

Testing Nutrient Limitation Status Along a Subalpine Pond Hydroperiod Gradient

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Abstract. In lentic aquatic ecosystems, benthic algae are a foundational primary producer, providing habitat and basal resources for higher trophic levels. However, many primary producers’ photosynthetic rates are determined by a limiting nutrient such as Nitrogen (N) or Phosphorus (P). Thus, determining the limiting nutrient of the benthic algae allows us to understand what chemical factors are most important to the productivity of an ecosystem. Furthermore, given that differences in species compositions among ponds of different hydroperiods provide different amounts of animal driven nutrient supply, ponds with different hydroperiods could have different limiting nutrients. Thus, the objective of this study was to determine if nutrient limitation status differed among pond hydroperiods. To do this, we measured limitation in twelve ponds at Mexican Cut: four permanent ponds that never dry, four semi-permanent that dry in some but not all years, and four temporary that dry every year. Six replicates of four Nutrient Diffusing Substrate (NDS) treatments (0.5M N, 0.5M P, 0.5M N and P, and a control without added nutrients) were installed in each pond. After two weeks, we removed the NDS, then measured the chlorophyll-a contents on their substrate to infer benthic algal biomass and determine nutrient limitation status. We found semipermanent and temporary ponds to be strongly nitrogen limited, however did not find any evidence of phosphorus algal limitation. We believe this might be due to a phosphorus limited bacteria which outcompeted the algae.

Introduction

Lentic algae produce much of the energy that fuels aquatic ecosystems, but, like all primary producers, the amount of energy it can create is restricted by the nutrients present, as well as light and space availability (Tank et al., 2017). When a scarcity of one nutrient is responsible for limiting the production, this is called a limiting nutrient. Therefore, the presence of the lentic algae’s limiting nutrient dictates the amount of energy that will be able to flow through the pond’s food web. Aquatic primary producers are typically limited by either nitrogen or phosphorous. Situations where both nutrients are limiting production are referred to as “colimitation” (Flecker et al., 2002).

The Mexican Cut Nature Preserve (39°10 N, 107°40 W; Figure 1) contains 62 subalpine ponds along a hydroperiod gradient of permanent ponds that never dry, semipermanent ponds that dry in some but not all years, and temporary ponds that dry every year. Although long term research on caddisfly and salamander populations is conducted at the site, which nutrients limit benthic algal production remains unknown. Understanding what limits primary production at the Mexican Cut is important because benthic algae acts as the foundation of the food web and imposes bottom up control of higher trophic levels and potentially ecosystem functions such as nutrient cycling (Flecker et al, 2002).

Due to the Mexican Cut Ponds’ close proximity and similar basin geomorphologies, they face very similar environmental pressures and receive similar nutrient loading from the landscape. One of these shared environmental factors is the absence of atmospheric nitrogen deposition as a major nutrient source (Burns 2002). However, differences in their hydroperiods cause them to function as very different habitats with different community compositions. For example, the permanent ponds are host to large larval and paedomorphic adult salamander

communities, but the temporary ponds dry up annually making them unable to support salamander communities. The lack of salamander predators causes semipermanent and temporary ponds to have different communities of invertebrates relative to permanent ponds. Due to the different community compositions among hydroperiods, their animal-driven nutrient supplies also differ (Balik et al. *in press*). Specifically, permanent and semipermanent ponds should have N surpluses and adequate P supply relative to demand estimates based on nutrient uptake rates, whereas temporary ponds could have large deficits of both nutrients. Thus, pond water column nutrient concentrations likely vary among hydroperiods, and consequently the nutrient limitation of the algae may also differ. In turn, the relationship between these ponds' hydroperiod and their limiting nutrient could also provide insight into nutrient limitation status at other similar locations. Thus, finding the limiting nutrients of the ponds at Mexican Cut not only provides insight into the other research being conducted there, but also inform on the relationship between pond permanence and limiting nutrients in general.

