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EVALUATING THE IMPACT OF HYDROGEN PEROXIDE ON THE PHYTOPLANKTON COMMUNITY: A BENCH SCALE STUDY

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MARIA APARECIDA MELO ROCHA

EVALUATING THE IMPACT OF HYDROGEN PEROXIDE ON THE PHYTOPLANKTON COMMUNITY: A BENCH SCALE STUDY

Master thesis presented to the Graduate program in Civil Engineering at Federal University of Ceará, as a partial requirement to obtain the title of Master in Civil Engineering concentration area: Environmental Sanitation.

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To God.

To my parents, Cícera and Flávio.

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RESUMO

Devido à capacidade de produção de metabólitos secundários tóxicos (cianotoxinas) e não tóxicos (compostos que fornecem odor e sabor à água), a presença de cianobactérias, em águas destinadas ao abastecimento público, deve ser controlada. Entretanto, a remoção de desses microrganismos nas estações de tratamento de água de ciclo completo é um grande desafio, surgindo assim a necessidade de técnicas alternativas de tratamento. Uma dessas técnicas é a pré-oxidação *in-situ* utilizando o peróxido de hidrogênio (H_2O_2). Este composto, além de atuar seletivamente sobre as cianobactérias, dissocia-se em água e oxigênio, não deixando subprodutos tóxicos na água. Apesar de existirem diversos estudos avaliando a atuação desse oxidante no fitoplâncton, muitos avaliaram sua atuação somente em espécies cultivadas em laboratório, não incluindo sua atuação em diferentes espécies do fitoplâncton de um reservatório tropical. Além disso, ainda existe uma lacuna quando se trata de estudos em escala de bancada considerando o impacto do peróxido de hidrogênio no recrescimento dos organismos presentes no reservatório. Portanto, o objetivo deste estudo é avaliar o impacto causado por diferentes concentrações do H_2O_2 no fitoplâncton de um reservatório tropical, além de verificar se a metodologia utilizada para avaliar seu impacto no recrescimento dos organismos é adequada e eficiente. Analisando a variação da concentração de clorofila dos diferentes organismos, observou-se que o H_2O_2 foi eficiente na supressão das cianobactérias por até sete dias após sua aplicação. Por meio da densidade celular, observou-se que o H_2O_2 diminuiu significativamente ($p<0.05$) a densidade celular total somente para a concentração de 2 $mg.L^{-1}$ e não para as concentrações de 5 e 10 $mg.L^{-1}$. Vale ressaltar que *Planktothrix sp*. que foi bastante resistente a esse oxidante, sua densidade permanecendo da ordem de 10^5 $cel.mL^{-1}$ após a aplicação do H_2O_2. Entretanto é necessária uma avaliação mais completa da estrutura dessa espécie de cianobactéria e de seus possíveis mecanismos de defesa. Quanto à metodologia de avaliação do recrescimento dos organismos, para a duração de 30 dias, faz-se necessária a utilização de compartimentos de maior capacidade e de uma luz mais intensa, provavelmente de cerca 40 $\mu mol.m^{-2}$ s^{-1}. Pode-se concluir, que a contagem de densidade de células do fitoplâncton não foi apropriada para avaliar o efeito do H_2O_2 logo após a sua aplicação, já que não notou-se mudanças significativas na densidade celular do fitoplâncton devido às diferentes doses de H_2O_2. Finalmente, para avaliar eficientemente o recrescimento dos organismos em escala de bancada, necessita-se

realizar ajustes na metodologia para proporcionar um ambiente ideal de crescimento para os organismos. Com o desenvolvimento dessa metodologia, pode-se avaliar de forma eficaz se o H_2O_2 atua no controle de cianobactérias a longo prazo, além de possibilitar a aplicação do H_2O_2 em escala real, por meio da pre-oxidação *in-situ*.

Palavras-chave: Eutrofização. Cianobactérias. Peróxido de hidrogênio. Pré-tratamento in-situ. Recrescimento do fitoplâncton.

ABSTRACT

Due to the ability to produce toxic (cyanotoxins) and non-toxic (metabolites that provide odor and flavor to water) secondary metabolites, the presence of cyanobacteria in waters destined for public supply must be controlled. However, the removal of these microorganisms in water treatment plants is a significant challenge, thus requiring alternative treatment techniques. One of these techniques is *in-situ* pre-oxidation using hydrogen peroxide (H_2O_2). In addition to acting selectively on cyanobacteria, this compound dissociates into water and oxygen, leaving no toxic by-products in the water. Although several studies have evaluated the effect of this oxidant on the phytoplankton, many have evaluated its performance only on species grown in the laboratory, not evaluating the effect on different species of phytoplankton in a tropical reservoir. Besides, there is still a knowledge gap when it comes to bench-scale studies considering the long-term impact of hydrogen peroxide on the regrowth of organisms from freshwater environments. Therefore, this thesis aims to evaluate the impact caused by different concentrations of H_2O_2 on the phytoplankton of a tropical reservoir and to verify whether the methodology used to evaluate the regrowth of the organisms is suitable and efficient. It was observed that H_2O_2 was efficient in suppressing cyanobacteria for up to seven days after its application, after analyzing the variation in the chlorophyll concentration of different phytoplankton groups. It was observed by the values of cell density counts that H_2O_2 caused a significant decrease ($p < 0.05$) in the total cell density only for the concentration of 2 $mg.L^{-1}$ and not for the concentrations of 5 and 10 $mg.L^{-1}$. It is noteworthy that *Planktothrix sp*., which was highly resistant to this oxidant since its cell density remained in the order of 10^5 $cells.mL^{-1}$ after the application of H_2O_2. However, further studies on the structure of *Planktothrix sp.* and its possible defense features are needed. As for the methodology that evaluates the organisms regrowth, for 30 days, it is necessary to use jars of higher capacity and more intense light, probably around 40 $\mu mol.m^{-2}$ s^{-1}. The experiments showed that the phytoplankton cell density count was not appropriated for a fast post-treatment analysis of the impact of H_2O_2, since this method did not detect significant changes in the cell density values due to different doses of H_2O_2. Finally, to efficiently evaluate the growth of organisms on a bench scale, the methodology needs adjustments to provide an ideal environment for the organisms to grow. This methodology can evaluate whether H_2O_2

acts in the long term control of cyanobacteria, in addition to enabling the application of H_2O_2 on a full scale, through *in-situ* pre-oxidation.

Keywords: Eutrophication. Cyanobacteria. Hydrogen Peroxide. *In-situ* pre-oxidation. Phytoplankton regrowth.

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1 INTRODUCTION

Eutrophication caused by accelerated changes in land use and by the increase in the nutrient loads due to human activities is increasingly becoming an environmental problem (YAN *et al*., 2017). The increase in primary production in lakes and rivers is mainly controlled by the phosphorus and nitrogen concentrations in water (HAAKONSSON *et al*., 2017) and therefore, monitoring the nutrient load is the key to manage and control eutrophication (PAERL, 2009; HAAKONSSON *et al*., 2017).

Eutrophication increases the chance of harmful algal blooms (HABs) (MINASYAN *et al*., 2018; CAO *et al*., 2016). Furthermore, HABs are more likely to occur due to increases in temperature due to climate changes (CAREY *et al*., 2012). HABs can have negative impacts on benthic organisms and can be harmful to animals and plants that use this contaminated water (SNEED *et al*., 2017; ZI *et al*., 2018).

Cyanobacteria, a major organism existing in HAB, are cosmopolitan microorganisms that can be found on all continents except in Antarctica (ZURAWELL *et al.*, 2005). Cyanobacteria are important primary producers for the aquatic ecosystem (CAO *et al*., 2016) since they are able to photosynthesize oxygen and can fix atmospheric nitrogen, such as *Dolichospermum sp.* (HAQUE *et al*., 2017). The increasingly more intense and constant presence of cyanobacteria in water bodies is a growing concern due to their capacity to produce and release cyanotoxins and other metabolites into the environment, such as taste and odor compounds (QUIN *et al*., 2015; MINASYAN *et al*., 2018). Secondary metabolites or cyanotoxins can be classified as hepatotoxins, neurotoxins and dermatoxins (CARMICHAEL, 2001).

The main problems caused by the non-toxic secondary metabolites are the suppression of other phytoplankton, the interruption of the germination and growth of macrophytes, and the impact on the genetics of zooplankton (SUKENIK, QUESADA, SALMASO, 2015; ZI *et al*., 2018). Toxic metabolites or cyanotoxins can bioaccumulate in fish disturbing the balance of the ecosystem and increasing the risks of human intoxication (SUKENIK; QUESADA; SALMASO, 2015).

Conventional water treatment (coagulation, flocculation, sedimentation and filtration) is widely used in the world but, due to the low density, negative charge, and other morphological properties of cyanobacteria, those processes may not be ideal for the efficient removal of cyanobacteria and their metabolites (HE *et al*., 2016). Another problem with conventional treatment is that the addition of chemicals necessary to the

water treatment may damage the cells and lead to their lysis, inducing the release of intracellular metabolites (QIAN *et al*., 2014). Furthermore, conventional treatment does not remove dissolved metabolites already in the water or released during the process, which makes the use of additional procedures such as the addition of powdered activated carbon necessary (ZAMYADI *et al*., 2015; HE *et al*., 2016).

Oxidants are an alternative to complement conventional water treatment and increase the efficiency of cyanobacteria and metabolites removal. Many oxidants such as ozone, chlorine, potassium permanganate, and chlorine dioxide have been used to oxide cyanobacteria and their intracellular compounds, however, their susceptibility to each oxidant depends on the physiology of the cells, oxidation conditions, and type of metabolite or toxin (FAN *et al.*, 2014).

The efficacy of chlorine as an oxidant agent of cyanobacteria and its toxins depends on the species and their sensibility to this agent, besides the kind of toxin produced. This oxidant has shown to be efficient on the oxidation of cylindrospermopsin but not on the removal of anatoxin (ZAMYADI et al., 2013; HE et al., 2016), and depending on the phase of the cells and the initial dose applied, the chlorine showed being efficient on the removal of microcystin (FAN et al., 2016).

The chlorine dioxide does not have enough information on its efficiency, probably because it is a less strong oxidant when compared to the other existent ones (HE et al., 2016). LIN et al. (2018) evaluated the efficiency of this compound associated to sodium hypochlorite on the removal of cells from Cylindrospermopsis raciborskii and Microcystis aeruginosa, concluding that they were effective on the cell removal but they caused cell lysis, which released disinfection byproducts such as allogenic organic matter.

As for the potassium permanganate, it can inactivate cyanobacteria, changing mainly the cell integrity with doses of 10 mg.L -1 . However, it showed being less efficient than the treatment with ozone and chlorine, and it has a highly apparent color therefore limiting its application on water treatment (HE et al., 2016). Also, this product can cause cell lysis, increasing the concentration of extracellular toxin (FAN et al., 2013).

Ozone is efficient on the removal of cyanobacteria cells and their toxins, however, just as the other mentioned treatments this is variable, also depending on the toxin and the dose applied (HE et al., 2016).

Due to the disadvantages on the use of those oxidants, it is apparent that

further study of alternative oxidation techniques that could efficiently be applied to water treatment is necessary. One technique is the *in-situ* pre-oxidation using hydrogen peroxide (H_2O_2), which needs to be performed in-situ due to its slower kinetics (CAPELO-NETO and NEYCOMBE, 2017).

H_2O_2 is a reactive species of oxygen that is produced in natural water by the photolysis of organic matter under UV radiation, or produced biologically as a byproduct of photosynthesis (COOPER and ZIKA, 1983). It is also industrially produced to be applied for disinfection and water treatment (BAUZÁ *et al*., 2014). The major advantage of this oxidant is that it degrades into water and oxygen in a few days, not forming by-products or leaving chemical residues in the environment (MATTHIJS *et al*., 2012). However, few studies have been undertaken on understanding the action of H_2O_2 itself on the inactivation of cyanobacteria, therefore, to understand the impact of H_2O_2 further investigation is needed (BARRINGTON and GHADOUANI, 2008; HE *et al*., 2016).

Fan *et al*. (2014) performed a laboratory study to assess the impact of copper sulphate, chlorine, potassium permanganate, hydrogen peroxide and ozone on a strain of *Microcystis aeruginosa*. Although all those agents promoted a loss of cyanobacterial membrane integrity, only H_2O_2 and chlorine did not cause an associated increase in dissolved toxins. However, chlorine application is limited to the cyanobacteria species and the growing phase of the cells (**Error! Reference source not found.**).

In the study of Barrington, Reichwaldt and Ghadouani (2013) the authors used H_2O_2 to remove cyanobacteria and microcystin from waste stabilization ponds. Their experiment had three phases: microcosms, mesocosms and full-scale in waste stabilization ponds. They obtained positive effects with H_2O_2 until three weeks after its application, after this period the cell density of the cyanobacteria returned to the initial values.

Matthijs *et al*. (2012) used a dose of 2 $mg.L^{-1}$ of H_2O_2 to control an intense bloom of *Planktothrix agardhii*. This compound decreased the population of cyanobacteria and the concentration of microcystin in the lake significantly, which remained low for the next seven weeks. The authors concluded that low concentrations of H_2O_2 can be used for selective suppression of cyanobacteria, not significantly harming eukaryotic phytoplankton species, zooplankton, and macrofauna.

The forms of preventing future cyanobacteria blooms mostly are based on

studies on temperate lakes, which limits the understanding on ecosystems with a higher temperature and a higher tendency to have eutrophic lakes (HAAKONSSON *et al.*, 2017). Furthermore, there are few studies that include the effect of H_2O_2 on different specific species, and which is more sensitive to this compound. Therefore, there is still a knowledge gap on the behavior of H_2O_2 application on the phytoplankton species that exist in the freshwater environment of tropical semiarid reservoirs. Furthermore, by filling this knowledge gap it is possible to evaluate an *in-situ* technique of water treatment so the water treatment plants are not overloaded, thus removing cyanobacteria efficiently.

This present master's thesis is organized on the following five parts:

1- General introduction;
2- General main and specific objectives;
3- Chapter I which presents the first paper entitled "Effects of Hydrogen Peroxide on the phytoplankton of a reservoir in the tropical semi-arid region of Brazil ";
4- Chapter II which presents the second paper entitled "Developing and evaluating the efficiency of the method to analyze the regrowth of phytoplankton after the application of H2O2 on laboratory cyanobacteria strains, and on the water from Gaviao reservoir";
5- A general conclusion, and;
6- The references of the General introduction

Each paper is a complete manuscript ready to be submitted to an international peer-reviewed journal.

2 AIM AND OBJECTIVES

2.1 Main aim

To analyze the impact of H_2O_2 on the phytoplankton community and water quality of Gavião reservoir

2.2 Specific objectives

- To evaluate the effect of different concentrations of H_2O_2 on the chlorophyll and phycocyanin concentration of the phytoplankton

community;

- To evaluate the effect of different concentrations of H_2O_2 on the cell density of the most dominant phytoplankton organisms in Gavião water at genus level;
- To develop a methodology to evaluate the long-term effect of the application of H_2O_2 on pigment concentrations and cell regrowth of environmental samples;
- To evaluate the cell regrowth of laboratory strains (*Microcystis aeruginosa* and *Dolichospermum circinale*) after the application of different doses of H_2O_2.

CHAPTER I

Effects of Hydrogen Peroxide on the phytoplankton community of a reservoir in the tropical semi-arid region of Brazil

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Effects of Hydrogen Peroxide on the phytoplankton community of a reservoir in the tropical semi-arid region of Brazil

Authors: Maria Aparecida Melo Rocha; Allan Clemente de Souza; Hilder Assunção; Jessica da Silva Melo; José Capelo Neto

Abstract

Due to anthropogenic activities, the balance in the nutrient loads is altered, which can cause harmful cyanobacteria blooms. This problem is intensified in tropical regions, where higher temperatures increase the HABs intensity and duration. Besides producing toxic (cyanotoxins) and non-toxic (taste and odor compounds) secondary metabolites, cyanobacteria are difficult to be removed by conventional water treatment processes. Hence the increasing need for complementary treatments, one of which is the pre-oxidation with hydrogen peroxide (H_2O_2). Even though H_2O_2 impact on cyanobacteria has been widely studied, there are few studies on how this compound affects different phytoplankton organisms on a genera level and which techniques are more effective to evaluate the impact of H_2O_2. Therefore, this paper aims to use different approaches to evaluate how H_2O_2 impacts on the phytoplankton (chlorophyll concentration and cell density changes), besides assessing the impact that this oxidant causes on different genera. Cyanobacterial chlorophyll decreased from 28 $\mu g.L^{-1}$ to undetectable four days after the application of H_2O_2 with a dose of 10 $mg.L^{-1}$, remaining undetectable until seven days after the dose application, not causing significant changes ($p > 0.05$) in the chlorophyll concentrations from green algae and diatoms. However, when analyzing the cell density, there was not a significant decrease due to the H_2O_2 application, except for the lowest dose (2 $mg.L^{-1}$). The cyanobacterium *Planktothrix sp.* appeared the most resistant in the reservoir studied, with cell densities remaining 10^5 $cells.mL^{-1}$ for all doses. Using different mathematical models and principal component analysis (PCA), it was demonstrated that, using cell density, the H_2O_2 caused significant changes, however, all H_2O_2 doses presented similar effects. Therefore, it is possible to conclude that H_2O_2 has significant impcat on cyanobacteria chlorophyll, but not green algae and diatoms. For the cell density, the lowest concentration used (2 $mg.L^{-1}$) presented the highest impact on cyanobacteria cell density. However, the PCA showed that, even though they caused significant impacts when compared to the control samples, the different doses of H_2O_2 had similar effect on the phytoplankton cell density. This

indicates that cell density may not be effective to evaluate the impact of H_2O_2 after a 48h contact time. Finally, it is necessary further investigation on the long term impact of H_2O_2 on the phytoplankton, so to avoid species selection that may bloom out of control.

