | | -0.101 | 0.023 | -0.146 | -0.056 | |
| Intercept | 293 | 0.216 | 0.066 | 0.086 | 0.345 | 0.000 |
| Temp | | 0.001 | 0.002 | -0.004 | 0.005 | |
| Intercept | 118 | 0.210 | 0.090 | 0.034 | 0.386 | 0.000 |
| LogVel | | -0.035 | 0.279 | -0.582 | 0.512 | |

**Table S1.7: NP-LRR results from univariable models with environmental predictors (see Table S1.1). Lines delineate separate models, where SE=standard error, CI L.B. = 95% confidence interval lower bound, and CI U.B. = 95% confidence interval upper bound.**

| **Coefficient** | **Sample Size** | **Estimate** | **SE** | **CI L.B.** | **CI U.B.** | **$R^2$ Index** |
|---|---|---|---|---|---|---|
| Intercept | 233 | 0.849 | 0.098 | 0.658 | 1.041 | 0.000 |
| LogAmmonium | | -0.063 | 0.017 | -0.097 | -0.030 | |
| Intercept(Closed) | 288 | 0.439 | 0.082 | 0.279 | 0.600 | 0.038 |
| Canopy_Qual(SlightShade) | | 0.331 | 0.594 | -0.833 | 1.495 | |
| Canopy_Qual(Open) | | 0.365 | 0.029 | 0.309 | 0.422 | |
| Intercept | 153 | 0.882 | 0.118 | 0.651 | 1.112 | 0.032 |
| Canopy_Quant | | -0.006 | 0.002 | -0.010 | -0.002 | |
| Intercept | 152 | 0.856 | 0.112 | 0.636 | 1.076 | 0.000 |
| LogDischarge | | -0.158 | 0.059 | -0.273 | -0.042 | |
| Intercept | 319 | 0.609 | 0.090 | 0.432 | 0.786 | 0.000 |
| LogN:P | | 0.014 | 0.020 | -0.026 | 0.054 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Intercept | 359 | 1.233 | 0.097 | 1.043 | 1.422 | 0.110 |
| LogNitrate | | -0.121 | 0.019 | -0.158 | -0.084 | |
| Intercept(Fall) | 576 | 0.862 | 0.064 | 0.736 | 0.988 | 0.000 |
| Season(Spring) | | -0.106 | 0.040 | -0.184 | -0.028 | |
| Season(Summer) | | -0.065 | 0.034 | -0.132 | 0.003 | |
| Season(Winter) | | -0.640 | 0.0440 | -0.726 | -0.554 | |
| Intercept | 371 | 1.175 | 0.096 | 0.988 | 1.363 | 0.091 |
| LogSRP | | -0.180 | 0.024 | -0.228 | -0.133 | |
| Intercept | 311 | 0.640 | 0.078 | 0.487 | 0.793 | 0.000 |
| Temp | | 0.004 | 0.002 | 0.0002 | 0.009 | |
| Intercept | 170 | 1.104 | 0.124 | 0.860 | 1.347 | 0.000 |
| LogVel | | -1.106 | 0.254 | -1.603 | -0.609 | |

**Table S1.8: LRR results from univariable models with geographic predictors (see Table S1.1). Lines delineate separate models, where SE=standard error, CI L.B. = 95% confidence interval lower bound, and CI U.B. = 95% confidence interval upper bound.**

| **Coefficient** | **Sample Size** | **Estimate** | **SE** | **CI L.B.** | **CI U.B.** | **$R^2$ Index** |
|---|---|---|---|---|---|---|
| Nitrogen | | | | | | |
| Intercept(Agriculture) | 259 | -0.062 | 0.169 | -0.393 | 0.269 | 0.087 |
| LU(Forest) | | 0.468 | 0.184 | 0.108 | 0.829 | |
| LU(Grassland) | | 0.479 | 0.197 | 0.093 | 0.866 | |
| LU(Pasture) | | 0.604 | 0.195 | 0.222 | 0.988 | |
| LU(Urban) | | 0.038 | 0.280 | -0.510 | 0.586 | |
| Intercept | 191 | 0.390 | 0.119 | 0.157 | 0.623 | 0.048 |
| Order | | -0.065 | 0.038 | -0.140 | 0.011 | |
| Intercept | 175 | 0.600 | 0.122 | 0.361 | 0.839 | 0.013 |
| LogWA | | -0.042 | 0.030 | -0.101 | 0.016 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Phosphorus | | | | | | |
| Intercept(Agriculture) | 231 | 0.343 | 0.166 | 0.019 | 0.668 | 0.101 |
| LU(Forest) | | -0.177 | 0.181 | -0.530 | 0.177 | |
| LU(Grassland) | | -0.411 | 0.200 | -0.802 | -0.019 | |
| LU(Pasture) | | -0.191 | 0.190 | -0.564 | 0.181 | |
| LU(Urban) | | -0.845 | 0.267 | -1.368 | -0.322 | |
| Intercept | 181 | -0.179 | 0.124 | -0.422 | 0.063 | 0.151 |
| Order | | 0.127 | 0.040 | 0.048 | 0.205 | |
| Intercept | 170 | 0.413 | 0.106 | 0.206 | 0.620 | 0.001 |
| LogWA | | -0.028 | 0.025 | -0.077 | 0.021 | |
| Nitrogen + Phosphorus | | | | | | |
| Intercept(Agriculture) | 237 | 0.297 | 0.225 | -0.144 | 0.737 | 0.120 |
| LU(Forest) | | 0.313 | 0.246 | -0.170 | 0.795 | |
| LU(Grassland) | | 0.379 | 0.264 | -0.138 | 0.897 | |
| LU(Pasture) | | 0.227 | 0.257 | -0.278 | 0.731 | |
| LU(Urban) | | -0.782 | 0.345 | -1.458 | -0.107 | |
| Intercept | 177 | 0.553 | 0.204 | 0.154 | 0.952 | 0.000 |
| Order | | 0.005 | 0.064 | -0.120 | 0.131 | |
| Intercept | 163 | 1.208 | 0.155 | 0.904 | 1.511 | 0.022 |
| LogWA | | -0.060 | 0.037 | -0.131 | 0.012 | |