Nutrient limitation can be inferred via the ratio of N to P in the water column, however, this method can be uninformative in aquatic environments which contain particularly high or low concentrations of nitrogen and phosphorous (Sanderson et al., 2009). This method can also be confounded by how accessible the measured nutrients are to the organisms which need them, differences in the forms of nitrogen and phosphorous measured, and the difference in nutrient needs between different algal species. Alternatively, Nutrient Diffusing Substrates (NDS) can provide direct measurements of algal growth under different nutrient conditions, rather than simply predicting how algae would react based on the current availability of nutrients in the system. Thus, we used NDS to measure nutrient limitation status along the hydroperiod gradient at Mexican Cut. Similar to Elser et al. (2007) who studied subalpine lakes near the Mexican Cut, we expected to find all ponds across the hydroperiod gradient are strongly P limited, and temporary ponds likely experience colimitation. Likewise, Balik et al. (*in press*) also found evidence for greater animal driven N supply, suggesting that phosphorus will be scarcer in the pond ecosystems. Furthermore, Balik et al. predict that as climate change causes pond hydroperiods to become increasingly temporary, declines in the animal community biomass will lead to decreases in animal-driven nutrient supply. This change will exacerbate nutrient limitation in these already relatively nutrient-poor ponds.

Pond Number	Hydroperiod Classification
1	Permanent
4	Permanent
5	Permanent
7	Semipermanent
8	Semipermanent
10	Semipermanent
12	Permanent
13	Temporary
15	Semipermanent
22	Temporary
42	Temporary
43	Temporary

Table 1: The twelve ponds used in the experiment and their hydroperiod classification.

Finally, this climate change driven shift in pond hydroperiods will also directly impact of the distribution of many different organisms, including range-expanding larval caddisflies, which could also affect the nutrient cycles of these ponds. Thus, this research will help illustrate the potential consequences on the nutrient load of more and more ponds becoming temporary.

Methods

Twelve ponds were selected at Mexican Cut: four temporary, four semipermanent, and four permanent (Figure 1, Table 1). NDS treatments were created by making four batches of agar: one containing nitrogen compounds, one containing phosphorous, one with both nitrogen and phosphorous, and one with no added nutrient. The compound NH_4NO_3 was used to prepare the 0.5M nitrogen treatment. Thus, our nitrogen treatment contained 0.25M NH_4 and 0.25M NO_3 . Next, Na_3PO_4 was used to create the 0.5 M phosphorous treatment. Both compounds were used to create the 0.5M N+P treatment. Once the agar had set in the NDS polycon cups, they were left to sit for one night to set completely.

The next day, we installed the NDS at Mexican Cut. In order to secure the position of the NDS cups, we tied each one to a length of bamboo, which prevented movement, ensured the NDS remain upright, and assisted us in locating the cups at the end of the two-week experiment.

Each treatment was placed as far apart as the size of the pond allowed, approximately 1 meter apart, in order to prevent cross contamination of different nutrients among the same set. The six replicates of NDS treatments were then spaced out evenly across the perimeter of the pond (Figure 2).

Additionally, we measured the water column phosphorus, in the form of soluble reactive phosphorus, ammonium, and nitrate regularly throughout the experiment in order to compare the measure of nutrient limitation we gather from the NDS to a simple N:P ratio prediction of

