

*Testing for nutrient limitation of algal biomass across
elevational and permanence gradients in high elevation ponds
near the Rocky Mountain Biological Lab*

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Abstract

We investigated nutrient limitation in high elevation ponds in the Gothic, Colorado, USA area and whether it varied with elevation and/or degree of permanence. Nitrogen (N) and phosphorus (P) are often the key limiting nutrients in aquatic systems that regulate algal biomass (Pick & Lean 1987; Heo & Kim 1995). Rock weathering is the primary way phosphorus enters a system. As in many location, the bedrock at subalpine and alpine ponds such as those at Mexican Cut are have little or no phosphorus, and I therefore predicted that this element would limit algal growth. In contrast, at montane elevations (e.g., Kettle Ponds below the Rocky Mountain Biological Lab (RMBL)) ponds should have a greater phosphorus supply from surface and subsurface water flow, and therefore be more likely to be nitrogen or nitrogen-phosphorus co-limited. We found a significant difference in algal biomass between elevational and permanence gradients, as well as in their interactions. However, even within the same permanence gradient nutrient limitation between the ponds varied. On average, permanent ponds showed no nutrient limitation, while semi-permanent and temporary ponds had lower mean chlorophyll *a* in the nutrient-addition treatments compared with the control. We believe that high control treatments may be due to extensive grazing on algae growing on nutrient addition treatments, or that the presence of the nutrients actually inhibited algal growth. Studying nutrient limitation is important in understanding the basics of this system, as well as providing useful preliminary data in understanding how this system might change as species migrate up-slope with globally warming temperatures, thereby altering the ecosystem structure currently in place.

Introduction

Across ecosystems, primary productivity rates are often similar; however aquatic and terrestrial ecosystems differ in nutrient availability and energy usage (Shurin et al. 2006). When water and light are available, nitrogen (N) and phosphorus (P) are the most common resources that limit primary productivity, and in aquatic systems this limitation is usually conceptualized relative to algae's cellular growth requirements (Dodds et al. 2002; Vitousek et al. 2010). The global reservoir of nitrogen (N) is in the air, and its availability to aquatic producers ultimately depends on microbial fixation, and, once in aquatic systems, on microbial regeneration (Batzler & Sharitz 2014). Phosphorus primarily originates from the weathering of rocks and is especially likely to be limiting in regions without sedimentary or P-rich igneous rocks (Rabalais 2002). In some cases, nitrogen is the primary limiting nutrient such as many streams and near-shore marine environments. Nitrogen predominantly functions as a way for primary producers to accumulate energy through photosynthesis (Vitousek & Howarth 1991).

However, phosphorus limitation, sometimes in unison with nitrogen limitation (e.g., "co-limitation" in lakes and wetlands), may also occur (Elser et al. 2009). It is possible to find varying degrees of nitrogen and phosphorus deficiency, and this variation is typically due to the amount of nitrogen versus phosphorus uptake and losses in individual systems (Gusewell 2004).

Understanding the unique nutrient composition of freshwater systems is essential to identifying the limiting nutrient(s) in a system. A freshwater ecosystems' main source of nutrients and energy comes from accrued organic matter, especially allochthonous detritus entering from outside the system (Klemmer et al. 2012). Primary producers (i.e. phytoplankton) rapidly take up N and P, making them mostly comprised of nutrient-rich photosynthetic material (Shurin et al. 2006). However, across systems, nutritional deficits are often the result of heterotrophs having high N and P demands that primary producers are unable to meet (Shurin et al. 2006). Relationships exist between total phosphorus and chlorophyll in standing freshwater ecosystems (lakes, ponds, wetlands) that make phosphorus commonly considered the limiting nutrient in these systems (Mischler et al. 2014). In summary, nitrogen and phosphorus are assumed to limit algal productivity and rates of detrital decomposition (Elwood et al. 1981).

Likewise, pond permanence also plays a crucial role in the nutrients readily available or limiting. Temporary ponds that dry and fill on a yearly basis are typically well oxygenated. In the presence of oxygen, iron present in the pond does not bind to phosphorus and leaves it readily usable; nitrogen would likely be the limiting nutrient in these ponds (Aziz et al. 2004). On the other hand, permanent ponds can often become anaerobic at the bottom; in this environment, iron binds to phosphorus in such a way that now makes phosphorus the limiting nutrient in the pond.

