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Effect of Hydrogen Peroxide on Algae and Microcystin in Control and Lake Waters

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Abstract

Cyanobacteria are photo-autotrophic organisms with a worldwide distribution, which can result in Harmful Algal Blooms (HABs) producing toxins. One of the most common strains of cyanobacteria is *Microcystis aeruginosa*, which produces the most abundant cyanotoxin, microcystin. In this study, we analyzed the effect of H_2O_2 on algae and microcystin using both lake and reagent grade water. The first objective was to determine the effect of H_2O_2 on algae and cyanobacteria in lake water that was nutrient enriched. The second objective was to detect the effect of H_2O_2 at oxidizing microcystin in spiked deionized water and lake waters. For the first experiment, H_2O_2 significantly reduced the concentrations of cyanobacteria as the doses were increased, and H_2O_2 was effective at low doses (e.g. 50 μL L^{-1}). The addition of 100 μL H_2O_2 L^{-1} or more showed reductions in cyanobacteria that were not significantly different from each other. The second experiment on deionized water and lake water did not work, and the results did not show microcystin reduction as doses were increased. The first results suggests H_2O_2 as an effective treatment for cyanobacteria algal blooms. However, the second experiment should be repeated in order to prove what other studies have suggested about H_2O_2 reducing microcystin concentrations.

Introduction

Eutrophication in bodies of water such as lakes, rivers, ponds, and reservoirs has increased mainly due to human activities. This process results from the excess of nutrients such as nitrogen (N) and phosphorus (P) that favor the growth of algae and cyanobacteria, causing potentially large blooms (Khan & Ansari, 2005). The proliferation of cyanobacteria can influence the beneficial uses of water resources, from drinking water, fishing, irrigation to recreational use. These algal blooms, particularly cyanobacterial, can become harmful when toxins like microcystin are produced (Chorus & Welker, 2021). The spread of these harmful cyanobacterial blooms in freshwaters has become a major concern for communities around the world.

Cyanobacteria produce, under certain conditions, a wide range of secondary metabolites that are toxic to eukaryotic organisms and potentially humans. These toxins are produced only by some genera of cyanobacteria, including *Microcystis, Anabaena, Planktothrix, Nostoc*, and others (Chorus & Welker, 2021). Some species are known to show high or low levels of toxicity under certain conditions including environmental parameters such as temperature, light intensity, and nutrients (Kaebernick & Neilan, 2001). These toxins have the ability to cause serious health and environmental problems.

One of the most common strains of cyanobacteria is *Microcystis aeruginosa* (Rinehart et al., 1994), which produces the most abundant hepatotoxin, microcystin. Nearly a hundred microcystins have been described, which are cyclic peptides in which non-protein amino acids intervene and whose synthesis is not carried out (Bouaïcha et al., 2019; Harada, 1996). It is not necessary for a large proliferation of cyanobacteria to occur for the population present to be toxic, since cyanotoxins are one of the most potent poisons (Chorus & Welker, 2021).

If present in water supplies microcystins require treatment in the drinking water plant. Conventional water purification systems such as coagulation, flocculation, or filtration can be effective for intracellular toxins where cells are removed, but they are less effective at removing extracellular toxins (Pietsch et al., 2002). Furthermore, these treatment options may simply transfer toxins from intracellular to free in the treated water. Chlorination has been used extensively in the USA (Driska et al., 2001) and has served as an accessible and easy way to oxidize microcystin in drinking water treatment (Zamyadi et al., 2013).

The effectiveness of control methods in natural waters varies according to type and size of the lake, retention time, degree of alteration, amount of nutrient load, quality and quantity of

sediments, amount of aquatic life, etc. (Bhateria & Jain, 2016). The reduction of nutrients, such as N and P, in non-point and effluent inputs is the ultimate preventive measure (Nagar et al., 1974). The reduction of nutrients would likely result in reduced algal growth and blooms, but these reductions may take a long time to show up in terms of chemical water quality in streams (Meals et al., 2010).