**Table S1.9: LRR results from univariable models using ecoregion as the predictor, where SE= standard error, CI L.B.= 95% confidence interval lower bound, and CI L.B.= 95% confidence interval upper bound.**

| Coefficient | Estimate | SE | CI L.B. | CI U.B. | $R^2$ Index |
|---|---|---|---|---|---|
| Nitrogen: n = 371 | | | | | |
| Intercept | 0.132 | 0.112 | -0.088 | 0.352 | 0.075 |
| Eco(Great Plains) | 0.406 | 0.208 | -0.001 | 0.813 | |
| Eco(Marine West Coast Forests) | 1.353 | 0.334 | 0.700 | 2.007 | |
| Eco(Mediterranean California) | 0.211 | 0.235 | -0.248 | 0.671 | |
| Eco(North American Deserts) | 0.086 | 0.404 | -0.706 | 0.879 | |
| Eco(Northern Forests) | 0.250 | 0.165 | -0.074 | 0.574 | |
| Eco(Northwest Forested Mountains) | 0.258 | 0.159 | -0.054 | 0.571 | |
| Eco(Temperate Sierras) | 1.183 | 0.496 | 0.251 | 2.155 | |
| Phosphorus: n = 374 | | | | | |
| Intercept | 0.024 | 0.112 | -0.194 | 0.243 | 0.083 |
| Eco(Great Plains) | 0.235 | 0.203 | -0.162 | 0.632 | |
| Eco(Marine West Coast Forests) | 1.243 | 0.328 | 0.601 | 1.886 | |
| Eco(Mediterranean California) | -0.303 | 0.229 | -0.752 | 0.147 | |
| Eco(North American Deserts) | 0.174 | 0.357 | -0.526 | 0.874 | |
| Eco(Northern Forests) | 0.435 | 0.160 | 0.121 | 0.750 | |
| Eco(Northwest Forested Mountains) | 0.400 | 0.153 | 0.100 | 0.700 | |
| Eco(Temperate Sierras) | 0.028 | 0.483 | -0.918 | 0.974 | |
| Nitrogen + Phosphorus: n = 393 | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Intercept | 0.384 | 0.137 | 0.116 | 0.651 | 0.162 |
| Eco(Great Plains) | 0.700 | 0.252 | 0.206 | 1.193 | |
| Eco(Marine West Coast Forests) | 1.434 | 0.757 | -0.050 | 2.919 | |
| Eco(North American Deserts) | 0.337 | 0.357 | -0.363 | 1.037 | |
| Eco(Northern Forests) | 0.701 | 0.199 | 0.310 | 1.091 | |
| Eco(Northwest Forested Mountains) | 1.094 | 0.189 | 0.725 | 1.464 | |
| Eco(Tundra) | 1.337 | 0.615 | 0.131 | 2.543 | |