The purpose of this study was to test for nutrient limitation (N, P, or N+P co-limitation) of benthic algae in high elevation ponds. Although nutrient limitation has been studied in alpine lakes, it is understudied in high elevation ponds and wetlands (Wissinger et al. 2016). Further, it tested whether nutrient limitation shifts along an altitudinal gradient (montane to subalpine), and along a habitat permanence gradient (temporary to permanent). This study was designed to test an assumption of a larger project that aimed to determine whether the regeneration of nutrients by detritivores can stimulate algal production (Klemmer et al. 2012). Understanding

which organisms control nutrient regeneration is important for understanding ecosystem function, therefore aids in understanding ecosystem services provided (Daily 1997).

Methods

Study System

This research question was focused on two context dependent variables: elevation (montane versus sub-alpine) and permanence (permanent, semi-permanent, and temporary ponds). At my study locations, permanent ponds are those with water in them every year, all year, semi-permanent ponds dry up in late summer every few years, and temporary ponds dry up every year (Wissinger et al. 1999).

This experiment took place near the Rocky Mountain Biological Lab in Gothic, Colorado, USA (Figure 1). The montane site (~2900m elev.) was located south of Gothic at the Kettle Ponds (Figure 2). We studied three semi-permanent ponds at this location, "K1", "K5", and "K6". No permanent ponds are found at this study site, and temporary ponds dried early in the season. The sub-alpine site (~3450m elev.) was located at the Mexican Cut Nature Preserve, approximately 6 km northwest of Gothic (Figure 3). Ponds at this site varied in permanence. The permanent ponds studied were "Pond 1" and "Pond 5"; the semi-permanent ponds were "Pond 6", "Pond 8", and "Pond 10"; the temporary ponds were "Pond 7" and "Pond 15"(see Fig. 1 in Wissinger et al. 2003).

The Nutrient-Diffusing Substrate

Nutrient-diffusing substrates (NDSs) were placed in ten ponds at varying elevational and permanence gradients (Flecker et al. 2002; Figure 4). To make the NDSs, agar was poured into polycons, allowed to solidify, and a fritted disc will be placed on top before covering with a lid that had a cut-out at the center to expose the disc. The four nutrient treatments were control (agar-only), nitrogen treatment, phosphorus treatment, and nitrogen with phosphorus in combination (to test for co-limitation). The control was made with deionized water and agar powder, ammonium chloride and sodium nitrate was added to make the nitrogen treatment, sodium phosphate was added to the agar solution for the phosphorus treatment, and ammonium chloride, sodium nitrate, and sodium phosphate were added to for the nitrogen with phosphorus treatment (Flecker et al. 2002).

Field Experiment

The NDSs were arranged randomly and firmly fastened along extruded angles of plastic angle iron using zip ties. Four replicates of NDSs were placed approximately 0.3m under the pond's surface in three kettle ponds (K1, K5, K6). In addition, four replicate NDSs were placed across a permanent (Mexican Cut ponds 1 & 5) to semi-permanent (Mexican Cut ponds 6, 8, & 10), to temporary ponds (Mexican Cut ponds 7 & 15), at a subalpine elevation. The NDSs were left in the ponds for approximately two weeks to accumulate algae growth, at which point we estimated that the nutrients are likely to have mostly diffused out (Rugenski et al. 2008). After the NDSs were retrieved, the fritted disk from each polycon was removed, placed in a Whirl-pak (Whirl-pak, Nasco, USA), and kept on ice; these were analyzed for chlorophyll *a* as a surrogate

measure for algal biomass (Buckley 2015).

Fluorometric Chlorophyll Analysis

To measure chlorophyll *a*, an extract of the sample will be measured on a fluorometer using an acidification method. To do this, a solution of ninety percent buffered ethanol was added to each Whirl-pak a minimum of four and maximum of twenty-four hours prior to analyzing the sample in a fluorometer (Wasmund et al. 2006). After the initial Relative Fluorescence Units (RFU) was recorded, a 0.1N HCl solution was added to each sample and re-analyzed to record a post-acid-addition RFU to account for phaeophytin (Mush 1980; Welschmeyer 1994).

Statistical Analyses

The statistics program JMP (Version 12.2.0, SAS Institute, Cary, NC) was used to perform a one-way analysis of variance (ANOVA) on Kettle Pond data to determine differences in nutrient limitation at the semi-permanent ponds. A two-way ANOVA was performed on Mexican Cut data to understand the impacts of treatment, permanence, and treatment/permanence interactions on mean chlorophyll-*a*. Two outliers were removed from the dataset because it is highly probable that they were due to experimental error. A Tukey-Kramer Post-Hoc test was performed to analyze differences between the treatments.

Results

For the semi-permanent ponds at subalpine and montane elevations, chlorophyll *a* levels on the NDS discs differed across nutrient type and elevation (Table 1). The significant nutrient by elevation interaction indicated the need to consider each elevation in greater detail.

