The impact nitrogenous additions have on microcystin production by cyanobacteria in a glyphosate treated reservoir.

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Abstract

Cyanobacterial harmful algal blooms are negatively impacting lakes globally. These bloom events can produce toxins that can have harmful implications for the wildlife and humans that interact with them. One of these toxins that can be produced is microcystin. The research question for this experiment is what impact does adding different nitrogenous compounds have on microcystin production in a reservoir previously treated with glyphosate. Nutrient enrichment experiments were conducted on water samples from the glyphosate treated reservoir. Nitrate (NO_3^-), ammonium (NH_4^+), urea, and a mixture of all of the aforementioned were added to collected water in triplicate. A control was also prepared. Phosphorus (PO_4^{3-}) was added to all of the samples, except for the control, to ensure that PO_4^{3-} would not be a limiting nutrient. I found that between treatments there was a significant difference between Chla concentrations but there was no significant difference between microcystin concentrations. However, there were differences between days for both microcystin and Chla. These results are significant because they can aid the world in understanding which nitrogenous compounds have the highest impact on the resulting toxin concentration, which can be useful for planning management strategies and understanding if preventing nitrogen compounds from entering water bodies is a good investment for lake managers.

Introduction

Cyanobacterial blooms in surface waters are a global problem caused by anthropogenic nutrient loading to lakes and rivers (Chaffin and Bridgeman, 2014; Chaffin et al., 2018). Algal blooms that cause damage to ecosystems are referred to as harmful algal blooms (HABs) and are fueled by eutrophication, which is the process of enriching algae with nitrogen (N) and phosphorus (P; Anderson et al., 2002). There are two types of HABs: toxic and non-toxic (Anderson et al., 2002) which can disrupt both ecological integrity and stability. They have the potential to cover water bodies and can become destructive to the ecosystem, particularly when dominated by cyanobacteria.

Cyanobacterial blooms are a natural occurrence; however, they have worsened in recent years due to anthropogenic pressures (Chaffin and Bridgeman, 2014) such as climate change, nutrient runoff, and agricultural practices (Paerl et al., 2016). Cyanobacteria grow optimally at high temperatures exceeding 25°C. Climate change can increase surface water temperatures, prolonging ice-free periods at higher latitudes, and ultimately increasing the seasonal duration of cyanobacterial blooms. Climate change also has the potential to alter regional precipitation patterns, increasing the intensity and duration of seasonal floods and promoting the growth of cyanobacterial HABs by nutrient enrichment of water bodies via runoff, erosion, and groundwater discharge from agricultural lands (Paerl et al., 2011). Agricultural non-point sources of nutrients such as pastures, crops, and animal feeding operations pose a challenge to controlling for N and P due to their diffuse nature (Paerl et al., 2016). The ecophysiological strategies for adapting to anthropogenic pressures exhibited by cyanobacteria has increased the likelihood of their presence in wetland environments (Paerl and Otten, 2013).

Cyanobacteria produce algal toxins such as microcystin, which can be extremely harmful to aquatic life within the lake as well as to the surrounding organisms. Microcystin has been detected in at least 108 countries worldwide (Chaffin et al., 2018) and several cyanotoxins have been proven to negatively impact the aquatic ecosystems by disrupting the food web and triggering fish kills (Chaffin et al., 2018). Microcystin has also caused fatalities in many land mammals such as livestock as well as humans who come into contact with or ingest the toxin (Chen et al., 2019).

Further research is required to understand the complex processes behind the production of toxins by cyanobacteria and the impacts of these toxins to the surrounding environment. Although both N and P can individually influence the production of algal biomass, the combination of N and P results in the highest growth response (Davis et al., 2015).