**Table S1.10: LRR results from univariable models using Köppen-Geiger climate classification (Peel, Finlayson & Mcmahon, 2007) as the predictor, where SE= standard error, CI L.B.= 95% confidence interval lower bound, and CI L.B.= 95% confidence interval upper bound.**

| Coefficient | Estimate | SE | CI L.B. | CI U.B. | $R^2$ Index |
|---|---|---|---|---|---|
| Nitrogen: n = 443 | | | | | |
| Intercept | 0.236 | 0.370 | -0.490 | 0.961 | 0.040 |
| KG(BSk) | 0.411 | 0.489 | -0.547 | 1.369 | |
| KG(BWh) | 1.134 | 0.728 | -0.293 | 2.561 | |
| KG(Cfa) | 0.073 | 0.378 | -0.668 | 0.813 | |
| KG(Cfb) | 0.125 | 0.380 | -0.620 | 0.869 | |
| KG(Csb) | 0.309 | 0.399 | -0.472 | 1.091 | |
| KG(Cwb) | -0.222 | 0.725 | -1.643 | 1.198 | |
| KG(Dfa) | -0.190 | 0.432 | -1.037 | 0.658 | |
| KG(Dfb) | 0.088 | 0.385 | -0.666 | 0.842 | |
| KG(Dfc) | -0.829 | 0.446 | -1.704 | 0.045 | |
| KG(Dsb) | 0.068 | 0.396 | -0.708 | 0.844 | |
| Phosphorus: n = 430 | | | | | |
| Intercept | 0.302 | 0.393 | -0.469 | 1.072 | 0.062 |

| | | | | | |
|---|---|---|---|---|---|
| KG(BSk) | -0.366 | 0.497 | -1.341 | 0.608 | |
| KG(Cfa) | -0.224 | 0.402 | -1.012 | 0.564 | |
| KG(Cfb) | -0.046 | 0.405 | -0.839 | 0.748 | |
| KG(Csb) | -0.399 | 0.425 | -1.232 | 0.434 | |
| KG(Cwb) | 0.207 | 0.780 | -1.322 | 1.734 | |
| KG(Dfa) | 0.094 | 0.461 | -0.810 | 0.999 | |
| KG(Dfb) | 0.109 | 0.407 | -0.689 | 0.907 | |
| KG(Dfc) | 0.193 | 0.459 | -0.706 | 1.092 | |
| KG(Dsb) | 0.418 | 0.420 | -0.406 | 1.242 | |
| Nitrogen + Phosphorus: n = 484 | | | | | |
| Intercept | 0.594 | 0.101 | 0.396 | 0.793 | 0.181 |
| KG(Cfb) | -0.104 | 0.148 | -0.393 | 0.186 | |
| KG(Csa) | 0.634 | 0.444 | -0.236 | 1.504 | |
| KG(Csb) | -0.231 | 0.203 | -0.628 | 0.167 | |
| KG(Cwb) | -0.0703 | 0.825 | -1.688 | 1.547 | |
| KG(Dfa) | 0.044 | 0.302 | -0.548 | 0.636 | |
| KG(Dfb) | 0.136 | 0.162 | -0.180 | 0.453 | |
| KG(Dfc) | 0.932 | 0.269 | 0.404 | 1.459 | |
| KG(Dsb) | 1.216 | 0.205 | 0.814 | 1.619 | |

**Table S2.1: Geographic characteristics of eight streams in the Cache la Poudre watershed. See Fig. S1 for abbreviations.**

| Site | Elev (m) | Lat | Long | For % | WA ($km^2$) | Slope % |
|---|---|---|---|---|---|---|
| BVR | 2590 | 40.9284 | -105.672 | 97.90 | 5.61 | 2.93 |
| EFSC | 3166 | 40.6236 | -105.708 | 65.80 | 1.29 | 15.90 |
| EK | 1992 | 40.6985 | -105.441 | 68.80 | 34.40 | 3.60 |
| KP | 2798 | 40.8132 | -105.709 | 81.00 | 3.04 | 9.27 |
| LB | 2443 | 40.6254 | -105.527 | 89.50 | 18.00 | 3.40 |
| PG | 2740 | 40.5713 | -105.591 | 39.10 | 15.10 | 2.65 |
| SM | 2212 | 40.7056 | -105.588 | 84.10 | 7.11 | 8.90 |
| WFSC | 3200 | 40.6079 | -105.725 | 64.40 | 1.20 | 5.90 |

**Table S2.2: Environmental characteristics at eight streams in the Cache la Poudre watershed during summer 2016 and fall 2016 NDS and algal accrual experiments. See Fig. S1 for abbreviations, and Q=discharge.**