Key words: Eutrophication, Selective suppression, Freshwater environments; Hydrogen peroxide.

3 INTRODUCTION

Cyanobacteria are one of the most abundant oxygenic phototrophs in salt and fresh waters, with some taxa being able to fix nitrogen under aerobic conditions, thus having a major role in the global carbon and nitrogen cycles (AGOSTONI *et al.*, 2016). In the case of lakes or reservoirs, an increase in nutrient load, mostly due to anthropogenic activities, can cause these organisms to bloom, increasing water turbidity, modifying the fish habitat, and harming other aquatic organisms (ZI *et al*., 2018). This problem is intensified in tropical regions since high temperatures can increase cyanobacterial blooms intensity and duration in eutrophic waters (HAAKONSSON *et al*., 2017). It is possible to find cyanobacteria in around 108 countries (ZI *et al*., 2018) since they are excellent competitors under low light condition (SCHEFFER *et al*., 1997) due to their large light-harvesting antenna that gives them an advantage over other phytoplankton in the environment (KIRST, FORMIGHIERI and MELIS, 2014).

Besides causing major difficulties in water treatment plants (WTPs), cyanobacteria blooms can produce high concentrations of toxins such as microcystin, saxitoxin, and cylindrospermopsin and non-toxic metabolites such as the taste and odor compounds 2methylisoborneol (MIB), and geosmin (WERT; DONG and ROSARIO-ORTIZ, 2013; WERT, 2014). Both, non-toxic and toxic metabolites can decrease consumers water safety perception and have negative effects on ecosystems such as suppression of other phytoplankton growth and damage to the genetic structure of zooplankton (SUKENIK; QUESADA; SALMASO, 2015; ZI *et al*., 2018).

Cyanobacteria are capable of causing more turbidity per unit of phosphorus than eukaryotic phytoplankton, promoting lower light availability in the water column (SCHEFFER *et al*., 1997). Therefore, nutrient reduction is generally unsuccessful as a means of controlling cyanobacterial blooms in eutrophic lakes (SAS, 1989; SCHEFFER *et al*., 1993). Even though conventional water treatment (coagulation, flocculation, sedimentation, and filtration) have been recognized as an efficient technology to remove suspended and colloidal particles, some recent studies have observed cells may break through, accumulate, or lyse in conventional Water Treatment Plants (WTPs) during cyanobacterial blooms, liberating intracellular metabolites, decreasing drinking water safety, and increasing the load of solids into WTPs (FAN *et al*., 2014; PESTANA *et al*., 2019). Hence, there is an increasing need for complementary treatments to help

conventional treatment to remove cyanobacterial cells and their metabolites efficiently in order to increase water safety and decrease the solid loads reaching WTPs.

Pre-oxidation is sometimes applied to help conventional WTPs to reach their full potential but its use is limited because most oxidants can generate harmful by-products or are effective against some cyanobacteria metabolites (ZAMYADI *et al*., 2013; FAN *et al*., 2016; HE *et al*., 2016). In that context, hydrogen peroxide (H_2O_2), an alternative oxidant and algicide (Barroin and Feuillade, 1986), has been receiving a lot of attention recently since it degrades into water and oxygen during the redox reaction and does not leave any chemical residues or disinfection byproduct in the environment (MATTHIJS *et al*., 2012). In addition, natural UV radiation increases the rate of H_2O_2 reaction, increasing the efficiency of natural organic matter oxidation (BARRINGTON, REICHWALDT; GHADOUANI, 2013).

Few studies have been performed to analyze the selective action of H_2O_2 and have indicated that cyanobacteria are more sensitive to this compound than the other phytoplankton due to their cellular structure and composition (MATTHIJS *et al*., 2012; SINHA; EGGLETON and LOCHMANN, 2018; YANG *et al*., 2018). Aguilera; Echenique and Giannuzzi (2014) studied the effect of H_2O_2 application on a hypereutrophic lake in Argentina by analyzing the changes in chlorophyll and cell density. The authors, however, did not observe a significant decrease in, cyanobacteria cell density after the analyzed period, but they observed cells of *Planktothrix sp.* in different stages of degeneration caused by the H_2O_2 application. On the other hand, Yang *et al*. (2018) observed a significant decrease in cyanobacteria densities in the hyper-productive catfish aquaculture ponds using doses of H_2O_2 higher than 1.3 $mg.L^{-1}$. Their study was applied to cyanobacteria strains cultivated in labs and to an aquaculture pond which differs significantly from reservoir water used for water supply. Matthijs *et al*. (2012) performed experiments using *Planktothrix agardhii* isolated from lake Koetshuis, the Netherlands, and cultivated in the laboratory. They performed bench microcosms experiments and, ultimately, applied H_2O_2 to the entire lake obtaining a significant decrease of cyanobacteria cell density of nearly one order of magnitude. It is important to quote that for the experiment in the lake, they did not evaluated the impact of H_2O_2 on the different phytoplankton genera, analyzing only at community level (cyanobacteria, green algae, diatom).

Most studies used laboratory strains to evaluate the performance of H_2O_2 (HO *et al*., 2010; CHANG; HUO; LIN, 2018; WANG *et al*., 2019). These cultured

strains can present different adaptations to the new environment when compared to the cyanobacteria living in the natural environments, the presence of cells that are able to fix nitrogen (Heterocyst), for instance (CAREY *et al.*, 2012), and thus their response to H_2O_2 may be misleading. Another knowledge gap observed in the recent literature is that the studies performed with water from natural lakes normaly do not focus on evaluating and comparing the action of different H_2O_2 concentrations on the different genera of the phytoplankton community, including green algae and diatoms. In addition, most reported studies on the use of H_2O_2 to cntrol cyanobacteria in lakes were performed in temperate climate regions (MATTHIJS *et al.*, 2012; WEENINK *et al.*, 2015; YANG *et al.*, 2018). High temperatures favor the formation of dense cyanobacteria blooms, especially in lentic systems (Munoz *et al.*, 2019) and therefore, it is necessary to evaluate the effect of different levels of concentration of H_2O_2 in tropical reservoir water where cyanobacteria are an urgent problem.

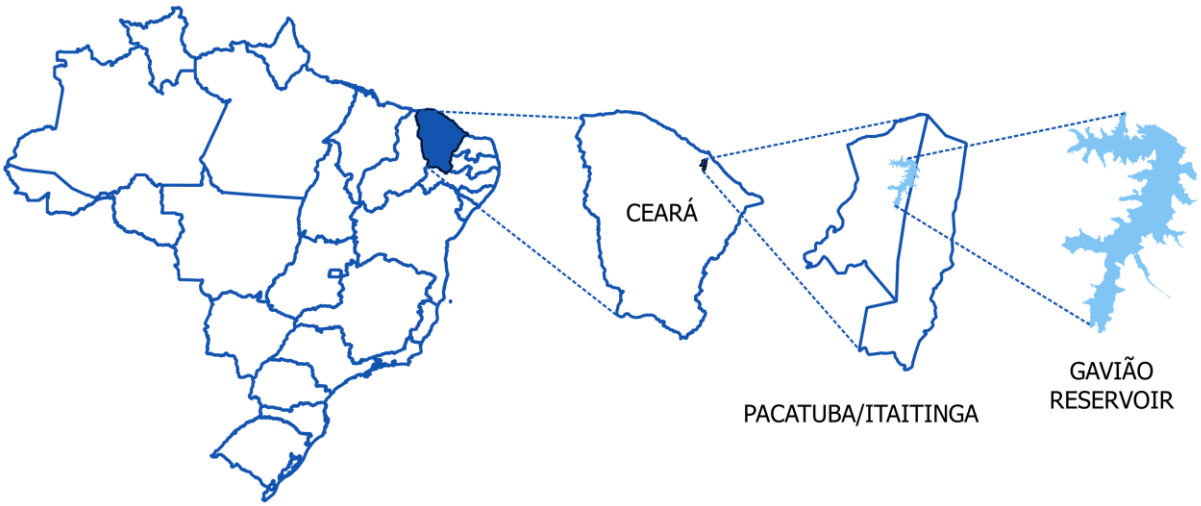
Therefore, this paper aims to evaluate the impacts of different H_2O_2 concentrations on pigments and cell density of different phytoplankton present in the water of a reservoir located in a semi-arid tropical region and used exclusively for human supply in order to access the viability of the use of H_2O_2 as *in-situ* control of cyanobacteria biomass.

4 MATERIALS AND METHODS

4.1 Study Area

The raw water used in this experiment was collected from Gaviao Reservoir (Figure 1) in the Northeast of Ceara (Brazil) at a 90 cm depth and next to the WTP intake. This reservoir has a storage capacity of 33.30 hm^3, an area of 5.9 km^2 (COGERH, 2018), and was chosen because it supplies water to more than 4 million inhabitants in the metropolitan region of Fortaleza (IBGE, 2018).

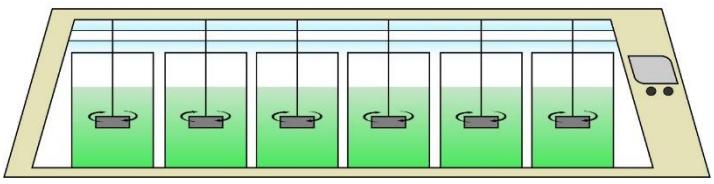
Figure 1 – Gaviao Reservoir



4.2 Design at bench-scale study

Two jar-testers with six containers each (1.5 L) and a stirring mechanism was used to perform the experiments (Figure 2). A propeller rotation of 100 rpm was maintained in order to provide homogeneous suspension. The H_2O_2 concentrations used were 0 (control), 2 $mg.L^{-1}$, 5 $mg.L^{-1}$, and 10 $mg.L^{-1}$. All the experimental procedures were performed in triplicates.

Figure 2 - Graphical representation of the jar-test equipment used to perform the experiment



The first set of the experiment focused on analyzing the effects of H_2O_2 (5 $mg.L^{-1}$, 10 $mg.l^{-1}$) on the chlorophyll concentration of the Phytoplankton community; cyanobacteria, green algae, and diatoms. The second set focused on analyzing the

effects of H_2O_2 (2 mg.L^{-1}, 5 mg.L^{-1}, 10 mg.L^{-1}) on phytoplankton cell density, this set of experiment was performed one month after the first set. Physico-chemical parameters (pH, temperature, conductivity, total and dissolved organic carbon) of water and phycocyanin concentration were also analyzed on both sets of experiments. Samples were collected when the H_2O_2 was not detected in the water, for both sets of experiments. To analyze the impact of the H_2O_2, the samples where the oxidant was applied were compared to the control samples from that same time. The control samples are the jars with the same raw water but with no H_2O_2, which were run simultaneously with each set of experiments in order to consider the effect of the laboratory environment on the water and phytoplankton. For both sets of the experiment, a photosynthetic photon flux density (PPFD) with an average of 10 µmol.$m^{-2}s^{-1}$ in a light/dark cycle of 12h:12h was used. A digital lux meter (YF – 1065F, Taiwan) was used to measure the light intensity and make sure it was nearly constant during the experiments.

4.3 Hydrogen peroxide quantification

Since the H_2O_2 is a highly reactive substance, the concentration of the stock solution had to be determined prior to the start of each set of experiments in order to dose the established concentration in each jar. For that propose, the iodometric method (SKELLON and WILLS, 2018) was used. To monitor the extinction of the H_2O_2 a semi-quantitative and simpler method (Quantofix® Peroxide 25) was used.

4.4 Chlorophyll concentration analysis

Chlorophyll is an essential pigment for the photosynthesis of the phytoplankton community, and it can be related directly to the biomass present in the water. In order to quantify chlorophyll and learn how H_2O_2 affected the phytoplankton community, the PHYTO-PAM II Phytoplankton Analyzer (Walz, Germany) was used. This equipment excites chlorophyll at four different wavelengths making it possible to distinguish phytoplankton with different types of light-harvesting pigment antenna (WALZ, 2003) and making it possible to obtain chlorophyll concentration values (µg.L^{-1}) from cyanobacteria, green algae, and diatoms.

4.5 Phycocyanin quantification

Cyanobacteria contain a high amount of phycobiliproteins, which encompass different types of phycocyanin (PATEL *et al*., 2018). These pigment transfers light energy to chlorophyll during photosynthesis, playing a crucial role in the survival of cyanobacteria (AGOSTONI *et al*., 2016). The method used to extract and quantify the Phycocyanin concentration on each sample was adapted from Bennett and Bogorad (1973). The adaptation consisted on macerating the filters so as to increase the contact of the material retained in the filter with the reagents used to extract the phycocyanin.

4.6 Phytoplankton cell density

The first step to evaluate the effect of H_2O_2 on cell density of different phytoplankton organisms was to analyze which genus showed the highest concentration in Gavião water, and therefore, would be the most important to be studied. Then, the cell counts were performed in duplicates for each jar using a Sedgewick-rafter chamber and optical microscope (Axio Vert.A1, Carl Zeiss, Germany), calibrated according to Apha *et al*. (2005) and Cetesb (1978) methods. Approximately 1 ml of the sample with lugol's iodine was transferred with a pasteur pipette to the cell count chamber, and cell density analysis was performed according to Apha *et al*. (2005).

The genus chosen to be considered were also historically dominant from 2016 to 2019 in Gavião water (Table 1) (Barros *et al*., 2017). In addition, the selected organisms followed a visual criterium where the most abundant ones in the water were considered, so as to facilitate the comparison with the samples after the application of H_2O_2. It is important to mention that *Chloro 1* was the designation given to a green alga which genus could not be identified but was highly present in some experiments and, therefore, was counted.

Table 1 – Organisms of the phytoplankton community in Gaviao reservoir that were considered for the cell counts.

GENUS	PHYTOPLANKTON GROUP
Planktothrix sp.	Cyanobacteria
Merismopedia sp.	Cyanobacteria
Pseudanabaena sp.	Cyanobacteria
Cylindrospermopsis sp.	Cyanobacteria
Crucigenia sp.	Green algae

GENUS	PHYTOPLANKTON GROUP
Planktothrix sp.	Cyanobacteria
Merismopedia sp.	Cyanobacteria
Pseudanabaena sp.	Cyanobacteria
Cylindrospermopsis sp.	Cyanobacteria
Crucigenia sp.	Green algae
Cyclotella sp.	Diatom
Coelastrum microsporum	Green algae
Fragilaria sp.	Diatom
Navicula sp.	Diatom
Scenedesmus sp.	Green algae
Monoraphidium contortum	Green algae
Tetraedron minimum	Green algae
Aulacoseira granulata	Diatom
Chloro 1	Green algae

4.7 Determination of regrowth potential

According to Matthijs *et al*. (2012), it is important to evaluate how long it takes for the cyanobacteria to regrow after the application of H_2O_2. Since there is no standard method in the literature, we proposed and implemented a test to evaluate this regrowth potential. Raw water from Gavião reservoir was filtered through a glass fiber filter (0.7µm, Millipore, Ireland) and autoclaved to be used as part of the matrix for the regrowth experiment. The matrix was composed of 80% of processed Gavião water and 20% of the ASM-1 medium (GORHAM *et al.*, 1964). This matrix was inserted to minimize the stress in the organisms and to minimize the effect of the species selection.

After the natural consumption of H_2O_2 in the first set of experiments, samples were taken from each jar to evaluate the impact caused by the application of this compound. Then, to promote a viable environment for the cells to recover, the same volume taken to perform the physico-chemical, pigments and cell density analyses was replenished with the proposed matrix. Seven days after the application of H_2O_2, all the

analyses were performed again to evaluate the regrowth and the changes in physic-chemical parameters.

4.8 Physico-chemical analyses

Physico-chemical analyses were performed using the methods and equipment presented in Table 2. The organic matter UV 254 nm can be utilized to determine the organic matter content (EDZWAKD *et al*., 1985). Total organic carbon (TOC) is a more precise method to indicate the concentration of organic matter that is present in the water and it can be used as an indicator of the water quality. For the dissolved organic carbon (DOC), the samples were prepared using the same method as the TOC (Table 2) but they were first filtered in a glass fiber filter (0.7 µm).