The use of chemical substances would be a more short-term management option acting with high impact on cyanobacteria. Copper has been one of the best algaecides, and it is generally applied as copper sulfate ($CuSO_4 \cdot 5H_2O$) (Murray-Gulde et al., 2002). However, the use of copper salts has some concerns such as non-selective toxic effect on non-objective aquatic organisms and potential negative consequences related to human health (Kenefick et al., 1993; Lam et al., 1995). However, this technique is still widely use but other methods with a less negative impact on the aquatic environment exits.

Hydrogen peroxide (H_2O_2) is a well-known agent with a high oxidizing capacity, which is commonly used in disinfection and water treatment (Wang et al., 2000). Some studies have suggested the use of low concentrations of H_2O_2 for the selective elimination of cyanobacteria in lakes. Although adding chemicals to natural waters seems like an unusual strategy, this method can be an effective and relatively harmless way to remove or reduce cytotoxins (Matthijs et al., 2012). Its use in lakes or reservoirs does not lead to the accumulation of trace elements like Cu in the environment. H_2O_2 has the property of degrading in water in a short time, which represents a great advantage when using it to suppress cyanobacteria blooms in lakes (Piel et al., 2020). The only challenge with H_2O_2 is that it is a non-discriminatory oxidant, but there is little chance of developing resistance like with other algicides.

In this study we analyzed the effect of H_2O_2 on algae and microcystin using both lake and reagent grade water. The first objective was to determine the effect of H_2O_2 on algae and cyanobacteria in lake water that was artificially nutrient enriched. The second objective was to detect the effect of H_2O_2 at oxidizing microcystin in spiked deionized water and lake waters. With the results obtained from this study, it would be possible to prescribe an effective treatment for algal blooms and toxic concentration of microcystin in local recreational lakes.

Material and Methods

Lake Fayetteville (36° 8′ 8″ N, 94° 8′ 21″ W) is within the limits of the City of Fayetteville, Arkansas. It has an area of approximately 0.78 km^2 and broadly serves as an

outdoor recreational place where activities such as fishing are very common. Lake Fayetteville also has a park with an area of around 1.85 km^2 surrounded by forests. There are some amenities in Lake Fayetteville including an 8.85 km nature trail around the lake, a disc golf course, and a boat bock marina (Fayetteville, AR - Official Website, 2021).

The routine monitoring of Lake Fayetteville by the Arkansas Water Resources Center (AWRC) has documented the occurrence of HABs every year since monitoring began. In 2019, the maximum levels reported was 11 $\mu g\ L^{-1}$ of microcystin on May 17, and another harmful algal bloom was reported during June 2020 with levels up to 30 $\mu g\ L^{-1}$ (Fayetteville, AR - Official Website, 2019). The City of Fayetteville has issued warnings to all residents when occurrences of HABs. All visitors were advised to take safety measures and avoid areas of algae accumulation when visiting the lake. The Environmental Protection Agency (EPA) suggested in 2019 that a total microcystin concentration of 8 $\mu g\ L^{-1}$ or below of microcystin keeps public health protected (EPA, 2019).

The water and algal samples used for this study were collected during October 2020 and March 2021 from this lake, and algal growth was enhanced in the lab with added nutrients and incubation in the sunlight. To start the first experiment, 100 ml of lake water was added to 250 ml flasks to be incubated on the windowpane at room temperature. The lake water in the flasks had initial measurements of the raw fluorescence (RFU) of phycocyanin and chlorophyll pigments using in vivo florescence with a CyanoFlour (Turner Designs, San Jose, Ca, USA). Phycocyanin and chlorophyll are pigments found in cyanobacteria and algae that capture energy from sunlight to carry out photosynthesis. Phycocyanin is produced exclusively by cyanobacteria while chlorophyll is found in other photosynthetic microorganisms like algae. Phycocyanin has been used as effective marker to detect cyanobacterial blooms in the fresh water (Woźniak et al., 2016). That is why phycocyanin: chlorophyll ratios are considered good indicators of the presence of cyanobacteria, where ratios over one suggest cyanobacteria are abundant and dominant in the water (Thomson-Laing et al., 2020).