| Site | Time | Q ($m^3$ $sec^{-1}$) | Depth (cm) | Vel ($cm^{-1}$) | Can (%) | pH | Con (µs $cm^{-1}$) | $NH_4^+$-N (µg $L^{-1}$) | $NO_3^-$-N (µg $L^{-1}$) | SRP-P (µg $L^{-1}$) | DIN:SRP Molar | T_Avg | T_CV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BVR | F16 | 0.03 | 21.8 | 6.6 | 49 | 7.83 | 105.10 | 3.09 | 20.17 | 2.12 | 20.45 | 8.25 | 0.20 |
| EFSC | F16 | 0.02 | 14.7 | 6.3 | 74 | 7.69 | 45.88 | 2.72 | 86.32 | 2.11 | 105.85 | 4.38 | 0.29 |
| EK | F16 | 0.01 | 28.8 | 1.3 | 70 | 8.48 | 217.30 | 2.80 | 2.50 | 2.06 | 6.29 | 11.43 | 0.16 |
| KP | F16 | 0.03 | 12.6 | 9.2 | 96 | 7.25 | 36.99 | 0.80 | 85.29 | 1.68 | 123.25 | 4.80 | 0.22 |
| LB | F16 | 0.11 | 22.2 | 13.3 | 5 | 7.90 | 47.96 | 2.69 | 0.56 | 1.67 | 5.17 | 8.68 | 0.42 |
| PG | F16 | 0.12 | 16.7 | 12.3 | 5 | 7.01 | 26.29 | 1.27 | 40.40 | 2.25 | 65.02 | 8.92 | 0.21 |
| SM | F16 | 0.01 | 10.3 | 4.2 | 97 | 8.62 | 110.50 | 2.86 | 37.50 | 2.15 | 53.90 | 8.35 | 0.16 |
| WFSC | F16 | 0.01 | 17.8 | 2.2 | 23 | 7.10 | 41.60 | 3.58 | 28.17 | 5.77 | 31.58 | 4.21 | 0.48 |
| BVR | S16 | 0.05 | 18.2 | 17 | 72 | 7.10 | 93.11 | 5.52 | 4.83 | 3.66 | 3.23 | 12.16 | 0.18 |
| EFSC | S16 | 0.07 | 24.8 | 14.3 | 57 | 6.92 | 33.18 | 2.45 | 90.55 | 6.48 | 39.34 | 7.69 | 0.26 |
| EK | S16 | 0.1 | 22 | 13 | 72 | 7.69 | 132.70 | 4.14 | 3.91 | 6.50 | 3.43 | 16.19 | 0.13 |
| KP | S16 | 0.11 | 22 | 18 | 93 | 6.74 | 34.40 | 2.00 | 33.50 | 2.17 | 35.59 | 6.97 | 0.21 |
| LB | S16 | 0.34 | 26.9 | 26 | 3 | 7.61 | 41.21 | 2.10 | 9.64 | 6.86 | 3.13 | 10.85 | 0.19 |
| PG | S16 | 1.1 | 30 | 50 | 0 | NA | 30.42 | 1.48 | 86.13 | 3.24 | 60.10 | 10.39 | 0.20 |
| SM | S16 | 0.05 | 11.5 | 20.3 | 97 | 7.87 | 98.62 | 4.38 | 11.47 | 4.77 | 5.34 | 11.92 | 0.13 |
| WFSC | S16 | 0.02 | 14 | 8 | 33 | 6.50 | 31.68 | 2.17 | 14.10 | 1.88 | 19.73 | 6.96 | 0.37 |

**Table S2.3: Correlations among stream characteristics. See Fig. S1 for abbreviations. p<0.001***, p<0.01**, p<0.05***

| | Elev | For | WA | Slope | Q | Depth | Vel | Can | pH | Con | $NH_4^+$-N | $NO_3^-$-N | SRP-P | DIN:SRP | T_Avg | T_CV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Elev** | 1 | | | | | | | | | | | | | | | |
| **For** | -0.331 | 1 | | | | | | | | | | | | | | |
| **WA** | -0.779*** | -0.101 | 1 | | | | | | | | | | | | | |
| **Slope** | 0.490 | -0.171 | -0.546* | 1 | | | | | | | | | | | | |
| **Q** | -0.167 | 0.116 | 0.292 | -0.273 | 1 | | | | | | | | | | | |
| **Depth** | -0.211 | 0.074 | 0.547* | -0.282 | 0.477 | 1 | | | | | | | | | | |
| **Vel** | -0.141 | 0.308 | 0.017 | -0.069 | 0.773*** | 0.188 | 1 | | | | | | | | | |
| **Can** | -0.235 | 0.257 | -0.154 | 0.468 | -0.501 | -0.375 | -0.118 | 1 | | | | | | | | |
| **pH** | -0.740*** | 0.315 | 0.504 | -0.126 | -0.107 | 0.057 | -0.273 | 0.234 | 1 | | | | | | | |
| **Con** | -0.769*** | 0.197 | 0.666** | -0.320 | -0.290 | 0.277 | -0.332 | 0.371 | 0.717** | 1 | | | | | | |
| **$NH_4^+$-N** | -0.320 | 0.391 | 0.116 | -0.191 | -0.213 | -0.012 | 0.095 | 0.217 | 0.209 | 0.450 | 1 | | | | | |
| **$NO_3^-$-N** | 0.598* | -0.319 | -0.545* | 0.824*** | -0.223 | -0.282 | -0.181 | 0.279 | -0.245 | -0.466 | -0.494 | 1 | | | | |
| **SRP-P** | -0.054 | -0.026 | 0.163 | 0.045 | 0.467 | 0.331 | 0.448 | -0.172 | -0.115 | -0.039 | 0.364 | -0.080 | 1 | | | |
| **NP** | 0.421 | -0.185 | -0.453 | 0.590* | -0.286 | -0.478 | -0.267 | 0.38 | -0.113 | -0.364 | -0.566* | 0.844*** | -0.437 | 1 | | |
| **T_Avg** | -0.795*** | 0.186 | 0.738** | -0.502 | 0.316 | 0.334 | 0.409 | 0.059 | 0.322 | 0.598* | 0.539* | -0.648** | 0.393 | -0.647** | 1 | |
| **T_CV** | 0.662** | -0.205 | -0.374 | 0.069 | -0.165 | -0.049 | -0.320 | -0.550* | -0.373 | -0.523* | -0.158 | 0.088 | -0.088 | 0.004 | -0.633* | 1 |