The results of the one-way ANOVAs for each Kettle Pond indicated that there is a significant difference in the mean chlorophyll *a* values between the treatments in K1 and K6 (Table 2; Figure 5). K5 did not show a significant difference between treatments.

When the mean chlorophyll *a* values were compared across the Mexican Cut ponds the two-way ANOVA showed that there was a significant difference in mean chlorophyll *a* between the treatments, permanence gradients, and the treatment/permanence gradient interaction (Table 3). A significant nutrient by permanence interaction indicated the need to consider each elevation separately for further statistics.

The mean chlorophyll *a* values were compared across the permanence gradient at the Mexican Cut and a significant difference was found between them (Table 4; Figure 6). Results from the one-way ANOVAs for each Mexican Cut pond revealed that for the permanent ponds, Pond 1 has no significant difference between the nutrient treatments, but Pond 5 had a significant difference between the treatments (Table 5; Figure 7). The semi-permanent ponds (Pond 6, 8, and 10) had significant differences among the nutrient treatments (Table 5; Figure 8). The temporary ponds (Pond 7 and 15) also showed a significant difference between the treatments (Table 5; Figure 9).

Discussion

Different Nutrients are Limiting Across the Kettle Ponds

Chlorophyll *a* differed between permanence and elevational gradients in this experiment. It was discovered that nutrients limited benthic algal biomass in the two of the three, semi-permanent Kettle ponds. However, each pond's change in algal biomass showed a different relationship to the nutrients; Pond K1 is nitrogen limited, pond K5 shows no nutrient limitation, and pond K6 reveals nitrogen and phosphorus co-limitation taking place (Figure 4). This variation in the nearby ponds could be due to different environmental impacts surrounding the ponds such as the yearly addition of cattle to part of the landscape in the late summer, which likely affects the amount of nitrogen and phosphorus present in the nearby soils and pond water through cattle wastes, feces, and urine (Gary et al. 1983).

Nutrient Limitation (or not) at Mexican Cut

A significant difference was found between the nutrient treatments, permanence categories, and nutrient treatment/permanence interactions at the Mexican Cut (Table 1). Ponds of all permanence types revealed significant differences between treatments, with permanent ponds on average showing no nutrient limitation, and semi-permanent and temporary ponds showing high controls, and therefore significantly low mean chlorophyll *a* for the N, P, and N+P treatments (Figure 4).

Differences in chlorophyll *a* between ponds within permanence gradients at the Mexican Cut also reveal contrasting results. For the permanent ponds (ponds 1 & 5) at the Mexican Cut, pond 1 has no limiting nutrient, while pond 5 nutrients appear to be significantly lower than the control (Figure 5). The semi-permanent and temporary ponds at the Mexican Cut (Semi-Permanent ponds 6, 8, and 10; Temporary ponds 7 and 15) show the control as being significantly higher than the nutrient addition treatments (Figure 6 & 7).

These significantly lower mean chlorophyll *a* values for the nutrient-addition treatments compared to the control suggest two possibilities: that grazing on the nutrient treatments has given us such low mean chlorophyll *a* or that the nutrients are inhibiting benthic algal biomass. Another essential element, such as iron, may be the limiting factor in the ponds that show low N:P ratios and high controls; if iron was limiting benthic algal biomass, addition of nutrients could potentially simply inhibit nutrient growth because it is not what is needed for the algae to grow (Rabalais 2002). Other abiotic factors such as temperature and mixing regimes can play a role in the productivity of an aquatic system, as well as the availability of iron or carbon (Pick & Lean 1987).

Additionally, it is not uncommon for grazers (e.g., snails and caddisflies) to play a significant role in the amount of algal biomass lost in a system (Rosemond et al. 1993). Previous studies have found that ungrazed experimental treatments had more algal biomass than grazed treatments (Rosemond et al. 1993). The addition of nitrogen and phosphorus may have had a positive effect on the algal biomass but grazing may have diminished the traceability of this effect.

Experimental Error

Steps were taken to check for experimental error in this experiment. At a later date than the initial analysis on the fluorometer, a subset of the pond samples were rerun through the fluorometric analysis method to check the relative values to the ones previously obtained; this was done to check for processing error and it was discovered that the relative relationships between the treatments held true to what was previously discovered. Additionally, during statistical analysis, the total photosynthetic pigmentation of the ponds (the relative fluorescence unit (RFU) before adding acid) as well as the calculated chlorophyll *a* value was used to compare relationships between the treatments; the relationship was found to be consistent.

