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Differential Effect of Hydroxen Peroxide on Toxic Cyanobacteria of Hypertrophic Mediterranean Waterbodies

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Abstract: Cyanobacterial blooms have been known since ancient times; however, they are currently increasing globally. Human and ecological health risks posed by harmful cyanobacterial blooms have been recorded around the world. These risks are mainly associated with their ability to affect the ecosystem chain by different mechanisms like the production of cyanotoxins, especially microcystins. Their expansion and their harmful effects have led many researchers to seek techniques and strategies to control them. Among them, hydrogen peroxide could be a promising tool against cyanobacteria and cyanotoxins and it is well-established as an environmentally friendly oxidizing agent because of its rapid decomposition into oxygen and water. The aim of the present study was to evaluate the effect of hydrogen peroxide on phytoplankton from two hypertrophic waterbodies in Greece. The effect of hydrogen peroxide on concentration of microcystins found in the waterbodies was also studied. Treatment with 4 mg/L hydrogen peroxide was applied to water samples originated from the waterbodies and Cyanobacterial composition and biomass, phycocyanin, chlorophyll-a, and intra-cellular and total microcystin concentrations were studied. Cyanobacterial biomass and phycocyanin was reduced significantly after the application of 4 mg/L hydrogen peroxide in water treatment experiments while chlorophytes and extra-cellular microcystin concentrations were increased. *Raphidiopsis (Cylindrospermopsis) raciborskii* was the most affected cyanobacterial species after treatment of the water of the Karla Reservoir in comparison to *Aphanizomenon favaloroi*, *Planktolyngbya limnetica*, and *Chroococcus* sp. Furthermore, *Microcystis aeruginosa* was more resistant to the treatment of Pamvotis lake water in comparison with *Microcystis wesenbergii* and *Microcystis panniformis*. Our study showed that hydrogen peroxide differentially impacts the members of the phytoplankton community, affecting, thus, its overall efficacy. Different effects of hydrogen peroxide treatment were observed among cyanobacerial genera as well as among cyanobacterial species of the same genus. Different effects could be the result of the different resistance mechanisms of each genus or species to hydrogen peroxide. Hydrogen peroxide could be used as a treatment for the mitigation of cyanobacterial blooms in a waterbody; however, the biotic and abiotic characteristics of the waterbody should be considered.

Keywords: harmful cyanobacterial blooms; microcystins; hydrogen peroxide; eutrophic waterbodies

Citation: Papadimitriou, T.; Katsiapi, M.; Stefanidou, N.; Paxinou, A.; Poulimenakou, V.; Laspidou, C.S.; Moustaka-Gouni, M.; Kormas, K.A. Differential Effect of Hydroxen Peroxide on Toxic Cyanobacteria of Hypertrophic Mediterranean Waterbodies. *Sustainability* **2022**, *14*, 123. https://doi.org/10.3390/su14010123

Academic Editor: Agostina Chiavola

Received: 30 November 2021
Accepted: 20 December 2021
Published: 23 December 2021

1. Introduction

Cyanobacterial blooms were first described in 1878 [1] and from then until today, mass cyanobacterial blooms have been reported in eutrophic lakes and reservoirs worldwide [2–4]. Major consequences of the global expansion of cyanobacterial blooms are associated with threats to the safety and sustainability of water supplies for human consumption, agriculture (irrigation), inland fisheries, as well as the recreational and ecological value of

impacted waters [5]. Moreover, cyanobacteria possess a range of unique and highly adaptable eco-physiological traits which enable them to dominate over other phytoplankton groups: the ability to grow in warmer temperatures, buoyancy, high affinity for and ability to store phosphorus, nitrogen-fixation, akinete production and associated life history characteristics, and light capture at low intensities and a range of wavelengths [6,7].

Another trait of cyanobacteria is the production of toxins. Scientific research has shown that 75% of the water containing cyanobacteria also contains toxic cyanobacterial metabolites [8]. The microcystins (MCYSTs), as the most cosmopolitan cyanotoxins, are accused of degrading water quality and also public health [9,10]. More than 90 variants of MCYSTs have been detected [11]. It is known that MCYSTs are hepatotoxins that act in the intense inhibition of intracellular serine/threonine phosphatases 1 and 2A (PP1 and PP2A) [12]. Because of this inhibition, an imbalance of cell phosphorylation occurs, culminating in a cell signaling disorder [13]. In addition, MCYSTs can cause oxidative stress due to the intracellular excess of reactive oxygen species (ROS) [14,15].

Cyanobacteria can influence the structure and dynamics of an aquatic ecosystem especially if the intensity and duration of cyanobacteria blooms increase along with the proceeding eutrophication [5]. The effect of cyanobacteria on grazers has generally been negative because of the toxins they produce, but also because cyanobacteria are difficult to manage because of their colonial form and because they have a low nutritional value [5]. Fish seem to be more sensitive to cyanobacteria than invertebrates [5]. Hepatotoxic MCYSTs can damage the gill structures of fish, exposing them to other dissolved toxins in the water [16]. Moreover, the oxidative stress caused by microcystins can also affect fish at the cellular level [17]. The negative effects on fish can also be caused by the low nutritional quality of cyanobacteria [5]. Community-level changes in fish populations may be possible as well. Decreased feeding, condition, and growth of fish larvae probably affect their survival rates, potentially leading to variations in year-class strength [18].

Numerous studies have shown that climate change probably favors the further expansion of cyanobacterial blooms [3,19,20], leading many researchers to find techniques and strategies to control them. The root cause of cyanobacterial blooms is usually nitrogen and phosphorous loading, so watershed management to reduce nutrient pollution of a waterbody is an effective strategy to prevent and mitigate cyanobacterial blooms [21]. However, such a solution is often difficult, expensive, and time consuming [22]. Biologically-driven control may be able to target cyanobacteria, although it poses a risk of release of toxins from cells after treatment and its toxicity towards other non-target species and organisms is unknown [23].

The hydrogen peroxide (H_2O_2) treatment has been proposed as an effective option of controlling cyanobacterial bloom formation [24,25]. Since H_2O_2 rapidly decomposes to H_2O and O_2 via biological, chemical, and photochemical mechanisms during oxidation, it does not leave harmful residues in the environment. H_2O_2 inhibits photosynthetic electron transfer in Photosystem II by inhibiting the ascorbate peroxidase system of hydrogen peroxide detoxication [26]. Because cyanobacteria are prokaryotic, H_2O_2 has an even more pronounced effect on cyanobacterial cells than eukaryotic phytoplankton groups, whose photosynthetic apparatus are contained within discrete organelles [27]. The application of H_2O_2 on cyanobacteria results in the lysis of their cells. When cyanobacterial cells are lysed, the possible intra-cellular cyanotoxins will be released. The fate of released MCYSTs after H_2O_2 treatment of natural cyanobacterial populations has only been studied in a few investigations and the results are conflicting [25,28–30]. It has been suggested that, under natural conditions, the amount of MCYSTs released after cell lysis induced by H_2O_2 may be reduced by environmental factors, such as microbiological degradation, UV radiation, photosensitized transformation in the presence of humic substances and pigments, or adsorption to particles [31]. However, more studies on degradation of MCYSTs in waterbodies are required since degradation of MCYSTs is essential for the proper functioning of waterbodies and for the protection of public health.