For the first experiments a total of ten flasks were labeled and treated with different rates of common 3% H_2O_2 stock solution. The proper volume of the stock H_2O_2 was added by pipet to the 100 ml of lake water in the flasks on October 29, 2020. Doses of 0, 50, 100, 200, 400, 600, 800, 1000, 2500, 5000 $\mu L\ L^{-1}$ of H_2O_2 were applied, respectively. These treatments were incubated at room temperature on the windowpane. The first RFU readings after H_2O_2 treatments

were taken on November 5, 2020 and the second readings on November 11, 2020. These RFU measurements were used as a proxy for cyanobacteria and algae content in the flasks. Data were entered into Excel and analyzed using analysis of variance (ANOVA) and least significant difference (LSD) with means separation.

For the second experiment the procedure followed was similar to the first experiment. Two set of treatments were prepared where one set was containing lake water and the other set was containing reagent grade water. The deionized water and lake water used was spiked with >5 $\mu g\ L^{-1}$ microcystin and then treated with various volume of 3% of H_2O_2. Doses containing 0, 5, 10, 20, 40, 60, 80, 100, 250, 500 $\mu L\ L^{-1}$ of H_2O_2 were added to the treatments in flasks of 100 ml and incubated at room temperature. The RFU readings for these treatments in lake water were taken the following day on March 19, 2021. Water from all treatments was set aside for total microcystin analysis following three freeze thaw cycles.

To measure total microcystin concentrations in the second experiment, enzyme-linked immunosorbent assay (ELISA) was used (Abraxis; EPA method 546). This method consisted in determine the concentration of microcystin based on color absorbance of samples. The protocol followed for this experiment was the same developed by Zaffiro et al. (2016). First, the treatments were placed on a 96-well microtiter plate where microcystin found in the samples would compete for binding to a primary detection antibody. The protocol was followed by two washes steps where after the first enzyme conjugated was added to bind to the primary antibody, and after the second wash tetramethylbenzidine substrate also was added to react and produce color on samples. The readings were taken using a plate reader from Abraxis (Warminster, PA, USA), and the method with a twofold dilution gave a maximum concentration of 10 $\mu g\ L^{-1}$.

Results

Experiment 1

Phycocyanin

In the first set of data (Table 1), the addition of 3% of H_2O_2 influenced the raw fluorescence of phycocyanin. On treatments from November 5, 2020, after seven days of incubation, the control had a mean value of 17747 RFUs for phycocyanin. On treatment with the least addition of H_2O_2 (50 $\mu L\ L^{-1}$), the phycocyanin RFUs was significantly less (5615 RFUs) than the control; this value corresponded to three time less compared to the control. Within the least doses (50-200 $\mu L\ L^{-1}$) the mean RFUs for phycocyanin were not different; however, these

treatments were all significantly less than the control. Mean RFUs of phycocyanin of treatments from 100 to 5000 μL L^{-1} were not significantly different from each other, but all were significantly less than the control (Table 1).

Treatments from November 11, 2020 were measured after 14 days of incubation in the laboratory. The mean RFUs of the control (8951) was approximately half what was measured on November 5, 2020, and all H_2O_2 treatment had mean RFUs less than the control after 14 days of incubation. Treatments with doses from 800 to 5000 μL L^{-1} were significantly different from the control, where 2500 μL L^{-1} had the least mean RFUs (162).

Chlorophyll

The raw fluorescence of chlorophyll was used as a proxy for algal biomass in the water, where greater RFUs suggested greater biomass (Table 2). The control for the treatments from November 5, 2020, had a mean value of 24558 after seven days. The least doses of 3% H_2O_2 (50-200 μL L^{-1}) had mean RFUs for chlorophyll that were not significantly different from the control (Table 2). However, H_2O_2 doses from 400-5000 μL L^{-1} had mean RFUs for chlorophyll that were significantly different from the control.