The herbicide glyphosate is toxic to many algal species, can serve as a P source, and is contributing to the rise of HABs around the globe (Dabney and Patiño, 2018). This is due to the herbicide's ability to degrade rapidly (mean half-life=10 days in water) which releases persistent P into the water column (Hébert et al., 2019). Glyphosate can enter aquatic waterways through a variety of ways, but the majority is from non-point agricultural runoff. Of the pesticides applied to croplands, only ~1% of the pesticide reaches the target organism, while the rest is distributed throughout the environment impacting non-target species (Grube et al., 2011). When low levels of glyphosate enter a water body, the chemical stress on cyanobacteria can lead to the release of microcystin (Martínez-Ruiz and Martínez-Jerónimo, 2018). For the reservoir sampled in this experiment, glyphosate was intentionally added to the water to minimize macrophytes disrupting angling. While fertilizers are highly regulated for P, herbicide-derived P loading is highly ignored in the regulation of P (Hébert et al., 2019).

This proposal aims to assess the impact additions of urea, nitrate, and ammonium have on cyanobacterial production of microcystin in a reservoir previously treated with glyphosate. Nutrient experiments were conducted over a period of nine days. It is expected that the addition of a combination of P, urea, nitrate, and ammonium will have the greatest microcystin concentration among all of the treatments.

Materials & Methods

Study Site:

I sampled Crow Pond, a small (surface area=12,626 m^2) reservoir located in the Prairie Fork Conservation Area in Williamsburg, Missouri (38°89'37.5"N; -91°73'76.9"W). This reservoir is surrounded by conserved prairie and is often used for education. Crow Pond was treated with 9.08 liters of glyphosate over a 4,047 m^2 treatment area on July 22, 2019, as well as treated with 3.03 liters of glyphosate over an area of 3,237 m^2 on August 20, 2019 (Niswonger D, Missouri Department of Conservation, Sep 2019, pers. comm.). The aim of this treatment was to manage the *American Lotus* species within the pond to facilitate angling and ease of access by school children.

Field Approach:

I sampled on September 9, 2019 from the deepest point in the reservoir (3.4 meters). I used a YSI EXO3 profiling sonde to collect temperature, pH, and dissolved oxygen data throughout the depth of the water column. The thermocline was defined as the depth where the temperature drops greater than 1°C m^{-1}. The thermocline of the reservoir when sampled (Zm) was calculated using the Lake Analyzer package on R(Winslow et al., 2019). This depth was 2.75 meters. Secchi disk depths were also recorded. A Li-Cor was used to measure

photosynthetically active radiation (PAR) throughout the water column. I then calculated the vertical attenuation coefficient (k_d), by finding the slope of the line when you regress PAR with depth (Guildford et al., 2000). We collected 75 liters of water using a peristaltic pump from the water surface to the thermocline to be used for nutrient addition experiments and an initial characterization of water quality in Crow Pond.

Experimental Approach:

Initial reservoir conditions were assessed via total N (TN) and total P (TP) samples which were preserved in the freezer in plastic bottles. Initial water was poured into an amber bottle and preserved with Lugols solution (1%) for phytoplankton analysis. Microcystin samples were collected in 8 mL glass vials and frozen to be analyzed at a later point. Chlorophyll A (Chla), and total suspended solids (TSS) samples were also collected on glass fiber filters (pore size 0.7 μm) in duplicate. The TSS filters were prewashed in ultra-distilled water and weighed for tare-weight prior to the experiment. These filters were then frozen in desiccant until analysis. The filtrate from the glass fiber (0.7 μm pore size) filters was stored frozen for NO_3^-, NH_4^+, urea, and SRP analysis.

Crow Pond water was equally dispersed into 18, 4 L cubitainers (6 treatments in triplicate). The cubitainers were enriched in triplicate to have the final added nutrient concentrations as follows: 5 μM PO_4^{3-}, 100 μM NO_3^-, 100 μM NH_4^+, 100 μM urea, and an addition that contains all of the preceding nutrients. 5 μM of PO_4^{3-} was also added to all of the treatments with the exception of the control to ensure that P would be in sufficient quantities. No nutrients were added to the control treatment. The cubitainers were incubated for nine days in three incubation ponds that had light and temperature conditions similar to the collection site.

