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Research Article

Cyanocidal Effect of H_2O_2 on the Bloom-Forming *Microcystis aeruginosa* and *Sphaerospermopsis aphanizomenoides*

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ABSTRACT

Cyanobacterial blooms are a global concern causing water quality problems that have serious effects on recreational activities, irrigation, and drinking water usage. Various approaches are available to control cyanobacterial blooms in which Hydrogen Peroxide (H_2O_2) emerges as a noteworthy environmentally safe oxidizing agent selectively inhibiting the growth of cyanobacteria and leaving no residue. The objective of this study was to assess how different concentrations of H_2O_2 (1, 2, and 4 mg L^{-1}) affect the growth of unicellular *Microcystis aeruginosa* and filamentous *Sphaerospermopsis aphanizomenoides* cultures obtained from inland waters in Türkiye and to compare the effectiveness of H_2O_2 application in monocultures and mixed cultures. For this purpose, the experimental setups were conducted in 96-well microtiter plates with eight replicates, and the growth of cultures during the experiment was monitored by measuring cell optical density at 665 nm (OD_{665}). The results showed that 1 mg L^{-1} H_2O_2 had a significant effect on the growth of monocultures of *Microcystis* with cell densities of 100x10^3 cell mL^{-1} ($p<0.05$) and *Sphaerospermopsis* with 50x103 cell mL^{-1}. The cell growth of Microcystis cultures with higher densities declined at 4 mg L^{-1} H_2O_2 addition, significantly. However, 4 mg L^{-1} H_2O_2 dosage was effective up to 200x10^3 cell mL^{-1} *Sphaerospermopsis*. In the mixtures of these species, 2 mg L^{-1} H_2O_2 application was effective to suppress the tested cell densities in the case of *Microcystis* dominance.

Keywords: Cyanobacteria bloom, hydrogen peroxide, freshwater management

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INTRODUCTION

Harmful cyanobacterial blooms are a globally major concern in freshwater resources (Mantzouki, Lürling, et al. 2018; Schuurmans et al. 2018; Svirčev et al. 2019). Cyanobacteria have unique and highly adaptable eco-physiological properties such as nitrogen fixation, buoyancy regulation, and toxin production (Carey et al. 2012; Ganf & Oliver 1982; Litchman & Klausmeier 2008; Mantzouki et al. 2016). These eco-physiological characteristics contribute to the competitiveness of cyanobacteria, allowing them to adapt rapidly to changing environmental conditions and outcompete other phytoplankton species (Mantzouki et al. 2018). They achieve high population densities within water bodies, and form dense blooms which are visible scums on the water surface and as mats along the edges of water bodies (Svirčev et al. 2019). Studies have shown that prolonged periods of stratification in water bodies, resulting from climate change, can benefit fast-growing and buoyant cyanobacteria (Carey et al. 2012; Mantzouki et al. 2018). As nutrient loadings and stratification increase in water bodies, cyanobacteria with access to well-lit surface waters or nutrient-rich hypolimnetic layers have a competitive advantage and can become dominant (Ganf & Oliver 1982; Mantzouki et al. 2018).

The occurrence of cyanobacterial proliferation

is promoted by eutrophication, increasing CO_2 levels, and global warming (Mantzouki et al. 2018; Paerl & Huisman 2009; Sandrini et al. 2020; Schuurmans et al. 2018). Cyanobacterial blooms can result in various water quality issues, including turbidity, nocturnal oxygen deficiency, undesirable appearance, and unpleasant taste and odor (Bláha et al. 2009; Kang et al. 2022), and the possibility of producing various cyanotoxins which also result in human health risks (Azevedo et al. 2002; Bláha et al. 2009; Köker et al. 2017). Thus, these blooms pose a significant risk to drinking water reservoirs, recreational waters, and overall ecological balance, resulting in economic and ecological damage (Sandrini et al. 2020). The impact of eutrophication and cyanobacterial blooms extends beyond the environment, affecting ecosystem services including the decline of commercial fisheries, aquaculture, and property values, as well as disruptions to recreational activities, irrigation, and drinking water usage (Akcaalan et al. 2014; Albay et al. 2003a; Kang et al. 2022).