**Table S2.4: Results of one-way ANOVAs testing nutrient limitation of algal biomass (i.e., significant difference between a treatment and control) at eight streams across two seasons. Sites are ordered from low to high elevation**

| Site | Status | F-value | Pr(>F) | Tukey's HSD P-value |
|---|---|---|---|---|
| **Summer** | | | | |
| EK | Primarily N-limited, Secondarily P-limited | 37.92 | <0.001 | N: 0.018<br>NP: <0.001 |
| SM | NP-limited | 3.587 | 0.032 | NP: 0.035 |
| LB | NP-limited | 38.55 | <0.001 | NP: <0.001 |
| BV | Primarily N-limited, Secondarily P-limited | 59.92 | <0.001 | N: <0.001,<br>NP: <0.001 |
| PG | NP-limited | 33.42 | <0.001 | NP: <0.001 |
| KP | None | 7.689 | 0.002 | |
| EFSC | None | 4.366 | 0.016 | |
| WFSC | Primarily N-limited, Secondarily P-limited | 13.21 | <0.001 | N: <0.001<br>NP: <0.001 |
| **Fall** | | | | |
| EK | Primarily N-limited, Secondarily P-limited | 30.33 | <0.001 | N: 0.016<br>NP: <0.001 |

| | | | | |
|---|---|---|---|---|
| SM | None | 2.652 | 0.077 | |
| LB | Primarily N-limited, Secondarily P-limited | 382.2 | <0.001 | N: <0.001<br>NP: <0.001 |
| BV | NP-limited | 9.483 | <0.001 | NP: 0.042 |
| PG | Primarily P-limited, Secondarily N-Limited | 21.26 | <0.001 | P: <0.001<br>NP: <0.001 |
| KP | None | 5.84 | 0.005 | |
| EFSC | None | 3.159 | 0.047 | |
| WFSC | None | 9.153 | <0.001 | |

**Table S2.5: Simple linear regression models quantifying the effects of geographic variables on nutrient diffusing substrate effect sizes (n=16 experiments) and algal accrual (n=15 experiments) at eight sites over two seasons in 2016. p<0.05 is denoted by "*". For = % watershed forest cover and WA = watershed area.**

| Response Variable | Coefficient | Estimate | T-value | P-value | $R^2$ |
|---|---|---|---|---|---|
| Accrual | Intercept | 0.027 | 1.806 | 0.096 | |
| | For | -0.000 | -0.506 | 0.622 | 0.021 |
| N-LRR | Intercept | -0.100 | -0.151 | 0.882 | |
| | For | 0.008 | 0.887 | 0.390 | 0.053 |

| | | | | | |
|---|---|---|---|---|---|
| P-LRR | Intercept | 1.636 | 1.817 | 0.091 | |
| | For | -0.025 | -2.076 | 0.057 | 0.235 |
| NP-LRR | Intercept | 1.322 | 1.201 | 0.250 | |
| | For | -0.002 | -0.134 | 0.895 | 0.001 |
| Accrual | Intercept | 0.017 | 2.744 | 0.018 | |
| | Slope | 0.000 | 0.503 | 0.624 | 0.021 |
| *N-LRR | Intercept | 0.924 | 3.820 | 0.002 | |
| | Slope | -0.069 | -2.234 | 0.043 | 0.263 |
| P-LRR | Intercept | 0.772 | 3.630 | 0.015 | |
| | Slope | -0.036 | -1.271 | 0.260 | 0.244 |
| *NP-LRR | Intercept | 2.023 | 5.491 | <0.001 | |
| | Slope | -0.129 | -2.741 | 0.016 | 0.349 |
| Accrual | Intercept | 0.002 | 4.003 | 0.002 | |
| | WA | 0.000 | -0.149 | 0.884 | 0.002 |
| *N-LRR | Intercept | 0.115 | 0.667 | 0.515 | |
| | WA | 0.033 | 2.919 | 0.011 | 0.378 |
| P-LRR | Intercept | -0.304 | -0.926 | 0.370 | |
| | WA | 0.011 | 0.514 | 0.615 | 0.019 |
| *NP-LRR | Intercept | 0.556 | 2.094 | 0.055 | |