November 11, 2020, the control had a mean value of 26467 RFUs. The three greatest doses (1000-5000 μL L^{-1}) had mean RFUs for chlorophyll that were significantly less than the control (Table 2). The rest of treatments did not show means significantly different from the control.

PC:CHL Ratios

On November 5th, the mean PC:CHL ratios, for the control had a value of 0.720. Most of the treatments did not show significant difference from each other after seven days of incubation in the laboratory. The least addition of H_2O_2 (50 μL L^{-1}) had a mean of 0.209 while the highest addition (5000 μL L^{-1}) had a mean of 1.03 (Table 3). However, the 1000 μL L^{-1} treatment was significantly different from the control and the rest of the treatments. It corresponded to the dose with the greatest mean value (3.21); this value was almost four times greater than the control mean RFUs.

For measurement on November 11th, 2020, the control had a mean PC:CHL ratio of 0.324 (Table 3). Treatments with 50, 400, 1000 μL L^{-1} H_2O_2 doses had mean PC:CHL ratios significantly different from the control, where 50 and 400 μL L^{-1} had lower mean ratios, and the 1000 μL L^{-1} treatment had the highest ratio for this set of measurements.

Experiment 2

Phycocyanin

The RFUs for phycocyanin were rather variable in this experiment. On March 19, 2021 (Table 1), the control had a mean RFUs of 21337. The addition of H_2O_2 did result in mean RFUs for phycocyanin that were different from the control, except for 10 and 20 μL L^{-1}. Mean RFU for phycocyanin at 20 μL L^{-1} treatment was less than the control, whereas mean RFU at 10 μL L^{-1} was greater than the control.

Chlorophyll

The chlorophyll RFU readings for treatments on March 19th, 2020 had a control with a mean value of 12434 (Table 2). The treatments did not have mean RFUs significantly different from the control, except for 100 μL L^{-1} which had a mean RFU of 63667 and was significantly greater from the rest of treatments.

PC:CHL Ratios

For PC:CHL ratios on March 19th, 2021, the control had a mean value of 1.46 (Table 3). Dose of 10 μL L^{-1} showed the greatest mean (4.23) but was not significantly different from the control. However, this dose had a mean PC:CHL ratio that was greater than that observed with treatments greater than 80 μL L^{-1}.

Microcystin

For treatment spiked with microcystin, it was expected to get decreasing concentrations as the doses of H_2O_2 treatment were increased. However, the results showed that the decrease in total microcystin concentrations was not consistent since there is not a pattern followed where higher H_2O_2 doses show more reduction in total microcystin concentrations. The control lake water had a total microcystin concentration of 9.086 μg L^{-1} (Table 4), which was almost twice that intended with the spike solution. The other H_2O_2 treatments of lake water had concentrations less than 1.5 μg L^{-1}, except two treatments which had total microcystin concentrations exceeding 10 μg L^{-1} (Table 4). All the treatments using deionized water spiked with the algal toxin had total microcystin concentrations less than 0.5 μg L^{-1}, including the control which had a total microcystin concentration less than the reporting limit (i.e., 0.3 μg L^{-1}).

Table1. ANOVA and LSD means separation results for raw fluorescent units (RFUs) of phycocyanin by treatment. Different lower-case letters indicate significantly different groupings (LSD test, p=0.05).

DATE	Treatment (µL H_2O_2 L^{-1})	Mean	SE	MS
	0	17747	771	c
Nov 5, 2020	50	5615	348	b
	100	3091	269	ab
	200	3402	788	ab
	400	1540	380	a
	600	1327	572	a
	800	1010	253	a
	1000	1215	150	a
	2500	767	121	a
	5000	723	59	a
	LSD= 3487	**F= 2.39**		
		***p*= 5.36 E-8**	**df=9**	
	0	8951	2508	c
Nov 11, 2020	50	1937	403	ab
	100	3239	1399	ab
	200	2692	1124	ab
	400	1192	390	ab
	600	5490	3273	b
	800	3139	1467	a
	1000	355	61	a
	2500	162	31	a
	5000	175	28	a
	LSD= 4445	**F= 2.39**		
		***p*= 0.0012**	**df=9**	
March 19, 2020	0	21387	11990	bc
	5	9631	2854	ab
	10	50334	42077	d
	20	32797	7808	c
	40	1327	4344	a
	60	13193	1812	ab
	80	8982	1275	ab
	100	5814	1567	ab
	250	3871	226	ab
	500	2838	186	ab
	LSD=19259	**F= 2.39**		
		***p*= 0.3960**	**df=9**	