Table 2 - Physico-chemical analyses performed in the study

PARAMETER	METHOD	EQUIPMENT	UNIT
pH	APHA 4500 H-B, (2005)	Probe meter YSI Model 60, USA.	-
Dissolved oxygen	APHA 4500 O-G, (2005	Probe meter YSI model 55, USA.	$mg.L^{-1}$
Conductivity	APHA 2510-A, (2005)	Probe meter ORION 105 A+, USA.	$\mu S.cm^{-1}$
Turbidity	APHA 2130-B, (2005)	Turbidimeter Hach® Sievers InnovOx Laboratory, USA.	NTU
Organic M. aeruginosatter UV 254 nm	APHA 5910-B, (2005)	Spectrophotometer Thermo-Scientific genesis 105 UV-VIS, USA.	$mg.L^{-1}$
True color – UV 455 nm	APHA 2120-C, (2005)		HU
Organic Carbon	APHA 5310, (2005)	Sievers InnovOx Laboratory TOC Analyzer, General Eletrics®, USA.	$mg.L^{-1}$

4.9 Statistical analyses

Shapiro-Wilk normality test was performed on the data of the two sets of the experiment. Normality was observed using a significance level of 5%. In order to test if the observed changes in the parameters due to the treatment with H_2O_2 were significant, nonparametric one-tailed pairwise Willcoxon rank-sum tests with Bonferroni correction was used using a level of significance of 5 or 10%.

To verify the causality between the H_2O_2 concentration and the cell density values, a linear regression analysis was performed by calculating R^2 and adjusted R^2. Then the linear piecewise regression and quadratic model were performed, using as the criteria to test the quality of the adjustment: R^2, Adj. R^2, Log-Likelihood and Akaike information criterion (AIC). Other criteria assumed to evaluate the fitting of the model were:

- Normally distributed dependent variable ($p > 0.05$) with the significance level of 5%;
- The coefficients of the independent variable were significant ($p < 0.05$) using t-test;
- The model shows significance with F-statistics ($p < 0.05$).

Additionally, the principal component analysis (PCA) was performed to identify possible correlated variables. Kaiser criterion was considered to choose the principal components that better explain the behavior observed.

5 RESULTS AND DISCUSSIONS

5.1 Effect of hydrogen-peroxide on phytoplankton chlorophyll

The results obtained from the first set of the experiment showed that the concentration of the chlorophyll from cyanobacteria decreased consistently with the 5 $mg.L^{-1}$ concentration but decreased significantly ($p < 0.05$) with the 10 $mg.L^{-1}$ concentration, going from around 28 $\mu g.L^{-1}$ in the control samples to non-detectable in the samples of 10 $mg.L^{-1}$ H_2O_2 concentration (Figure 3). In addition, green algae and diatoms were less affected by the application of H_2O_2 than the cyanobacteria, since there was no significant variation ($p > 0.05$) in their chlorophyll concentration.

The application of H_2O_2 also suppressed cyanobacteria regrowth (Figure 4). On the other hand, it did not significantly harm the green algae and diatom communities in the regrowth experiment, since their chlorophyll concentrations were not significantly different ($p > 0.05$) to the control samples.

Figure 3 – Effect of hydrogen peroxide on the chlorophyll of phytoplankton community four days after the application of the H_2O_2. (A) variation of the chlorophyll from cyanobacteria. (B) variation of the chlorophyll from green algae. (C) variation of the chlorophyll from diatoms. Letters on each bar graph means that there is

no significant difference (p> 0.05) between the chlorophyll concentration due to the different concentrations of H_2O_2.

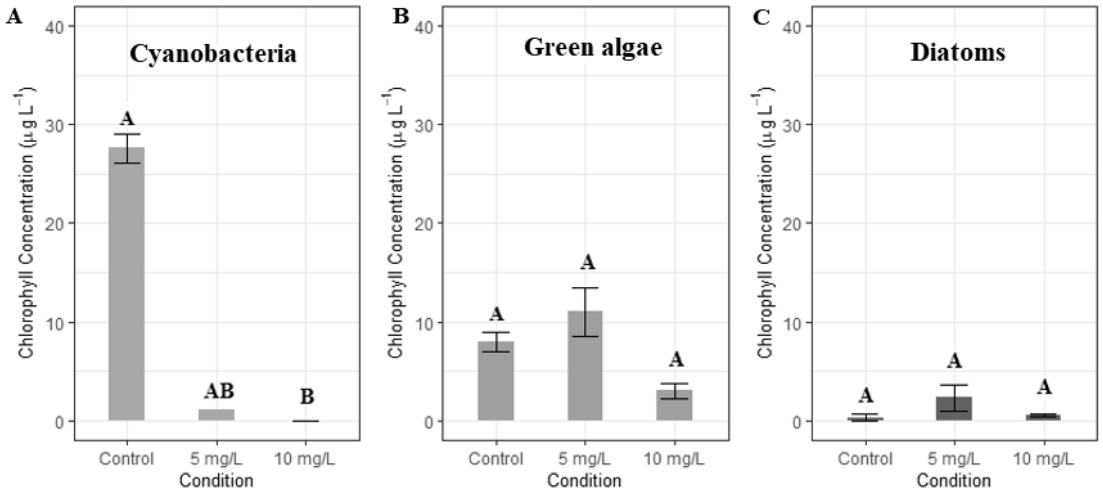
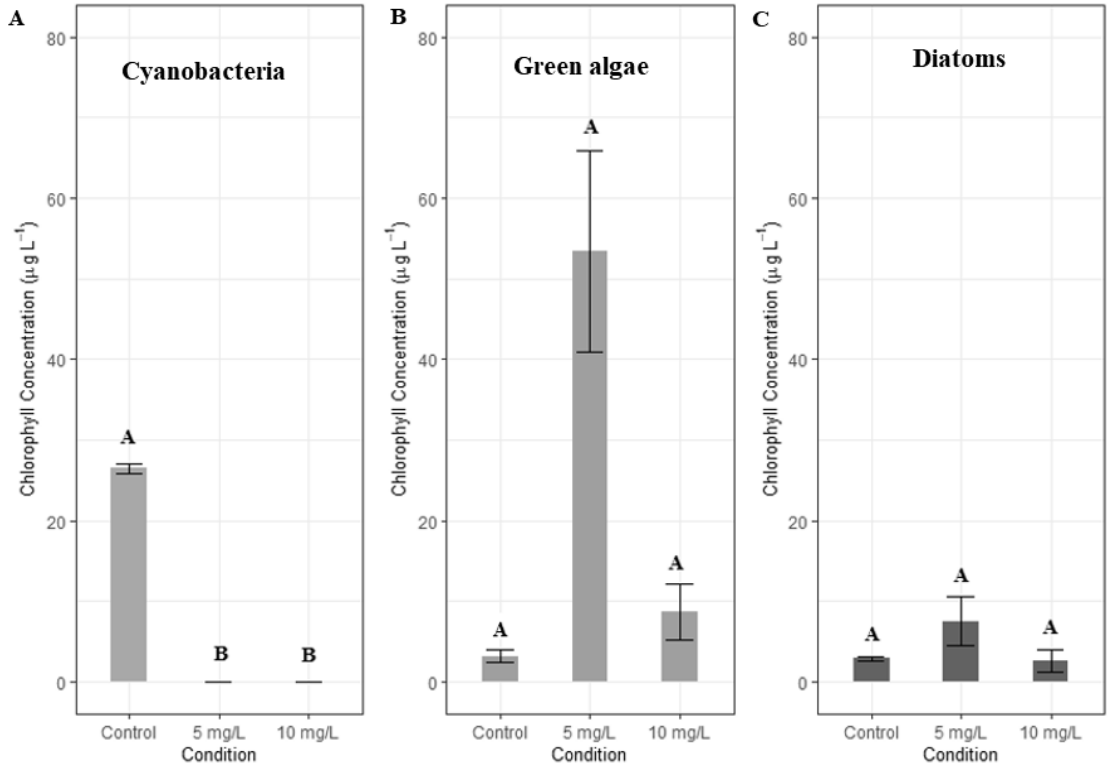


Figure 4 – Chlorophyll concentration from different organisms seven days after the application of the H_2O_2. (A) variation of the chlorophyll from cyanobacteria. (B) variation of the chlorophyll from green algae. (C) presents the variation of the chlorophyll from diatoms. Letters on each bar graph means that there is no significant difference (p> 0.05) between the chlorophyll concentration due to the different doses of H_2O_2.



The concentrations of 5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ of H_2O_2 allowed a strong regrowth of green algae after the regrowth experiment (Figure 4B) as compared to its initial concentration (control). Even though both concentrations of H_2O_2 appeared to

have similar effects on the chlorophyll from the phytoplankton community, the concentration of 10 $mg.L^{-1}$ decreased the cyanobacteria chlorophyll to an undetectable level faster than the concentration of 5 $mg.L^{-1}$. Since the primary purpose of this study is to decrease the amount of phytoplankton in the raw water, the concentration of 10 $mg.L^{-1}$ should be a better option for that purpose. However, the use of H_2O_2 in a concentration higher than 5 $mg.L^{-1}$ is not recommended since it can damage zooplankton and other organisms, and the local directives in the Netherlands, for instance, do not allow the application of this higher concentration (WEENINK *et al*., 2015).

The results obtained in this first stage corroborate with studies that found evidence that cyanobacteria are more sensitive to H_2O_2 than green algae and diatoms (MATTHIJS *et al*., 2012; SINHA; EGGLETON; LOCHMANN, 2018). Cyanobacteria do not produce enough enzymes to eliminate reactive oxygen species (ROS), such as the H_2O_2, which stimulate the oxidation of lipids and proteins in the cells, leading to loss of cellular membrane integrity and inactivation of enzymes and, ultimately, in cell death (SINHA, EGGLETON; LOCHMANN, 2018). The main substances responsible for ROS degradation are ascorbate peroxidase (not present in cyanobacteria cells) and the haem peroxidase family (found in a few cyanobacteria but not in *Planktothrix sp*.) (BERNROITNER *et al*., 2009).

Although Matthijs *et al*. (2012) also corroborate this hypothesis, they add that another possible explanation for cyanobacteria sensitivity to H_2O_2 could be related to the Mehler reaction. Mehler reaction is a side activity of photosynthetic electron transfer that produces H_2O_2 in eukaryotic phytoplankton and higher plants when exposed to high light intensity. This reaction does not occur in cyanobacteria cells, suggesting that cyanobacterial cells are not adapted to these higher levels of oxidative stress.

In environments with high concentrations of organic matter, very commonly found in tropical regions, a decrease in the effectiveness of selective suppression of cyanobacteria has been observed due to an increase in the consumption rate of H_2O_2 (WEENINK *et al*., 2015). In this sense, the contact time is an important factor that can influence H_2O_2 effectiveness in controlling cyanobacteria blooms (MATTHIJS *et al*., 2012; BURSON *et al*., 2014). For instance, Barrington *et al*. (2013) recommended the addition of H_2O_2 at a ratio proportional to the chlorophyll concentration in the water. Even though this method proved successful on the phytoplankton suppression, it could

be harmful to the zooplankton community and, therefore, low doses of H_2O_2 are recommended (Reichwaldt *et al*., 2012). Thus, the ideal procedure to apply H_2O_2 in a reservoir would be first to evaluate the general characteristics of the water and the organisms present, and then decide which is the best concentration to achieve the desired objectives.

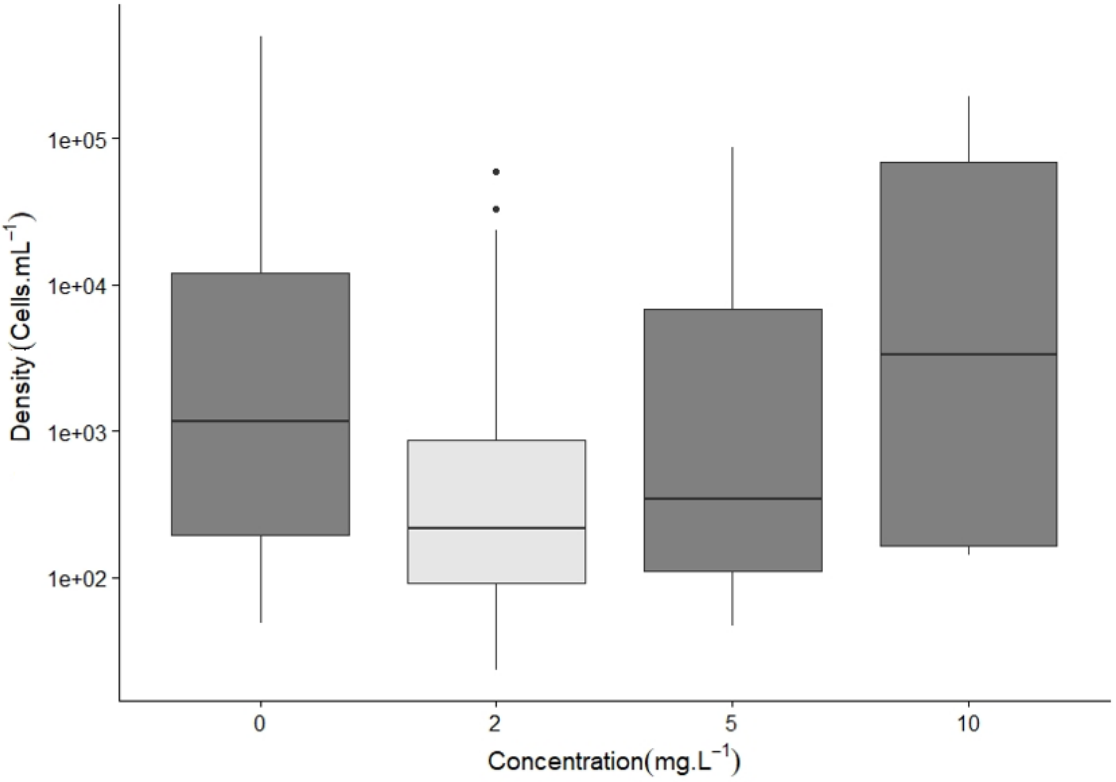
To understand how various concentrations of H_2O_2 affect different phytoplankton organisms and to provide additional tools to aid operators to decide which is the best concentration to control cyanobacteria in Gavião reservoir, we performed another set of experiment adding a lower concentration of H_2O_2 and replaced chlorophyll analysis by cell density counts, evaluating the organisms at the genus level.

5.2 Determination of the effects of H_2O_2 on phytoplankton cell density

In order to verify the effect of H_2O_2 on different genera of the phytoplankton community of Gavião reservoir water, the cell density changes at different H_2O_2 doses were analyzed. All cell counts were performed after H_2O_2 extinction or two days after its application. The species considered in the cell counts for this set of experiments were presented in Table 1. Since they visually seemed the most frequent in the water from Gaviao reservoir, it is expected that they may be the most resistant genus in that water.

Figure 5 shows that there was a significant ($p = 0.077$) reduction in cell density of the studied organisms only samples where a H_2O_2 dose of 2 $mg.L^{-1}$ was applied. On the other hand, the results obtained on the interference of H_2O_2 in the phytoplankton chlorophyll (first set of experiments), showed that H_2O_2 acted in a similar way in each group under all concentrations used. In order to verify which organism interfered the most with this pattern, the organisms considered on the cell count were divided into two groups to be compared: cyanobacteria (Figure 6A) and non-cyanobacteria, which includes green algae and diatoms (Figure 6B).

Figure 5 – The effect of H_2O_2 concentrations on the cell density of all the studied organisms. Different grey scale indicate significant differences ($p<0.10$) comparing with the control (no application of H_2O_2).