| | | | | | |
|---|---|---|---|---|---|
| | WA | 0.058 | 3.302 | 0.005 | 0.438 |

**Table S2.6: Simple linear regression models quantifying the effects of environmental variables on nutrient diffusing substrate effect sizes (n=16 experiments) and algal accrual (n=15 experiments) at eight sites over two seasons in 2016. p<0.05 is denoted by "*". See Fig. S1 for abbreviations, and Q = discharge.**

| Response Variable | Coefficient | Estimate | T-value | P-value | $R^2$ |
|---|---|---|---|---|---|
| Accrual | Intercept | 0.032 | 3.921 | 0.002 | |
| | Ammonium | -0.003 | -1.650 | 0.125 | 0.185 |
| N-LRR | Intercept | 0.181 | 0.446 | 0.662 | |
| | Ammonium | 0.080 | 0.775 | 0.451 | 0.041 |
| P-LRR | Intercept | 0.154 | 0.235 | 0.818 | |
| | Ammonium[log] | -0.283 | -0.552 | 0.589 | 0.021 |
| NP-LRR | Intercept | 1.231 | 1.838 | 0.087 | |
| | Ammonium | -0.015 | -0.085 | 0.933 | 0.001 |
| Accrual[log] | Intercept | -3.936 | -8.465 | <0.001 | |
| | Can | -0.006 | -0.797 | 0.441 | 0.050 |
| N-LRR | Intercept | 0.773 | 2.976 | 0.010 | |
| | Can | -0.006 | -1.395 | 0.185 | 0.122 |
| *P-LRR | Intercept | 0.541 | 1.550 | 0.144 | |
| | Can | -0.014 | -2.501 | 0.025 | 0.309 |

| | | | | | |
|---|---|---|---|---|---|
| *NP-LRR | Intercept | 1.925 | 5.069 | <0.001 | |
| | Can | -0.014 | -2.367 | 0.033 | 0.286 |
| Accrual | Intercept | 0.028 | 1.242 | 0.238 | |
| | Con[log] | -0.002 | -0.370 | 0.718 | 0.011 |
| N-LRR | Intercept | -0.905 | -0.961 | 0.353 | |
| | Con[log] | 0.341 | 1.479 | 0.161 | 0.135 |
| P-LRR | Intercept | 2.480 | 1.831 | 0.088 | |
| | Con[log] | -0.659 | -1.991 | 0.066 | 0.221 |
| NP-LRR | Intercept | 0.187 | 0.116 | 0.910 | |
| | Con[log] | 0.245 | 0.619 | 0.546 | 0.027 |
| Accrual | Intercept | 0.002 | 1.919 | 0.079 | |
| | Depth | 0.000 | -0.177 | 0.863 | 0.003 |
| N-LRR | Intercept | -0.421 | -0.898 | 0.385 | |
| | Depth | 0.045 | 1.989 | 0.067 | 0.220 |
| P-LRR | Intercept | -1.006 | -1.304 | 0.213 | |
| | Depth | 0.042 | 1.113 | 0.284 | 0.081 |
| *NP-LRR | Intercept | -0.590 | -0.836 | 0.417 | |
| | Depth | 0.090 | 2.619 | 0.020 | 0.329 |

| | | | | | |
|---|---|---|---|---|---|
| *Accrual | Intercept | 0.013 | 3.404 | 0.005 | |
| | DIN:SRP | 0.002 | 2.668 | 0.020 | 0.372 |
| *N-LRR | Intercept | 1.348 | 4.114 | 0.001 | |
| | DIN:SRP[log] | -0.306 | -2.882 | 0.012 | 0.372 |
| P-LRR | Intercept | -0.160 | -0.488 | 0.633 | |
| | DIN:SRP | -0.001 | -0.108 | 0.916 | 0.001 |
| NP-LRR | Intercept | 2.281 | 3.865 | 0.002 | |
| | DIN:SRP[log] | -0.385 | -2.013 | 0.064 | 0.225 |
| Accrual | Intercept | 0.011 | 1.379 | 0.193 | |
| | Q[log] | -0.003 | -1.227 | 0.243 | 0.112 |
| N-LRR | Intercept | 0.768 | 2.052 | 0.059 | |
| | Q[log] | 0.099 | 0.863 | 0.403 | 0.051 |
| *P-LRR | Intercept | 0.990 | 2.111 | 0.053 | |
| | Q[log] | 0.393 | 2.735 | 0.016 | 0.348 |
| NP-LRR | Intercept | 1.938 | 3.335 | 0.005 | |
| | Q[log] | 0.254 | 1.427 | 0.175 | 0.127 |
| Accrual | Intercept | 0.015 | 3.197 | 0.008 | |
| | Nitrate | 0.000 | 1.451 | 0.172 | 0.149 |