Current management methods for algal blooms include using activated carbon, membrane filtration, or UV disinfection (Akcaalan et al. 2006; Westrick et al. 2010). Using oxidants like copper-based substances is one of the most common procedures for combating algal blooms (Albay et al. 2003b). However, due to its detrimental effect on the ecosystem, alternative methods that involve the use of various oxidizing agents are being considered (Huang & Zimba 2020). Among these agents, Hydrogen Peroxide (H_2O_2) stands out as an environmentally friendly oxidant. The major advantages of H_2O_2 are the rapid degradation into water and oxygen through chemical and biological oxidation-reduction reactions which do not persist in the environment and result in no traces, as well as selective suppression of cyanobacteria (Matthijs et al. 2016; Piel et al. 2019).

Studies have shown that the use of low concentrations of H_2O_2 ranging from 2-10 mg L^{-1} effectively induces oxidative stress to suppress cyanobacterial proliferations. As opposed to other phytoplankton groups, such as green algae, phycobilisomes of cyanobacteria are located directly on the outside of the membranes (Bauzá et al. 2014), which are highly sensitive to the oxidizing agents. In contrast, green algae and diatoms experience significantly less impact from this treatment (Sandrini et al. 2020). Although there are many variables to affect H_2O_2 efficiency, H_2O_2 dose, and algal biomass are of great importance (Jia et al. 2014; Liu et al. 2017).

Cyanobacterial blooms are a global phenomenon; *Microcystis* spp., *Anabaena* spp., *Dolichospermum* spp., *Sphaerospermopsis* spp., and *Planktothrix* spp. are the most commonly found cyanobacteria in the world that are known as actual or potential cyanotoxin producers (Svirčev et al. 2019). *Microcystis* spp. and *Sphaerospermopsis* spp. are also widely encountered cyanobacteria in Turkish freshwater resources (Koker et al. 2017). Although the first cyanobacterial bloom records in Türkiye started in the 1980s, monitoring studies started in the 1990s. Since then, cyanobacterial blooms have been a growing concern in Turkish freshwater resources (Akcaalan et al. 2006; Albay et al. 2003a; Albay et al., 2005; Köker et al. 2022). It is observed that most of the cyanobacteria species proliferate from May to September, whereas *Planktothrix rubescens* can be found in high numbers throughout the year in some lakes in Türkiye. Following the results of the aforementioned study and the increase in problems related to cyanobacteria and their toxins, guidelines for cyanobacterial toxins have been prepared for drinking and recreational waters (Anonymous 2019a; 2019b).

The purpose of the current study was to determine the response of various densities of unicellular *Microcystis aeruginosa* and filamentous *Sphaerospermopsis aphanizomenoides* cultures isolated from Turkish inland waters to 1, 2, and 4 mg L^{-1} H_2O_2 concentration and to compare the efficiency of H_2O_2 application in terms of monocultures and their mixtures.

MATERIALS AND METHODS

Cultivation of cyanobacteria

Two different species of cyanobacteria isolated from freshwater sources in Türkiye were used. The filamentous cyanobacterium *Sphaerospermopsis aphanizomenoides* was isolated from Lake Iznik and the unicellular cyanobacterium *Microcystis aeruginosa* was isolated from Küçükçekmece Lagoon.

Media and flasks were sterilized in an autoclave at 121° C for 20 min before starting the experimental sets. For the cultivation of *S. aphanizomenoides,* a medium without nitrogen was used, while *M. aeruginosa* was cultivated in the BG11 medium with nitrogen. The specific contents of the BG11 media are presented in Table 1.

Table 1. Media used for cyanobacteria cultures.

Component	BG-11	$BG\text{-}11_0$
	M. aeruginosa	*S. aphanizomenoides*
$NaNO_3$	1.5 g L^{-1}	-
$CaCl_2$	0.0272 g L^{-1}	0.0272 g L^{-1}
Ferric ammonium citrate	0.012 g L^{-1}	0.012 g L^{-1}
Na_2EDTA	0.001 g L^{-1}	0.001 g L^{-1}
K_2HPO_4	0.04 g L^{-1}	0.04 g L^{-1}
$MgSO_4$	0.0361 g L^{-1}	0.0361 g L^{-1}
Na_2CO_3	0.02 g L^{-1}	0.02 g L^{-1}
Sodium citrate	0.00882 g L^{-1}	0.00882 g L^{-1}
Trace minerals	1 mL stock L^{-1} *	1 mL stock L^{-1} *

*Trace minerals contain of 2.86 g of H_3BO_3, 1.81 g of $MnCl_2.4H_2O$, 0.39 g of $NaMoO_4.2H_2O$, 0.079 g of $CuSO_4.5H_2O$, and 0.494 g of $Co(NO_3)_2.6H_2O$ in 1 L of ultrapure water.

