Title

Addressing Confounding Factors in Algal Phosphorus Limitation Experiments

Running Title

Algal Phosphorus Limitation

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Abstract

Assessing algal nutrient limitation is critical for understanding the interaction of primary production and nutrient cycling in stream ecosystems. Nutrient diffusing substrates (NDS) have been used for over thirty years to quantify nutrient limitation. However, many NDS studies have shown a decrease in algal biomass on phosphorus (P) treatments compared to controls, which is inconsistent with the paradigm of freshwater P-limitation. While direct P toxicity may occur, P-inhibition of algal growth could also be the result of NDS preparation artifacts or stream environmental factors. We first used a quantitative review to determine how often NDS P treatments inhibit algal biomass. We then designed and deployed targeted experiments to determine whether algal growth inhibition with P additions 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 as an artifact of NDS preparation. In addition, we completed a quantitative review of published data to determine the potential for heterotrophic microbial competition or selective grazing to explain decreases in algal biomass with P additions. We found that 13.7% of NDS P experiments showed significant inhibition of algal growth, as compared to just 5.1% and 3.9% of N and NP experiments. Our field experiments did not show inhibition of autotrophic growth with P additions as is routinely reported. However, we did see increased heterotrophic biomass on treatments that used monobasic phosphate salts as compared to those that used dibasic phosphate salts. The quantitative review did not show significantly enhanced heterotrophy on NDS P treatments, nor did we find consistent evidence for grazer selection of P-rich algae. Past laboratory experiments and meta-analyses support the plausibility of direct P toxicity, cation toxicity, or phosphate form (monobasic vs. dibasic) leading to inhibition of algal growth. Given that multiple mechanisms may be acting simultaneously, we recommend practical, cost-effective steps for designing NDS experiments to avoid the potential

for P-inhibition due to artifacts of the experimental design. We also outline future research that could lead to a better understanding of how resources interact with environmental conditions to regulate algal growth in stream ecosystems.

Keywords: algae, phosphorus, toxicity, nutrient diffusing substrate, nutrient limitation, microbial

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 which nutrients limit algal productivity for 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). NDS 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 response metrics can then be compared across the NDS treatments. 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 treatments as compared to controls (e.g., Bernhardt & Likens 2004, Sanderson et al. 2009), and it is unclear how often and why this phenomenon occurs.

Several hypotheses have been introduced to explain why addition of a common limiting nutrient (i.e. P) in NDS would result in a decrease of algal biomass. Hypotheses focused on P treatment experimental design include: 1) P toxicity, 2) phosphate cation form (K vs. Na) and toxicity, 3) phosphate form (monobasic vs. dibasic), and 4) H_2O_2 production from phosphate reacting with agar during autoclaving. As a macronutrient, P is required for algal growth and maintenance (Borchardt 1996), but high P concentrations from NDS have been hypothesized to lead to toxicity (Fairchild et al. 1985). While mechanisms for this toxicity in algae are unclear, the terrestrial plant literature has shown that excessive P concentrations in growth media 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. Algal studies have rarely tested whether P itself is toxic, or whether high concentrations of the phosphate salt cations (K^+, Na^+, H^+) may inhibit algal growth. However, laboratory studies have shown that algae have lower toxicity thresholds for K phosphates as compared to Na phosphates, and for KCl as compared to NaCl (Chu 1943, Lehman 1976). The form of the phosphate salt 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 by contributing to H^+ or metal toxicity or altering nutrient availability (Planas 1996). Finally, NDS construction methods could mediate the effect of P treatments on 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).

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 stimulate heterotrophic microbes and increase competition between autotrophs and heterotrophs for other limiting nutrients, ultimately suppressing algal growth. Heterotrophs could increase if they disproportionately benefit from P additions (Bechtold et al. 2012), or if they are less susceptible to P toxicity. 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 an observed algal biomass decrease on NDS P treatments (Bernhardt & Likens 2004). Nutrient-specific foraging was supported in a theoretical analysis (Neeson et al. 2013), and additional laboratory experiments have found support for P-specific foraging (Hood et al. 2014, Mooney et al. 2016).

Determining why P-inhibition of algal growth occurs is an important methodological question with implications for our ecological understanding of lotic ecosystems. For instance, if P toxicity is the driving mechanism, then future studies should measure in-stream P concentrations to inform the most effective P concentrations to use in NDS agar. If cation toxicity or pH alteration are the inhibition mechanisms, then many previous studies may be underestimating algal P-

limitation and future experiments should attempt to control for these factors. If H_2O_2 production is the mechanism (Tanaka et al. 2014), then studies have also likely been underestimating algal P-limitation due to an experimental artifact, but this mechanism can be avoided by a simple methodological change (i.e., boiling agar and phosphate separately before mixing, Tank et al. 2017). If heterotrophic microbial competition is the mechanism, then studies should consider measuring additional response variables to capture more comprehensive characteristics of the biofilm including heterotrophic dynamics. Finally, if grazers are selecting for P rich algal resources, then future studies should consider employing grazer exclusions (Pringle & Blake 1994) to account for biomass losses due to grazing. Each of these 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. A further challenge is that the hypothesized mechanisms are not mutually-exclusive.

We used field experiments and quantitative analyses of existing data to investigate how often and why NDS P treatments inhibit algal growth. First, we quantitatively analyzed past experiments to determine the frequency of significant inhibition on NDS P, N, and NP treatments. We also reviewed the existing literature to determine if there was consistent evidence for heterotrophic microbial competition or top-down grazing control that could be leading to algal P-inhibition across multiple study systems. Finally, we completed field experiments to better understand the effects of P treatment experimental design on several indices of algal growth: chlorophyll *a* (a measure of algal biomass), ash-free dry mass (AFDM, a measure of organic matter), a calculated index of autotrophy (AI), gross primary productivity (GPP), chlorophyll-specific GPP (GPP/chlorophyll *a*), net primary productivity (NPP), and respiration.

Methods

Quantitative Review

To determine 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 chlorophyll *a* as a response variable. Results were extracted from the primary literature, along with a suite of experimental, environmental, and geographic factors. We used separate one-tailed t-tests for each NDS experiment to determine how often N treatments (n=553), P treatments (n=534), and NP treatments (n=591) produced significantly lower chlorophyll *a* means than controls. Although we could have used a meta-analysis approach, we chose to use t-tests because they better represent how investigators analyze data in individual studies.

To assess whether P additions changed algal-heterotrophic competitive interactions, we identified a subset of experiments from Beck et al.'s (2017) database that also reported AFDM as a response variable, as a proxy for total biofilm biomass. We extracted AFDM data using Webplot Digitizer version 3.12 (Rohatgi 2015), and calculated an autotrophic index (AI) as follows:

$$AI = AFDM / \text{chlorophyll } a \qquad \text{Eqn. 1}$$

AFDM and chlorophyll *a* were both recorded in ug cm^{-2}, and lower values of the index were interpreted as a higher proportion of autotrophy in the microbial community (Bechtold et al. 2012). We used ANOVAs with nutrient treatment as a factor and study as a fixed effect block to determine whether AFDM and AI differed by nutrient treatment.

To determine whether P additions influence grazer selection of algal biofilms, we identified NDS experiments that also incorporated grazer exclusion treatments. As the effect size, we

calculated the log response ratio (LRR) of adding P separately for exclusion plots and grazed plots:

$$\text{P-LRR} = \ln (Y_1 / Y_2) \qquad \text{Eqn. 2}$$

where Y_1 is the NDS P treatment chlorophyll *a* mean, and Y_2 is the NDS control chlorophyll *a* mean. LRRs greater than zero indicate there is a positive treatment effect on the response variable of interest, but LRRs less than zero indicate an inhibitory effect of the treatment as compared to controls. For reference, a meta-analysis of NDS P effect sizes found that the average LRR was 0.26 (Beck et al. 2017).