These incubation ponds were continually monitored for changes in temperature and light using HOBO Pendant Temperature/Light 8K Data Loggers. Sub-samples were collected every three days during the incubation period (e.g., day 3, 6, and 9) for microcystin, Chla, TSS, phycocyanin, and pH. pH was measured on a Fisher Scientific accument AE 150 pH meter. On day nine of the incubation, nutrient samples (SRP, TP, TN, NO_3^-, NH_4^+, and urea) were also collected.

Chla samples were inserted into tubes with 90% ethanol and placed in a hot water bath for 15 minutes. The samples were analyzed using a Turner Design fluorometer (TD700). These samples were all individually corrected for pheophytin (Knowlton, 1984; Sartory, 1986).

TP and total dissolved P (TDP) samples were measured spectrophotometrically (Crumpton et al., 1992; Greenberg et al., 1997) with a detection limit of 0.0054 g L^{-1}. TN and total dissolved N (TDN) were measured in triplicate using the second derivative spectroscopy procedure with a detection limit of .45 g L^{-1}. NO_3^- and NH_4^+ samples were analyzed using a Lachat QuikChem Flow Injection Analyzer. The detection limit for NO_3^- was .0648 g L^{-1} and .1278 g L^{-1} for NH_4^+. SRP samples were analyzed using a Cary60 spectrometer (Hales et al., 2004). The detection limit for SRP was .0054 g L^{-1}.

Microcystin samples underwent a freeze/thaw cycle three times to extract intracellular microcystin from the whole water samples. The samples were filtered through 0.45 µm glass fiber filters. The filtered water was analyzed using indirect competitive ELISA (Enzyme Linked Immunosorbent Assay) kits from Abraxis LLC. These kits had a detection limit of 0.15 µgL^{-1}.

TSS filters were analyzed using a four-step process of drying the filters at 105 °C, then weighing the dry weight of the filters while negating the initial weight of the filter prior to

processing. They were ashed at 550 °C for 20 minutes to burn off the organic material to be weighed again and quantify that organic and volatile matter. Particulate organic matter was calculated by finding the difference between TSS weight and particulate inorganic matter weight.

Statistical Approach:

Statistics were found using R statistical software. The differences between days and treatments were found using a Kruskal-wallace test. If a statistical difference was found (<0.05), then a post-hoc dunn's test would be performed to see where those differences were between the various factors (Dinno, 2017).

Results

Initial reservoir characteristics:

The surface of area of Crow Pond was calculated using bathymetric maps, that resulted in a surface area of 12,626 m^2 (Figure 1). Before glyphosate was added to the reservoir, a microcystin sample was collected on June 19, 2019. This sample was ran in duplicate and was below the detection limit for microcystin. However, the average microcystin concentration was 1.742 $\mu g L^{-1}$ for the control on the initial day of sampling, which is more than 11 times higher than the detection limit of 0.15 $\mu g L^{-1}$. The mean PAR value (initially measured as lux) measured from the HOBOs in the incubation tanks was 138.28 $\mu mol\ m^{-2}\ s^{-1}$. This value is slightly higher than the site measured PAR from the epilimnion of 100.26 $\mu mol\ m^{-2}\ s^{-1}$. The average temperature measured by the HOBOs in the incubation tank over the incubation period was 28.14 °C. This is also slightly higher than the average temperature measured at the site from the water surface to the epilimnion depth, which had an average value of 21.42 °C.

Chla concentrations:

To determine if there was a significant difference between days for Chla concentrations, a Kruskal-Wallis rank sum test was used to obtain a p-value of 1.896e-05, showing there was some statistical difference between the days. From there, a Dunn's Test was used to determine which days were significantly different from one another. There was a statistical difference between day 3 and 9, as well as day 6 and 9. However, there was no statistically significant difference between day 3 and 6 for all of the treatments combined (Table 2).