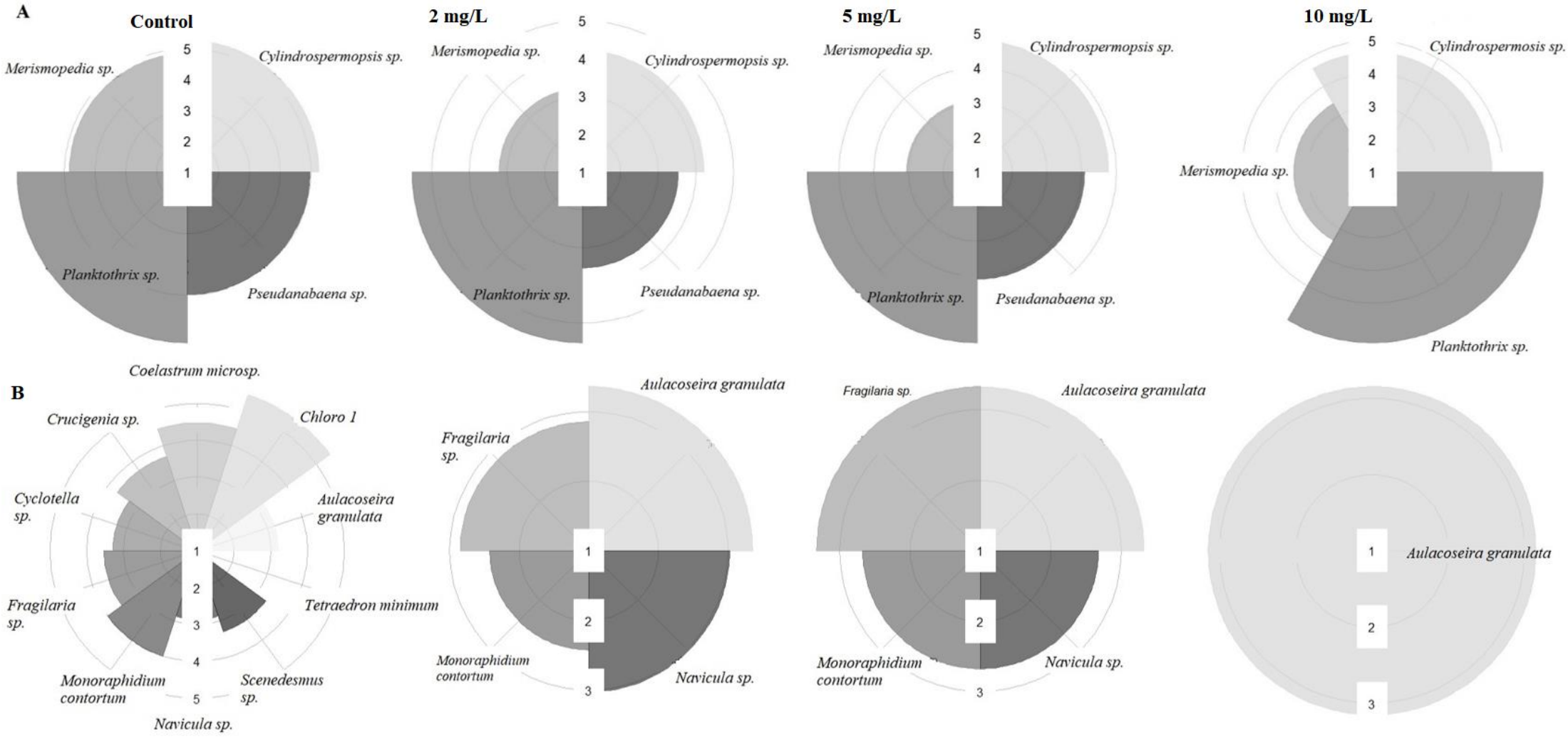


As presented in Figure 6, *Planktothrix sp*. continued to dominate with median cell density higher than 10^5 cells mL^{-1} in all H_2O_2 concentrations used, just as in the control water. *Cylindrospermopsis sp*., *Merismopedia sp*., and *Pseudanabaena sp*. presented more sensibility to the oxidant, mainly in the 2 mg L^{-1} concentration, with reductions of approximately 1 log, 2 logs, and 2 logs, respectively. This high reduction of the cell density from *Cylindrospermopsis sp*., *Merismopedia sp*., and *Pseudanabaena sp* contributed to the significant total cell density reduction ($p = 0.045$) at the 2 $mg.L^{-1}$ concentration (Figure 6A), confirming the behavior observed in Figure 5. However, in the samples with 5 ($p = 0.329$) and 10 mg L^{-1} ($p = 0.993$), there was not a significant reduction of cell densities for those three species. *Cylindrospermopsis sp*. cell density was approximately 10^5 cells mL^{-1}, close to the values observed in the control samples. *Merismopedia sp*. cell density observed in the 2 $mg.L^{-1}$ samples remained unchanged, around 10^3 cells mL^{-1}. *Pseudanabaena sp*. had similar behavior as *Merismopedia sp.*, except for the 10 $mg.L^{-1}$, where it was not detected.

There was no apparent change in *Planktothrix sp* cell density during the experiment (Figure 6A). This effect may be due to the selective effect of H_2O_2 on

decreasing the cell density of other taxa (Figure 6B), selecting the most resistant species. With less competition, the most resistant taxa (*Planktothrix sp)* probably managed to survive. In addition, Figure 7 shows the values of the cyanobacteria density, confirming that they were the ones that most contributed to the behavior of total cell density shown in Figure 5, where only the concentration of 2 $mg.L^{-1}$ had a significant impact.

Figure 6 – Cell density of each studied taxa and H_2O_2 concentration two days after the application of H_2O_2. (A) represents the median of cyanobacteria cell density; (B) represents the other phytoplankton taxa, including green algae and diatoms. The median is in the logarithm scale and each concentric circle represents 1 log.

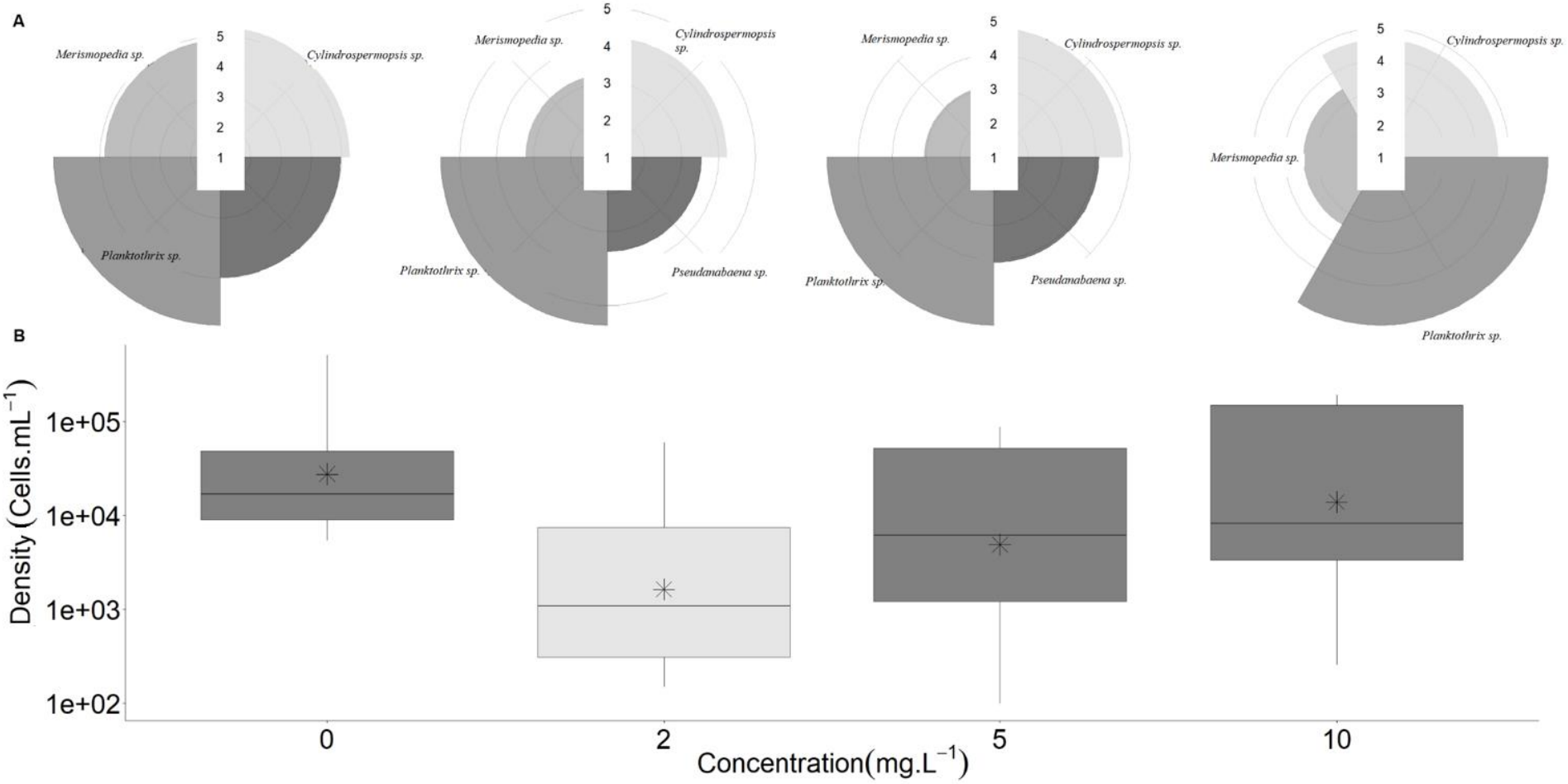


When analyzing the non-cyanobacteria group, the genus that appeared to be resistant to the H_2O_2 were *Fragilaria sp*., *Monoraphidium contortum*, *Navícula sp*., and *Aulacoseira granulatta* (Figure 6B) among the eight genera evaluated. This appears to contradict the effect presented in the first part of the experiment (Figure 3), where the green algae and diatoms were not significantly affected based on their chlorophyll concentration. The diatom *Aulacoseira granulatta* and the cyanobacteria *Merismopedia sp*., *Cylindrospermopsis sp*., and *Planktothrix sp*. were the only genera detected when 10 $mg.L^{-1}$ of H_2O_2 was applied.

Since the chlorophyll concentration decreased significantly after the application of H_2O_2, the expectation was that the same would happen with cell density, especially of cyanobacteria. Even though cell density decreased significantly (except for *Planktothrix sp*.) the total cyanobacteria cell density is still higher than 10^5 $cells.mL^{-1}$ (Figure 6A). With this cell density value, the cyanobacteria continue to be a concern for water companies, since Brazilian legislation establishes that when cyanobacteria cell density exceed 20,000 cells mL^{-1}, they must include weekly monitoring for cyanotoxins in the reservoir and the collection point (PRC n° 5, 2017).

Aguilera, Echenique, and Giannuzzi (2014) obtained results similar to the ones found in our study. They observed a chlorophyll decreas of 89% in a hypereutrophic lake, but the cell density did not decrease significantly ($p>0.05$) after 48 h of the application of H_2O_2 idespite the fact that *Planktothrix agardhii* filaments and cells were in different stages of degeneration, such as swollen cell walls and cytoplasmatic alteration, which could explain why the chlorophyll decreased but the cell densities did not.

Figure 7 – Diversity analysis of cyanobacteria and its cell density two days after the different doses of H_2O_2 applied to the samples. (A) represents the median of the cell density, and it is in logarithm scale, each concentric circle represents one log. (B) where different colors represents significant differences ($p<0.05$) concerning the control samples.



Differently from other researches on this topic, our study did not use of UV light in association with H_2O_2, which could improves the reaction performance (BARRINGTON; REICHWALDT; GHADOUANI, 2013). Wert *et al*. (2014) used H_2O_2 with a UV lamp emiting out 254 nm. UV light was no used in this experiment in order to evaluate the isolated performance of H_2O_2 as an algicide in regions of the water column were UV light does not penetrate. The light used in this study had a mean intensity of 10 $\mu mol.m^{-2}s^{-1}$. This light intensity was lower than the one utilized by Yang *et al*. (2018) that used a photosynthetic photon flux density (PPFD) of 40 $\mu mol.m^{-2}s^{-1}$. Matthijs *et al*. (2012) used ambient daylight and H_2O_2 in the laboratory in water dominated by *Planktothrix agardhii* but with a cell density (1.2×10^6 cells.mL^{-1}) higher than in the current study ($3.45x10^5$ cells.mL^{-1}). Matthijs *et al*. (2012) obtained a cell density decrease of two logs, higher than in our study.

Filamentous cyanobacteria appear to have a higher resistance to H_2O_2, specially *Planktothrix sp*. Wert, Dong, and Rosario-Ortiz (2013) performed a study in which they evaluated how different oxidants interfered with cyanobacteria such as *Microcystis aeruginosa*, *Oscillatoria*, and P*lanktolyngbya*. They concluded that filamentous cyanobacteria were less affected by the treatment. In addition, Sinha, Eggleton, and Lochmann (2018) measured cell densities 10 days after the application of different H_2O_2 concentrations on cultured *Planktothrix sp.* All the concentrations above 2.0 mg.L^{-1} (in a range from 1.5 to 8.0 mg.L^{-1}) resulted in no significant difference in cell densities. However, just like in the current study, Wert, Dong, and Rosario-Ortiz (2013) obtained a reduction in cell density by one order of magnitude after the application of H_2O_2 (from $1.1x10^6$ to $0.1x10^6$ cells mL^{-1}) supporting the hypothesis that *Planktothrix sp*. is very resistant to H_2O_2.

The cell density of *Planktothrix sp*. presented a particular behavior where its cell density decreased the most under the concentration of 2 mg L^{-1} and the least under the 10 mg L^{-1} (Figure 7). Yang *et al*. (2018) studied the effect of H_2O_2 on water dominated by *Planktothrix* after three days of application, close to the time-lapse of our study (two days). For that time frame, the concentration that caused a higher reduction on the *Planktothrix sp.* biovolume was not the highest one used (20 mg L^{-1}) but the 6.7 mg L^{-1} concentration. However, seven days after the treatment with H_2O_2, *Planktothrix sp.* biovolume submitted to different concentrations of H_2O_2 decreased to similar values, implying that it can take a longer period for the different concentrations of H_2O_2 to result in a similar effect on cyanobacteria cell density.

This extra period for the H_2O_2 to impact *Planktothrix sp* cell density appears to be linked to the mechanism where higher concentrations of H_2O_2 would trigger protective features exhisting in some organisms. Some studies suggest that *Planktothrix* filaments can become thicker in some instances, such as to protect against grazing or as a response to other stress conditions (GER *et al*., 2016; WEJNEROWSKI *et al*., 2018). Even though direct contact with a grazer stimulates these mechanisms, some examples show the expression of these strategies after a stimulation caused by chemicals (WEJNEROWSKI *et al.*, 2018).

Other studies discuss how *Microcystis aeruginosa* forms colonies or increases the production of polysaccharides, considered a defense feature (BURKERT *et al*., 2001; YANG *et al*., 2006; YANG). Huo *et al*. (2015) suggested that the mucilaginous layer produced by *Microcystis aeruginosa* can react with the OH radicals to protect the cellular membrane from H_2O_2. Xu *et al*. (2019) also observed a period, called lag phase, before the H_2O_2 causes significant cell lysis of a *Pseudanabaena* sp.

Fiałkowska and Pajdak-Stós (2014) added that this ability to recognize a sign of grazer and accelerate sheath-building materials production is a characteristic that gives an advantage for solitary trichomes. Cerbin *et al*. (2013) found an example of this case when the filamentous cyanobacteria *Aphanizomenon gracile* reacted to infochemicals (chemical compounds that carry information between organisms) released by *Daphnia* by shortening and thickening their filaments, therefore becoming more resistant to grazing. Therefore, higher concentrations of H_2O_2 may take longer to decrease *Planktothrix sp*. cell density but a more detailed study is needed to confirm this hypothesis.

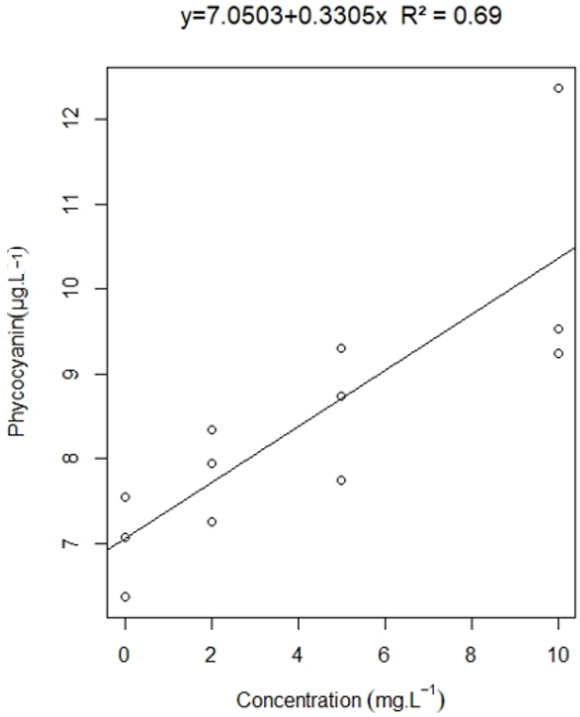
Another possible explanation is that microscopy may not be the ideal method for fast-post treatment analysis because the integrity of some cyanobacterial cells remains visibly unaffected while viable green algae cells can show a temporary wrinkled appearance until some time after treatment (Weenink *et al*., 2015; Calomeni and Rodgers, 2015).

5.3 Effect of H_2O_2 on phycocyanin concentration in cyanobacteria

In the second set of experiments, in addition to the cell counts, the total phycocyanin concentration was also analyzed, since this pigment is mainly associated with cyanobacteria. Phycocyanin was also analyzed two days after the H_2O_2 application and a slight increase in concentration when compared to the control jars (Figure 8) was

noticed. This increase may be due to the intracellular content release, from the cells to the solution. Yang *et al*. (2018) observed that after five days of the H_2O_2 application, the phycocyanin concentration remained low for the H_2O_2 doses of 2.7, 8.1, and 24.3 $mg.L^{-1}$. Weenink *et al*. (2015) reported that there was a higher presence of detached phycobilin pigments in relation to the H_2O_2 dose applied, disappearing only after four days from the application, supporting the idea that the phycocyanin in our experiment would probably be degraded some later time.

Figure 8 – Phycocyanin concentration changes due to the application of different doses of Hydrogen Peroxide



5.4 Evaluation of different models to explain the behavior of the phytoplankton community after the H_2O_2 application

In order to verify if the effects on the cell density of the different organisms could be explained by the increase in H_2O_2 concentrations, a series of mathematical models was applied to the data. Different models were tested until the one that best fitted the experimental data was found. Finally, PCA was performed to confirm the results obtained by the selected model.