Experiments

To explore how experimental design influences 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 treatments (KH_2PO_4, K_2HPO_4, NaH_2PO_4, or Na_2HPO_4 all at 0.1 M concentrations) with two agar preparation methods (boiling agar and phosphate together vs. separately) for a total of eight treatments (Table 1). In a third experiment, we again crossed the four phosphate compounds with two laboratory heating methods but in order to assess direct toxicity of excess P, we also used two different concentrations of P (0.05 M and 0.5 M, Fairchild et al. 1985) for a total of 16 treatments (Table 1).

We constructed NDS according to the most commonly used approach (Tank et al. 2017). Briefly, we boiled 2% agar with deionized water, poured the solutions into 6 replicate 30 mL vials (Item #66159, U.S. Plastic Corps, Lima OH), and capped the cooled agar with fritted glass

discs (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 for all other treatments. 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 intermediate response variables to test pH alteration as a putative mechanism for P-inhibition. 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 Ziploc® bags with 1 L of distilled water. We used a multimeter and probes (Thermo Fisher Scientific Orion Star™ A329, Waltham, MA) to measure the starting pH and remeasured the values 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). Experiments 1 and 2 were deployed in summer 2016, while experiment 3 was deployed in summer 2017 (Table 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 chlorophyll *a* relative to the control (Beck, unpublished). We randomized the treatments and attached 6-8 individual NDS vials to plastic L-shaped bars (Item #45031, U.S. Plastic Corp, Lima, OH). 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 (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 MiniWater® 20 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 (U.S. EPA 1993) and SRP using the ascorbic acid method (Murphy & Riley 1962) on an ALPKEM® Flow Solution IV autoanalyzer (O.I. Analytical, College Station, TX).

We analyzed primary production and respiration 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 (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 NPP as the increase in oxygen during the light incubations, correcting for the change in the blank streamwater tubes. We calculated respiration as the decrease in oxygen during the dark incubations, correcting for the change in the blank streamwater tubes. GPP was calculated as follows, then all variables were standardized by disc area and time:

$$GPP = NPP + |Respiration| \qquad \text{Eqn. 3}$$

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 (U.S. EPA 1997) using an Aquafluor® fluorometer (Turner Designs, San Jose, CA). We calculated chlorophyll-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, 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 re-hydrated 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 also combined the chlorophyll *a* and AFDM measurements to calculate AI as described in Equation 1.

Because experimental durations differed (14-18 day in-stream incubations), we compared measurements that were standardized and unstandardized by number of days for the chlorophyll *a* and AFDM accrual variables. Statistical analyses using standardized versus unstandardized metrics did not differ and thus we used variables that were unstandardized by days of incubation so they could be directly comparable to the other response variable rates (GPP, NPP, and respiration) and all calculated responses (AI and chlorophyll-specific GPP).

Statistical Analyses for Experiments

To test the effect of treatments on the pH of the NDS agar and water incubated with the NDS vials, we used three-way ANOVAs with phosphate compound cation, phosphate form (monobasic vs. dibasic), and heating method as factors. Because the field experiments comprised an incomplete factorial design (i.e., crossed treatments and a separate control), we used two separate approaches to analyze the results. First, to determine whether treatments significantly stimulated or inhibited response variables relative to controls, we used separate one-way ANOVAs pairing each main treatment class (cation, phosphate form, heat) with each response variable (chlorophyll *a*, AFDM, AI, GPP, NPP, respiration, chlorophyll-specific GPP). Second, to determine whether treatments interactively influenced NDS P effect sizes, we ran three-way ANOVAs with cation form, phosphate form, and heat as predictor variables. The model response variables were P treatment values of chlorophyll *a*, AFDM, AI, GPP, NPP, respiration, and chlorophyll-specific GPP divided by their respective experimental control means (Tank et al. 2017). For the third experiment (summer 2017) we used four-way ANOVAs to also test the effect of concentration. In the ANOVAs, we used experiment as a fixed effect blocking factor to account for temporally-variable stream environmental factors that may have affected microbial responses to NDS. Although we recognize that family-wise error rates may be high because

multiple F-tests are computed for multi-factor ANOVAs, our research questions required testing both main effects and interactions since P-inhibition mechanisms were not mutually exclusive.

Results

Quantitative Review

In our analysis of hundreds of experiments from the literature we found that algal biomass was more commonly inhibited by NDS P treatments than either N or NP treatments. Results showed a significant negative effect of P additions in 13.7% of experiments (Figure 1). However, we found a significant negative effect of N and NP additions in only 5.1% and 3.9% of experiments, respectively (Figure 1).

To address the potential for heterotrophic suppression of autotrophs in microbial communities on NDS P treatments, we identified 45 experiments from 11 studies that reported both chlorophyll *a* and AFDM in response to NDS experiments with P as a treatment. Our analysis of this data showed chlorophyll *a* and AFDM were both significantly affected by study ($F_{10,178}$=40.78, $p<0.001$ and $F_{10,178}$=62.72, $p<0.001$) and nutrient treatment ($F_{3,178}$=14.14, $p<0.001$ and $F_{3,178}$=3.29, $p=0.022$), with significantly higher chlorophyll *a* and AFDM on N+P treatments than controls (Tukey's HSD, $p=0.004$ and $p=0.042$). Study also had a significant effect on AI ($F_{10,178}$=44.073, $p<0.001$) but nutrient treatment did not ($F_{3,178}$=1.626, $p=0.185$).

We found only three studies in the literature that used NDS with P treatments in conjunction with grazer exclusions, and we re-analyzed data from those studies to quantify potential grazer selection of algae on NDS P treatments. Of the three studies, one found that the effect of P on algal growth in NDS experiments (measured as LRRs, Eqn. 2) was greater when fish were present (LRR=0.069) than when they were absent (LRR=-0.202, Flecker et al. 2002). Another

study that examined the interaction between invertebrate grazers and P on algal growth found that the P effect size was greater in plots where invertebrate grazers were excluded (LRR=0.173) compared to those where invertebrates were present (LRR=-0.06395, Lourenço-Amorim et al. 2014). A third study found that the mean effects of P on algal growth were similar for plots with (LRR=0.060) and without (LRR=0.075) snail grazing (Winterbourn & Fegley 1989). Taken together, these five experiments from three independent studies showed similar P effect sizes for grazed (mean LRR=0.037, median LRR=0.013) and ungrazed plots (mean LRR=0.039, median LRR=0.038) and no consistent support for the selective feeding by grazers on P-enriched algal biomass.

Experiments

In our field experiments we found no significant differences between P treatments (Table 1) and controls (all $p > 0.05$) across all response variables, indicating neither inhibition nor stimulation of algal growth was induced by P treatments. In our analysis testing the effect of phosphate treatments and their interactions on NDS effect sizes (Table 1), many response variables showed a significant effect of experimental block (i.e., treatment effects differed between experiments) including AFDM, AI, GPP, NPP, and chlorophyll-specific GPP (all Tukey's HSD $p<0.05$). Environmental conditions were generally similar across the three experiments, except discharge was considerably higher during experiment 3 compared to experiments 1 and 2 (Table 2). GPP and chlorophyll-specific GPP showed no significant main effects or interactions related to the treatments ($p>0.05$, Figures 2-3). However, chlorophyll *a* did show a significant three-way interaction between cation, phosphate form (monobasic vs. dibasic), and heat ($F_{1,183}=4.799$, $p=0.030$), but no significant lower order effects (Figure 4). AFDM ($F_{1,168}=5.666$, $p=0.018$), AI ($F_{1,166}=6.079$, $p=0.015$), and NPP ($F_{1,180}=4.235$, $p=0.041$) all

showed a significant effect of phosphate form, whereby monobasic treatments had higher responses than dibasic treatments (Figures 5-7). Respiration was influenced by a significant two-way interaction between cation and heat ($F_{1,181}$=5.622, p=0.019, Figure 8). Responses to K phosphates were stronger than responses to Na phosphates, but only in the treatments where they were boiled together with agar. The third experiment (that also assessed the effect of P concentration on algal growth) showed no significant effect of concentration ($p>0.05$) on any of the response variables.