Another Kruskal-Wallis rank sum test was performed to determine if there was a statistical difference between Chla concentrations between treatments on day 9 of the experiment, which resulted in a p-value of 0.03423. Since there was a significant difference between treatments, another Dunn's Test was used to observe where the differences were. The control was significantly lower from all treatments except for the Phosphorus addition treatment. The P treatment was significantly lower than every treatment apart from the control and the urea treatment. Apart from those, there was no significant differences among the different nitrogenous compound additions for day 9 (Table 3).

Over 9 days the Chla concentrations appear to be increasing in the treatments (Figure 2). For the P and control treatments, Chla concentrations rise at a lower rate (around 30%) compared to the rest of the treatments, which nearly triple in Chla concentrations from day 3 to day 9 (Tables 2 & 3). It is notable that the initial lake water had Chla concentrations that were nearly four times higher than the day 3 control incubated water measurements.

Microcystin concentrations:

The Kruskal-Wallis rank sum test was used to determine if there is statistical difference in microcystin concentrations between days, which resulted in a p-value of 1.375e-07. A Dunn's Test was used to determine which days were statistically difference, which showed there were significant differences between every one of the days measured (Table 4).

Another Kruskal-Wallis test was used to determine if there was a statistical difference between treatments for microcystin concentration. This test resulted in a p-value of 0.177, showing there is no statistical difference for microcystin concentrations between the treatments for day 9. There was an increase in the microcystin concentration for each treatment over the incubation period (Figure 3).

Discussion

Summary of Results:

Chla concentrations were significantly different between days, with increasing concentration over time. Chla was also significantly different between treatments, with all of the nutrient additions being significantly higher than the control, except for the phosphorus addition. Microcystin concentrations were not significantly different between treatments, however they were significantly higher each consecutive measurement date.

Chla:

The day zero value for Chla was nearly four times higher than the average control values on day 3.This may be due to grazing by zooplankton in the system (Klein et al., 1986). Zooplankton were not filtered out from the initial lake water, in order to preserve the microcystin colonies already be in the water. However, because of this it may have given zooplankton the

opportunity to consume lots of the algae initially, potentially leading to this drop in Chla concentrations from day 0 to day 3 of the experiment. Pheophytin concentrations can be an indicator of grazing because the pheophytin *a* pigment can be found within and in the fecal matter of zooplankton (Jeffrey, 1974). However, the pheophytin concentrations did not change drastically over the course of this experiment.

The results show that in Crow Pond, P is not a limiting nutrient for Chla production because there was no significant difference between the PO_4^- treatment and the control on day nine of the experiment. The TN:TP ratio from the initial lake water was right around 15. This also indicates the reservoir in deficient in N (TN:TP ratio <20; Guildford and Hecky, 2000). However, every nitrogenous addition was significantly different from the control, suggesting that nitrogen is in fact a limiting nutrient in this system for Chla. However, it is interesting to note that in Missouri reservoirs are typically P deficient (Petty et al., forthcoming).

Microcystin:

Microcystin concentrations did not have a statistical difference between treatments on day 9 of the experiment. However, microcystin did have a significant difference between days. Every consecutive day of measurements for microcystin, the concentrations were significantly higher than the previous measurements. This microcystin may come either actively in relation to algal production, or from the degradation of *Microcystis* releasing microcystin (Maruyama et al., 2003). This may be due to being in a confined system, where there is no natural dilution of the water from outside sources. Dilution is a major contributor to lowering microcystin concentrations in reservoirs (Tsuji et al., 2001).

Impact of glyphosate on mc concentrations:

While nutrient measurements were not taken before glyphosate was added to the reservoir, it is interesting how P was not a limiting nutrient in this system. Since glyphosate can be a source of P in aquatic systems (Dabney and Patiño, 2018), it is not surprising that there appears to be an abundance of phosphorus already in the system. This would suggest that the likelihood of a HAB event is more likely when glyphosate is loaded into reservoirs, either intentionally or from outside sources. This is particularly a risk in Missouri, which typically has P limited reservoirs (Petty et al., forthcoming).