Table 2. ANOVA and LSD means separation results for raw fluorescent units (RFUs) of chlorophyll- a by treatment. Different lower-case letters indicate significantly different groupings (LSD test, p=0.05).

DATE	Treatment (µL H_2O_2 L^{-1})	Mean	SE	MS
Nov 5, 2020	0	24558	1442	c
	50	22762	9742	c
	100	18538	2526	bc
	200	20828	2520	bc
	400	10922	1979	ab
	600	7258	4895	a
	800	1539	804	a
	1000	490	140	a
	2500	566	34	a
	5000	717	43	a
	LSD= 10969	**F= 2.39**		
		***p*= 0.00012**	**df=9**	
Nov 11, 2020	0	26467	4921	b
	50	12750	4221	ab
	100	13906	5292	ab
	200	10859	3054	ab
	400	12670	3888	ab
	600	20351	9281	b
	800	19979	9897	b
	1000	613	9.3	a
	2500	429	25	a
	5000	446	5.7	a
	LSD=15537	**F= 2.392**		
		***p*= 0.02**	**df=9**	
March 19, 2020	0	12434	3937	a
	5	10507	591	a
	10	10866	635	a
	20	14538	568	a
	40	13142	1489	a
	60	15372	1947	a
	80	13110	965	a
	100	63667	40427	b
	250	12610	2874	a
	500	8018	355	a
	LSD= 17536	**F= 2.393**		
		***p*= 0.18**	**df=9**	

Table 3. ANOVA and LSD means separation results for ratios of raw fluorescent units (RFUs) of chlorophyll- a and phycocyanin by treatment. Different lower-case letters indicate significantly different groupings (LSD test, p=0.05).

DATE	Treatment (µL H_2O_2 L^{-1})	Mean	SE	MS
Nov 5, 2020	0	0.720	0.02	a
	50	0.209	0.044	a
	100	0.169	0.011	a
	200	0.163	0.028	a
	400	0.137	0.012	a
	600	0.331	0.15	a
	800	1.13	0.47	a
	1000	3.21	1.32	b
	2500	1.34	0.142	a
	5000	1.03	0.14	a
	LSD=1.33	**F= 2.39**		
		***p*= 0.0028 df=9**		
Nov 11, 2020	0	0.324	0.037	b
	50	0.178	0.039	a
	100	0.227	0.037	ab
	200	0.228	0.035	ab
	400	0.126	0.052	a
	600	0.231	0.055	ab
	800	0.254	0.098	ab
	1000	0.576	0.094	c
	2500	0.373	0.0543	b
	5000	0.393	0.0683	b
	LSD=0.18	**F= 2.392**		
		***p*= 0.0023 df=9**		
March 19, 2020	0	1.46	0.624	ab
	5	1.23	0.406	ab
	10	4.23	3.573	b
	20	1.20	0.543	ab
	40	1.00	0.324	ab
	60	0.864	0.069	ab
	80	0.711	0.145	a
	100	0.408	0.119	a
	250	0.361	0.0412	a
	500	0.273	0.028	a
	LSD=3.46	**F= 2.393**		
		p= 0.503 df=9		

Table 4. Treatments with respective concentration of microcystin. Left column correspond to concentration of microcystin (µg L^{-1}) in lake water while right column shows concentrations in deionized water.