We found that phosphate form (monobasic vs. dibasic) was the only significant treatment factor influencing agar pH ($F_{1,24}$=1408.333, $p<0.001$) and water pH ($F_{1,8}$=496.508, $p<0.001$) in the laboratory experiments. For both response variables (Figure 9, Tukey's HSD $p<0.001$ for all comparisons), monobasic chemicals lowered pH means (agar pH =4.81, water pH =5.45) and dibasic chemicals raised pH means (agar pH=8.88, water pH =8.28) relative to controls (agar pH =7.25, water pH =6.35).

Discussion

Our experiments and quantitative literature analyses did not identify a clear mechanism to explain why 13.7% of past NDS experiments have shown a significant negative effect of P treatments on algal biomass. In fact, on average we observed slight P stimulation rather than inhibition in our experiments, consistent with the paradigm of P-limitation in inland waters. However, we did find that past laboratory experiments (e.g., Chu 1943, Lehman 1976) and meta-analyses (Beck et al. 2017, Keck & Lepori 2012) support the plausibility of direct P toxicity, cation toxicity, or phosphate form leading to inhibition of algal growth. Given that multiple mechanisms may be acting simultaneously, and some mechanisms are most likely ecosystem dependent, we recommend a series of practical steps for future NDS experiments to reduce the

likelihood of P-inhibition resulting from artifacts of experimental design and preparation. We also suggest promising avenues for future ecological research to improve our understanding of how resources and environmental conditions interact to affect algal growth in natural ecosystems

P toxicity

While our experiments did not show evidence of P toxicity, previous research supports the possibility of P toxicity occurring across a range of stream ecosystems. In contrast with previous research (Fairchild et al. 1985), we saw no significant difference in chlorophyll *a* between the low (0.05 M) P concentrations and high (0.5 M) P concentrations. However, we generally found higher values of autotrophic and heterotrophic metrics on the lower P concentration (0.05 M) treatments as compared to the higher P concentration (0.5 M) treatments (Figures 2-8). P toxicity thresholds are dictated by biological and environmental context (see below), and we may not have exceeded the threshold with the concentrations of P we used in this experiment. There is evidence that NDS experiments in other systems may commonly exceed toxicity thresholds, as a meta-analysis of 534 NDS experiments showed that higher P concentrations in NDS treatments significantly decreased P effect sizes (Beck et al. 2017). Physiological mechanisms for P toxicity of algae are not well defined, but some insight can be gained from the terrestrial plant literature.

Multiple terrestrial plant studies have demonstrated P toxicity in the form of leaf necrosis and discoloration (Musick 1978), reduced growth rates (Christie & Moorby 1975), and plant death (Heddle & Specht 1975, Groves & Keraitis 1976). These 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). For instance, a soybean study used KCl and NaCl controls to show that P rather than K or Na was toxic to the plant (Foote & Howell 1964). P toxicity may occur because algae (Stevenson & Stoermer 1982)

and terrestrial plants (Chapin et al. 1986) can take up excess P for storage (i.e. "luxury P uptake"), a strategy which may allow communities to take advantage of nutrient pulses in infertile environments. The terrestrial plant literature has shown that luxury P uptake can lead to toxic accumulation in fertile environments (Chapin et al. 1986), possibly because excess P within a cell can induce Fe (Christie & Moorby 1975), K (Christie & Moorby 1975), or Zn (Loneragan et al. 1982) deficiencies. The mechanisms and potential consequences of luxury P consumption for algae are less clear, but it is plausible that P accumulation could induce other elemental deficiencies as has been shown for terrestrial plants. However, the question remains as to whether NDS experiments diffuse at high enough rates that P could accumulate in the water-cell boundary layer or biofilm and exceed toxicity thresholds.

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 mM KH_2PO_4 can release 0.321 mM PO_4^{3-}-P·hr^{-1} at day 0, but that diffusion rate declines to 0.001 mM PO_4^{3-}-P·hr^{-1} by day 14 (Capps et al. 2011). Clay pot NDS (86.8 cm^2 area) constructed with 50 mM KH_2PO_4 can release 0.113 mM PO_4^{3-}-P ·hr^{-1} P at day 0, with a diffusion rate that declines to 0.011 mM PO_4^{3-}-P ·hr^{-1} by day 14. An additional study showed that 50 mM KH_2PO_4 plastic vial NDS (2.6 cm^2 area) diffused just 0.011 mM PO_4^{3-}-P ·hr^{-1} after being submerged in water for 96 hours (Rugenski et al. 2008). In general, diffusion rates decline in a log-linear fashion over time. Comparing these diffusion rates to reported laboratory toxicity thresholds (e.g., 0.019 mM PO_4^{3-}-P, Lehman 1976) shows that algal populations may initially experience concentrations of P from NDS that are sufficiently high to induce toxicity and inhibition of growth, but that influence likely declines over time as diffusion rates decrease. To optimize NDS experiments, future studies could attempt to calculate diffusion rates to

determine experimental lengths that are long enough to surpass the P toxicity stage, but short enough to maintain P stimulation levels from NDS vials. More oligotrophic streams may require less P to elicit a growth response and could also be more susceptible to toxicity if the P concentration is too high (Keck & Lepori 2012). Furthermore, it is important to consider that diffusion rates depend on experimental P concentrations, stream velocity (Corkum 1996), and temperature (Rugenski et al. 2008) which should be informed by background stream conditions and an understanding of the hydrograph in the ecosystem of study.

There is also evidence that the form and availability of other nutrients can influence P toxicity. For example, a laboratory study showed that P toxicity in diatoms and green algae was higher when NO_3^- was the source of N as compared to NH_4^+ (Chu 1943). Furthermore, laboratory experiments testing P toxicity thresholds generally supply an unlimited amount of N and light, but these resources are commonly limiting under field conditions. N or light limitation could potentially induce toxicity at lower concentrations of P, if luxury P accumulates without being used to support cellular functions due to limitation of biomass production by other resources. Terrestrial plant studies have shown a positive relationship between P toxicity thresholds 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). This is consistent with NDS studies in aquatic ecosystems where N+P treatments show less inhibition and higher stimulation as compared to treatments with P alone (Francoeur 2001). This is most likely because N does not become secondarily limiting and thus P is less likely to accumulate within the cell, avoiding the potential for P toxicity. Furthermore, P-inhibition tends 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 these conditions are likely to change among different ecosystems and conditions within an ecosystem.

Cation and Phosphate Form

When measuring P stimulation or toxicity, few studies have controlled for or even explored the effects of cation or phosphate form (monobasic vs. dibasic). However, there is some experimental evidence suggesting that toxicity thresholds differ based on the cation in the phosphate salt (Lehman 1976), whereby K leads to more rapid toxicity. *Dinobryon sociale* growth was maintained at P concentrations of 0.032 mM PO_4^{3-}-P for NaH_2PO_4 but growth declined when P concentrations were raised from 0.005 mM PO_4^{3-}-P to 0.019 mM PO_4^{3-}-P for KH_2PO_4 (Lehman 1976). Similarly, a study on *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. Field studies have not explicitly tested for cation toxicity, but Beck et al.'s (2017) meta-analysis of field studies found increased P effect sizes when NDS experiments used K phosphates as compared to Na phosphates (Beck et al. 2017). Taken together these results suggest K is unlikely to be toxic to algae in field experiments, and laboratory experiments do not support the likelihood of Na toxicity.