The potential of H_2O_2 to control cyanobacteria has been studied by several researchers. Drabkova et al. [27] showed that cyanobacteria were negatively affected by H_2O_2 at concentrations 10 times less than that of green algae and diatoms. Matthijs et al. [32] showed that H_2O_2 can be used to selectively suppress cyanobacteria in natural waters without affecting other organisms. Yang et al. [33] concluded that H_2O_2 treatment promotes chlorophytes over toxic cyanobacteria in a hyper-eutrophic aquaculture pond. They also showed that H_2O_2 treatment was less effective in the degradation of *Microcystis* compared with *Anabaena*, *Cylindrospermopsis*, and *Planktothrix* under culture conditions. Most studies used laboratory strains to evaluate the performance of H_2O_2 [34–36]. These cultured strains might pose present different adaptations to the new environment when compared to the cyanobacteria living in natural environments, and thus their observed response to H_2O_2 may be not realistic under natural conditions [6]. Studies related to the effect of H_2O_2 on different cyanobacterial genera or species of natural communities are rather scare [37,38]. Since cyanobacterial algal blooms can be dominated by one or more cyanobacterial taxa, it would be useful to know if the effects of H_2O_2 are genus and/or species dependent in natural communities. The aim of the present study was to evaluate the possible differential effect of H_2O_2 on phytoplankton communities, in abstracted water from a hypertrophic lake and a hypertrophic reservoir, suffering from prolonged cyanobacterial blooms. Furthermore, the effects of H_2O_2 on MCYSTs concentrations in the abstracted waters were also evaluated.

2. Materials and Methods

2.1. Study Areas

To study the effects of H_2O_2 on phytoplankton focusing on cyanobacteria and microcystins, water samples were collected from two different waterbodies: Karla Reservoir (39°29′27″ N 22°49′19″ E) and Lake Pamvotis (39°40′0″ N and 20°52′60″ E) (Figure 1).

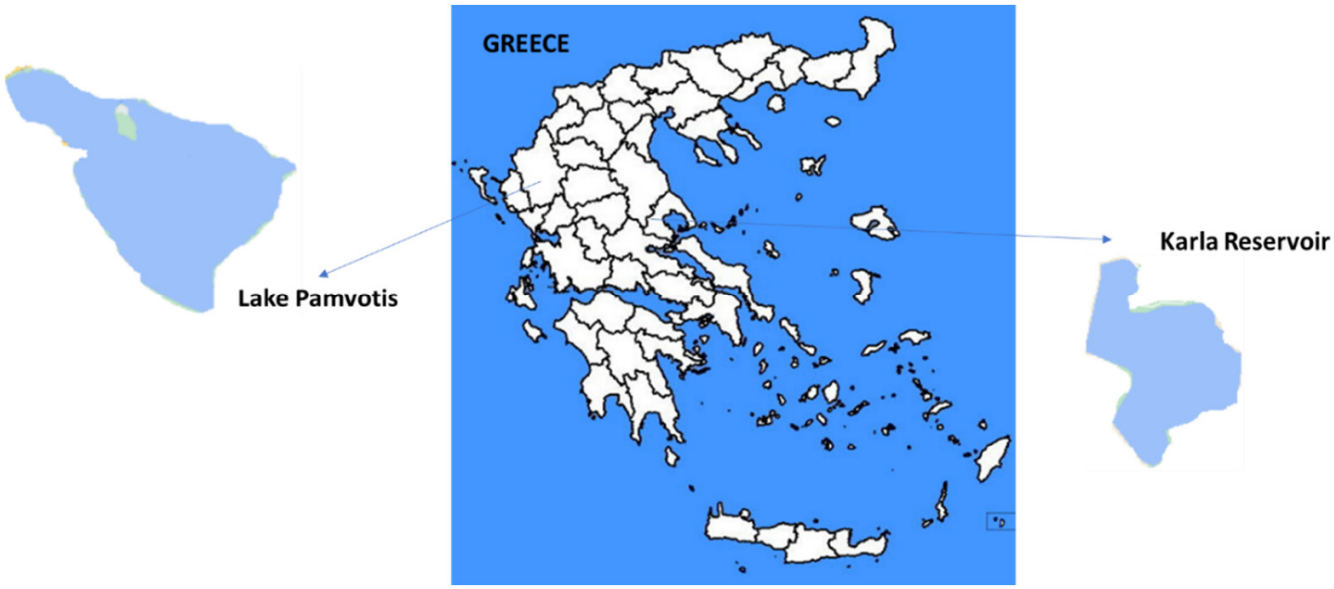

Figure 1. Map of Greece with study areas (Karla Reservoir and Lake Pamvotis) by Google Earth.

Karla Reservoir used to be a natural lake with important biodiversity; however, the lake was intentionally dried in 1960s. The reconstruction of Karla Reservoir in 2010 was a public request for the local area to be upgraded environmentally, socially, and economically. The reconstructed reservoir has a surface of 38 km^2, while its maximum depth has not exceeded 2 m. Karla Reservoir has already been adversely affected by both agricultural and industrial pollution from the surrounding area resulting to eutrophic-hypertrophic conditions. During the time from re-establishment until now, extensive cyanobacterial and algal blooms were recorded, dominated by toxin-producing species [39,40]. During the

same period, episodes of fish and bird mortality were also recorded [40,41]. Water from the Karla Reservoir is used for irrigation needs for the surrounding cultivations and is planned to be used for supply needs for the city of Volos. Recent studies showed that the cyanotoxins from the Karla Reservoir are transferred via irrigation to edible vegetables [42,43].

Pamvotis is an urban, shallow eutrophic-hypertrophic lake (mean depth, 4.3 m, maximum depth of 7.5 m) and occupies an area of 22.8 km^2 [44]. During the last three decades, Pamvotis Lake was exposed to many activities such as irrigation, discharge of domestic sewages, and sediment deposit, affecting its trophic status [44,45]. Cyanobacterial blooms have been recorded almost throughout the year, whereas high concentrations of microcystins have been recorded in water (0.29–15.83 μg/L) and tissues of animals (45.35–1200 ng/g) living in the lake [46]. Water from Lake Pamvotis is used for recreation, fisheries, and irrigation.