A linear regression model (Figure 9) was applied to help to describe the behavior of the most dominant phytoplankton species considered. Observing the slopes in Figure 9, it´s possible to identify that the concentration of H_2O_2 seems not to

influence the cell densities of *Planktothrix sp*., *Cylindrospermopsis sp*., and *Aulacoseira granulatta* but appears to influence *Merismopedia sp*. and *Pseudanabaena sp*. The linear regression model could not be calculated for the remaining organisms because they were not detected at the higher H_2O_2 concentrations.

The linear regression coefficients that support this information are presented in Table 3, which contains the logarithm of the cell density of each species as a function of the H_2O_2 concentration. Also in Table 3, the F and T statistics for each slope are presented (which presented 0 on the 95% estimated confidence interval), indicating that all the slopes were non-significant ($p<0.05$) and that it was not possible to identify significant reductions of cell density of the organisms *Pseudanabaena sp*., *Merismopedia sp*., *Cylindrospermopsis sp*., and *Planktothrix sp*., as well as *Fragilaria sp*., *Monoraphidium contortum*, *Navícula sp*., and *Aulacoseira granulatta*. This fact is also corroborated by the values of R^2 and adjusted R^2 (Table 3). Therefore, this model is not well suited to analyze the relationship between the cell densities of each organism and the concentration of H_2O_2.

Figure 9 –Effect of hydrogen peroxide on cell density of the studied genera (logarithm scale). The straight lines and the 95% confidence bands were obtained through linear regression.

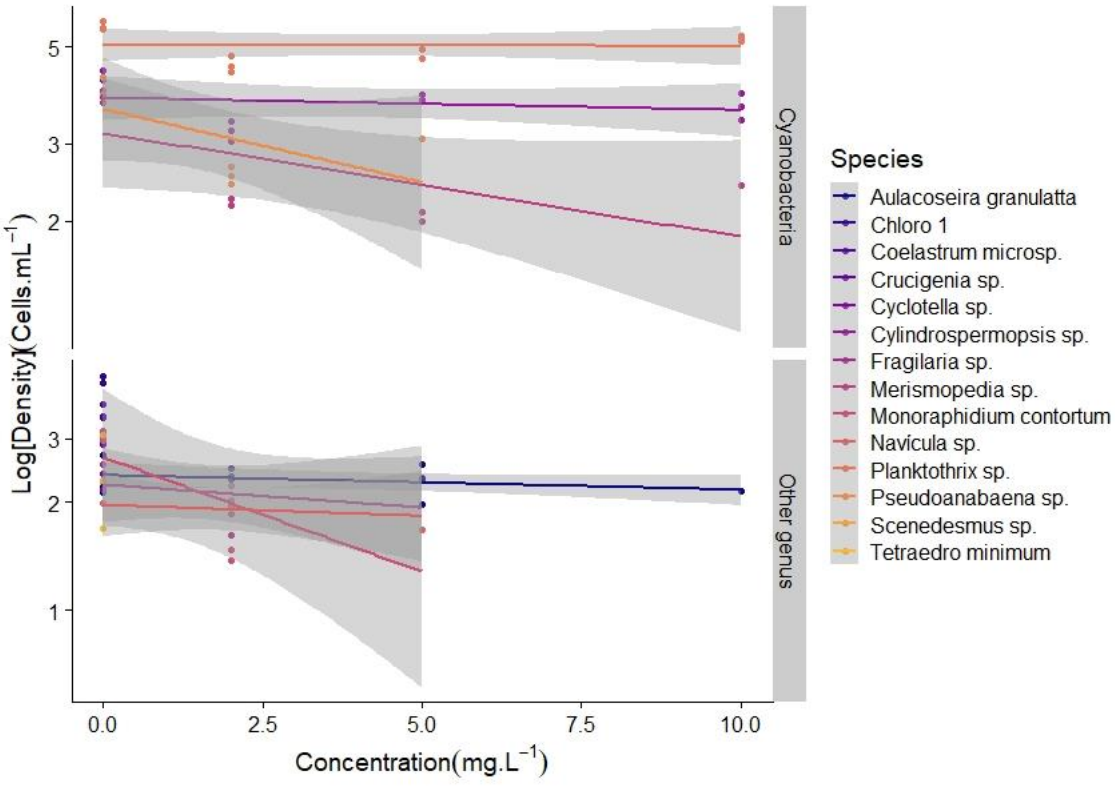


Table 3 – Coefficients of the linear regression models for the logarithm of cell density for each species in

relation to the hydrogen peroxide concentration.

Genus	Species	Intercept p-value (t-test)	Angular coefficient p-value (t-test)	p-value (F-test)	R^2	Adj. R^2
Other genus	*Aulacoseira granulatta*	2.38 [<0.001]	-0.0219 [0.175]	0.175	0.18	0.09
	Fragilaria sp.	2.26 [<0.001]	-0.0646 [0.473]	0.473	0.11	-0.07
	Monoraphidium contortum	**2.77 [0.001]***	**-0.3290 [0.077]***	**0.077 ***	**0.58**	**0.48**
	Navícula sp.	1.96 [<0.001]	-0.0252 [0.674]	0.674	0.05	-0.19
	*Ohter species**	not found from 2 $mg.L^{-1}$				
Cyanobacteria	*Cylindrospermopsis sp.*	3.86 [<0.001]	-0.0279 [0.421]	0.421	0.07	-0.03
	Merismopedia sp.	**3.30 [<0.001]***	**-0.1630 [0.077]***	**0.077 ***	**0.43**	**0.34**
	Planktothrix sp.	5.08 [<0.001]	-0.0062 [0.856]	0.856	0	-0.1
	Pseudanabaena sp.	3.68 [<0.001]	-0.2640 [0.132]	0.132	0.39	0.27

* Cyclotella sp., Crucigenia sp., Tetraedro minimum, Coelastrum micro sp., Scenedesmus sp. and Chloro 1

Apart from cyanobacteria, the most resistant organism to H_2O_2 after 48 h was the diatom *Aulacoseira granulatta*. This organism showed a considerable variation in cell size and morphology related to adaptation to changes in the environmental conditions (O'FARRELL; TELL; PODLEJSKI, 2001). Viana and Rocha (2005) evaluated the toxicity of copper sulphate and atrazine on *Aulacoseira granulatta* and found that the reason for its adaptability is its short lifecycle. This characteristic imposes a short response time to changes in habitat conditions, population and productivity reduction (RAND, 1995).

Since the linear fitting did not describe well ($p \approx 0.21$) the behavior of cell density in relation to the H_2O_2 concentration, we applied the linear piecewise regression model (Equation 1), and the quadratic model (Equation 2) to the experimental data. The parameters used for those models are presented in Table 4.

$$Density = \begin{cases} \beta_0 + \beta_1 Concentration, & x \leq \theta \\ \beta_0 + \beta_2 Concentration + \theta(\beta_1 - \beta_2), & x > \theta \end{cases} \quad (1)$$

$$Density = \beta_0 + \beta_1 Concentration + \beta_2^2 Concentration \quad (2)$$

Where θ is the breakpoint that determines the change in the behavior of the cell density for the piecewise regression model (Equation 1), defined as 2 $mg.L^{-1}$, according to the behavior presented in Figure 10. β_0, β_1 and β_2 are the coefficients of each model. Based on Table 4, the model that best fitted our data was the piecewise regression model (Equation 1), considering the criteria used to test the quality of the

model, as follows: R^2, Adj. R^2, Log-likelihood, and AIC. It is possible to observe in Figure 10, two well defined trends in the 2 $mg.L^{-1}$ concentration on the piecewise regression model:

- Initially, the graph decreases until the breakpoint (2 $mg.L^{-1}$), with the lowest level of the cell density significantly smaller ($p = 0.045$) than the control samples, according to the Wilcoxon statistical test.
- The second part of the graph ascends from the breakpoint, suggesting an increase in cell density. Only the most resistant species were found in the samples treated with higher H_2O_2 concentrations (Figure 10) and, therefore, few organisms remained to be compared, limiting the use of the Wilcoxon statistics. Thus, to better understand what happens after the breakpoint, PCA was used with two groups of variables. Where the bold parameters are the components chosen.
    - Group one: pH, turbidity, true color, conductivity, light intensity, **percentage of cyanobacteria, and cell density**
    - Group two: **percentage of cyanobacteria, cell density**, organic matter (UV_{254}), total organic carbon, inorganic carbon, total carbon, dissolved organic carbon, dissolved inorganic carbon, dissolved total carbon, Phycocyanin.

The percentage of cyanobacteria and cell density are present in both groups in order to evaluate their weight on each component and their interaction with the other variables.

Figure 10 – Comparison of the linear regression model with the non-linear models piecewise and quadratic.

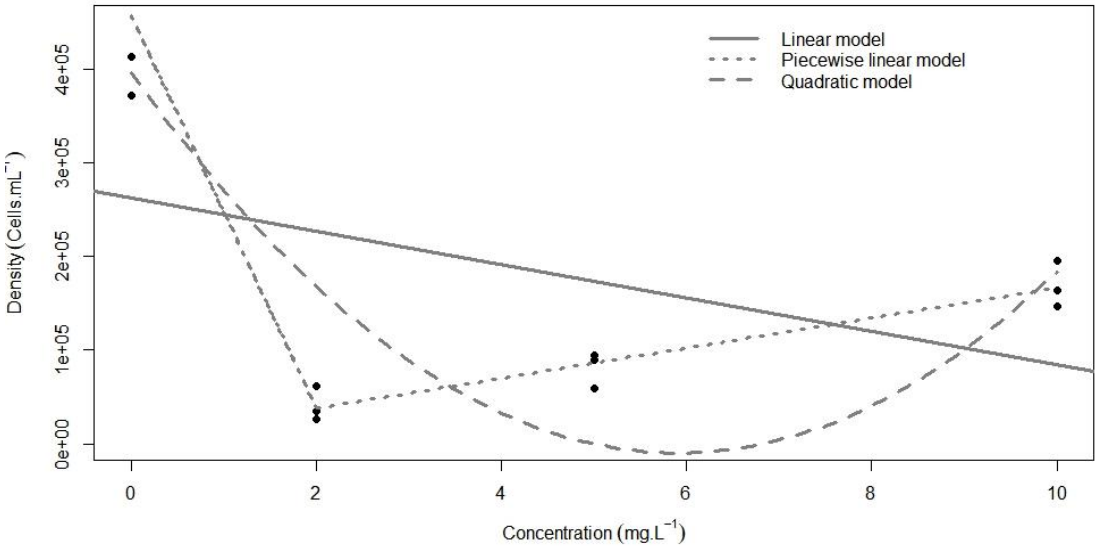


Table 4 – Parameters considered for the linear piecewise linear model and the quadratic model

Model	β_0 [CI] t-test p-value	β_1 [CI] t-test p-value	β_2 [CI] t-test p-value	R^2	Adj R^2	Log-L	AIC
Piecewise linear	457,479,0 [383230.2; 531727.1] p<0.001	-209,776.0 [-258,814.8; -160,736.7] p<0.001	225,869.0 [169,367.7; 282,370.0] p<0.001	0.91	0.91	-146.7	301.3
Quadratic	396,478.0 [263050.9; 529905.4] p<0.001	31,832.0 [4,873.413; 18,350.39] P=0.002	11,612.0 [4,873.4; 18,350.4] p = 0.004	0,68	0,68	-154.6	317.2

The two principal components were chosen for Group one (Figure 11A) by using the Kaiser criterion. The first component is the most important since it accounts for 65.24% of the data variability. Together, the two principal components express 81% of the data set total inertia, which represents the total variability of the observations (Figure 11B). Therefore, the plane defined by these two components represents a significant part of the data variability.

Figure 12A shows how distant the observations 1, 2, and 3 of the control experiments (0 mg L^{-1}) are from the samples where H_2O_2 was applied. In addition, the samples with H_2O_2 are close to each other and on the opposite side to the control experiments. The points that represent the concentrations of 2, 5, and10 mg L^{-1} have

low and close values for cell density, pH, conductivity, turbidity, and true color but high and close values of cyanobacteria percentage (Figure 12B). It is important to highlight how the group of 10 mg L^{-1} presented high values for conductivity, resulting in a slight distance between points 11 and 12.

Figure 11 – Definition of the number of principal components for Group one. (A) Variance in relation to the number of components, and (B) decomposition of the total inertia. The dashed line represents the limit established for the variance according to the Kaiser criterium.

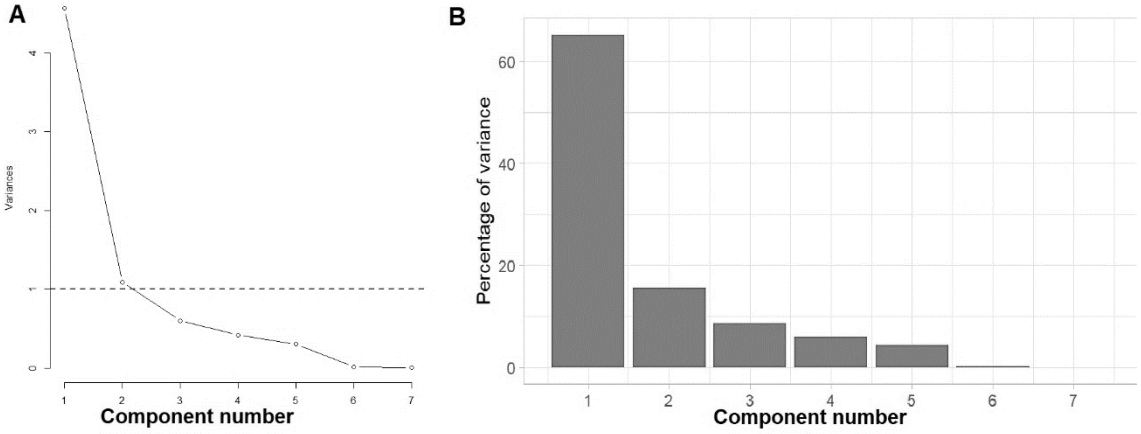
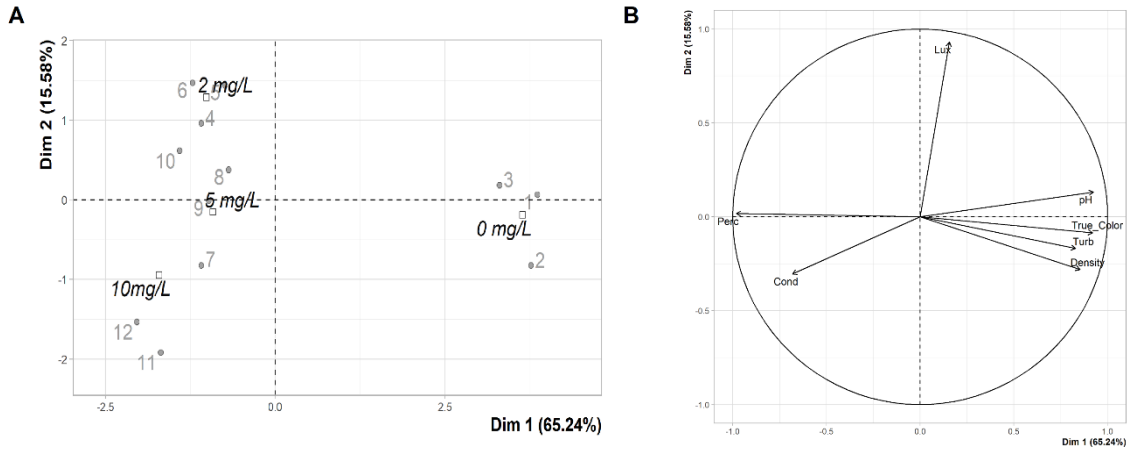


Figure 12 – PCA Graph for Group one. In (A) the points represent the triplicates of the experiment – performed with 0, 2, 5 and 10 mg.L^{-1}. In (B) is the loading plot. Each vector represents a variable from Group one. The loading plot can range from -1 to 1. Where loadings close to 0 indicate that the variance has a weak influence on the component.



For Group 2 (Figure 13A), three components were chosen using the Kaiser criterium. Since the components 2 and 3 have close variance percentages (14.74%, and 12.63%, respectively – Figure 13B), two planes were analyzed: the first plane delimited by the component 1, which explains 58.13% of the data variance, and component 2 (Figure 14A and Figure 14B). The second plane is delimited by components 1 and 3 (Figure 14C and Figure 14D). Both planes express more than 70% of the total inertia

from the data set, which means they represent the total variability of the observations well.

Figure 13 – Definition of the number of principal components for Group two. (A) Variance in relation to the number of components, and (B) decomposition of the total inertia. The dashed line represents the limit established for the variance according to the Kaiser criterium.