Although the cation in the phosphate salt does not appear to influence the effect of NDS P treatments on algal growth, it is likely that the form of phosphate (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 monobasic agar treatments (pH 4.81 ± 0.09) but was comparable to the dibasic agar treatments

(pH 8.88 ± 0.06). In this study we reported an increase in AFDM and AI for monobasic treatments compared to the dibasic treatments, suggesting that autotrophic microbes comprised a lower proportion of the community biomass. This is consistent with Beck et al.'s (2017) meta-analysis which showed P and NP effect sizes for chlorophyll *a* were significantly reduced in monobasic NDS treatments relative to those effects in dibasic treatments. Multiple lines of evidence suggest that the effect of pH from phosphate form has a stronger effect than the cation in the phosphate salt. For instance, we found P-inhibition is reported more commonly than N-inhibition, which could be driven by P compounds containing easily disassociated H^+ ions while N compounds often do not. Furthermore, algae clearly exhibit pH preferences that differ by species (Planas 1996), and it is plausible that the monobasic, acidifying treatments may inhibit algae and promote heterotrophic microbes that are more plastic in their pH requirements. 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. This simple step would avoid the confounding influence of pH on P effects on benthic algae. Future experiments could also test the effect of non-phosphate induced pH changes on autotrophic and heterotrophic growth to further test this mechanism.

Heating Method

The production of H_2O_2 during the preparation of NDS could also create an artifact in P-limitation experimental results. 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. Until further research is completed in this area, a simple solution would be to heat P and agar separately (described by Tank et al. 2017) and avoid the possibility of H_2O_2 interference with algal growth.

Microbial Competition

Indirect mechanisms may also 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 carbon that fuels heterotrophy, leading to a coupling of the two communities (Scott et al. 2008, Hoagland et al. 1993). When nutrients are added, these community dynamics are altered affecting the biomass and diversity of each pool (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 are influenced by P additions (i.e., no algal biomass inhibition and no change in AI), but future studies should consider P dynamics along with C and N dynamics to more comprehensively uncover the nature of nutrient-mediated microbial autotrophic and heterotrophic interactions.

Grazer Selection

In addition to heterotrophic microbes affecting algal growth through ecological interactions, we hypothesized that P effect sizes (measured as LRRs) on grazer exclusion treatments may be larger than on grazed treatments because grazers selectively consume P enriched algae. Our review of the literature did not produce consistent evidence to suggest that P effect sizes differed between grazed and ungrazed plots. However, in one primarily N-limited stream (Lourenço-Amorim et al. 2014) and one primarily P-limited stream (Winterbourn & Fegley 1989) the P effect sizes for ungrazed treatments were clearly higher than for grazed treatments. Because of the difficulty of conducting grazer exclusion NDS studies, only a few studies to date have investigated resource selectivity in benthic stream invertebrates. The few studies that have, however, do provide some insight into the possibility that P-enriched algae are preferentially grazed.

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 in California showed that despite low algal productivity, >50% of invertebrate biomass depended at least partially on algal food resources (McNeely 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* caddisflies (Mooney et al. 2016), a result that 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* that reduced available P for periphyton. 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. One option is to construct electrical exclusion treatments which can prevent grazing from

macroinvertebrates and vertebrates across a wide range of body sizes (Moulton et al. 2004, Pringle & Blake 1994). Although technically challenging, electrical exclusions appear to effectively exclude grazers, and facilitate field-scale experiments that avoid confounding effects that might be produced by physical barriers, such as sedimentation or velocity alterations.

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, and that have previously been observed at our study site (Beck, unpublished). 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, cation and P concentration treatment effects have been identified using controlled laboratory experiments that can achieve a wider and more precise concentration gradient as compared to field experiments. For example, NDS diffusion rates (Corkum 1996) and environmental characteristics in the field are clearly variable over 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.

Conclusion

Clarifying the role of cation form, secondary limiting nutrients, microbial competition, and grazer nutrient selection requires additional research but understanding these dynamics may have ecological implications beyond NDS methodological considerations. First, determining K and Na toxicity thresholds could inform ecological studies on how the increasing prevalence of road salt pollution from agricultural or impervious surface runoff is affecting algal growth (Cañedo-Argüelles et al. 2016). Second, NDS experiments could be completed along light or N gradients

under field conditions to determine whether P toxicity is influenced by other limiting resources, which could inform predictions about ecological effects of riparian canopy cover, sedimentation, N inputs, or other principal environmental controls on benthic algal growth. Additionally, it is clear that the autotrophic and heterotrophic components of biofilm communities may quickly shift between mutualism and competition depending on limiting resources like P (Shantz et al. 2016). NDS provide a promising experimental design that could be more commonly used to study ecological interactions between the heterotrophic and autotrophic components of stream biofilms. Finally, studies have not definitively demonstrated that grazers select for P-rich resources within algal mats, but such a feedback could drastically impact the structure and function of algal communities.

Considering that multiple mechanisms may be operating simultaneously to inhibit algal growth on NDS P treatments, we recommend several low effort, cost-effective steps 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 molarities for NDS construction. This would help limit molarities to an appropriate concentration to avoid the potential for toxicity to algae. Furthermore, measuring NDS diffusion rates under conditions that mimic natural systems would allow investigators to determine appropriate experimental lengths. 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 consistent P diffusion from NDS and avoid establishment by large grazer populations. 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 heating methods outlined by Tank et al. (2017) to avoid the potential for H_2O_2 production that inhibits microbial growth. We have outlined a series of practical steps to improve NDS studies that seek to understand P-limitation of benthic algae, with the goal of ensuring that studies are not underestimating P-limitation of primary producers in aquatic ecosystems. Avoiding confounding factors in NDS experiments will also improve our understanding of how resources and environmental conditions interact to affect algal growth in natural stream ecosystems.

Author Contributions

WSB conceived, designed, and implemented the study; analyzed and interpreted data; and wrote the manuscript. EKH interpreted data and wrote the manuscript.