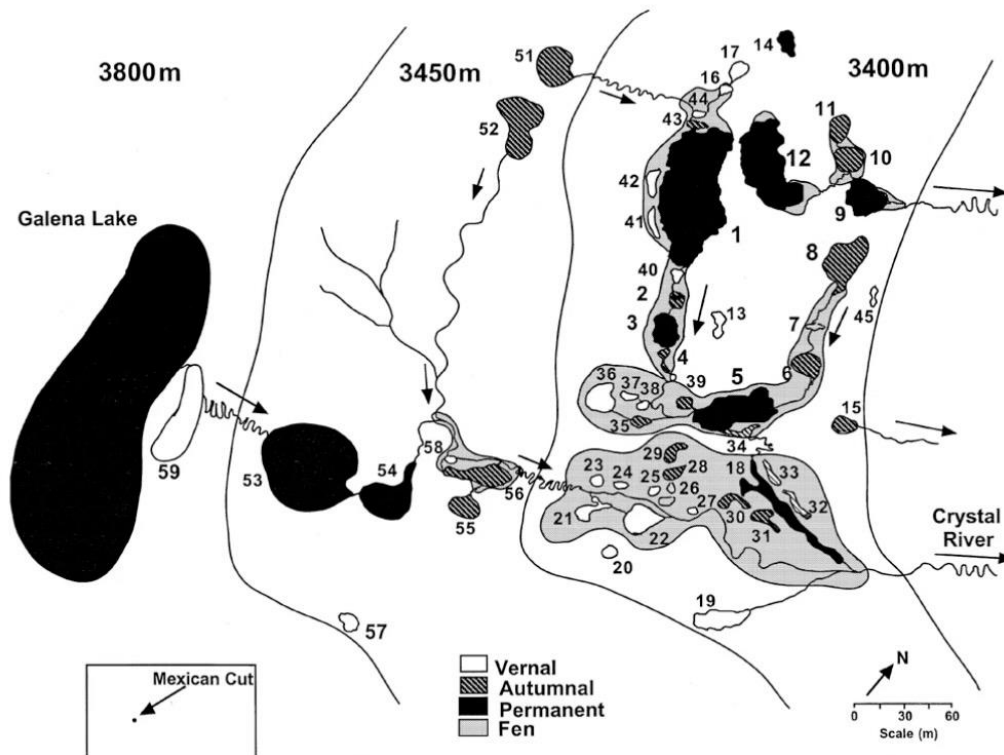


Figure 1: A map of the ponds at Mexican Cut from Wissinger et al., 1999.



Figure 2: On the left, an image of a pond containing 24 NDS treatments. On the right, an image of one set of 4 NDS treatments, marked with red arrows.

limitation status. We also measured the Benthic Organic Matter (BOM) chlorophyll-a content throughout the season, which allows us to plot how the different hydroperiod's algal biomass changed throughout the season.

Two weeks after initial installation of the NDS, or on the event of a temporary pond drying, the NDS were collected and we removed their filters, placed each filter in individual bags clearly marked with the pond number and treatment type, and froze them. Then, we returned to the lab with the filters, where we added an ethanol solution to each bag and let it sit for 5 hours in order to lyse algal cells and release their chlorophyll. Finally, we used a fluorometer to measure the chlorophyll concentration following standard methods (Agar, Elizabeth J., 1997)

Each NDS cup has two independent variables: the nutrient it contained, and the permanence of the pond where it was placed. These independent variables will affect the amount of chlorophyll and biomass which are extracted from its filter at the end of the experiment. Data was analyzed in R version 4.1 (R Core Team, 2020) using the package “nlme” (citation; Model: $\text{Chl-A} = \text{Nutrient Treatment} * \text{Hydroperiod}$, with Pond nested within Hydroperiod). Tukey's HSD post-hoc test was used to detect differences among levels of the nutrient treatment & hydroperiod interaction. The phosphorus and colimitation treatments were dropped from this analysis due to the fact that they did not accrue any chlorophyll a (90% of samples ≤ 0 ug Chl-A)

After completing analysis of our initial data, we chose to carry out a further experiment in order to gauge the impacts of bacteria on our NDS substrates. To do this, we installed 6 new replicates of each NDS treatments in Pond 1 and allowed them to remain for another two weeks. At the end of this two-week period, we collected our NDS and separated them into buckets of water by treatment: 6 control cups in one bucket, 6 nitrogen cups in another, and so on. Then, we put dissolved oxygen probes into the buckets and left them in the dark for approximately 5 hours. By measuring the change in dissolved oxygen we can determine if there are differences in bacterial growth between nutrient treatments because algae produces O_2 , while bacteria consumes it. Area under the curve (AUC) will be calculated and used to compare treatments.