2.2. Design of the Experiment

The experimental design consisted of the following steps:

Step 1: Sampling and water sample analysis. Water samples were collected from Karla Reservoir and Lake Pamvotis (200 L from each waterbody) on 17 September 2017. Water was collected in polyethylene containers, which were placed 10–20 cm below the water surface. The containers were slowly immersed in the water so that the surface of the water was disturbed as little as possible. Water samples were transferred to the laboratory within 3 h after sampling. At each sampling site, water temperature, pH, dissolved oxygen (the in situ oxygen saturation was measured in the morning), and conductivity were measured in situ by electrode probes (YSI, Yellow Springs, OH, USA). Concentrations of nitrate, nitrite, ammonium, and soluble reactive phosphorus (SRP) in the water of the Karla Reservoir and Lake Pamvotis were analyzed according to standard methods [47]. Before the H_2O_2 treatment (time zero), sub-samples were collected from the containers and initial phytoplankton composition, chlorophyll–a, phycocyanin, and extra-cellular and intracellular MCYSTs were analyzed.

Step 2: Treatment of the samples with H_2O_2. Sixty L of water collected from Karla Reservoir and 60 L of water collected from Lake Pamvotis were placed into transparent jars. The experiment consisted of two conditions, with three replicate jars each: a control (0 mg/L H_2O_2) and 4 mg/L H_2O_2 treatment achieved by the addition of a 30% *w*/*w* H_2O_2 solution. Jars were put in an incubation cabinet (50 μmol photon/m^2/s) and temperature of 24 ± 1 °C. A light/dark cycle of 12 h:12 h was used. An air pump was placed in each jar and jars were mixed daily by hand. The experiment lasted six days. Subsamples were collected from each jar at 5, 72, and 144 h. Subsamples were used for phytoplankton identification and biovolume determination as well as for the analysis of chlorophyll-a, phycocyanin, and intra-cellular and extra-cellular microcystins concentrations. Our previous results showed that a dose of 4 mg/L H_2O_2 resulted in a significant decrease of phycocyanin concentration in samples of the Karla Reservoir [48] and doses higher than 4 mg/L had a negative impact on the dynamics of zooplankton living in the Karla reservoir (unpublished data). Therefore, we chose the concentration of 4 mg/L H_2O_2 as the appropriate concentration of H_2O_2 to be used in the present study.

Step 3. Hydrogen peroxide degradation assay. In order to assess whether there is a difference between H_2O_2 degradation in a sample that contains phytoplankton and one that does not, we filtered (GF/C filter, nominal filtering size of 1.2 μm) water samples (5 L each) from the Karla Reservoir and Lake Pamvotis and added H_2O_2 to a concentration of 4 mg/L. The jars containing the filtered water samples were used to assess the chemical degradation of H_2O_2 over time and were incubated under the same conditions as step 2. Subsamples of 50 mL were collected from each jar (filtered and unfiltered) at the beginning (t = 0) and at 30, 90, 180, and 300 min after the addition of H_2O_2. Two replicates were included. Subsamples were only analyzed for H_2O_2 concentration. H_2O_2 concentrations were measured according to Allen et al. [49], who used the spectrophotometric determination of triiodide complex for

the detection of H_2O_2 concentrations. Degradation rate of H_2O_2 was calculated according to the following equation:

$$\text{Rate} = -\Delta[H_2O_2]/\Delta t \text{ (mg/L/min)}$$

2.3. Phytoplankton Analysis

Live and preserved samples were examined in sedimentation chambers using an inverted microscope with phase contrast (Nikon SE 2000). Phytoplankton in composite samples of three replicates were counted. Phytoplankton species were identified using taxonomic keys [50]. Phytoplankton counts (cells, colonies, and filaments) were performed using the Utermöhl sedimentation method [51]. For biovolume estimation, the dimensions of 30 individuals (cells, filaments, or colonies) of the dominant species were measured a digital microscope camera (Nikon DS-L1), while mean cell or filament volume estimates were calculated using the appropriate geometric formulae [52].

2.4. Pigment Analysis

To analyze pigments, the water subsamples were processed at low-intensity light.

2.4.1. Chlorophyll-a (Chl a) Analysis

To determine the concentration of Chl a (µg/L), a volume of 200 mL of water subsamples was passed through Whatman GF/C filters (0.45-µm) in vacuum. Chl- a was then extracted from the filter with 95% acetone solution [47]. The solution absorbance was then recorded at 630 nm, 647 nm, 664 nm, and 750 nm [47]. The final concentration of Chl- a was determined according to the equation of Jeffrey and Humphrey [53]:

$$\text{Chla} = \text{Ca} * \text{Vex} * \text{Vsample}^{-1},$$

where,

$$\text{Vex} = \text{measured sample volume}$$

$$\text{Vsample} = \text{volume of sample filtered}$$

and

$$\text{Ca} = 11.85 * (\text{OD'664}) - 1.54 * (\text{OD'647}) - 0.08 * (\text{OD'630})$$

where

$$\text{OD'664} = \text{Absorption 664} - \text{Absorption 750}$$

$$\text{OD'647} = \text{Absorption 647} - \text{Absorption 750}$$

$$\text{OD'630} = \text{Absorption 630} - \text{Absorption 750}$$

2.4.2. Phycocyanin Analysis

The quantitation of phycocyanin (µg/L) was performed fluorometrically according to the methods of Sarada et al. [54] and Mellios et al. [55]. Water volume (200 mL) was passed through cellulose acetate membrane filters (Whatman glass fiber filters) (0.22 µm, 47 mm) in vacuum. The extraction of phycocyanin from the filters with a solution of phosphates 10 mM (pH = 7) was the next step. The determination was performed on a fluorometer (Perkin Elmer, LS45) with excitation wavelength of the molecule at 630 nm and emission wavelength of the molecule at 660 nm. The construction of the standard curve was carried out using standard solutions of pure phycocyanin isolated from the cyanobacterium Spirulina sp. (Sigma, P2172).