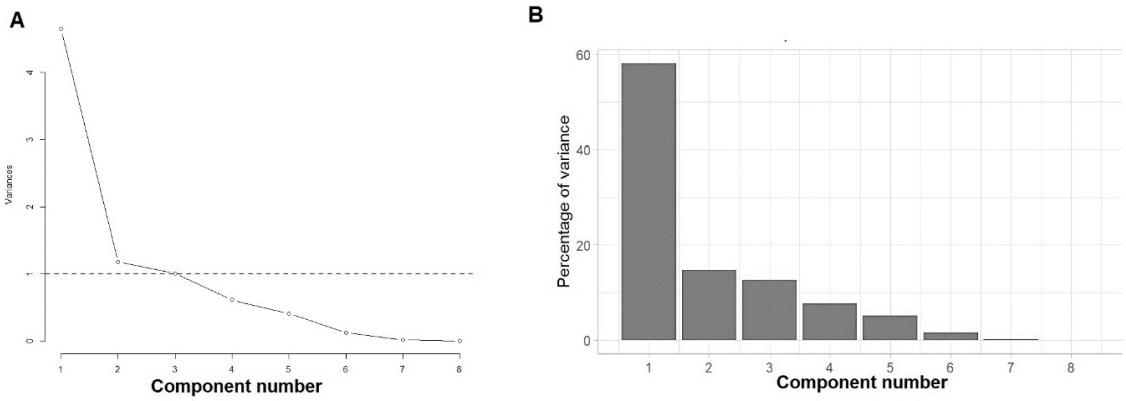
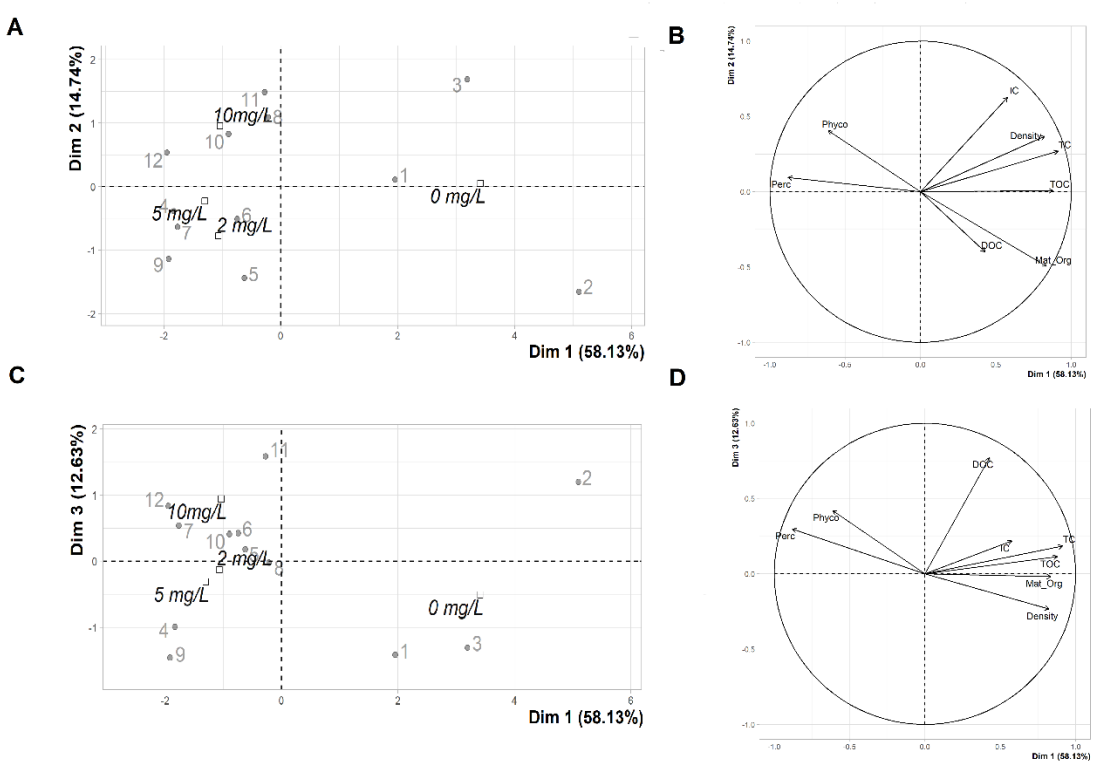


Figure 14 shows a similarity both in the scattering of the triplicates observation and in the tendencies of the vectors. Additionally, Figure 14A and Figure 14C present a similar tendency observed in Group 1 (Figure 12), where the control samples were distant from the other observations. The observations referring to the samples with 2, 5 and 10 mg.L^{-1} of H_2O_2 were also close to each other and on the opposite sides of the control samples. Therefore, based on Figure 14B and Figure 14D, the samples impacted by H_2O_2 present lower values for most variables of Group 2, and higher values of phycocyanin and percentage of cyanobacteria. This indicates that in this phase of the experiment, the cyanobacteria prevailed in comparison to the control samples where a higher diversity was observed. This tendency corroborates with the increase of phycocyanin concentration presented in Figure 8.

Figure 14 - PCA Graph for Group two. In (A) the points represent the triplicates of the experiment – performed with 0, 2, 5 and 10 mg.L^{-1}. In (B) Loading plot. Each vector represents a variable from Group two. The loading plot can range from -1 to 1. Where loadings close to 0 indicate that the variance has a weak influence on the component.



With the modeling and the PCA performed in the second set of experiments, it is possible to infer that after 48h of contact time, the different H_2O_2 concentrations caused a similar effect on the parameters analyzed. This pattern was also observed by Yang *et al.* (2018) where the application of different concentrations of H_2O_2 resulted in similar values of biovolume of *Planktothrix agardhii* after the end of the experiment. This may corroborate the idea that counting cell density using an optical microscope is not the best method to observe the effect of the H_2O_2 right after the treatment.

6 CONCLUSION

The impact on cyanobacteria chlorophyll was significant, indicating that cyanobacteria are more sensitive to the H_2O_2 when compared to the green algae and diatom chlorophyll. However, when analyzing the impact on phytoplankton cell density, our study was not conclusive. The lowest concentration used (2 mg.L^{-1}), appeared to have the highest impact on cyanobacteria cell density, different from expected. Even though the concentration of 2 mg.L^{-1} presented a significant cell density reduction, it did

not decrease to a level recommended by cyanobacteria and cyanotoxins Brazilian guidelines.

The results obtained from the cell density counts indicate either that the H_2O_2 needs a longer contact time to effectively impact cyanobacteria or that the cell count using microscopy is not an efficient way to evaluate the oxidant effect for 48h contact time. Also, the filamentous cyanobacterium that dominates the Gaviao reservoir – *Planktothrix sp*. – appear to be highly resistant to oxidation by H_2O_2.

The PCA analysis confirmed that the control samples were significantly different from the samples with H_2O_2. However, the observed pattern indicates that the effects caused by the different concentrations of H_2O_2 were not significantly different from each other.

The most resistant filamentous cyanobacteria in Gaviao reservoir should be further studied since *Planktothrix sp.* might survive the treatment and, without competition, may become dominant. One option to investigate this hypothesis is to investigate the regrowth potential in the laboratory, but a proper technique is still not available and and needs to be developed.

CHAPTER II

Assessment of phytoplankton regrowth after the use of hydrogen peroxide: A bench-scale study including laboratory cyanobacterial strains and phytoplankton from a freshwater environment.

Assessment of phytoplankton regrowth after the use of hydrogen peroxide: A bench-scale study including laboratory cyanobacterial strains and phytoplankton from a freshwater environment

Authors: Maria Aparecida Melo Rocha; Allan Clemente de Sousa; Ismael Carloto Lopes; Marianna Correia Aragao; José Capelo Neto

Abstract

The concern with cyanobacteria has increased through the years, and conventional water treatment processes are not always suitable for an effective removal of those organisms. Therefore, the need for alternative treatments increased, such as pre-oxidation with hydrogen peroxide (H_2O_2). However, there are not many studies on the impact of this oxidant on the regrowth of the phytoplankton organisms (cyanobacteria included), both from laboratory or freshwater environments. This paper aims to apply and evaluate a methodology for analyzing the regrowth potential of the laboratory strains *Dolichospermum circinale* and *Microcystis aeruginosa*, and the phytoplankton organisms from a tropical reservoir. The Hydrogen Peroxide (5 and 10 $mg.L^{-1}$) successfully controlled *D. circinale* and *M. aeruginosa* cell densities for a 30-day period. However, when analyzing the changes in cell density of the phytoplankton from the freshwater, the experiment itself caused a significant impact, decreasing the cell densities of the organisms in the control samples the same amount as the one caused by the H_2O_2 application (a one log decrease for *Planktothrix sp*., for instance). Some adaptations may improve this methodology such as increasing the light intensity and the capacity of the jars used in the experiment. This methodology showed potential for successfully evaluating the phytoplankton regrowth from freshwater enironments and, after the suggested adaptations, may be applied for full-scale regrowth studies in reservoirs.

Key words: Cyanobacteria. Phytoplankton regrowth. Hydrogen peroxide.

7 INTRODUCTION

Cyanobacteria play an important role in the production of oxygen and the fixation of nitrogen and carbon (AGOSTONI *et al*., 2016). However, when the nutrient cycle is disrupted, usually by anthropogenic interference, cyanobacteria are prone to cause harmful blooms (HABs). These HABs can disrupt the aquatic ecosystem (ZI *et al*., 2018) and impact the quality of drinking water due to the production of toxic and taste and odorous metabolites (WERT *et al*., 2014). The most significant risk associated with cyanobacteria and their metabolites is the intoxication of humans, which can lead to diseases such as liver damages or even death. Exposure do metabolites normaly happen during a recreational activity or by drinking contaminated water (MCQUAID *et al*., 2011).

Conventional water treatment processes (coagulation, flocculation, sedimentation, and filtration), normally used in drinking water treatment plants (WTP), have been used as effective technologies to remove suspended and colloidal particles (FAN *et al*., 2014). However, phytoplankton cells are not easily removed by those treatments due to their low electronegativity and apparent densities which makes the coagulation/flocculation and sedimentation processes inefficient, allowing cells to overburden the sand filters (JIAN *et al*., 2019).

Due to these difficulties of conventional WTPs, alternative and more efficient treatments have been pursued, including many studies using H_2O_2 (MATTHIJS *et al*., 2012; AGUILERA; ECHENIQUE; WEENINK *et al*., 2015; SINHA; EGGLETON; LOCHMANN, 2018; YANG *et al*., 2018). CHEN *et al*. (2016) found that this oxidant acts selectively on the control of cyanobacteria, and the dose required to inhibit their growth is ten times lower than the dose required to inhibit green algae. MATTHIJS *et al*. (2012) proposed that a possible explanation for that is that cyanobacteria lack of production of enzymes that eliminate reactive oxygen species, such as the H_2O_2. Chen *et al*. (2016) suggested that the best period to control cyanobacteria blooms is during the overwintering, applying H_2O_2 during the resting stage. They simulated a regrowth experiment and, after seven days, the H_2O_2 applied proved to be effective. Even though developed at laboratory scale, this strategy may not be suitable for reservoirs in tropical regions, since the seasons are not clearly defined in the Brazilian northeast and, therefore, cyanobacteria do not undergo overwintering, blooms are perennial.

Although there is a growing interest in investigating the effectiveness of H_2O_2 in inactivating cyanobacteria cells (HE *et al*., 2016), little research has been performed in regards to the regrowth potential of cyanobacteria after the application of H_2O_2 in laboratory scale, both for laboratory and freshwater strains. Matthijs *et al*. (2012) applied H_2O_2 to a lake and observed that cyanobacterial cell density remained low over a recovery period of seven weeks. In the control lake, the cell density was around 10^6 cells.mL^{-1} while the treated lake presented a cell density of around 10^5 cells.mL^{-1} for the same period. In addition, the negative impacts on other organisms in the treated lake appeared mild. Yang *et al*. (2018) performed a seven-day study also using H_2O_2, and after this period, the *Planktothrix sp.* biovolume remained low (0.1 x 10^7µm^3.mL^{-1}, while in the control the biovolume remained 1 x 10^7µm^3.mL^{-1}), promoting the growth of chlorophytes.

Coral *et al*. (2013) studied oxidation of the laboratory strains *M. aeruginosa* and *Anabaena flos-aquae*, using ozone instead of H_2O_2. Although observing a complete loss of integrity of their cells, they did not focus on studying the regrowth potential of those species. Zamyadi *et al.* (2012) performed a similar study but they observed the impact of the chlorination in the laboratory cultures of *M. aeruginosa, D. circinale* (previously known as *Anabaena circinalis)*, *Cylindrospermopsis raciborskii*, and *Aphanizomenon issatsckenka*. However, this study also did not focus on evaluating the regrowth potential of laboratory cyanobacteria strains.

This paper aims to study the regrowth ability of cultivated strains the *Microcystis aeruginosa* and *Dolichospermum circinale* after the application of two different concentrations of H_2O_2 and to develop and apply a bench-scale methodology to evaluate the recovery of cyanobacteria from a freshwater environment after the application of doses of the H_2O_2.

8 MATERIALS AND METHODS

8.1 Cyanobacteria cultivation

Both *Microcystis aeruginosa* and *Dolichospermum circinale* strains were isolated and cultivate at the Selaqua Laboratory at the Federal University of Ceará (Brazil). The strains were grown in the ASM-1 medium (GORHAM *et al.,* 1964; ZAGATTO and ARAGAO, 1992) at 25°C, with a constant light intensity of 10 µmol.$m^{-2}s^{-1}$ (YF – 1065F, Taiwan), and with an automated light/dark cycle of 12/12 h.

8.2 Study area

The raw water used to perform the second and third set of this experiment was collected from Gavião Reservoir, Northeast of Ceará (Brazil) at a 90 cm depth and next to the WTP intake. With a storage capacity of 33.30 hm^3, and an area of 5.9 km^2 (COGERH, 2018), this reservoir plays an essential role on supplying water to more than 4 million inhabitants in the Metropolitan region of Fortaleza (IBGE, 2018).

8.3 Hydrogen peroxide quantification and dilution

To determine the concentration of the H_2O_2 stock solution and to monitor the natural consumption of this oxidant applied into the jars, the iodometric method (PERÓXIDOS DO BRASIL, 2018) and the Quantofix® Peroxide 25 semi-quantitative method were used, respectively.

8.4 Determination of cyanobacterial regrowth analysis

The experiment to evaluate phytoplankton regrowth was divided into three parts. The first one was performed after applying doses of 5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ of H_2O_2 on cell cultures of *M. aeruginosa and D. circinale,* diluted to a cell density of 10^5 $cells.mL^{-1}$ to a volume of 1.5 L and a propeller rotation of 100 rpm to maintain the suspension homogeneous.

To assess the cell regrowth, aliquots of 80 mL were collected from the jars two days after the application of H_2O_2 and placed in 250 mL sterile erlenmeyers. Two and four weeks after that, the cell densities were counted again. Samples were kept at the same condition as during cultivation. The experiment was performed in triplicates for all H_2O_2 concentrations and for both species.

The second and third sets of experiments were performed using jartest (1.5 L) themselves as the regrowth vessel, also using a propeller rotation of 100 rpm to maintain the suspension homogeneous. The H_2O_2 concentrations used were 0 (control), 5, and 10 $mg.L^{-1}$, for the second set of experiment and 0 (control), 2, 5, and 10 $mg.L^{-1}$, for the third set of experiment, all performed in triplicates. In order to evaluate the regrowth using different approaches, the second experiment used PHYTO-PAM II Phytoplankton Analyzer (Walz, Germany) to estimate chlorophyll concentration after one week.

The third experiment used cell density counts by optic microrscopy, performed two days and four weeks after the application of the H_2O_2. In both set of experiments, a mix of 20% of ASM-1 (GORHAM *et al.*, 1964; ZAGATTO and ARAGAO, 1992) medium and 80% of Gaviao reservoir water filtered (glass fiber 0.7 µm, Milipore, Ireland) and autoclaved was added to the jars topping up the 1.5 L volume every time a sample was subtracted from the jar so as to provide nutrients for an optimum regrowth. This mix was used in an attempt to provide an optimum growth environment and to replace the volume of water removed for analyses.

Both, the second and the third experiment the jars were maintained under a constant light intensity of 10 $\mu mol.m^{-2}s^{-1}$ (YF – 1065F, Taiwan), and with an automated light/dark cycle of 12/12 h.

8.5 Analysis

8.5.1 Pigments analysis

Chlorophyll is known to be an essential pigment for the photosynthesis of phytoplankton and it can relate to the biomass in the water (PIEHLER, CURRIN and HALL, 2010). Chlorophyll was estimated using the PHYTO-PAM II Phytoplankton Analyzer (Walz, Germany). This equipment distinguishes phytoplankton with different types of light-harvesting pigment antenna by exciting chlorophyll at four different wavelengths (WALZ, 2003), which provides chlorophyll concentration values from cyanobacteria, green algae, and diatoms. Thus, obtaining chlorophyll concentration values ($\mu g.L^{-1}$) for cyanobacteria, green algae, and diatoms on each condition. The extraction method to estimate the phycocyanin concentration was adapted from Bennett and Bogorad (1973), where the adaptation was to macerate the filters in order to increase the contact of the material retained in the filter with the reagents used to extract the phycocyanin.

8.5.2 Determination of phytoplankton cell density

The cell counts were performed in duplicate using Sedgewick-Rafter chamber and inverted optical microscope (Axio Vert.A1, Carl Zeiss, Jena, Alemanha) calibrated according to APHA *et al*. (2005) and CETESB (1978) methods. The samples with Lugol's iodine (1 mL) were transferred to the chamber and then counted according to APHA *et al*. (2005).