Future Studies

Future work on this experiment is recommended in order to better understand this system. Modifications to the protocol are suggested to include a grazing versus non-grazing factor in order to explore whether the high mean chlorophyll *a* in the control treatments of the Mexican Cut ponds was due to grazing on the N, P, and N+P treatments. Previous studies show that when a large amount of the primary productivity is removed (such as by herbivores) only a small fraction of the remaining nutrients remain, thereby having a significant effect on nutrient storage and usage within the pond (Cebrian & Lartigue 2004). Caddisflies were seen to be grazing on these treatments, but this was not a measured variable in this particular experiment.

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Figures and Graphs



Figure 1. Vicinity map of the montane Kettle Ponds and subalpine Mexican Cut Nature Preserve in Colorado, USA.

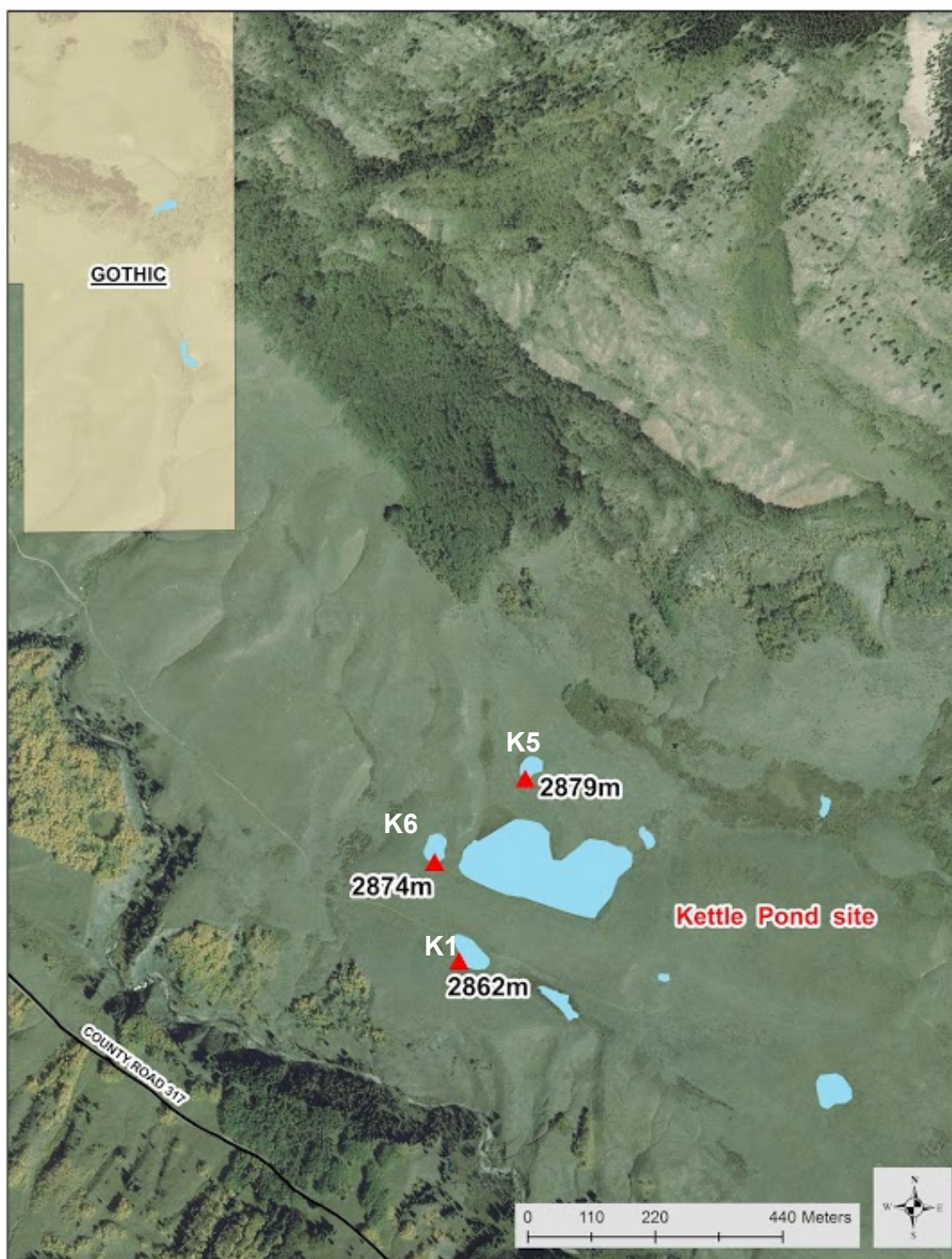


Figure 2. Map of the montane Kettle Ponds in Colorado, USA. Numbers beside the pond represent the number of meters in elevation.



Figure 3. Map of the subalpine Mexican Cut Nature Preserve ponds in Colorado, USA. Numbers beside the pond represent the number of meters in elevation.