2.5. MCYSTs Analysis

Three forms of MCYST were investigated: extra-cellular, cell-bound (intra-cellular), and Total MCYSTs (the sum of extra-cellular and intra-cellular). For intra-cellular MCYSTs, subsamples were filtered on a Whatmann GF/C filter which was immediately frozen

at −20 °C. MCYSTs were extracted from the filter after placement in 100% methanol and stirred overnight at room temperature followed by centrifugation at 1300× *g* for 15 min. This extraction procedure was repeated three times and the three supernatants were pooled. The organic solvent was removed by placing the extract under nitrogen-stream. The remaining concentrated sample was subjected to enzyme-linked immunosorbent assay (ELISA). Results are expressed as micrograms of cellular MCYST equivalents per liter. For analysis of dissolved MCYST, the filtered water was applied directly to ELISA. A commercial ABRAXIS Microcystin ELISA kit was used (520011, USA) following the instructions of the manufacturer. Results are expressed as micrograms of cellular MCYSTs equivalents per liter [56]. For each ELISA run, the negative control and four calibrates were assayed at least in duplicate.

2.6. Statistical Analysis

Analysis of variance (ANOVA) and Fisher LSD test were carried out using the software package SPSS 20. The statistical requirements for the ANOVA (normal distribution, homogeneity of variance) were performed.

3. Results

3.1. Water Physical—Chemical Parameters

Physical and chemical parameters measured in the lake water are presented in Table 1. Temperature in the Karla Reservoir (23.5 °C) and Lake Pamvotis (22.4 °C) was within expected values for the month of sampling (September). Table 1 shows that pH values were relatively high in both areas (8.85 in Lake Pamvotis and 9.04 in the Karla Reservoir), suggesting high photosynthetic activity. Furthermore, the electrical conductivity was significant higher in the Karla Reservoir (3.21 mS/cm) than in Lake Pamvotis (1.97 mS/cm). High Dissolved Inorganic Nitrogen (DIN) concentrations in both areas are associated with very high concentrations of ammonium found in both areas (Table 1). SRP high values were similar in both water bodies (0.32 mg/L in the Karla Reservoir and 0.25 in Lake Pamvotis. High values of phycocyanin were measured in both areas (Karla Reservoir: 456 μg/L; Lake Pamvotis: 1256 μg/L). The values of the above parameters were in addition to the high concentrations of Chl a (Karla Reservoir: 250 μg/L; Lake Pamvotis: 570 μg/L. Both areas are characterized by significant concentrations of intra-cellular MCYSTs (Karla Reservoir: 5.45 μg/L; Lake Pamvotis: 8.10 μg/L) and extra- cellular MCYSTs (Karla Reservoir: 3.65 μg/L; Lake Pamvotis: 5.21 μg/L).

Table 1. Chemical-physical parameters and pigment and MCYST content of the Karla Reservoir and Lake Pamvotis before the experiment.

Parameters	Karla Reservoir	Lake Pamvotis
Temperature (°C)	23.5	22.4
Dissolved Oxygen (mg/L)	8.00	8.10
pH	9.04	8.85
Conductivity (m S/cm)	3.21	1.97
Nitrate (mg/L)	0.57	0.68
Nitrite (mg/L)	0.02	0.03
Ammonium (mg/L)	1.56	1.28
SRP (mg/L)	0.32	0.25
Pigments		
Chl a (μg/L)	250	570
Phycocyanin (μg/L)	456	1256
MCYSTs		
Intra-cellular (μg/L)	5.45	8.10
Extra-cellular (μg/L)	3.65	5.21

Natural Phytoplankton Communities of the Waterbodies

Phytoplankton blooms occurred in both the Karla Reservoir and Lake Pamvotis. Almost 99% of the total phytoplankton biomass in Karla Reservoir consisted of cyanobacteria, while chlorophytes (the most abundant was *Monoraphidium* spp.) were counted in low biomass, consisting of less than 1% of the total phytoplankton biomass (Table 2). The cyanobacterial species forming the bloom in the Karla Reservoir were *Aphanizomenon favaloroi* (biomass 23.88 mg/L), *Raphidiopsis (Cylindrospermopsis) raciborskii* (biomass 21.72 mg/L), *Chroococcus* sp. (biomass 1.91 mg/L), and *Planktolyngbya limnetica* (biomass 1.55 mg/L). Additionally, in Lake Pamvotis, cyanobacteria were forming a heavy bloom with biomass of 150 mg/L (Table 2). *Microcystis aeruginosa* was the species with the highest biomass (81.88 mg/L), followed by *Microcystis wesenbergii* and *Microcystis panniformis* (42.60 and 17.35 mg/L, respectively). Chlorophytes, diatoms, and cryptophytes were also observed in Lake Pamvotis in low biomass (0.086, 0.65, and 0.68 mg/L, respectively) (Table 2).

Table 2. Total biomass (mg/L) of phytoplankton in the Karla Reservoir and Lake Pamvotis.

	Lake Pamvotis (mg/L)	**Karla Reservoir (mg/L)**
Cyanobacteria		
Microcystis spp. (single cells)	8.17	
Microcystis aeruginosa (colonies)	81.88	
Microcystis wesenbergii (colonies)	42.60	
Microcystis panniformis (colonies)	17.35	
Raphidiopsis (Cylindrospermopsis) raciborskii		21.72
Aphanizomenon favaloroi		23.88
Planktolyngbya limnetica		1.55
Chroococcus sp.		1.91
Chlorophytes		
Scenedesmus spp.	0.07	<0.01
Monoraphidium contortum	0.01	
Monoraphidium minutum	0.006	
Monoraphidium spp.		0.01
Diatoms		
Small centric diatoms	0.08	<0.01
Aulacoseira granulata	0.20	
Nitzschia spp.	0.37	<0.01
Cryptophytes		
Rhodomonas minuta	0.68	<0.01

3.2. H_2O_2 Degradation

To explore the role of biological processes in H_2O_2 degradation in water samples from the Karla Reservoir and Lake Pamvotis, degradation rates of H_2O_2 in both filtered and unfiltered samples from the waterbodies were calculated. According to results, degradation rates of H_2O_2 in unfiltered samples (Biological and Chemical degradation) of both waterbodies were quite fast (Figure 2A). The biological and chemical degradation rate in water samples from Lake Pamvotis was significantly higher ($p < 0.05$) than the corresponding rate in water samples from the Karla Reservoir (Figure 2A). On the other hand, degradation rates in both waterbodies appeared significantly lower ($p < 0.05$) for filtered samples (chemical processes). Figure 2B shows that all the added H_2O_2 in water samples from Lake Pamvotis was degraded completely in 3 h, different from the added H_2O_2 in water samples from the Karla Reservoir, which was dissipated completely in 5 h.