Before the glyphosate addition, the microcystin sample collected was below detection limits. However, all microcystin concentration measurements taken after the glyphosate addition were detectable. Based on the excess of phosphorus that glyphosate may have released into the system, this suggests that phosphorus may have been a limiting nutrient for microcystin in the system prior to the addition of glyphosate. The initial addition of glyphosate may of also triggered apoptosis to the cyanobacteria cell, killing the cells and releasing microcystin (Wu et al., 2016). The combination of these factors should deter lake managers from intentionally adding glyphosate into reservoirs to lower the likelihood of a HAB occurring.

In Missouri, the use of N based fertilizers continues to increase despite environmental concerns (Motavalli et al., 2008). Based on the results, this in combination with glyphosate use may be the perfect storm for microcystin production within reservoirs. Best management practices (BMPs) should be incentivized and utilized by farmers to minimize their loss of N from fields into reservoirs (Ruidisch et al., 2013). This prevents the likelihood of eutrophication and HABs.

These results also show us the potential impact glyphosate runoff may have in reservoirs. This broken down glyphosate in reservoirs may lead to eutrophication. Eutrophication may lead to HABs, fish kills, and the release of algal toxins. It is important to manage for glyphosate before it even reaches our reservoirs, to prevent disturbing these habitats.

Conclusion

Based on the increase of both Chla and microcystin concentrations over the course of the experiment, it shows that N was a limiting nutrient for cyanobacteria within this system. As glyphosate use and eutrophication continues to occur at increasing rates, it may have disastrous consequences for midwestern reservoirs. Reduction of the use of glyphosate as well as incentivizing BMPs in the future will help to protect reservoirs from these nutrient loads (Ruidisch et al., 2013).

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List of Tables

Table 1: Initial water chemistry measurements for Crow Pond. These were measured on September 9, 2019. The surface area was found using a bathymetric map (Figure 1). The pH, total phosphorus (TP), total nitrogen (TN), nitrate (NO_3^-), urea, ammonium (NH_4^+) were all calculated in the lab from the collected water. The mixing depth of the water (Zm) was calculated using the RLakeAnalyzer package on R (Winslow et al., 2019).

Crow Pond Water Characteristics	
Surface area	12,626 m^2
Max depth	3.409 m
Epilimnetic water temperature	21.42 °C
pH	7.09
TP (μgL^{-1})	0.117
TN (μgL^{-1})	1.76
$NO3^-$(μgL^{-1})	0.025
urea (μgL^{-1})	0.2378
$NH4^+$ (μgL^{-1})	0.025
Zm (m)	3.40
PAR from epilimnion ($\mu mol\ m^{-2}\ s^{-1}$)	100.26

345 **Table 2:** P-values between days for Chlorophyll A concentrations determined through Dunn's
346 statistical test. Significant differences between treatments (**) are those with a value less than
347 0.05.

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Comparison of Chla concentrations by day		
	3	**6**
6	0.1076	
9	0.0000**	0.0005**

Table 3: P-values between treatments for final Chlorophyll A concentrations on day 9 determined through Dunn's statistical test. Significant differences between treatments (**) are those with a value less than 0.05.

Comparison of Chla concentrations by treatment					
	All	**Ammonium**	**Control**	**Nitrate**	**Phosphorus**
Ammonium	0.4093				
Control	0.0089**	0.0047**			
Nitrate	0.5000	0.4093	0.0089**		
Phosphorus	0.0332**	0.0195**	0.2962	0.0332**	
Urea	0.2511	0.2703	0.0234**	0.3511	0.0731

353 **Table 4:** P- values between days for microcystin concentrations determined through Dunn's
354 statistical test. Significant differences between treatments (**) are those with a value less than
355 0.05.