Figure 4. Photo of nutrient-diffusing substrates used in this experiment.

Table 1. Two-way ANOVA statistics for semi-permanent ponds at subalpine and montane elevations

Source	F Ratio	df	p-value
Nutrient Treatment	18.0266	3	<.0001
Elevation	18.8580	1	<.0001
Treatment*Elevation	47.3141	3	<.0001

Table 2. One-way ANOVA statistics for nutrient treatment differences in montane ponds

Source	F Ratio	df	p-value
Pond K1	16.7265	3	<0.0001
Pond K5	2.6011	3	0.0718
Pond K6	6.5486	3	0.0017

Table 3. Two-way ANOVA statistics for subalpine nutrient treatment by permanence interaction

Source	F Ratio	df	p-value
Nutrient Treatment	45.4555	3	<.0001
Permanence	156.8053	2	<.0001
Treatment*Permanence	6.5493	6	<.0001

Table 4. One-way ANOVA statistics for nutrient treatment differences in subalpine permanence gradient

Source	F Ratio	df	p-value
Permanent	4.4630	3	0.0068
Semi-Permanent	58.9326	3	<0.0001
Temporary	14.2932	3	<0.0001

Table 5. One-way ANOVA statistics for nutrient treatment differences in subalpine ponds

Source	F Ratio	df	p-value
Pond 1 (permanent)	0.7872	3	0.5112
Pond 5 (permanent)	6.2099	3	0.0024
Pond 6 (semi-permanent)	15.7899	3	<0.0001
Pond 8 (semi-permanent)	24.9879	3	<0.0001
Pond 10 (semi-permanent)	29.2607	3	<0.0001
Pond 7 (temporary)	57.2947	3	<0.0001
Pond 15 (temporary)	4.4787	3	0.0109

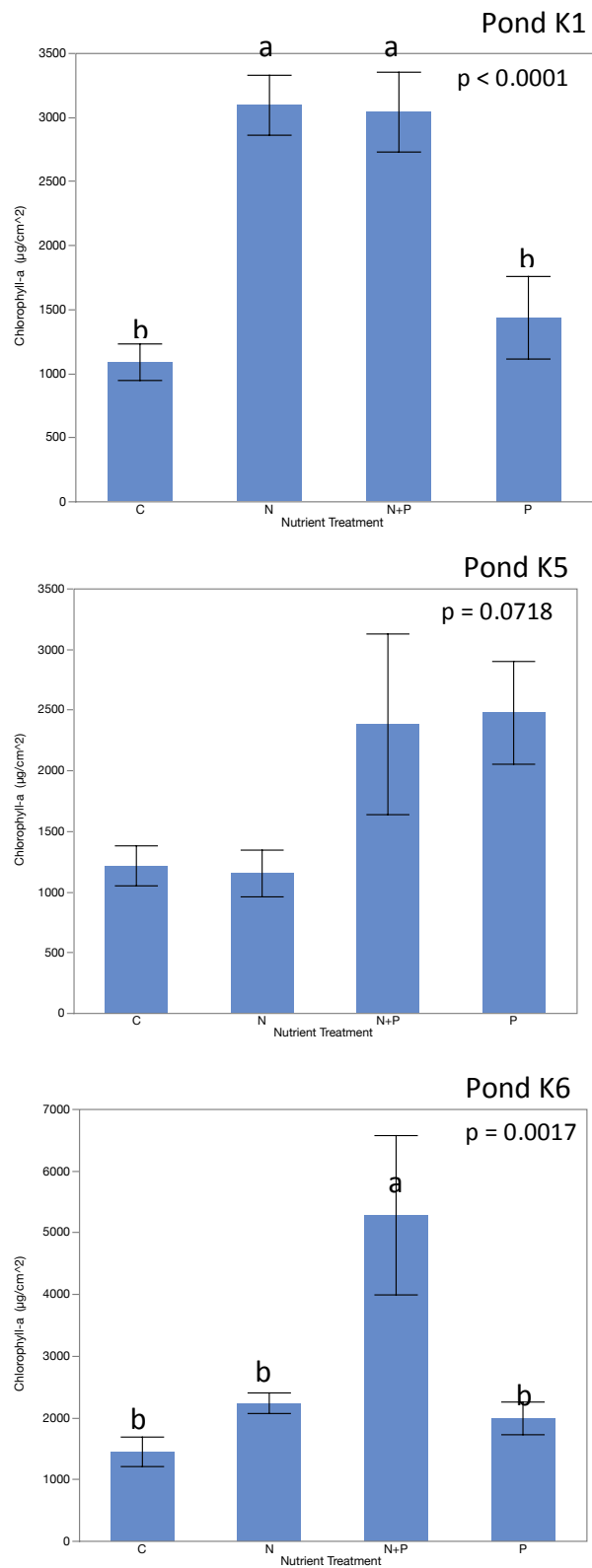


Figure 5. Comparison of mean chlorophyll-a between the four nutrient treatments (control (C), nitrogen (N), phosphorus (P), nitrogen and phosphorus co-limitation (N+P) in the three, montane, semi-permanent Kettle ponds, "K1", "K5", and "K6", using the Tukey-Kramer method. Error bars represent $\pm$ one standard error of the mean. Means that do not share a letter are significantly different.