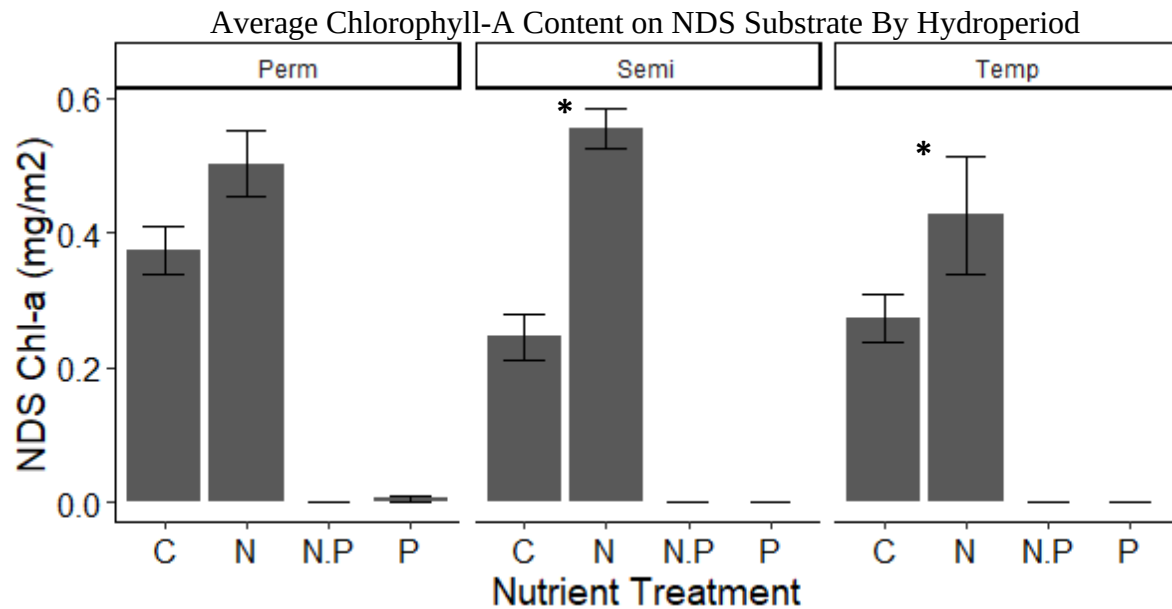


Figure 3: The average chlorophyll concentration on NDS Substrate by pond type. No phosphorous or colimitation treatments had an average above 0. * are added above bars that are statistically different (Tukey's HSD < 0.05).

Results

Algal biomass accrual differed among nutrient treatments (Figure 3; Nutrient: $F_{1,129}=43.69$, $p < 0.001$). Algal biomass accrual did not differ significantly among hydroperiods (Hydroperiod: $F_{2,9}=0.18$, $p=0.835$). Most importantly, however, different nutrient treatments accrued different amounts of algal biomass within each hydroperiod (Nutrient*Hydroperiod: $F_{2,129} = 4.31$, $p = 0.016$). Specifically, in semipermanent and temporary ponds, the N