To calculate the initial cell count was in the experiment, the samples from cultures were fixed by Lugol's iodine solution for the enumeration. For the abundance analysis of *S. aphanizomenoides*, the Utermöhl (1958) method was employed using a Zeiss Axiovert inverted microscope, and the cell counting of *M. aeruginosa* was done using a Neubauer chamber (hemocytometer).

The growth of cultures during the experiment was monitored by measuring cell optical density at 665 nm (OD_{665}) by using a micro-

plate reader (Thermo Scientific Multiskan FC, Waltham, MA). Measurements were taken prior to H_2O_2 addition (t=0) and t=1, 2, 4, and 6 h after H_2O_2 addition, and then densities were monitored on a daily basis.

Experimental setup

Two different experimental setups were conducted within the scope of this study. All treatments were performed with eight replicates. First, the sensitivity of various densities of monocultures of *M. aeruginosa* and *S. aphanizomenoides* to 1, 2, and 4 mg L^{-1} H_2O_2 was determined in 96-well microtiter plates. After the first experiment, the second experiment was conducted according to the appropriate dose. Since zooplankton is sensitive to H_2O_2 in a concentration exceeding 2.5 mg L^{-1}, it is not applicable to use H_2O_2 in higher concentrations (Matthijs et al. 2012). Thus, the second experiment was prepared to demonstrate the various densities of *M. aeruginosa* and *S. aphanizomenoides* mixtures to 2 mg L^{-1} H_2O_2 dosage. The control groups were also included in the experiment without H_2O_2 addition (0 mg L^{-1} H_2O_2). The experimental setups were summarized in Table 2. For each species and mixture, 96-well plates were inoculated with 340 µL cultures with different densities and 10 µL of H_2O_2 was added at different concentrations. Well-plates were placed in the Plant Growth Chamber (MLR-351, Sanyo) at 21 °C temperature with an 18:6 hour day cycle (day: night) and the tests were continued for 6 days.

Statistics

All the experimental sets were replicated and the mean values were used. The differences between controls and H_2O_2 added cultures were assessed by the Student's t-test.

RESULTS AND DISCUSSION

Controlling cyanobacterial blooms with H_2O_2 treatment needs to be thoroughly studied, since there are so many physical (light intensity), chemical (concentration of dissolved organic materials), and biological factors (algal density, cyanobacterial biomass) at play (Agustina et al. 2005; Chen et al. 2021; Matthijs et al. 2012). This study was designed to assess the effects of low H_2O_2 dosages on the presence of various densities of *S. aphanizomenoides* and *M. aeruginosa*.

The sensitivity of the monoculture of *S. aphanizomenoides* to H_2O_2 dosages is given in Figure 1. 1 mg L^{-1} H_2O_2 concentration was only effective for the cell density of 50x10^3 cell mL^{-1} ($p<0.05$) and cell growths were not affected by 1 mg L^{-1} H_2O_2 at higher cell densities ($p>0.05$). The growth of *S. aphanizomenoides* was significantly decreased at 2 mg L^{-1} and 4 mg L^{-1} H_2O_2 addition at 100x10^3 cell mL^{-1} ($p<0.05$). For higher cell densities, 4 mg L^{-1} H_2O_2 dosage was effective at 150 x10^3 cell mL^{-1} ($p<0.05$) and 200 x10^3 cell mL^{-1} ($p<0.05$). Higher cell densities were resistant to assessed H_2O_2 dosages ($p>0.05$).

The sensitivity of the monoculture of *M. aeruginosa* to H_2O_2 dosages is depicted in Figure 2. 1 mg L^{-1} H_2O_2 had a significant effect on 100x10^3 cell mL^{-1} and a slightly increase in biomass was observed at 250x10^3 cell mL^{-1} and 500x10^3 cell mL^{-1} ($p<0.05$). Although the addition of 1 mg L^{-1} H_2O_2 did not affect the cell growth of *M. aeruginosa* cultures with higher densities (750 x10^3 cell mL^{-1}, 1000x10^3 cell mL^{-1}, and 1750x10^3 cell mL^{-1}) ($p>0.05$), the cell growth declined significantly at 2 mg L^{-1} and 4 mg L^{-1} H_2O_2 addition ($p<0.05$). The results of this study are in line with a recent study carried out by Kang et al. (2022), in which the authors found that the addition of 1, 3, and 10 mg L^{-1} H_2O_2 decrease *M. aeruginosa* growth. In another study, Wang et al. (2022) investigated the effectiveness of H_2O_2 on *Microcystis* with a cell density of 1.5x10^{10} cells L^{-1}. The authors found that 5.4 mg L^{-1} H_2O_2 dosage caused a decrease in chl-*a* concentration and lower dosages did not reveal any significant effects on *Microcystis* cells.