The taxa considered in the cell density counts (Table 5) were historically dominant in Gaviao reservoir from a period of 2016 to 2019 (BARROS *et al.*, 2017). The denomination *Chloro 1* represents green algae present in Gavião raw water which could not be identified.

Table 5 – Organisms considered in the cell counts that are part of the phytoplankton community in Gaviao reservoir.

GENUS	**PHYTOPLANKTON GROUP**
Planktothrix sp.	**Cyanobacteria**
Merismopedia sp.	**Cyanobacteria**
Pseudanabaena sp.	**Cyanobacteria**
Cylindrospermopsis sp.	**Cyanobacteria**
Crucigenia sp.	**Non-cyanobacteria**
Cyclotella sp.	**Non-cyanobacteria**
Coelastrum microsporum	**Non-cyanobacteria**
Fragilaria sp.	**Non-cyanobacteria**
Navicula sp.	**Non-cyanobacteria**
Scenedesmus sp.	**Non-cyanobacteria**
Monoraphidium contortum	**Non-cyanobacteria**
Tetraedron minimum	**Non-cyanobacteria**
Aulacoseira granulata	**Non-cyanobacteria**
Chloro 1	**Non-cyanobacteria**

8.6 Data analysis

Shapiro-Wilk normality test was used to verify the normality of the chlorophyll data with a significance level of 5%. This same significance level was considered to test whether the changes in the chlorophyll data due to the treatment were significant using the nonparametric one-tailed pairwise Willcoxon rank-sum tests with Bonferroni correction. For the remaining data, the difference in the results was graphically observed, and the error bars in each bar graph represents the standard error of the triplicate samples.

9 RESULTS AND DISCUSSION

The regrowth experiment was performed in three different steps. First, with the laboratory strains of *M. aeruginosa* and *D. circinale*, by analyzing cell density two and four weeks after the application of H_2O_2. The second set was performed using the raw water from Gaviao reservoir and analyzed the regrowth of cyanobacteria, green algae, and diatoms using chlorophyll seven days after the application of H_2O_2,. Finally, the third set of experiments, also using the water from Gaviao, evaluated the regrowth potential after four weeks analyzing cell density.

9.1 *Microcystis aeruginosa* and *Dolichospermum circinale* regrowth

Hydrogen proxide successfully controlled both species (*D. circinale* and *M. aeruginosa*) for the entire four week period. However, *D. circinale* was more sensitive than *M. aeruginosa*, since its cells were not detected immediately after the extinction on both concentrations, 2 and 5 $mg.L^{-1}$ (Figure 15A). As for *M. aeruginosa*, the cells were undetectable at the 2 $mg.L^{-1}$ concentration only after the two-week period (Figure 15B). This may indicate that the *M. aeruginosa* strain used for this experiment has a more resistant structure to the H_2O_2 than the *D. circinale*. This higher resistance can be due to the *M. aeruginosa* ability to form mucilage or sheaths to defend itself from nutrient deficiencies, grazing or environmental stress (FAN *et al.*, 2016), which the *D. circinale* does not usually produce. On the other hand, *M. aeruginosa* laboratory strains appear to be less resistant than environmental strains since they mainly exist as individual cells and tend not to form colonies (FAN *et al*., 2016) or produce as much mucilage as the *M. aeruginosa* from freshwater environments (ZHANG *et al.*, 2007)

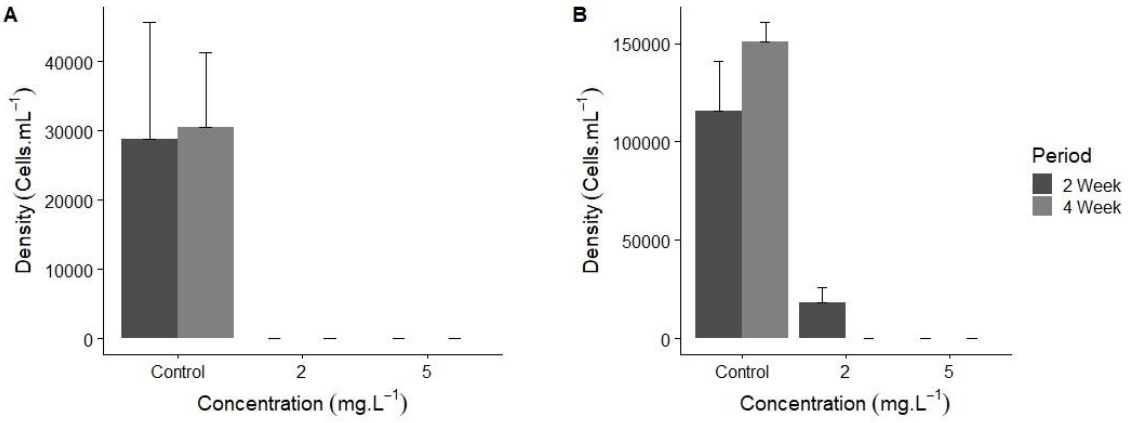
Wang *et al.* (2019) performed an experiment with a *M. aeruginosa* strain using different concentrations of H_2O_2 to examine the changes in cyanobacteria biomass for a period of nearly three days. The concentration of 5.4 $mg.L^{-1}$ effectively reduced the percentage of cyanobacteria biomass from the total phytoplankton biomass, going from around 90% to close to 10%. In contrast, there are not many recent studies evaluating the control of *D. circinale* species.

Velzeboer *et al.* (1995) compared the effect of copper and aluminum sulfate in a strain of *D. circinale* (former *Anabaena circinalis*). A dose of 0.25 $mg.L^{-1}$ of copper sulfate did not leave any visible cells after four hours while a dose of 0.10 $mg.L^{-1}$ of aluminum sulfate did not cause a significant reduction in *D. circinale* cell density. These

experiments did not evaluate the cell regrowth potential and, when they did, they did for a shorter period (24 hours) when compared to our method.

Lin *et al*. (2009) studied the effects of chlorination on the cell integrity of the *M. aeruginosa* and *D. circinale* laboratory strains. They found that the cell integrity of both species was highly affected by chlorination, but the cells rupturing rate of *D. circinale* was up to five times higher than that for *M. aeruginosa*, showing that *D. circinale* is more susceptible to oxidation than the *M. aeruginosa*. Another study that supports this fact was performed by Zamyadi *et al*. (2012) who also evaluated chlorination effects on cell integrity of *M. aeruginosa* and *D. circinale* strains. They found that *M. aeruginosa* strain needed a contact time higher than 31 mg.min.L^{-1} to affect the integrity of 99% of its cells, on the other hand, to achieve a similar effect with *D. circinale* strain a contact time of approximately 8 mg.min.L^{-1} was needed.

Figure 15- Cell density on the 2 and 4-week period after the application of H_2O_2 at 2 and 5 mg.L^{-1} for (A) *D. CIRCINALE* and (B) for *M. AERUGINOSA*.

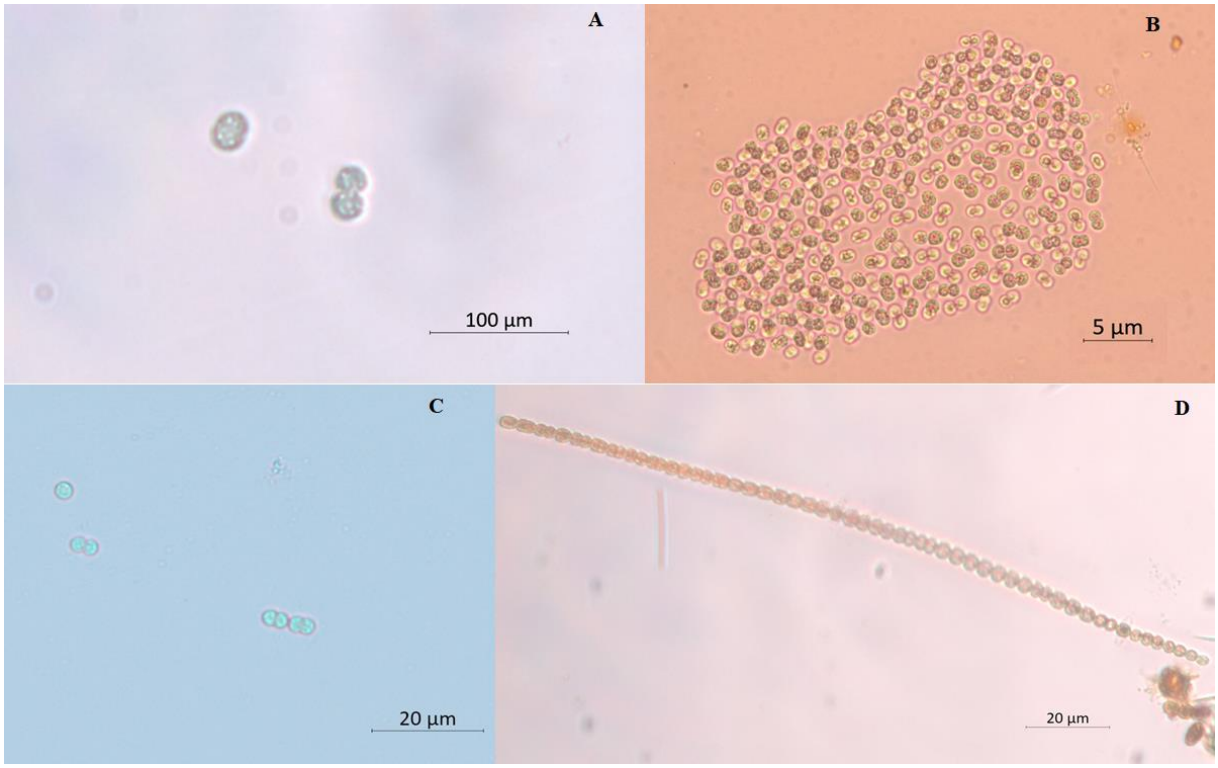


However, this set of experiments was not conclusive, since there are many differences between cyanobacteria from laboratory strains and freshwater environments, as mentioned before. In Figure 16B is possible to see how *M. aeruginosa* from a freshwater environment forms dense colonies which can increase their buoyancy and floating velocity, besides acting as a protective layer from environmental stresses (Reynolds *et al*., 1981; Fan *et al*., 2016). Also, *D. circinale* from our laboratory strain (Figure 16C) present small trichomes and it has no differentiated cells that are present at the trichomes that exist in Gaviao reservoir (Figure 16D). Those trichomes present heterocysts – which are able to fix molecular nitrogen (akinetes) sustain long-term

dormancy and enable DC to survive under extreme conditions such as dry seasons, and finally, both species have gas vesicles that allows them to have buoyancy regulation.

Because of the differences between cultivated and environmental strains, we decided to perform a regrowth experiment in a microcosm with freshwater from Gaviao reservoir, in order to verify if the cyanobacteria that dominate this water are able to regrow after the application of H_2O_2. To answer this question, we decided to use a similar methodology from the regrowth experiment presented above.

Figure 16 – Differences between cyanobacteria between environment and laboratory cultivated strains. (A) the laboratory strain of *M. aeruginosa* used in this study. (B) *M. aeruginosa* colony from Gaviao reservoir. (C) the laboratory strain of *D. circinale* used in this study. (D) a *D. circinale* trichome from Gaviao reservoir.



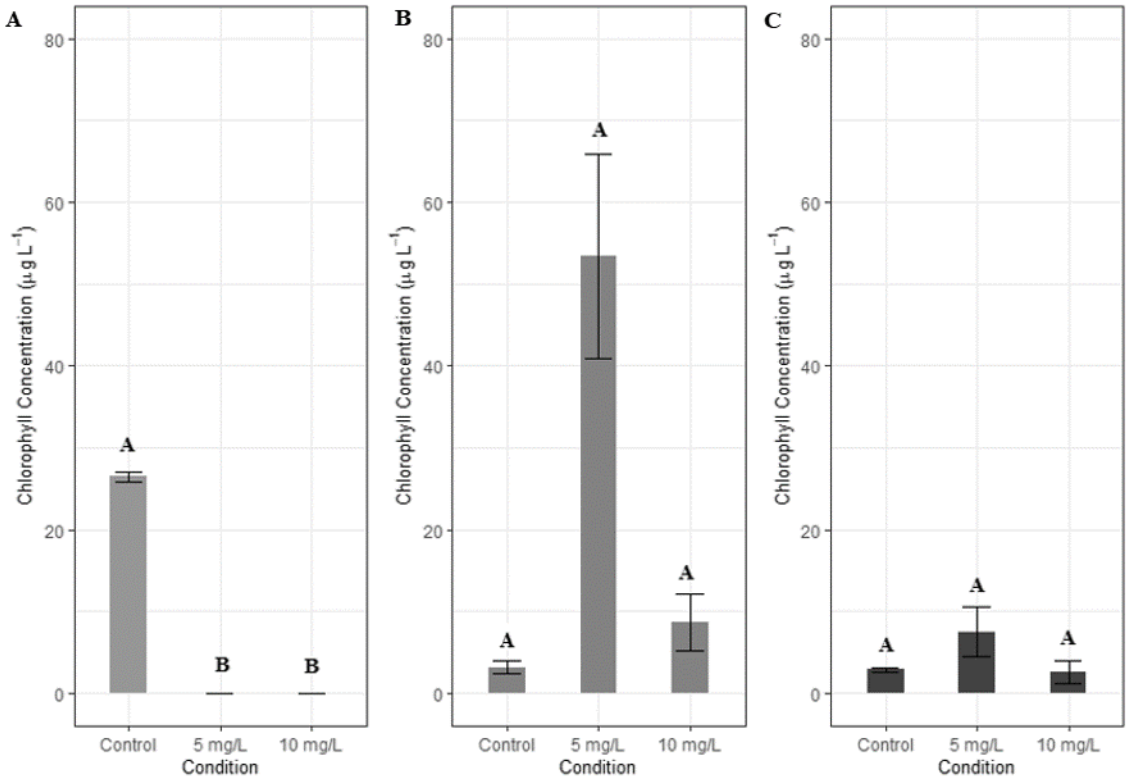
Source: SELAQUA laboratory.

9.2 Phytoplankton regrowth analysis using pigments as a parameter

The changes in chlorophyll concentration of cyanobacteria, green algae and diatoms were analyzed in order to evaluate the effect of H_2O_2 after the regrowth experiment. Using this parameter, the concentrations of 5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ successfully suppressed cyanobacteria recovery for seven days (Figure 17A). As for the green algae and the diatoms, the H_2O_2 did not significantly interfere with their regrowth (Figure 17B and Figure 17C), since their chlorophyll concentrations were not significantly different from the control samples ($p > 0.05$).

Fan *et al.* (2019) performed a mesocosm study in a Chinese lake evaluating how a H_2O_2 concentration of 10 $mg.L^{-1}$ controlled cyanobacteria for a 15-day period. They evaluated the phycocyanin concentration, associated with the cyanobacteria community, and the chlorophyll-*a* concentration, associated with other phytoplankton organisms. The results showed that the chlorophyll-*a* concentration remained low for six days but then recovered to a level close to the control samples. On the other hand, phycocyanin remained low for the entire 15 days of the experiment. Which corroborates the results obtained for our study, and confirms that cyanobacteria are more sensitive to the H_2O_2 when considering their recovery capacity.

Figure 17 – Chlorophyll concentration of the phytoplankton community after the application of 5 and 10 $mg.L^{-1}$ and the regrowth experiment. (A) cyanobacteria. (B) green algae. (C) diatoms. Similar letters presented over each bar graph means that there is no significant difference ($p > 0.05$) between the chlorophyll concentration.



Yang *et al.* (2018) observed the effect of different concentrations of H_2O_2 (ranging from 0.3 to 24.3 $mg.L^{-1}$) on the phycocyanin concentration and the photosynthetic activity of the cyanobacteria *Dolichospermum, Cylindrospermopsis, Planktothrix,* and *Microcystis.* The concentrations of 0.3 and 0.9 $mg.L^{-1}$ did not effectively control the phycocyanin concentration and did not decrease the

photosynthetic activity during the entire five-days experiment. However, for concentrations higher than 0.9 $mg.L^{-1}$, H_2O_2 was able to control phycocyanin of the filamentous cyanobacteria (*Dolichospermum, Cylindrospermopsis,* and *Planktothrix*) and decrease their photosynthesis, while for *Microcystis,* only concentrations higher than 8.1 $mg.L^{-1}$ were able to control their phycocyanin for the entire experiment. Phytoplankton responds differently to the concentrations of H_2O_2 when considering their regrowth. With that in mind, the third set of experiments was designed to study the regrowth using the cell density count on a genera level.