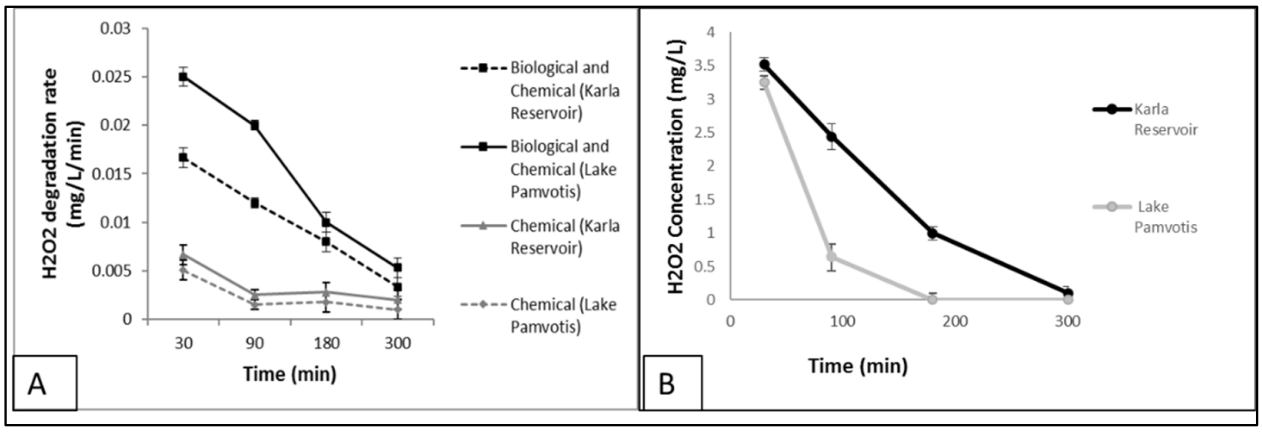

Figure 2. (**A**) Degradation rates of H_2O_2 over time in unfiltered and filtered water samples from the Karla Reservoir and Lake Pamvotis. (**B**). H_2O_2 concentration in water samples from the Karla Reservoir and Lake Pamvotis over time.

3.3. Effects of H_2O_2 on Karla Reservoirs' Phytoplankton

Cyanobacterial biomass in Karla Reservoir samples treated with H_2O_2 decreased over time (Figure 3). At the end of the experiment (144 h), in samples treated with H_2O_2, the initial cyanobacterial biomass (time zero) decreased by 93% in Karla's samples. In contrast, in the control samples, initial cyanobacterial biomass increased by 140% at the end of the experiment.

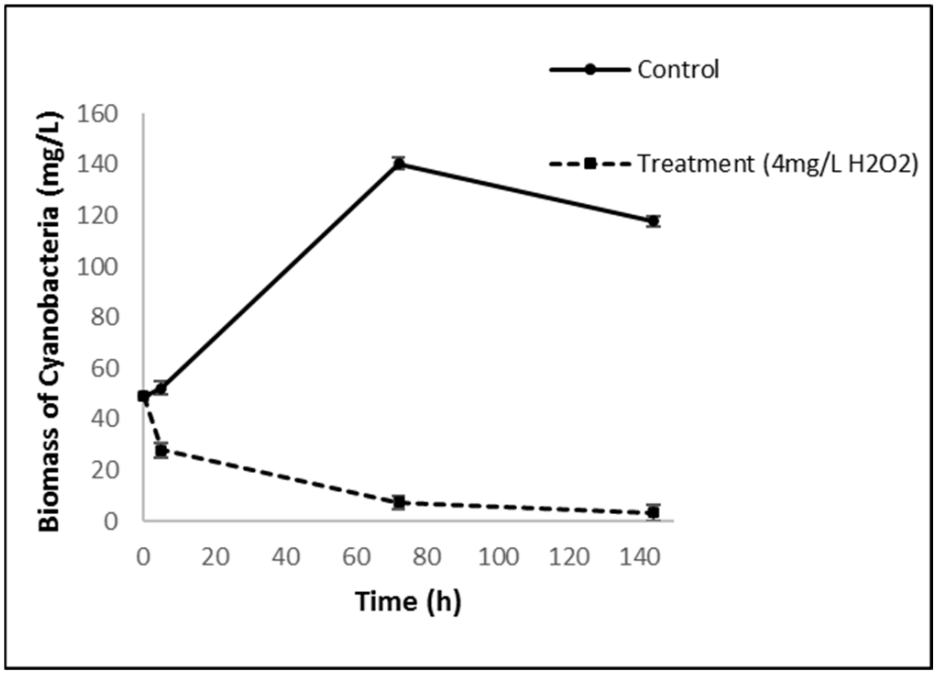

Figure 3. Biomass (mg/L) of cyanobacteria in the control and treated samples (4 mg/L H_2O_2) over time (hours) in Karla Reservoir water samples.

Figure 4 shows biomass variation of different cyanobacterial species in control and treated samples over time in Karla Reservoir water samples. *R. raciborskii*, *A. favaloroi*, *P. limnetica*, and *Chroococcus* sp. were the dominant cyanobacterial species identified in water samples of the Karla Reservoir. Among them, *R. raciborskii* was affected the most by the application of H_2O_2. Specifically, *R. raciborskii*'s biomass dropped from 21.72 mg/L in the control samples to non-detectable in the samples treated with 4 mg/L H_2O_2 (Figure 4A).

Figure 5 shows micrographs of water samples taken 5 h after the beginning of the experiment. Micrograph of the control sample (Figure 5A) and the samples treated with H_2O_2 5 h (Figure 5B), 72 h (Figure 5C), and 144 h (Figure 5D). In Figure 5B, broken filaments of *R. raciborskii* are depicted. In the micrographs from 72 (Figure 5C) and 144 h (Figure 5D)-treated samples, no *R. raciborskii* are present, while the filaments of *A. favaloroi* and *P. limnetica* and small-number colonies of *Chroococcus* are shown. It is noteworthy that *R. raciborskii*'s biomass increased from 21.72 mg/L to 100.45 mg/L in the control samples (Figure 4A).