Comparison of mc concentrations by day		
	3	**6**
6	0.0001**	
9	0.0000**	0.0330**

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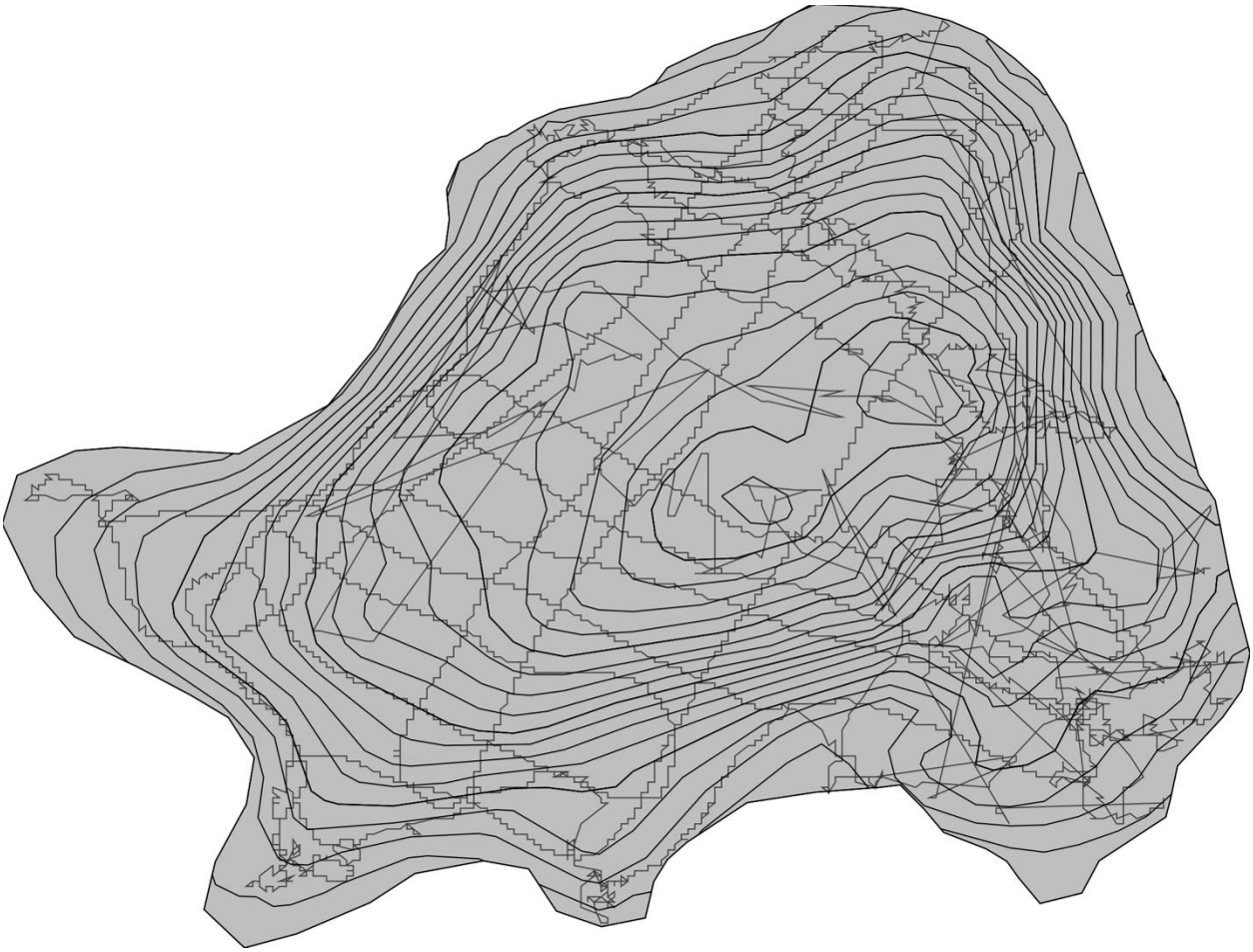
Figure 1.