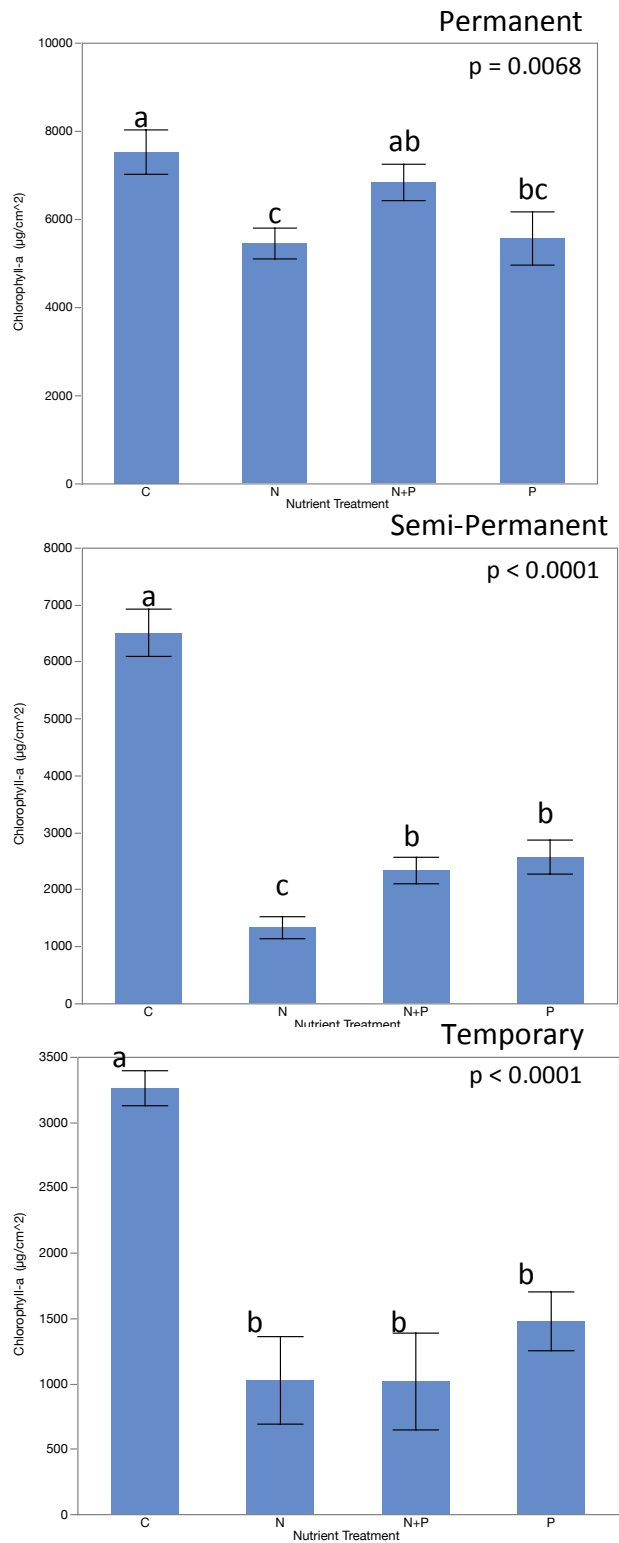


Figure 6. Comparison of mean chlorophyll-a between the four nutrient treatments (control (C), nitrogen (N), phosphorus (P), nitrogen and phosphorus co-limitation (N+P) for ponds in the three permanence categories (permanent, semi-permanent, and temporary) at the sub-alpine Mexican Cut, using the Tukey-Kramer method. Error bars represent +/- one standard error of the mean. Means that do not share a letter are significantly different.