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Figures

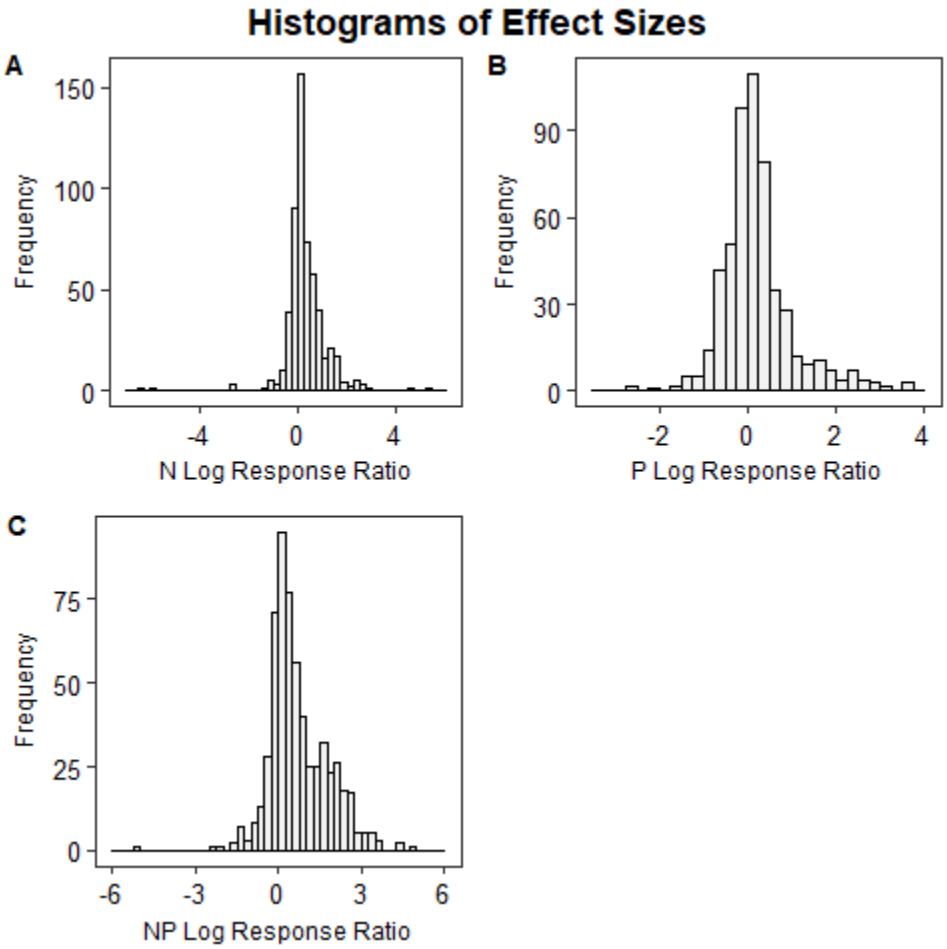

Figure 1: Histograms of NDS N (A), P (B), and N+P (C) effect sizes from the database of Beck et al. (2017), representing 67 studies and 649 experiments. Effect sizes are calculated as log response ratios (LRRs): ln(treatment chlorophyll a *mean/ control chlorophyll* a *mean). 5.1% of experiments showed a significant negative effect of adding N (n=553), 13.7% showed a significant negative effect of adding P (n=534), and 3.9% showed a significant negative effect of adding N+P (n=591).*

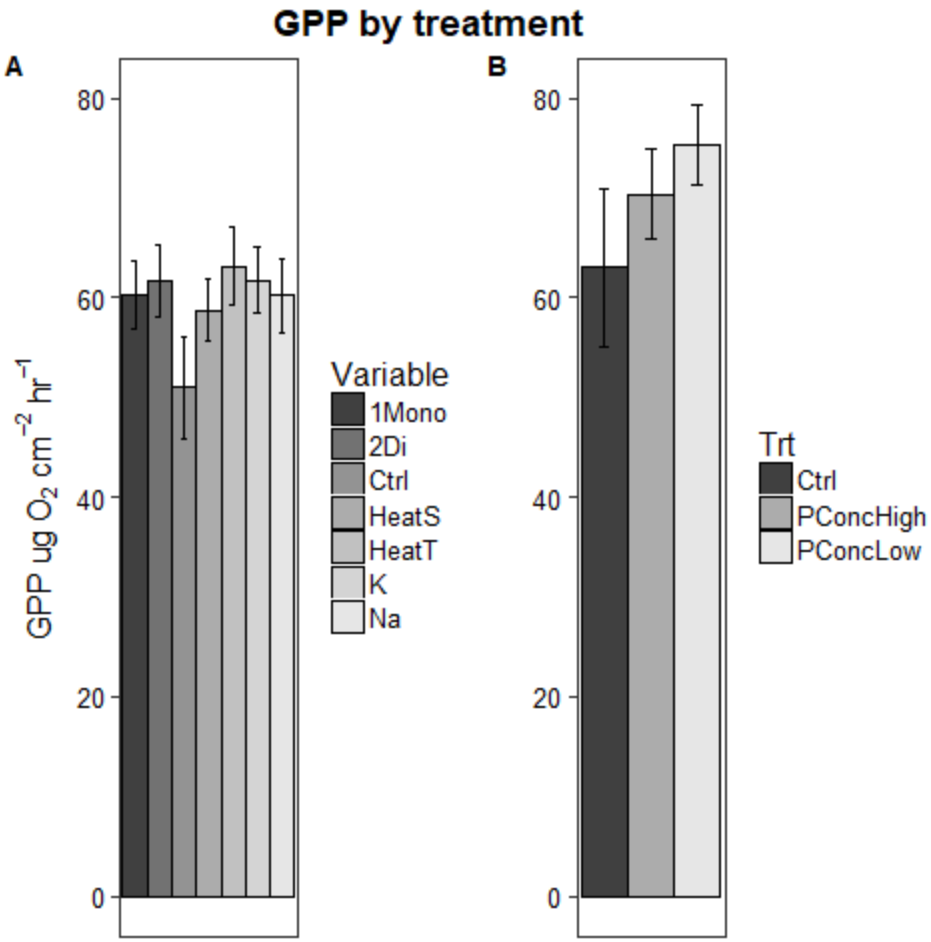

Figure 2: Gross Primary Productivity (GPP) means +/- 1 standard error from NDS experiments completed in July 2016, August 2016, and August 2017 in Little Beaver Creek, CO. Treatments consisted of crossing monobasic (KH_2PO_4 and NaH_2PO_4) vs. dibasic (K_2HPO_4 and Na_2HPO_4) chemical compounds (1Mono vs. 2Di), phosphate and agar heated together vs. separately (HeatT vs. HeatS), and K vs. Na phosphates (K vs. Na). A control was included in each experiment (Ctrl), and 0.5 vs. 0.05 M phosphorus was added as an additional factor in experiment three only (PConcHigh vs. PConcLow). For all experiments (A), total n=206, with treatments represented as follows: n= 96 (1Mono), n=92 (2Di), n=19 (Ctrl), n=93 (HeatT), n=95 (HeatS), n=90 (K), n=98 (Na). For experiment 3 (B), n=6 (Ctrl), n=47 (PConcHigh), n=50 (PConcLow).