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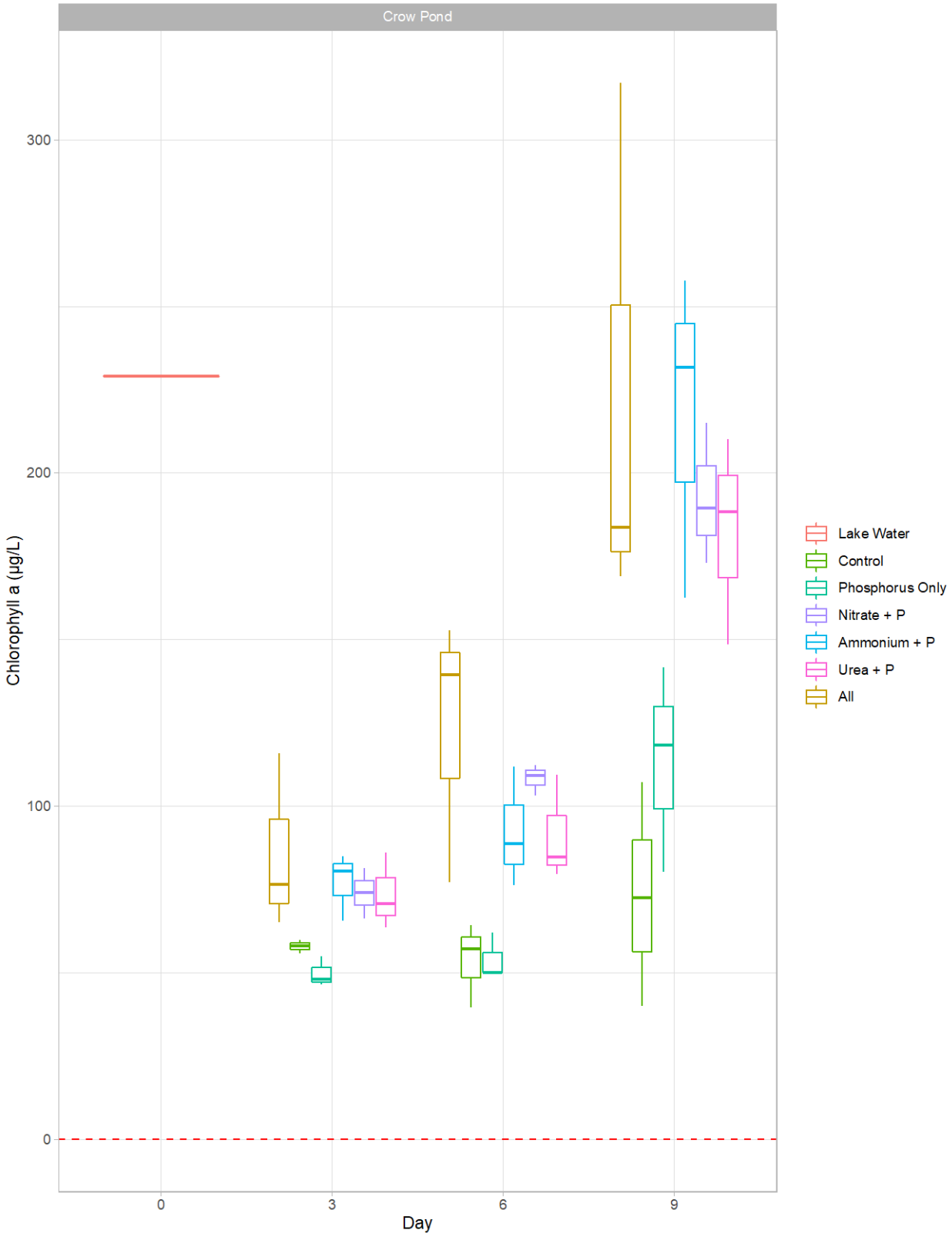