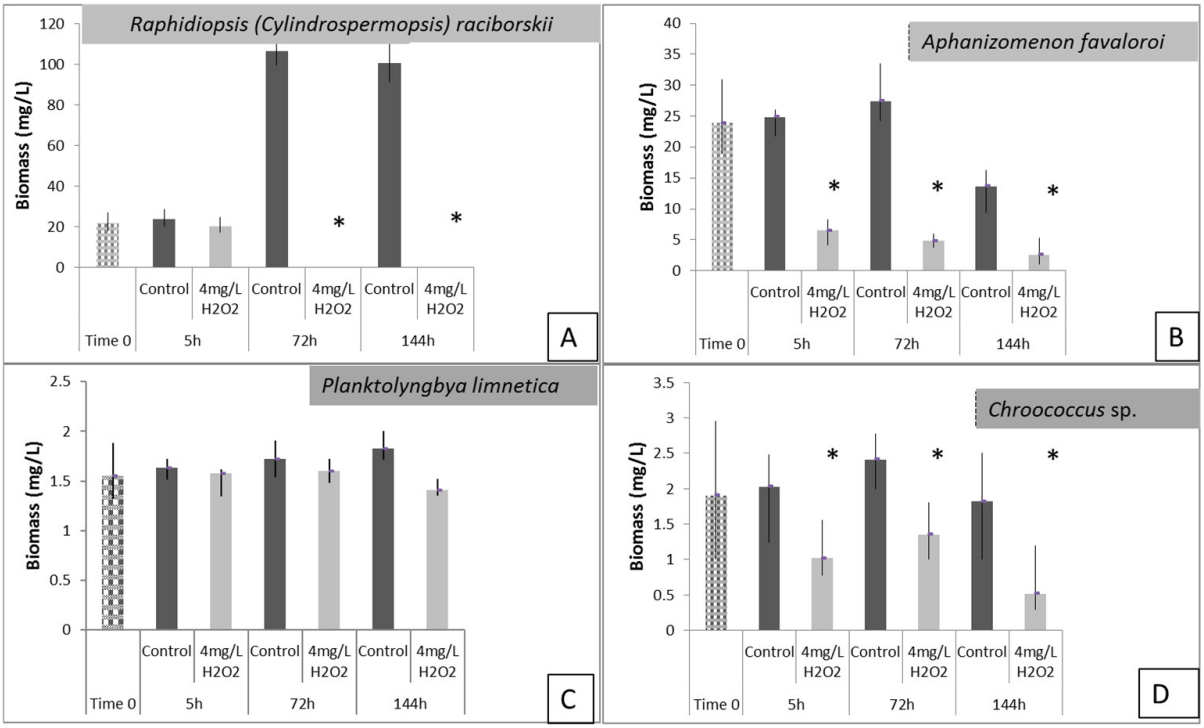

Figure 4. Initial biomass (time zero) and biomass (mg/L) of (**A**) *Raphidiopsis (Cylindrospermopsis) raciborskii*, (**B**) *Aphanizomenon favaloroi*, (**C**) *Planktolyngbya limnetica*, and (**D**) *Chroococcus* sp. in the control and treated samples (4 mg/L H_2O_2) over time (hours) in Karla Reservoir water samples. The asterisk indicates statistically significant differences in comparison with the control ($p < 0.05$).

A. favaloroi's biomass decreased significantly ($p < 0.05$) from 23.88 mg/L in the control samples to 2.58 mg/L in the samples treated with 4 mg/L H_2O_2 (Figure 4B). The *A. favaloroi* biomass decreased not only due to filament numbers decreasing, but also due to the filament size decreasing. In the treated samples *A. favaloroi* filaments were half in length and 10–20% thinner. *A. favaloroi* biomass in the control samples increased 72 h after the beginning of the experiment; however, a significant decrease ($p < 0.05$) occurred at the end of the experiment (144 h) (Figure 4B).

P. limnetica biomass was not affected by the application of H_2O_2 since its biomass remained constant in the samples treated with 4 mg/L H_2O_2 throughout the experiment. Additionally, its biomass remained constant in control samples throughout the experiment. *Chroococcus sp.*'s biomass reduced significantly ($p < 0.005$) by 26% in the samples treated with 4 mg/L H_2O_2 at the end of the experiment (144 h), in contrast with control samples, in which the biomass of *Chroococcus sp.* did not change significantly ($p > 0.05$) in comparison with the biomass that this species had at time zero (Figure 4D).

The remaining phytoplankton taxa and particularly chlorophytes, the most abundant group contributing less than 1% to the total phytoplankton biomass in the lake water, changed very little during the experiment between <1% and <2% in all the treatments and controls, while an individual of euglenophytes was observed only at the end of the experiment (144 h).

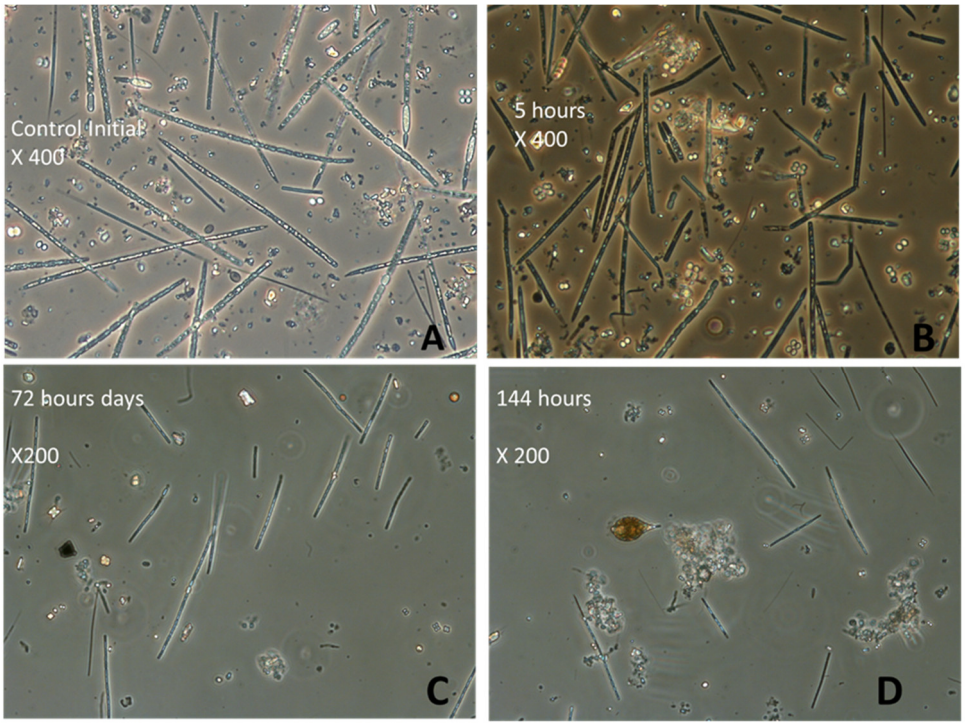

Figure 5. Micrographs of the Karla Reservoirs' water samples: (**A**) Control sample (initial), (**B**) Treated sample, 5 h after the beginning of the experiment, (**C**) Treated sample, 72 h after the beginning of the experiment, (**D**) Treated sample, 144 h after the beginning of the experiment.