| | | | | | |
|---|---|---|---|---|---|
| *N-LRR | Intercept | 1.447 | 7.671 | <0.001 | |
| | Nitrate[log] | -0.341 | -5.772 | <0.001 | 0.704 |
| P-LRR | Intercept | -0.222 | -0.652 | 0.525 | |
| | Nitrate | 0.001 | 0.153 | 0.881 | 0.002 |
| *NP-LRR | Intercept | 2.480 | 6.103 | <0.001 | |
| | Nitrate[log] | -0.456 | -3.576 | 0.003 | 0.477 |
| Accrual | Intercept | -0.022 | -0.465 | 0.651 | |
| | pH | 0.006 | 0.887 | 0.394 | 0.067 |
| N-LRR | Intercept | -2.754 | -1.455 | 0.169 | |
| | pH | 0.429 | 1.703 | 0.112 | 0.182 |
| *P-LRR | Intercept | 5.671 | 2.203 | 0.046 | |
| | pH | -0.791 | -2.306 | 0.038 | 0.290 |
| NP-LRR | Intercept | -2.216 | -0.682 | 0.507 | |
| | pH | 0.450 | 1.039 | 0.318 | 0.077 |
| Accrual | Intercept | 0.029 | 4.307 | 0.001 | |
| | SRP | -0.003 | -1.588 | 0.138 | 0.174 |
| N-LRR | Intercept | 0.651 | 2.039 | 0.061 | |
| | SRP | -0.052 | -0.637 | 0.534 | 0.028 |

| | | | | | |
|---|---|---|---|---|---|
| P-LRR | Intercept | -0.307 | -0.628 | 0.54 | |
| | SRP | 0.035 | 0.285 | 0.78 | 0.006 |
| NP-LRR | Intercept | 1.505 | 2.923 | 0.011 | |
| | SRP | -0.094 | -0.722 | 0.482 | 0.036 |
| *Accrual | Intercept | 0.040 | 4.914 | <0.001 | |
| | T_Avg | -0.002 | -2.661 | 0.021 | 0.371 |
| N-LRR | Intercept | -0.319 | -0.777 | 0.450 | |
| | T_Avg | 0.089 | 2.041 | 0.061 | 0.229 |
| P-LRR | Intercept | -0.555 | -0.793 | 0.441 | |
| | T_Avg | 0.042 | 0.561 | 0.584 | 0.022 |
| NP-LRR | Intercept | 0.121 | 0.174 | 0.865 | |
| | T_Avg | 0.119 | 1.609 | 0.130 | 0.156 |
| Accrual | Intercept | 0.011 | 1.275 | 0.227 | |
| | T_CV | 0.038 | 1.202 | 0.253 | 0.108 |
| N-LRR | Intercept | 0.418 | 1.045 | 0.314 | |
| | T_CV | 0.226 | 0.146 | 0.886 | 0.002 |
| P-LRR | Intercept | -0.442 | -0.734 | 0.475 | |
| | T_CV | 1.080 | 0.463 | 0.650 | 0.015 |

| | | | | | |
|---|---|---|---|---|---|
| NP-LRR | Intercept | 1.091 | 1.684 | 0.114 | |
| | T_CV | 0.365 | 0.145 | 0.887 | 0.002 |
| Accrual | Intercept | 0.034 | 4.258 | 0.001 | |
| | Vel[log] | -0.006 | -1.950 | 0.075 | 0.241 |
| N-LRR | Intercept | 0.417 | 0.982 | 0.343 | |
| | Vel[log] | 0.024 | 0.138 | 0.892 | 0.001 |
| P-LRR | Intercept | -1.258 | -2.224 | 0.043 | |
| | Vel[log] | 0.468 | 2.036 | 0.061 | 0.229 |
| NP-LRR | Intercept | 1.048 | 1.522 | 0.150 | |
| | Vel[log] | 0.057 | 0.203 | 0.842 | 0.003 |