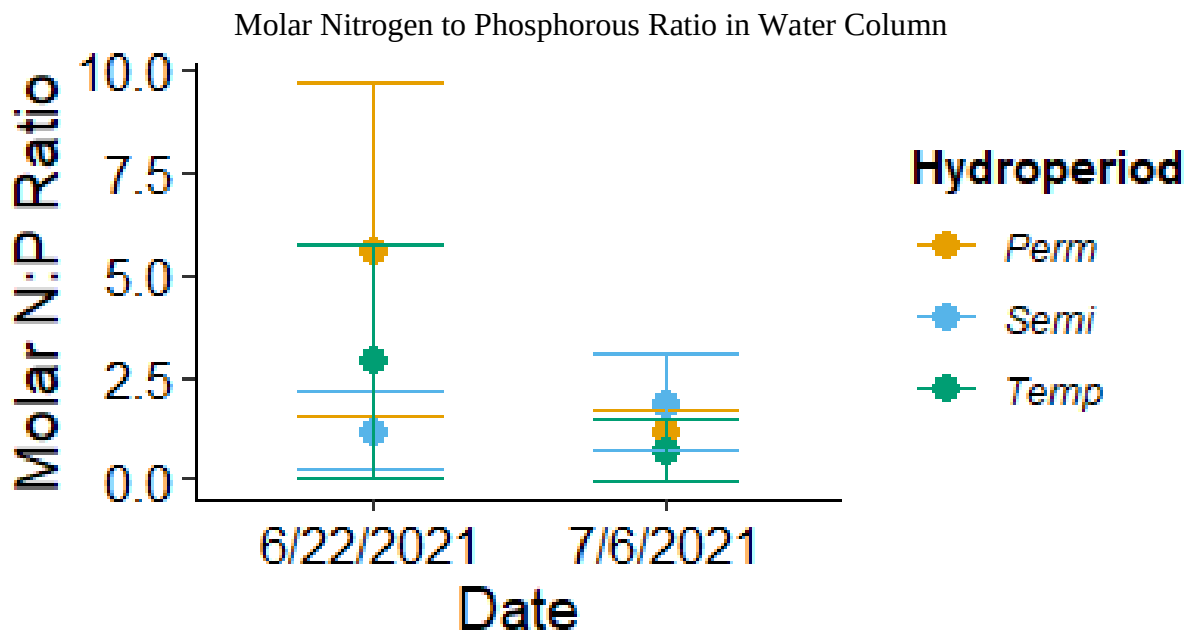


Figure 4: The molar ratio of Nitrogen to Phosphorous measured in the water column on June 22nd, 2021, and July 6th, 2021.

treatments accrued significantly more Chl-A than the control treatments ($HSD < 0.05$). However, in the permanent ponds, the two treatments did not differ. Therefore, algal growth is N limited in the semipermanent and temporary ponds, but not in the permanent ponds.

We utilized our measurements of Nitrogen and Phosphorous in the water column to calculate the molar ratio between the two nutrients throughout our experiment. These ratios showed that molar N:P did not vary significantly between hydroperiods on the dates measured (Figure 4). Additionally, over the course of the experiment, the ratio of N:P declined significantly in both permanent and temporary ponds and had converged by the end of the experiment.

To analyze the results of our dissolved oxygen experiment, we plotted dissolved oxygen concentration over time for each bucket, and then calculated the area under the curve, or AUC (Figure 5). The lower AUC values indicate higher oxygen demand in their respective bucket.

Lastly, results on our Benthic Organic Matter samples are still forthcoming.

Discussion

Our NDS experiment showed two surprising findings: firstly, nitrogen was a limiting nutrient only in semipermanent and temporary ponds, and secondly, the phosphorous and colimitation treatments had even lower chlorophyll concentrations than the control treatment across all hydroperiods.

The first result, which showed that only permanent ponds were not limited by Nitrogen, is supported by research conducted by Balik et al., which predicted a that permanent ponds were receiving a large nitrogen surplus from the animal community. However, Balik et al.'s research also predicted a nitrogen surplus in Semipermanent ponds, which we did not find. This is likely because last year in 2020, Mexican Cut experienced severe drought conditions, and most of the semipermanent ponds dried completely. Thus, because animal communities change drastically the following year after ponds dry, it stands to reason that semipermanent ponds' current biotic