Treatment (µL H_2O_2 L^{-1})	**Lake Water Microcystin (µg L^{-1})**	**Deionized Water Microcystin (µg L^{-1})**
0	9.086	0.284
5	1.456	0.238
10	0.000	0.050
20	0.094	0.102
40	0.098	0.000
60	> 10.0	0.472
80	> 10.0	0.062
100	1.088	0.000
250	0.896	0.000
500	0.690	0.058

Discussion and Future Opportunities

Hydrogen peroxide has become a popular control mechanism to treat cyanobacterial harmful algal blooms (Guo et al., 2021). However, the required doses to control these blooms varies depending on environmental conditions (Neilan et al., 2013). In this study, the data obtained showed that H_2O_2 may reduce cyanobacteria, based on the decrease in mean RFUs for phycocyanin observed in experiment 1. In fact, the addition of any amount of H_2O_2 (50-500 µL L^{-1}) reduced phycocyanin RFUs in the first experiment. These results support previous studies where H_2O_2 was proven as an effective treatment against cyanobacterial harmful algal blooms. Barrington et al. (2013) suggests H_2O_2 as algicide able to reduce cyanobacteria and microcystin effectively and fast. Also, a more recent study showed H_2O_2 can remove and inhibit the growth of cyanobacteria in water bodies (Kansole & Lin, 2017).

Furthermore, the results from RFUs for chlorophyll-a also showed a decrease in means as the doses of H_2O_2 were increased in experiment 1. This suggests that H_2O_2 reduced the amount of total algae in the water not just cyanobacteria. Drábková et al.(2007) mentions that H_2O_2 could have an effect on reducing algae; therefore, H_2O_2 would likely be an effective algicide at Lake Fayetteville.

On the other hand, experiment 2 did not follow expected results. The concentration of microcystin in control from lake water was 9.086 µg L^{-1} , which corresponded to an amount almost twice higher than expected originally (5 µg L^{-1}). This suggests that of the spike solution had almost twice the concentration of total microcystin, i.e. 1000 µg L^{-1} . Despite this, the treatments did not show decreasing total microcystin concentration with increasing H_2O_2 dose. In fact, two of the treatment had total microcystin concentrations greater than 10 µg L^{-1}.

The lake water used in the second experiment was different from the first, because it has larger groups of filamentous algae, which sometimes clogged the spout when dispensing lake water. The variability in algal content of the lake water might explain why phycocyanin and chlorophyll RFUs did not decrease with increasing H_2O_2 dose, like observed in the first experiment. This experiment was focused on the second objective, so the lake water variability was not an initial concern. In hindsight, the long filamentous algae should have been broken and mixed thoroughly in the lake water so this could not have introduced variability.

Also, the concentrations of microcystin in deionized water did not logically decrease as H_2O_2 treatments are increased. This observation was puzzling, and it was not the influence of the

algal content since it was deionized water. The treatment was incubated in the sunlight in both experiments, but maybe sunlight degraded the H_2O_2 in the reagent grade H_2O much faster and it did not have the expected effect on reducing microcystin concentrations (Cooper et al., 1988). Also, it is possible that sunlight could degrade microcystin and, therefore, alter the concentrations in the reagent grade water (Schmidt et al., 2014).

For future research and experiments, the experiment 2 could be repeated making sure that a hand mixed is used to break apart long filamentous chains of algae in water. This will help to reduce variability in the results. Also, it is important to consider the exposure to sunlight since it could be a factor that could inhibit the effects of H_2O_2 on treatments. Before spiking water with microcystin, it should be well mixed without any algae chain left. Then, it is important to make sure to incubate the treatments in a place where exposure to sunlight could not affect the results. This could help to improve the results for next experiments.

Conclusion

All in all, the first experiment showed that H_2O_2 has effect on reducing cyanobacteria numbers as the doses of H_2O_2 were increased. This supports previous studies (Barrington et al., 2013) that demonstrated H_2O_2 as an effective algicide.

The second experiment did not work, but research has shown H_2O_2 remediates cyanotoxins like microcystin (Huo et al., 2015). This experiment should be repeated making sure to remove factors that introduce variability like samples with chains of algae on water and sunlight exposure.

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