3.4. Effects of H_2O_2 on Lake Pamvotis' Phytoplankton

Cyanobacterial biomass in Lake Pamvotis samples treated with H_2O_2 decreased over time (Figure 6A). At the end of the experiment (144 h), in samples treated with H_2O_2, the initial cyanobacterial biomass (time zero) decreased by 71% in Pamvotis' samples. In contrast, in the control samples, initial cyanobacterial biomass increased by 49% at the end of the experiment (Figure 6A). In contrast with cyanobacteria, chlorophytes were positively affected by the application of H_2O_2 by increasing their biomass significantly ($p < 0.05$) throughout the experiment. In control samples, biomass of chlorophytes remained constant throughout the experiment ($p > 0.05$) (Figure 6B).

Biomass of diatoms increased significantly ($p < 0.05$) 72 h after the application of H_2O_2. In the control samples, biomass of diatoms decreased significantly ($p < 0.05$) by 17% at the end of the experiment (Figure 6C).

Biomass of cryptophytes was not affected by the treatment of H_2O_2 ($p > 0.05$); however, in control samples their biomass decreased significantly over time ($p < 0.05$) (Figure 6D).

3.5. Effects of H_2O_2 on Different Species of Microcystis in Lake Pamvotis

Microcystis aeruginosa was the least affected species by H_2O_2 compared with *Microcystis panniformis* and *Microcystis wesenbergii* (Figure 7). Biomass of *M. aeruginosa* in treated samples decreased significantly ($p < 0.05$) 72 h after the beginning of the experiment. However, an increase was observed in its biomass at the end of the experiment compared to its biomass at 72 h. Overall, at the end of the experiment, biomass of *M. aeruginosa* was significantly lower ($p < 0.05$) than the initial biomass (biomass at time zero). *M. aeruginosa*'s biomass increased significantly in the control condition ($p < 0.05$) (Figure 7A). *M. wesenbergii*'s biomass decreased in treated samples to undetectable cell numbers at the end of the experiment (144 h), although conspicuous remnants of its colony mucilage were observed (Figure 8B). Interestingly, its biomass in control samples increased significantly ($p < 0.05$) at the end of the experiment (Figure 7B). *M. panniformis* was the most affected species by the H_2O_2 treatment. Its biomass decreased

to undetectable levels as early as 72 h of the experiment. In contrast, *M. panniformis*'s biomass increased significantly ($p < 0.05$) over time (Figure 7C) in the control condition.

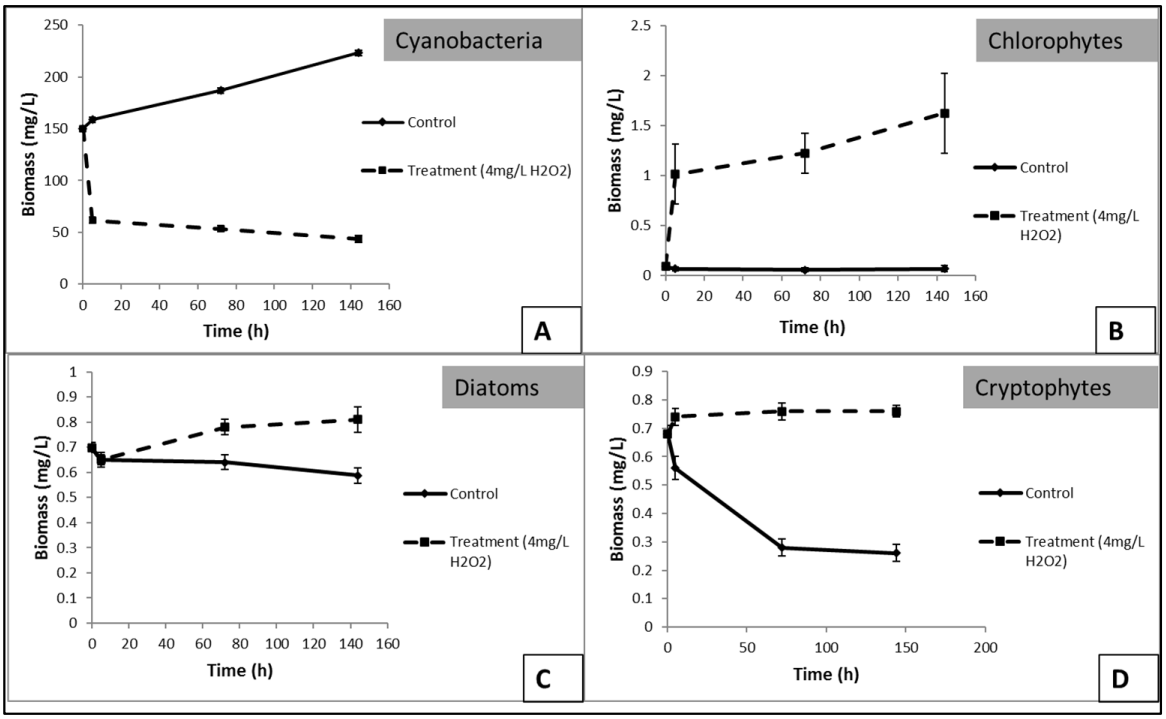

Figure 6. Biomass (mg/L) of (**A**) Cyanobacteria, (**B**) Chlorophytes, (**C**) Diatoms, and (**D**) Cryptophytes in the control and treated samples (4 mg/L H_2O_2) over time (hour) in water samples from Lake Pamvotis.