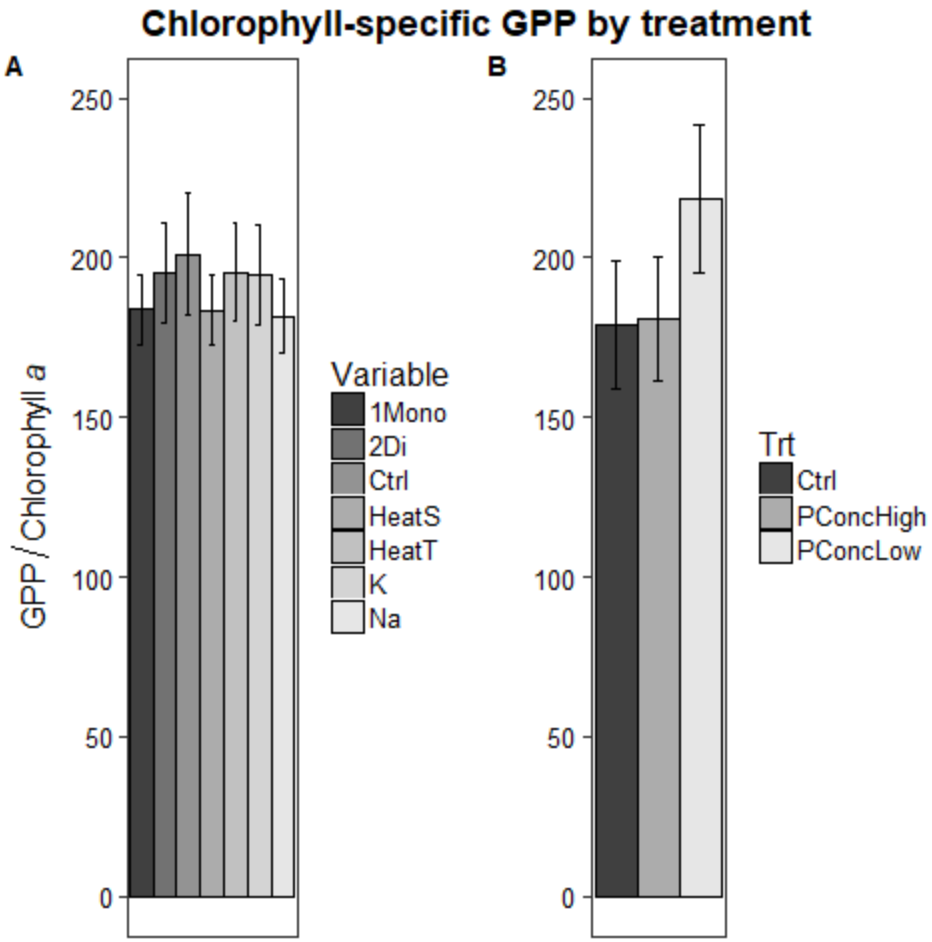

Figure 3: Chlorophyll-specific GPP means +/- 1 standard error from NDS field experiments (see Figure 2 caption). For all experiments (A), total n=205, with treatments represented as follows: n= 95 (1Mono), n=92 (2Di), n=18 (Ctrl), n=92 (HeatT), n=95 (HeatS), n=90 (K), n=97 (Na). For experiment 3 (B), n=6 (Ctrl), n=47 (PConcHigh), n=50 (PConcLow).

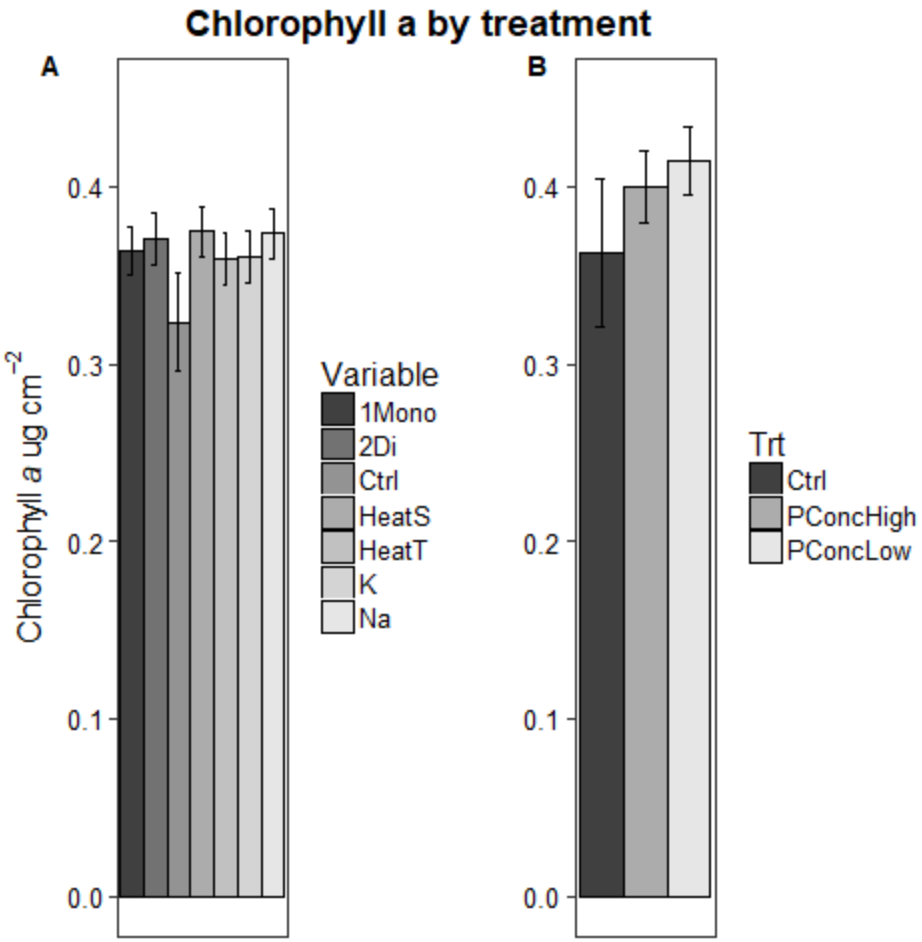

Figure 4: Chlorophyll a *means +/- 1 standard error from NDS field experiments (see Figure 2 caption). For all experiments (A), total n=212, with the treatments represented as follows: n= 98 (1Mono), n=95 (2Di), n=19 (Ctrl), n=95 (HeatT), n=98 (HeatS), n=94 (K), n=99 (Na). For experiment 3 (B), n=6 (Ctrl), n=49 (PConcHigh), n=50 (PConcLow).*

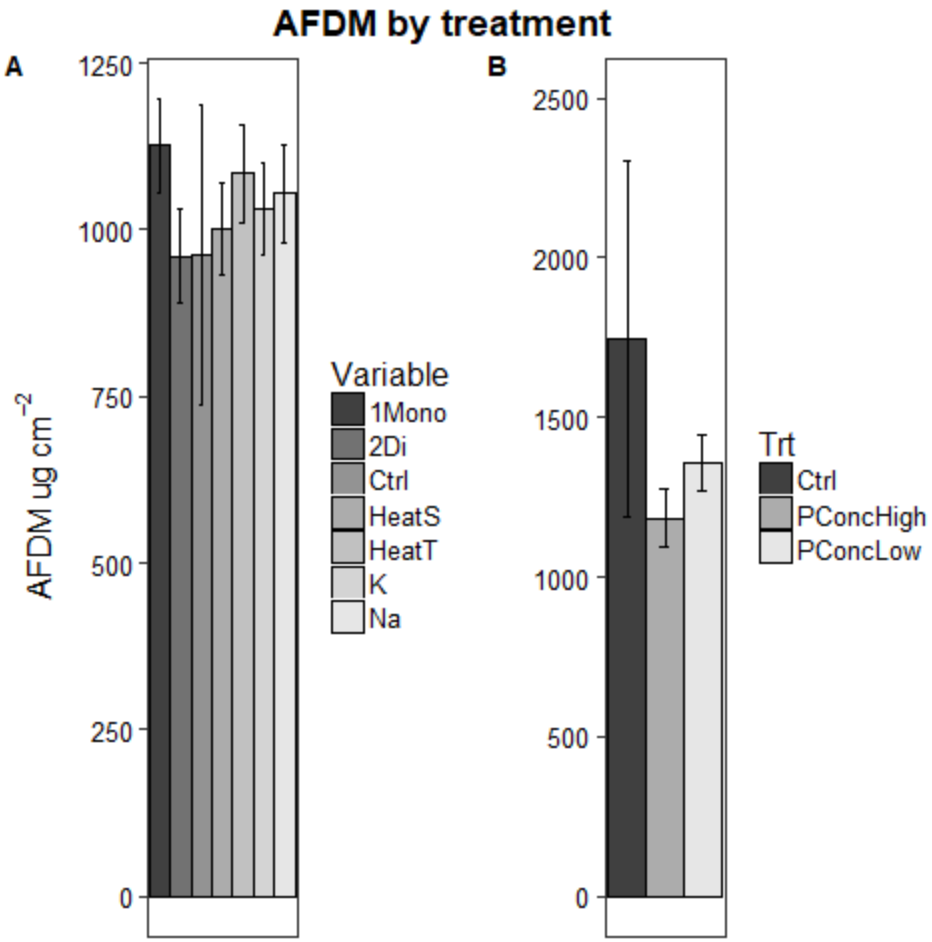

Figure 5: Ash-free dry mass (AFDM) means +/- 1 standard error from NDS field experiments (see Figure 2 caption). For all experiments (A), total n=197, with the treatments represented as follows: n= 90 (1Mono), n=89 (2Di), n=18 (Ctrl), n=89 (HeatT), n=90 (HeatS), n=86 (K), n=93 (Na). For experiment 3 (B), n=6 (Ctrl), n=49 (PConcHigh), n=49 (PConcLow).