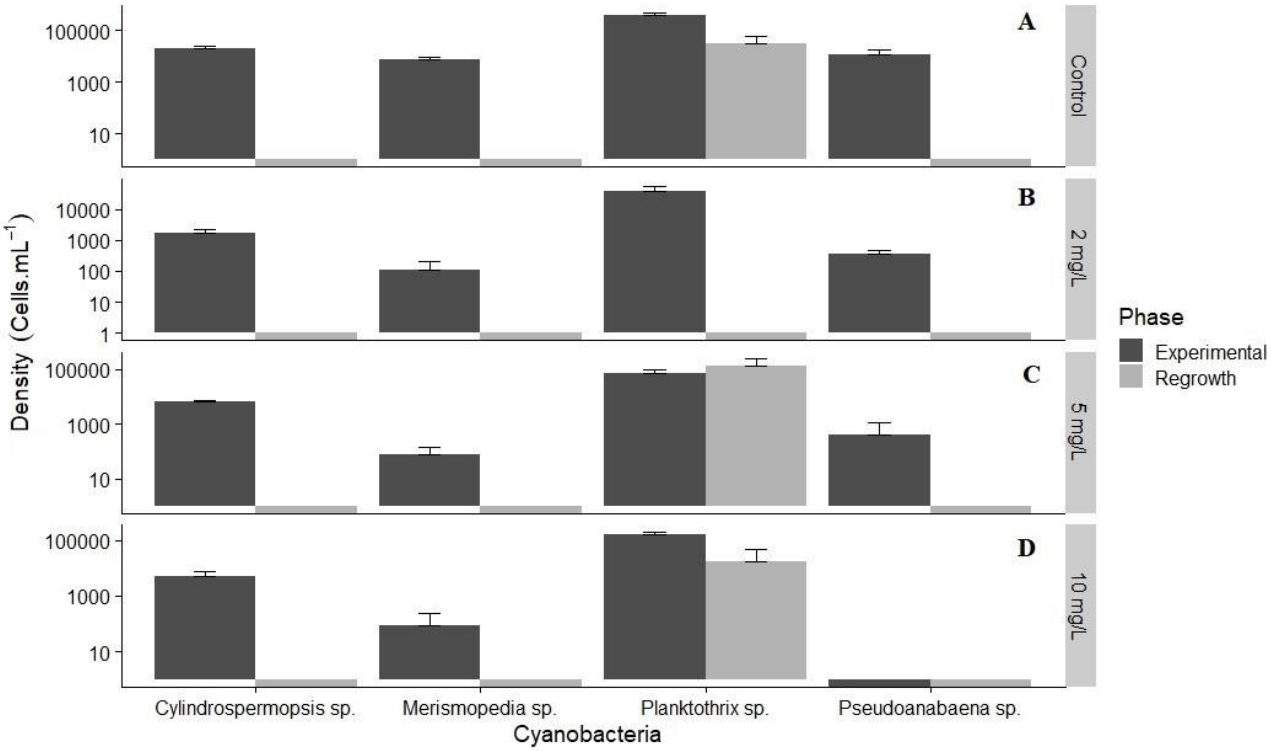
9.3 Analysis of phytoplankton regrowth using cell density

The primary aim of this third set of experiment was to apply the same methodology of the two sets of experiment presented previously, but this time considering the cell densities from different organisms. The cell counts were performed at two periods: two days after the application of the H_2O_2 and four weeks after the application of H_2O_2. The doses applied were: 2 $mg.L^{-1}$, 5 $mg.L^{-1}$, and 10 $mg.L^{-1}$.

Analyzing the cyanobacteria regrowth (Figure 18) it is possible to see how the stress of the experiment itself interfered on the cyanobacteria cell density. Since in the control samples (0 $mg.L^{-1}$ of H_2O_2) the cell densities of *Cylindrospermopsis sp.*, *Merismopedia sp.,* and *Pseudanabaena sp.* decreased from around 10^4 $cells.mL^{-1}$ to almost an undetectable level (Figure 18A). Which was different from the behavior of *Planktothrix sp.* that appeared not to suffer as much stress as the other cyanobacteria species, since its cell density decreased from 10^5 to near 10^4 $cells.mL^{-1}$. This tendency observed in the control samples occurred similar to the samples with the H_2O_2 affect; except for the 2 $mg.L^{-1}$ samples (Figure 18B).

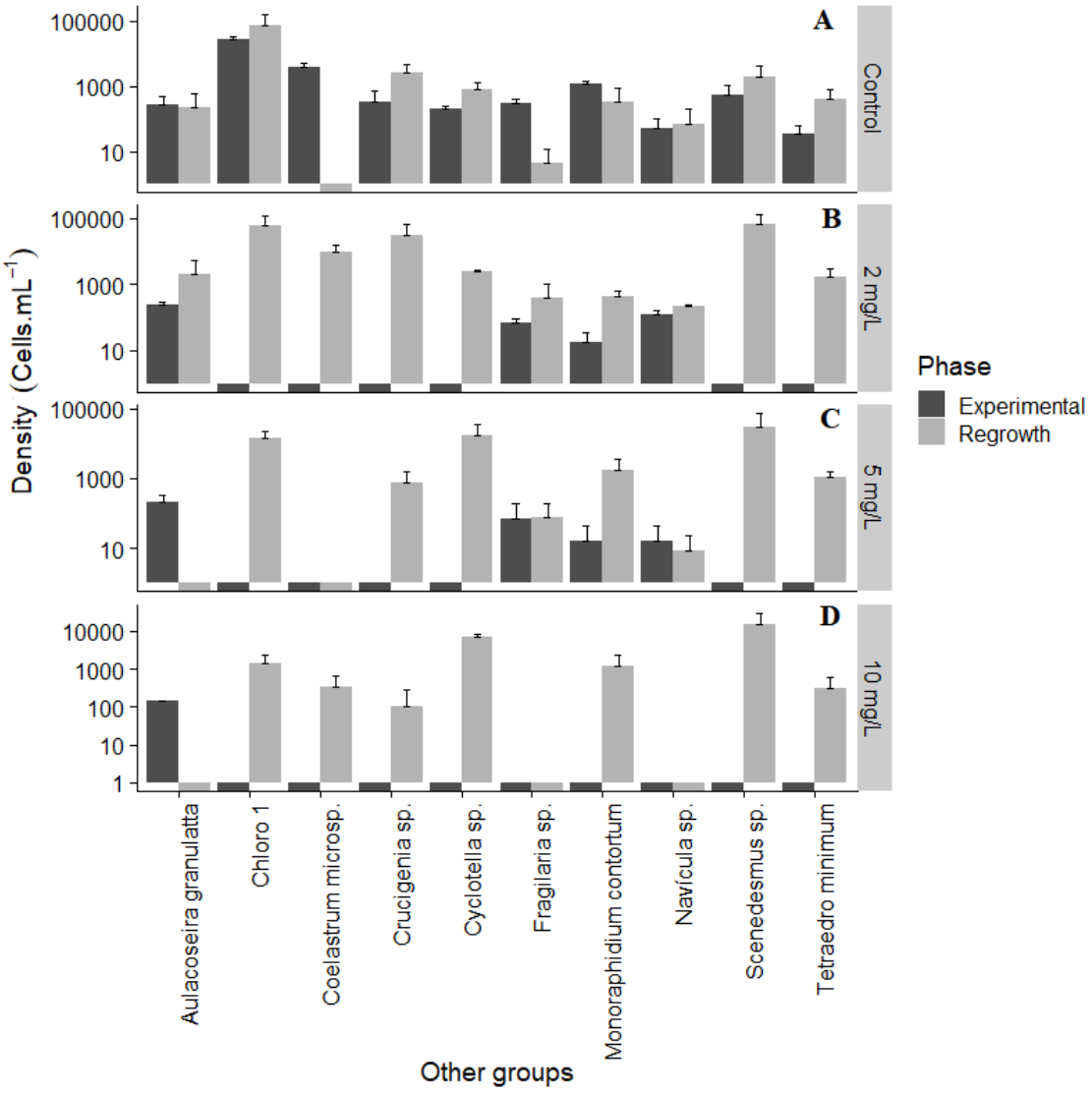
In the 2 $mg.L^{-1}$ samples all cyanobacteria genera, including *Planktothrix sp.* decreased to an almost undetectable level. This can be explained by a higher dominance of the other phytoplankton organisms, for example the species *Coelastrum microsporum* and the non-identified species *Chloro 1* (Figure 19). Highlighting the species *Coelastrum microsporum* that was almost undetectable in all samples, both for the experimental and regrowth phase, except in the samples with the 2 $mg.L^{-1}$ concentration, were it had a cell density close to 10^4 $cells.mL^{-1}$. This prominent competition, allied to the application of H_2O_2 could explain why the genus *Planktothrix sp*. presented this high decrease on its cell density.

Figure 18 – Cell density changes in the different cyanobacteria genera due to different doses of hydrogen peroxide. The experimental bars are form samples taken two days after the application of H_2O_2 and the regrowth bars are four weeks after the application of H_2O_2 (A) presents the changes in the control. (B) the changes in the 2 mg.L^{-1} concentration. (C) the changes in the 5 mg.L^{-1} concentration. (D) the changes in the 10 mg.L^{-1} concentration



Examining the regrowth of the green algae and diatoms considered in this study (Figure 19), the concentrations of 2 mg.L^{-1} and 5 mg.L^{-1} enabled more their regrowth than the concentration of 10 mg.L^{-1}. This probably happened because H_2O_2 causes killing of cyanobacteria and it results in a temporary increase of the nutrient availability in the water, which becomes a food source for the other taxa that were not affected by it (WEENINK *et al.*, 2015). However, *Aulacoseira granulata* and *Navicula sp.* presented a reduced regrowth potential in the samples with 5 and 10 mg.L^{-1}, where it decreased its cell density from 10^3 cells.mL^{-1} to undetectable. This contradicts what O'farrell; Tell and Podlejski, (2001) reported about *Aulacoseira granulata* of how this species can adapt to stresses in the environment due to its short life cycle in addition to its capacity to increase biomass when cyanobacteria competition is eliminated (YANG *et al.*, 2017). This may have happened because diatoms need more carbon than other phytoplankton, and, since green algae organisms increased their cell density, the *Aulacoseira granulata* did not have as much nutrient as it needed, thus presenting a reduced regrowth potential (CHRACHRI *et al.*, 2018).

Figure 19 - Cell density changes in the different green algae and diatom genera due to different doses of hydrogen peroxide. (A) presents the changes in the control. (B) the changes in the 2 mg.L^{-1} concentration. (C) the changes in the 5 mg.L^{-1} concentration. (D) the changes in the 10 mg.L^{-1} concentration



Comparing the resistance to the environmental stress caused by the microcosm regrowth experiment itself, it is possible to see in Figure 18A and Figure 19A how the cyanobacteria cell density in the control samples changed visibly more than the green algae and diatoms. Where even the most resistant analyzed cyanobacteria (*Planktothrix sp.*) presented a decrease close to one log in its cell density, when comparing the experiment and the regrowth analysis in the control samples. The explanation for that can be associated with the experimental set up used in this study.

One possible factor that interfered with the regrowth analysis was the limited volume. The jars used for this study had the capacity of 1.5 L. Since this volume was much limited than the natural environment where the organisms lived, this probably

disturbed the balance in the community. Mainly because the jars could not provide a stratified environment, which is known to favor cyanobacteria blooms in freshwater environments (YANG *et al.*, 2017). Fact also corroborated by Yang *et al.* (2016), that mentions how cyanobacteria have more advantages in the competition with green algae and diatoms in warmer and more stratified environments. For instance, *Planktothrix sp.* uses vertical migration along the water column to control the light-harvesting processes (KURMAYER, DENG; ENTFELLNER, 2016) or even *Microcystis sp.* which uses its mucilaginous layer in their colonies to search for nutrient-rich places in the water column (REYNOLDS, 2007).

This limitation could be solved with two options. The first one is, when the regrowth experiment is performed for more than 20 days, it is performed at a mesocosm scale, which provides conditions closer to the freshwater environment and, therefore, would decrease the stress caused in the phytoplankton (YANG *et al.*, 2018). Another possible alternative is to use a experimental set up with a bigger capacity than the one used in this present study greater than 1.5 L.

However, the factor that possibly interfered the most with this set-up of the regrowth experiment was the light intensity used. This factor is known to interfere, along the nutrient availability changes, in the shift in the phytoplankton community (YANG *et al.*, 2016) Since we wanted to use a similar methodology to the previous mentioned set-ups of this study, a light intensity of 10 $\mu mol.m^{-2}\ s^{-1}$ was used, intensity also used in the laboratory of cultivation of cyanobacteria. Even though effective for the survival of the laboratory cyanobacteria strains, for the regrowth experiment at the seven-day period using chlorophyll, this light did not seem to provide an ideal environment for the balance in the phytoplankton community.

Some studies evaluated the interference of H_2O_2 on the phytoplankton using UV-light (BARRINGTON; REICHWALDT; GHADOUANI, 2013), used a light intensity of around 40 $\mu mol.m^{-2}\ s^{-1}$ in their experimental set-up, which is higher than the one used in our experimental set-up.

Another alternative is to perform the experiment under natural sunlight as in the experiment performed by Xu *et al.* (2019). Where they monitored the percentage of live cells for two days after the application of H_2O_2 and all concentrations used (ranging from 3 to 20 mg.L-1) reduced this percentage to nearly undetectable for the samples treated with H_2O_2 under direct sunlight, with a light intensity up to 1800 µmol photons

m^{-2} s^{-1}. This alternative is the most common found in the literature (MATTHIJS *et al.*, 2012; FAN *et al.*, 2019; XU *et al.*, 2019).

Chen *et al.* (2016) actually used a light intensity of 10 µmol.m^{-2} s^{-1} but to simulate the *in-situ* conditions at the bottom of the lake that the studied cyanobacteria were sampled. Then, after a three-day period to allow the H_2O_2 to act, they increased the light intensity to 40 µmol.m^{-2} s^{-1} for the regrowth experiment, which lasted seven days. However, *Planktothrix sp.* was the least affected cyanobacterium when comparing the control samples from the experiment and from the regrowth stages (Figure 18A). This may be due to the fact that *Planktothrix sp.* requires low energy values to maintain its metabolism, therefore, it can sustain a stable growth rate in low light intensities, which is the case of this study (KURMAYER, DENG; ENTFELLNER, 2016).

Therefore, it is important to improve the method presented in this study of phytoplankton regrowth evaluation after the application of H_2O_2, which can be achieved by incorporating the solutions presented above. This can improve the understanding of the effectiveness of H_2O_2 as an *in-situ* pre-treatment to control cyanobacteria blooms thus helping the water treatment plants.

10 CONCLUSION

This study presented different evaluations of phytoplankton regrowth after the application of different doses of H_2O_2. First, using the laboratory strains of *D. CIRCINALE* and *M. AERUGINOSA*, the application of H_2O_2 has proven to be efficient in the suppression of *D. CIRCINALE* and *M. AERUGINOSA* even after four weeks of the application.

The method used for the laboratory strains regrowth experiment was adapted to a similar experiment but with the Gaviao reservoir water, using chlorophyll from cyanobacteria, green algae, and diatoms as a parameter of H_2O_2 effectiveness. After seven days, the application of 5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ proved efficient in reducing the cyanobacteria chlorophyll concentrations to an undetectable value, but it did not interfere significantly with the recovery of green algae and diatoms. Once more proving that the H_2O_2 treatment was effective to control cyanobacteria without significantly harming the other phytoplankton community.

Finally, applying the same regrowth methodology, but for a longer period (four weeks) and using cell density counts, the impact caused by the experiment itself proved to be high, even without the application of H_2O_2. The cyanobacteria cell densities decreased at the same level as the one caused by the application of the H_2O_2. However, after applying the modifications suggested, the methodology presented in this study has great potential on evaluating, in a microcosm scale, how long can the H_2O_2 treatment control the cyanobacteria and if it promotes changes in the dominance dynamics in the environment.

11 CONCLUSIONS

The impact caused by H_2O_2 on cyanobacterial chlorophyll was significant, but not significant in the green algae and diatoms. This fact supports the idea that cyanobacteria are more sensitive to the H_2O_2. However, when analyzing the impact on phytoplankton cell density, our study was not conclusive. The lowest concentration used (2 $mg.L^{-1}$), presented the highest impact on cyanobacteria cell density, different from the expected. Even though significant, this cell density reduction did not decrease to a level recommended by Cyanobacteria and cyanotoxins Brazilian guidelines.

The filamentous cyanobacterium that dominates the Gaviao reservoir – *Planktothrix sp*. – presented high resistance to oxidation by H_2O_2. This species needs to be further investigated, since it can survive the treatment and, without competition, it can bloom.

In addition, the results obtained in the cell density counts indicate that the H_2O_2 may need a longer contact time to effectively impact cyanobacteria or that microscopy is not suitable to evaluate the oxidant effect for 48h contact time. It was possible to conclude using PCA that even though the control samples were significantly different from the samples with H_2O_2, the different doses of H_2O_2 did not cause significantly different effects amongst them.

The regrowth of the laboratory strains of *D. circinale* and *M. aeruginosa* was successfully controlled by the application of H_2O_2 for a thirty day period. However, the method used for the regrowth experiment still needs improvement so it can evaluate the regrowth of the phytoplankton from Gaviao reservoir. Analyzing cell density counts, the impact caused by the experiment itself proved to be high, even without the application of H_2O_2. The cyanobacteria cell densities decreased at the same level as the one caused by the application of the H_2O_2. The factors that probably interfered were the small experimental volume, the light intensity, but the methodology used in this study, after the modifications suggested, may help to evaluate the behavior of the phytoplankton community in full-scale reservoirs after the application of H_2O_2, providing better comprehension of the impacts of the H_2O_2 application on the phytoplankton.

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