Table 2. Experimental setup.

Experiment 1	**Monoculture**					
	***S. aphanizomenoides* (S)**		***M. aeruginosa* (M)**			**H_2O_2 addition**
	10^3 x cell mL^{-1}					
	50		100			H_2O_2 was added as 0, 1, 2, and 4 mg L^{-1}.
	100		250			
	150		500			
	200		750			
	250		1.000			
	500		1.750			
Experiment 2	**Mixture**					
	***S. aphanizomenoides* (S)**	**+**	***M. aeruginosa* (M)**	**Total cyanobacteria**		
	10^3 x cell mL^{-1}					
	25	+	100	125	Set I	H_2O_2 was set as 0 and 2 mg L^{-1}
	25	+	150	175	Set II	
	25	+	200	225	Set III	
	25	+	250	275	Set IV	
	50	+	100	150	Set V	
	50	+	250	300	Set VI	

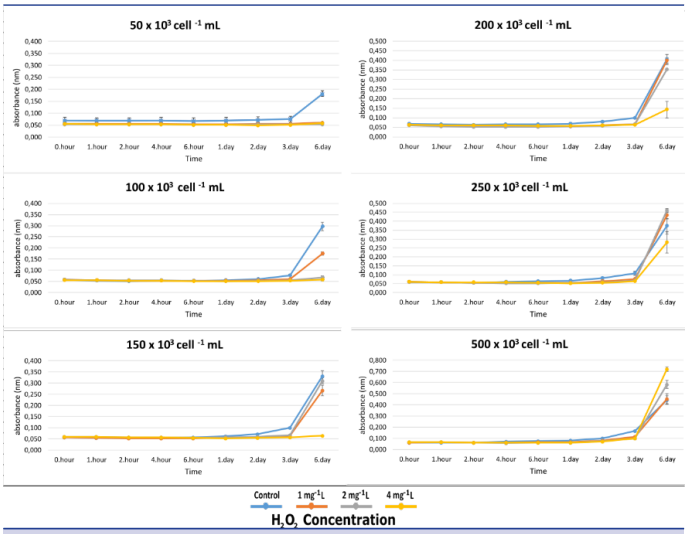

Figure 1. The cell densities of *S. aphanizomenoides* after the addition of 1, 2, and 4 mg L^{-1} H_2O_2 addition.

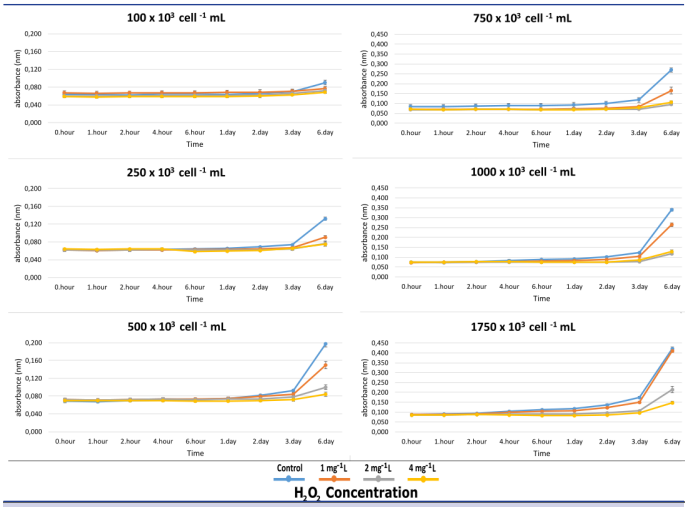

Figure 2. The cell densities of *M. aeruginosa* after the addition of 1, 2, and 4 mg L^{-1} H_2O_2 addition.