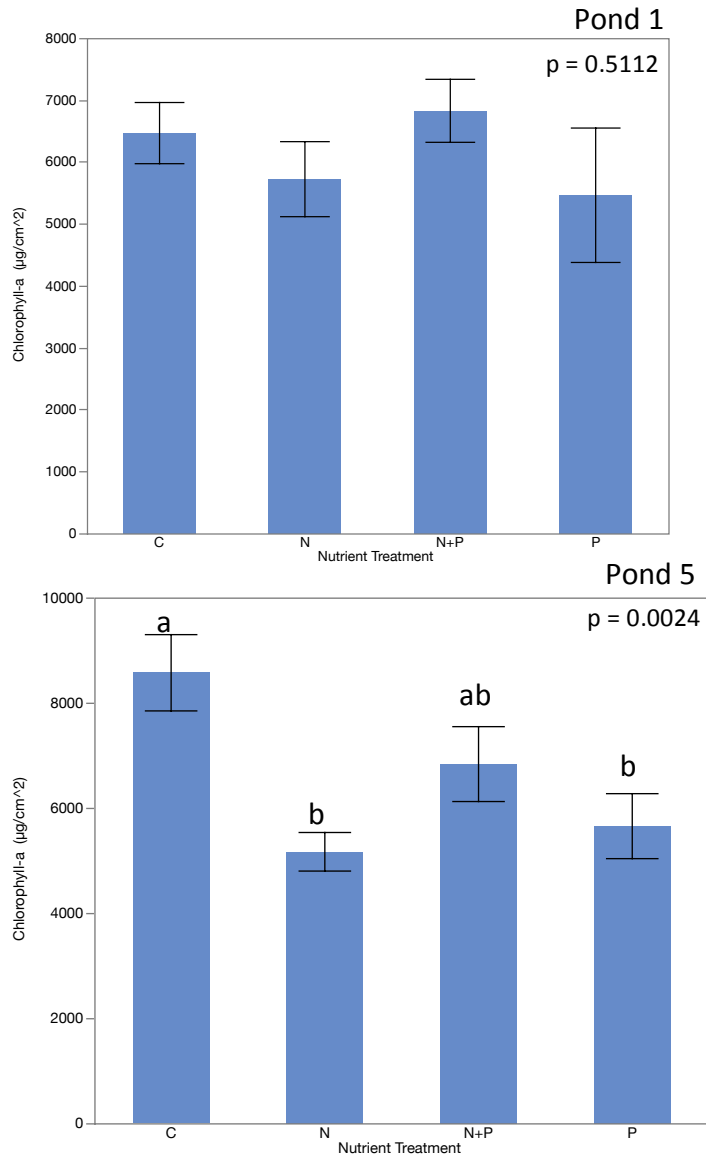


Figure 7. Comparison of mean chlorophyll-a between the four nutrient treatments (control (C), nitrogen (N), phosphorus (P), nitrogen and phosphorus co-limitation (N+P) for the two permanent ponds (Pond 1 & 5) at the sub-alpine Mexican Cut, using the Tukey-Kramer method. Error bars represent $\pm$ one standard error of the mean. Means that do not share a letter are significantly different.