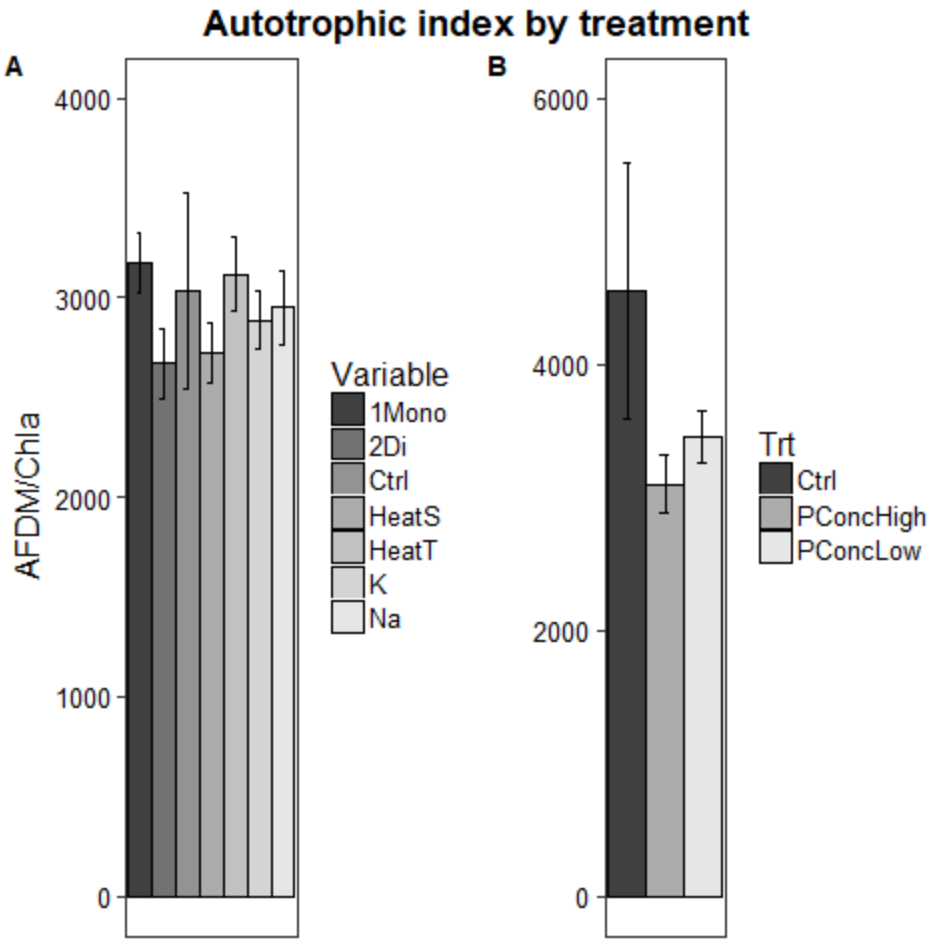

Figure 6: Autotrophic Index (AI) means +/- 1 standard error from NDS field experiments (see Figure 2 caption). For all experiments (A), total n=196, with treatments represented as follows: n= 89 (1Mono), n=89 (2Di), n=18 (Ctrl), n=88 (HeatT), n=90 (HeatS), n=86 (K), n=92 (Na). For experiment 3 (B), n=6 (Ctrl), n=49 (PConcHigh), n=49 (PConcLow).

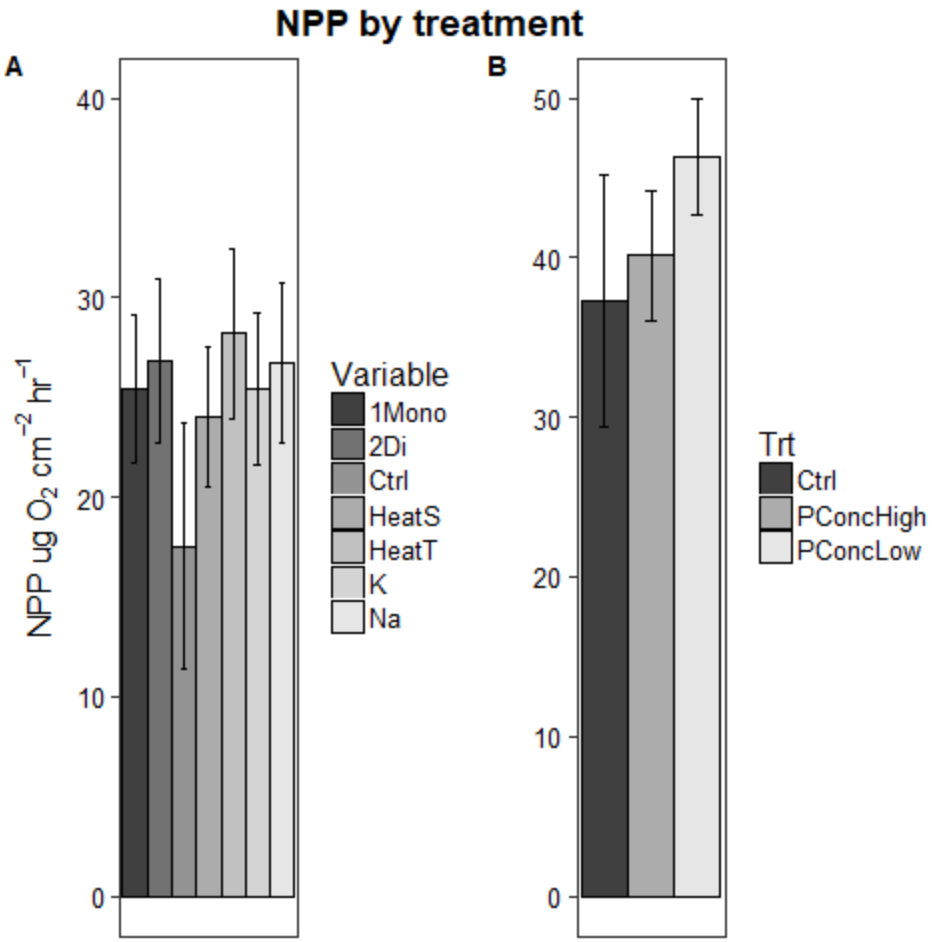

Figure 7: Net Primary Productivity (NPP) means +/- 1 standard error from NDS field experiments (see Figure 2 caption). For all experiments (A), total n=210, with treatments represented as follows: n= 97 (1Mono), n=94 (2Di), n=19 (Ctrl), n=95 (HeatT), n=96 (HeatS), n=91 (K), n=100 (Na). For experiment 3 (B), n=6 (Ctrl), n=48 (PConcHigh), n=50 (PConcLow). Note: Monobasic forms are <u>higher</u> than dibasic forms in the standardized dataset used for statistical analyses, despite this unstandardized dataset showing the opposite trend.