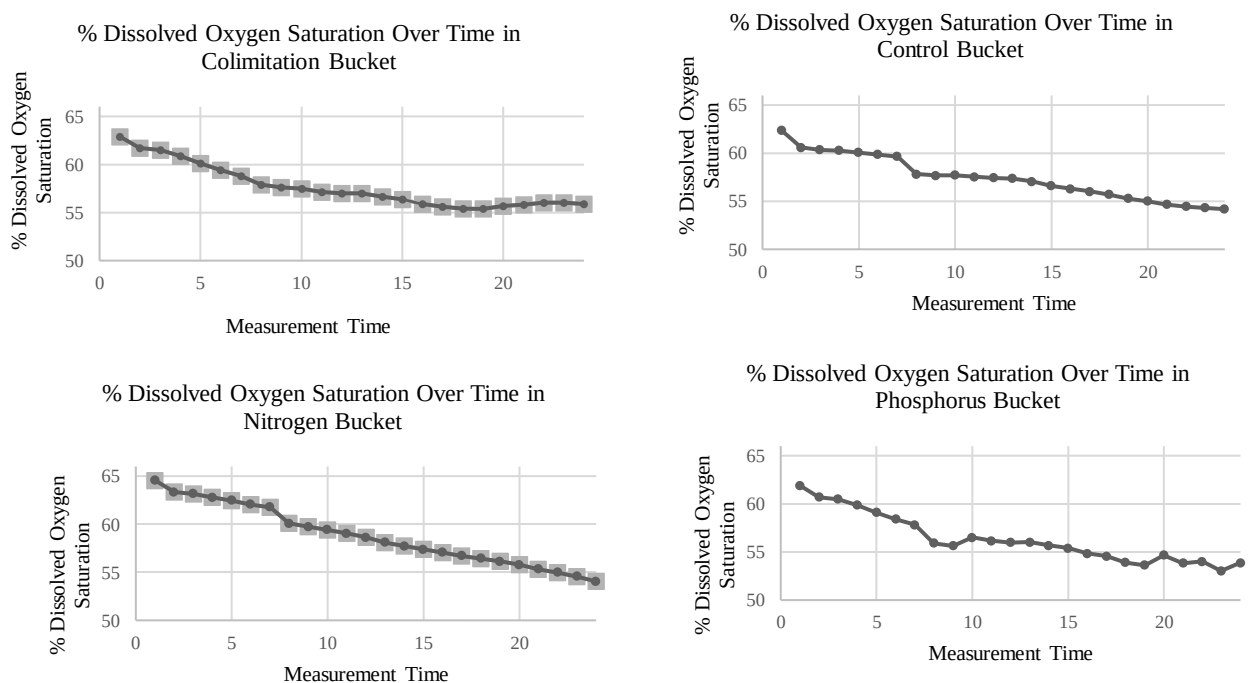


Figure 5: The % Dissolved Oxygen Saturation Over time for all four buckets of the dissolved oxygen experiment.

conditions would more closely resemble those of a temporary pond rather than a permanent pond. In turn, their animal driven nutrient supply this year may more closely resemble that of a temporary pond. Balik et al. predicted that temporary ponds may have large deficits in both nutrients. Lastly, it makes sense that temporary, and, this year, semipermanent, ponds would exhibit nitrogen limitation, due to the overall low nutrient contents of these ponds.

Furthermore, the presence of nitrogen limitation in both semipermanent and temporary ponds is supported by our ambient water chemistry measurements of the nitrogen to phosphorous ratio. The convergence of these ratios over time suggested that similar nutrient limitation statuses would be seen across the hydroperiod gradient.

However, the lack of algae on the NDS substrates which contained phosphorous or both nitrogen and phosphorous was unexpected and surprising. Even if phosphorous was not at all limiting to the algae, the colimitation NDS cups should have grown algae simply due to how much the nitrogen cups encouraged algal growth. We considered several possibilities for this inconsistency, including that perhaps the phosphorous compound we used in our P treatments was contaminated with an element that would restrict or inhibit algal growth. However, research into this possibility suggested that this was extremely unlikely, as added sodium concentrations were relatively low relative to values found to be toxic in other NDS experiments (Beck and Hall 2018) and the P salt manufacturer's reported purity was >99% (Sigma Aldrich). Additionally, on a few phosphorous NDS filters, there were small amounts of algae, which would be unlikely to occur if the phosphorous compound we used was truly toxic to algae. Finally, our lab group has used this same phosphorus compound in prior studies where P uptake rate was found to be related to algal biomass (Balik et al. 2021).

Alternatively, there could be bacteria which thrived in the presence of phosphorous and outcompeted algae on the NDS substrates containing P. In our initial experiment, we would not have been able to detect the bacteria due to the fact that they don't contain chlorophyll. Additionally, bacteria can reproduce and proliferate much faster than algae, which would allow it to take over the surface of the NDS filter very quickly and completely inhibit any algal growth.

This hypothesis led us to our follow up experiment measuring dissolved oxygen content, additional results of which are forthcoming. In this experiment, the phosphorous treatment did have by far the lowest AUC. Lower AUC correlates to more oxygen consumed, and a higher oxygen demand, which supports our hypothesis that bacteria proliferated on our phosphorous NDS cups. Furthermore, the nitrogen treatment had the highest AUC, which suggests that the bacteria were least attracted to the nitrogen NDS cups, or that the algae on the N cups continued to supply oxygen that they had produced while in light. This further explains why the algae was so successful on the nitrogen treated NDS cups.

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