**Table S3.1. Environmental variables measured during nutrient diffusing substrate experiments at Little Beaver Creek, CO. Multiple measurements are recorded as mean ± standard deviation. Several nutrient measurements were below detection. pH was not measured during experiment 3 because of an instrument malfunction.**

| Expt | Dates | Temp (°C) | pH | Cond ($\mu S\ cm^{-1}$) | SRP-P ($\mu g\ L^{-1}$) | $NO_3^-$-N ($\mu g\ L^{-1}$) | Discharge ($m^3\ sec^{-1}$) | Velocity ($cm\ sec^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | July 29 – Aug 14, 2016 | 11.55 ± 1.91 | 7.79 | 43.52 ± 2.05 | <1 | <10 | 0.13 | 45.12 ± 15.33 |
| 2 | Aug 24 – Sept 7, 2016 | 10.05 ± 1.77 | 8.1 ± 0.28 | 46.22 ± 2.47 | 1.67 ± 0.97 | <10 | 0.07 | 49.07 ± 8.54 |
| 3 | July 17 – Aug 5, 2017 | 11.7 | -- | 30.51 | 2.27 ± 2.11 | <10 | 0.4 | 49.31 ±13.3 |

**Table S3.2. Results of ANOVAs testing the effect of three treatment classes on P effect sizes. Four different phosphate chemicals ($KH_2PO_4$, $K_2HPO_4$, $NaH_2PO_4$, $Na_2HPO_4$) crossed with laboratory heating methodology were deployed in two NDS experiments in 2016. Treatment classes in the statistical analyses included phosphate cation, phosphate form, and heating method. ANOVA results testing the effect of each treatment class on each response variable's P treatment effect size are presented, with experiment included as a fixed effect block in all models. P<0.05 is indicated as "*".**

| | Chl *a* | | AFDM | | AI | | GPP | | GPP/Chla | |
|---|---|---|---|---|---|---|---|---|---|---|
| Factor | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* |
| Cation | 0.341 | 0.561 | 0.352 | 0.555 | 0.407 | 0.526 | 0.167 | 0.684 | 0.632 | 0.429 |
| Expt | 0.059 | 0.809 | 0.665 | 0.417 | 0.887 | 0.349 | 6.368 | 0.013* | 6.385 | 0.013* |
| Form | 0.571 | 0.452 | 1.552 | 0.217 | 2.262 | 0.137 | 5.057 | 0.027* | 5.578 | 0.020* |
| Expt | 0.080 | 0.778 | 0.673 | 0.415 | 0.913 | 0.342 | 6.417 | 0.013* | 6.448 | 0.013* |
| Heat | 0.498 | 0.482 | 0.262 | 0.610 | 0.528 | 0.470 | 0.351 | 0.555 | 0.011 | 0.917 |
| Expt | 0.080 | 0.779 | 0.591 | 0.444 | 0.789 | 0.377 | 6.449 | 0.013* | 6.589 | 0.012* |

**S3 Table. Results of ANOVAs testing the effect of four treatment classes on P effect sizes. Four different phosphate chemicals ($KH_2PO_4$, $K_2HPO_4$, $NaH_2PO_4$, $Na_2HPO_4$) crossed with laboratory heating methodology and two phosphate concentrations were deployed in an NDS experiment in 2017. Treatment classes in the statistical analyses included phosphate cation, phosphate form, and heating method, along with concentration as a potential modifier. Two-way ANOVA results are presented where each treatment class was crossed with concentration, to determine effects on each response variable's P treatment effect size. P<0.05 is indicated as "*".**

| | Chla | | AFDM | | AI | | GPP | | GPP/Chla | |
|---|---|---|---|---|---|---|---|---|---|---|
| Factor | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* |
| Cation | 0.124 | 0.725 | 0.275 | 0.601 | 0.079 | 0.800 | 0.044 | 0.835 | 0.234 | 0.630 |
| Conc | 0.263 | 0.609 | 1.231 | 0.270 | 0.901 | 0.345 | 0.767 | 0.384 | 0.107 | 0.744 |
| Cation*Conc | 1.507 | 0.223 | 0.060 | 0.806 | 0.311 | 0.578 | 0.858 | 0.357 | 0.000 | 1.000 |
| Form | 0.124 | 0.725 | 4.129 | 0.450 | 3.636 | 0.060 | 2.850 | 0.095 | 2.091 | 0.152 |
| Conc | 0.291 | 0.591 | 1.239 | 0.269 | 0.828 | 0.365 | 0.817 | 0.369 | 0.105 | 0.747 |
| Form*Conc | 0.586 | 0.446 | 0.097 | 0.756 | 0.008 | 0.927 | 0.475 | 0.493 | 1.045 | 0.309 |
| Heat | 0.700 | 0.405 | 0.005 | 0.944 | 0.296 | 0.588 | 2.234 | 0.138 | 5.540 | 0.021* |
| Conc | 0.359 | 0.550 | 1.233 | 0.270 | 0.790 | 0.376 | 0.690 | 0.408 | 0.050 | 0.823 |
| Heat*Conc | 0.516 | 0.474 | 1.199 | 0.276 | 2.875 | 0.093 | 1.159 | 0.284 | 1.156 | 0.285 |