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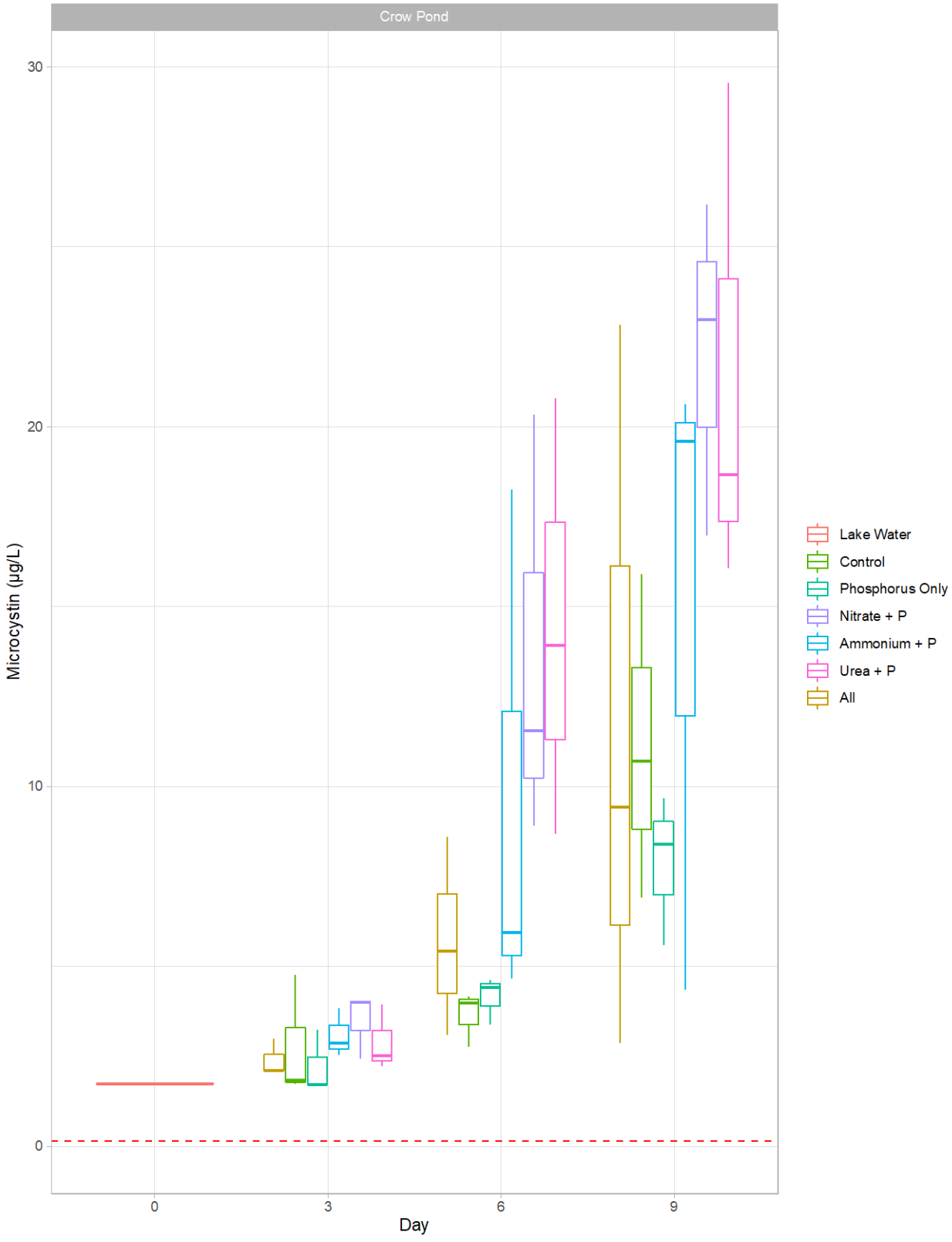
Figure 3.



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