The results revealed that *M. aeruginosa* cultures were more sensitive to H_2O_2 compared to *S. aphanizomenoides* culture. The potential reason for the relatively higher resistance to H_2O_2 in *Sphaerospermopsis* compared to *Microcystis* species might be explained in this way: *M. aeruginosa* has lost its colony structure in the culture conditions and has been exposed to H_2O_2 more intensely because it is in the form of a single cell. Since *Sphaerospermopsis* has a filamentous structure, it has higher resistance to H_2O_2 application. A possible explanation for the biomass decrease might be related to the loss of membrane integrity and cell lysis of *M. aeruginosa*. Studies showed that higher than 2 mg L^{-1} H_2O_2 concentrations effectively killed cyanobacteria, but can cause the substantial release of toxins into the water (Lürling et al. 2014). Furthermore, since zooplankton is sensitive to H_2O_2 in a concentration exceeding 2.5 mg L^{-1}, it is not applicable to use H_2O_2 in higher concentrations (Matthijs et al. 2012). Thus, in the following experiment, we decided to apply a dosage of 2 mg L^{-1} H_2O_2. Since freshwater algal blooms can be predominantly composed of a single cyanobacterial genus or multiple genera, it is important to evaluate the effects of H_2O_2 application to different species' interspecific variations (Yang et al. 2018). Thus, in Experiment 2, we assessed a mixture of *S. aphanizomenoides* and *M. aeruginosa* cultures with different cell densities and tried to see the interspecific effect of 2 mg L^{-1} H_2O_2. Figure 3 shows the cell densities of *S. aphanizomenoides* and *M. aeruginosa* after the addition of 2 mg L^{-1} H_2O_2 dosage. H_2O_2 addition had significant effects on cell densities ($p<0.05$). The increase in each group did not reveal resistance to H_2O_2. When the mixture of these two species was compared, effective suppression was observed at 125, 175, and 225 x10^3 cell mL^{-1}. 2 mg L^{-1} H_2O_2 application is effective on the coexistence of *S. aphanizomenoides* and *M. aeruginosa* in the case of *M. aeruginosa* dominance.

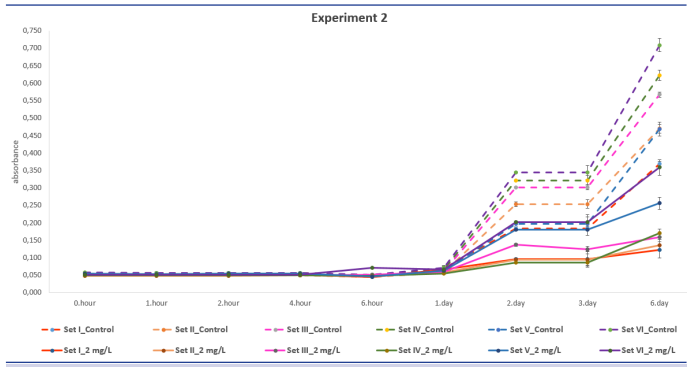

Figure 3. The cell densities of *M. aeruginosa* and *S. aphanizomenoides* mixtures after the addition of 2 mg L^{-1} H_2O_2.

Taken together, we can recommend that the application of H_2O_2 to control cyanobacterial blooms should be carried out in the early stage of a bloom, when algal density is relatively low (Chen et al. 2021; Fan et al. 2013; Liu et al. 2017). It should be noted that different cyanobacteria species exhibit varying responses to the application of H_2O_2, which can be attributed to their distinct survival strategies (Lusty & Gobler 2020). Thus, the application of H_2O_2 should be specifically optimized.

CONCLUSION

The main goal of the current study was to assess the response of various densities of two cyanobacteria species that were commonly found in Turkish freshwater, *M. aeruginosa* and *S. aphanizomenoides*, to 1, 2, and 4 mg L^{-1} H_2O_2 addition and evaluate the efficiency of H_2O_2 application in terms cell growth of monocultures and their mixtures. This study has found that H_2O_2 treatment is much more effective for generally low cell densities and *M. aeruginosa* cultures were more sensitive to H_2O_2 application compared to *S. aphanizomenoides* culture. More information on the application of H_2O_2 in larger-scale experiments would help to establish a greater degree of accuracy on this matter. A further study could assess the effects of H_2O_2 addition in the lake using mesocosm experiments.

Conflict of Interest: The author has no conflicts of interest to declare.

Ethics committee approval: Ethics committee approval is not required.

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