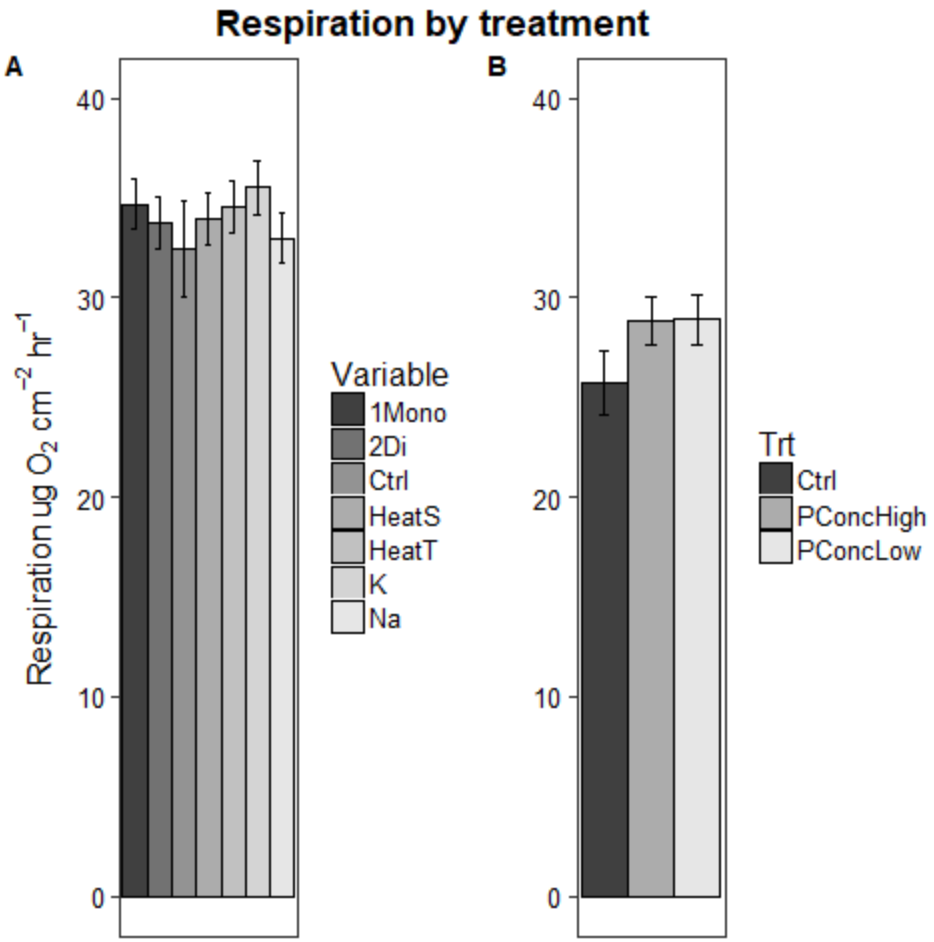

Figure 8: Respiration means +/- 1 standard error from NDS field experiments (see Figure 2 caption). For all experiments (A), total n=209, with treatments represented as follows: n= 98 (1Mono), n=93 (2Di), n=18 (Ctrl), n=95 (HeatT), n=96 (HeatS), n=92 (K), n=99 (Na). For experiment 3 (B), n=6 (Ctrl), n=48 (PConcHigh), n=50 (PConcLow).

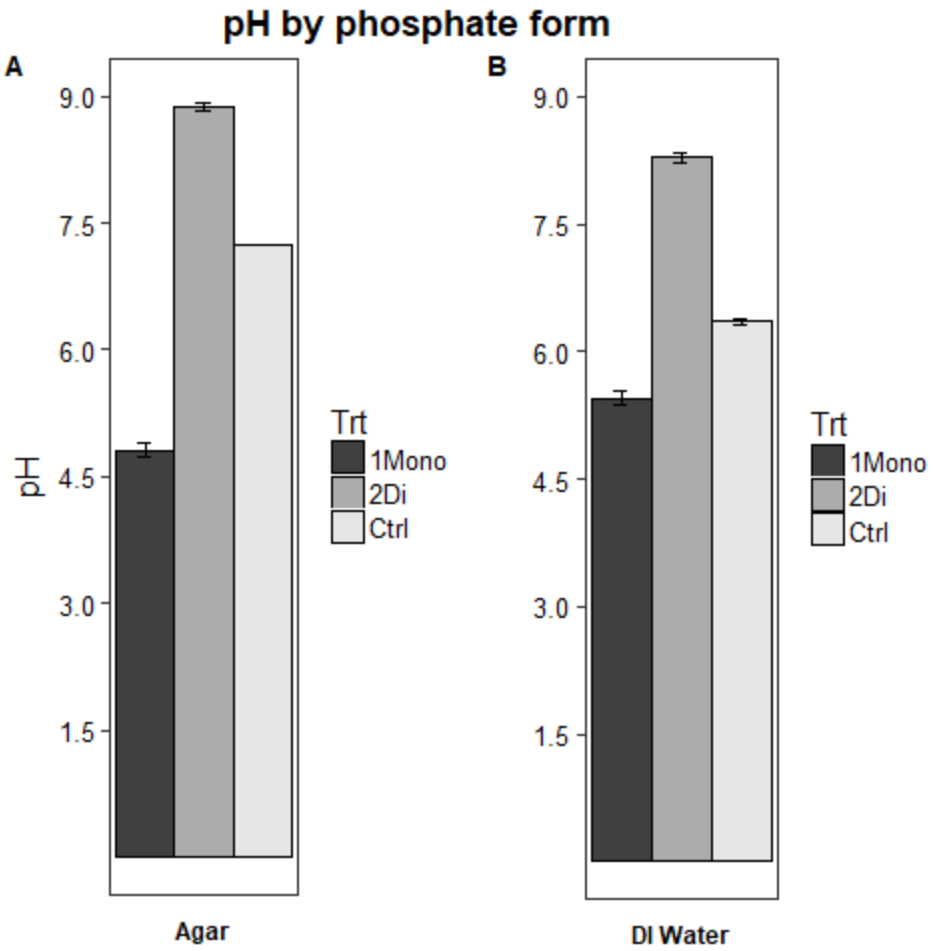

Figure 9: Influence of monobasic and dibasic phosphates on agar pH during NDS construction (A), and on distilled water pH after 24-hours (B). For agar pH, n=16 for each phosphate treatment and n=2 for the control. For distilled water pH, n=8 for each phosphate treatment and n=2 for the control.

Table 1: Four different phosphate treatments (KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4) were deployed in NDS experiments during three separate time periods, crossed with heating methodology and P concentration (PConc) treatments. Heated Together = phosphate and agar boiled together, Heated Separately = agar boiled and phosphate added at pouring temperature of 55-65° C.

Expt	**Dates**	**Cation**	**Form**	**Heat**	**PConc**
1	July 29 – Aug 14, 2016	KH_2PO_4 and K_2HPO_4 vs. NaH_2PO_4 and Na_2HPO_4	KH_2PO_4 and NaH_2PO_4 (Monobasic) vs. K_2HPO_4 and Na_2HPO_4 (Dibasic)	Together vs. Separately	0.1 M
2	Aug 24 – Sept 7, 2016	KH_2PO_4 and K_2HPO_4 vs. NaH_2PO_4 and Na_2HPO_4	KH_2PO_4 and NaH_2PO_4 (Monobasic) vs. K_2HPO_4 and Na_2HPO_4 (Dibasic)	Together vs. Separately	0.1 M
3	July 17 – Aug 5, 2017	KH_2PO_4 and K_2HPO_4 vs. NaH_2PO_4 and Na_2HPO_4	KH_2PO_4 and NaH_2PO_4 (Monobasic) vs. K_2HPO_4 and Na_2HPO_4 (Dibasic)	Together vs. Separately	0.5 M (High) vs. 0.05 M (Low)

Table 2: 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 because of an instrument malfunction in Experiment 3.

Expt	**Temp (°C)**	**pH**	**Conductivity (uS cm^{-1})**	**SRP-P (ug L^{-1})**	**NO_3^--N (ug L^{-1})**	**Discharge (m^3 sec^{-1})**	**Velocity (cm sec^{-1})**
1	11.55 ± 1.91	7.79	43.52 ± 2.05	<1	<10	0.13	45.12 ± 15.33
2	10.05 ± 1.77	8.1 ± 0.28	46.22 ± 2.47	1.67 ± 0.97	<10	0.07	49.07 ± 8.54
3	11.7	--	30.51	2.27 ± 2.11	<10	0.40	49.31 ±13.3