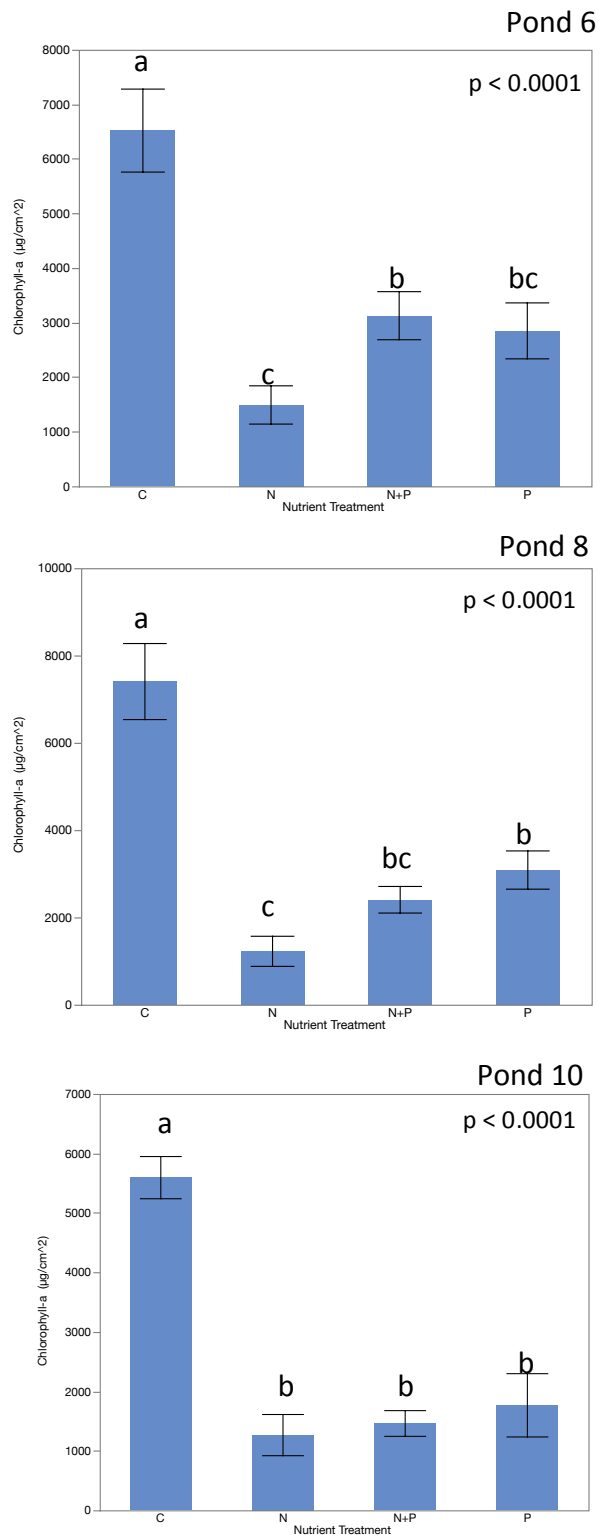


Figure 8. Comparison of mean chlorophyll-a between the four nutrient treatments (control (C), nitrogen (N), phosphorus (P), nitrogen and phosphorus co-limitation (N+P) for three semi-permanent ponds (Ponds 6, 8, & 10) at the sub-alpine Mexican Cut, using the Tukey-Kramer method. Error bars represent $\pm$ one standard error of the mean. Means that do not share a letter are significantly different.

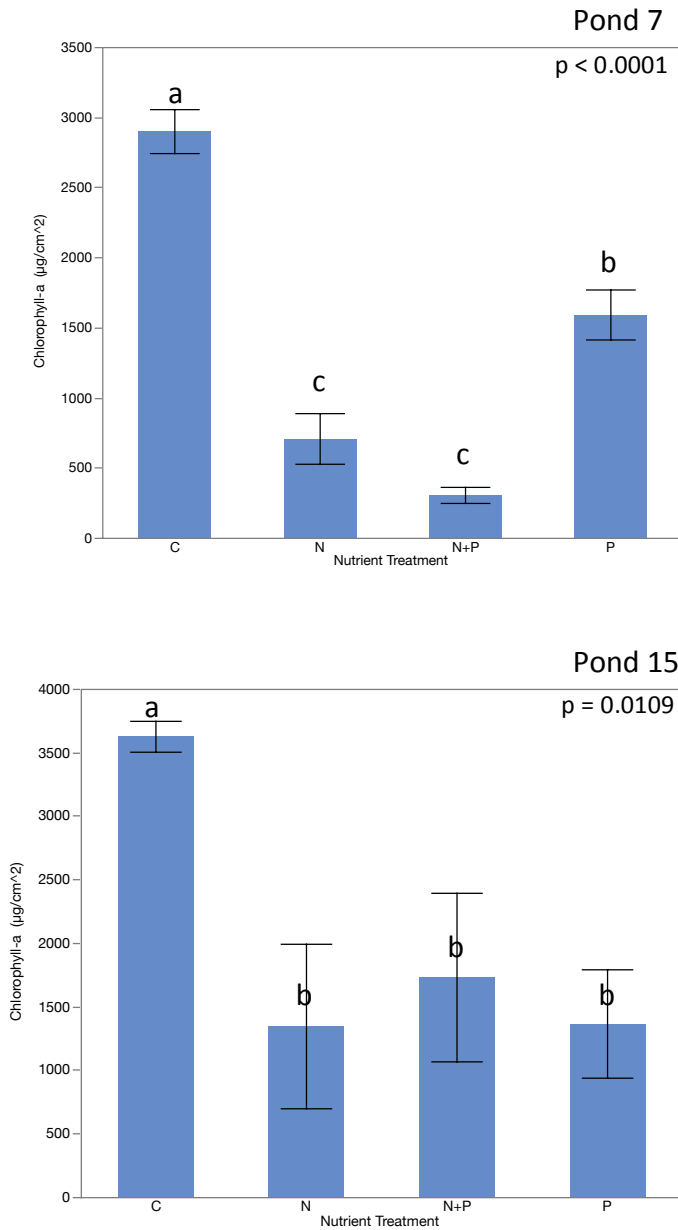


Figure 9. Comparison of mean chlorophyll-a between the four nutrient treatments (control (C), nitrogen (N), phosphorus (P), nitrogen and phosphorus co-limitation (N+P) for two temporary ponds (Ponds 7 & 15) at the sub-alpine Mexican Cut, using the Tukey-Kramer method. Error bars represent $\pm$ one standard error of the mean. Means that do not share a letter are significantly different.