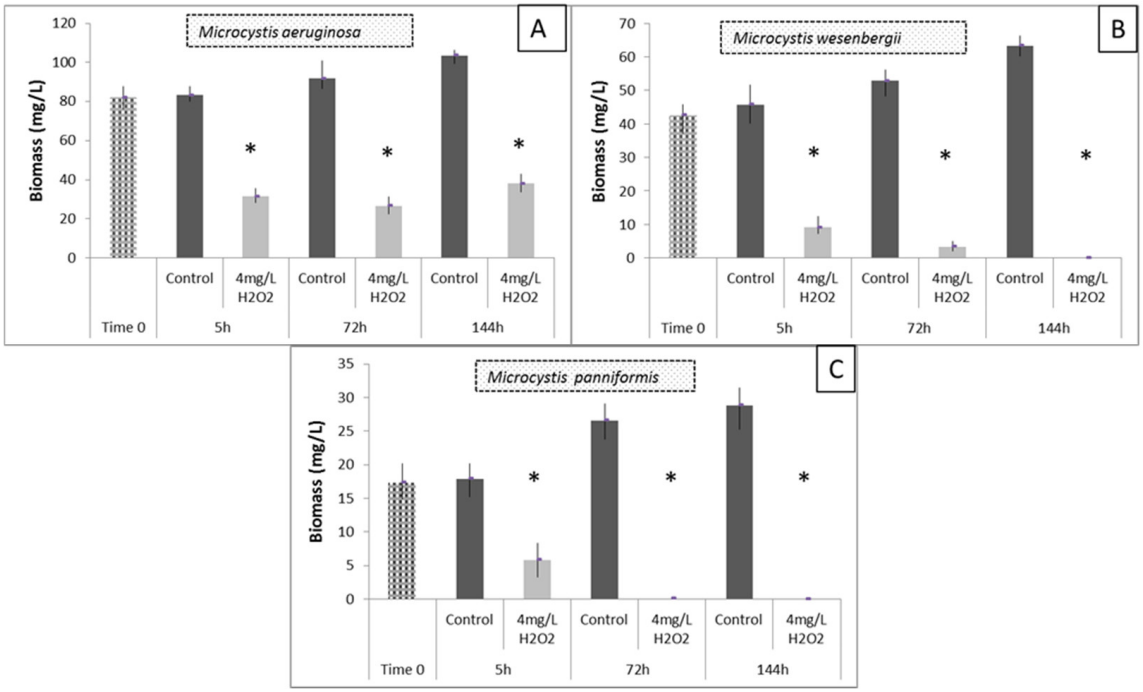

Figure 7. Initial biomass (time zero) and biomass (mg/L) of different species of *Microsystis*: (**A**) *Microcystis aeruginosa*, (**B**) *Microcystis wesenbergii*, (**C**) *Microcystis panniformis*, in the control and treated samples (4 mg/L H_2O_2) over time (hour) in water samples from Lake Pamvotis. The asterisk indicates statistically significant differences in comparison with the control ($p < 0.05$).

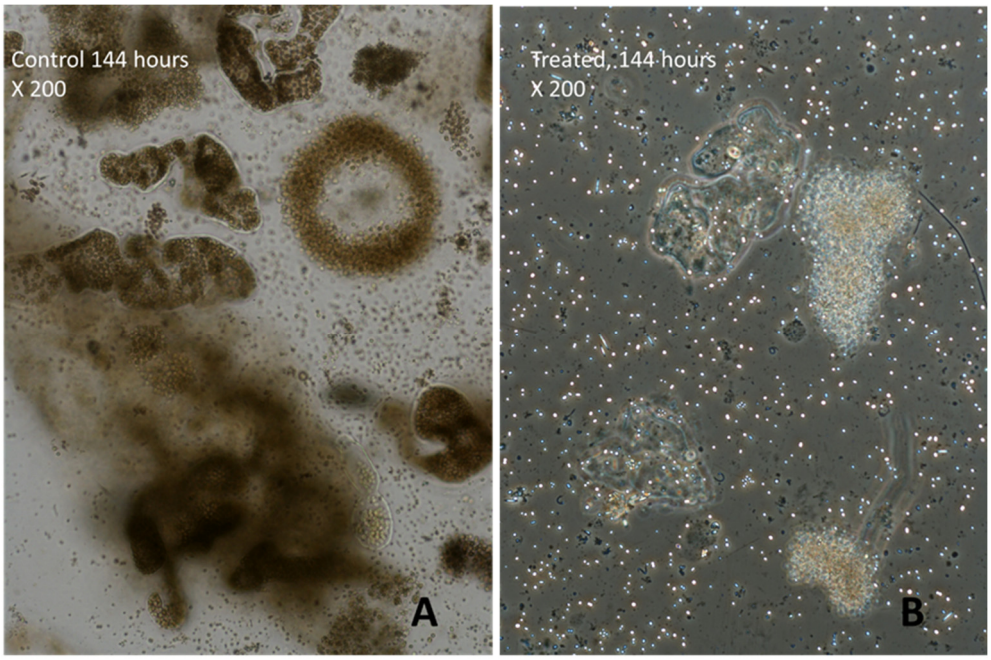

Figure 8. Micrographs of Lake Pamvotis' water samples: (**A**) Control sample (initial) and (**B**) Treated sample 144 h after the beginning of the experiment.

3.6. Effects of H_2O_2 on Chl-a and Phycocyanin

According to Figure 9, the application of H_2O_2 resulted in a decrease in Chl a and phycocyanin concentration in water samples from both waterbodies examined. In contrast, in control water samples of the Karla Reservoir and Lake Pamvotis, both pigments increased. In the Karla Reservoir, phycocyanin concentration decreased by 78% and it was significantly different ($p < 0.05$) from the corresponding concentration in Lake Pamvotis, which decreased by 63%.

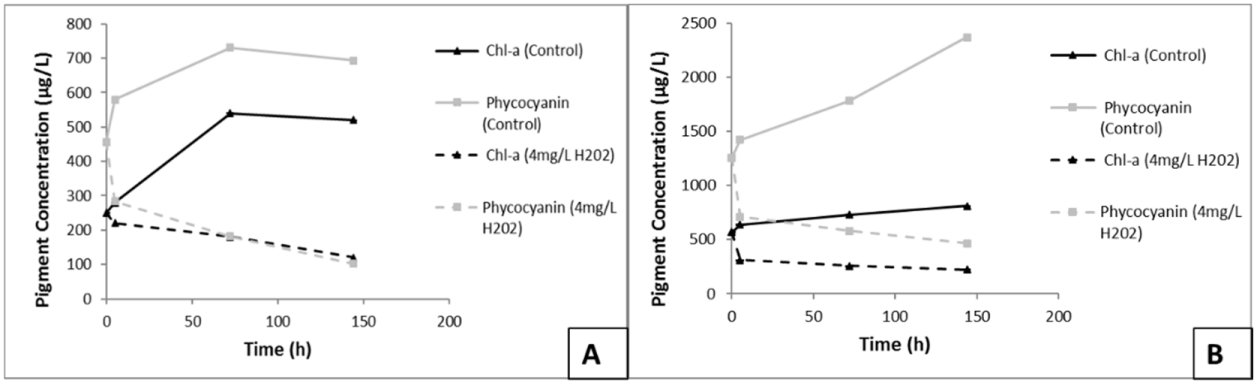

Figure 9. Chl a and phycocyanin concentrations in control and H_2O_2-treated water samples (4 mg/L) from (**A**) the Karla Reservoir and (**B**) Lake Pamvotis over time.

3.7. Effects of H_2O_2 on MCYSTs

According to Figure 10, intra-cellular and extra-cellular MCYSTs concentrations increased significantly ($p < 0.05$) over time in the control samples from both waterbodies. Intra-cellular MCYSTs concentration decreased significantly ($p < 0.05$) over time in the treated samples from both waterbodies. In contrast, extra-cellular MCYSTs concentration increased significantly ($p < 0.05$) over time in the treated samples from both waterbodies studied. Total MCYSTs concentration decreased significantly ($p < 0.05$) in the treated samples from both waterbodies.

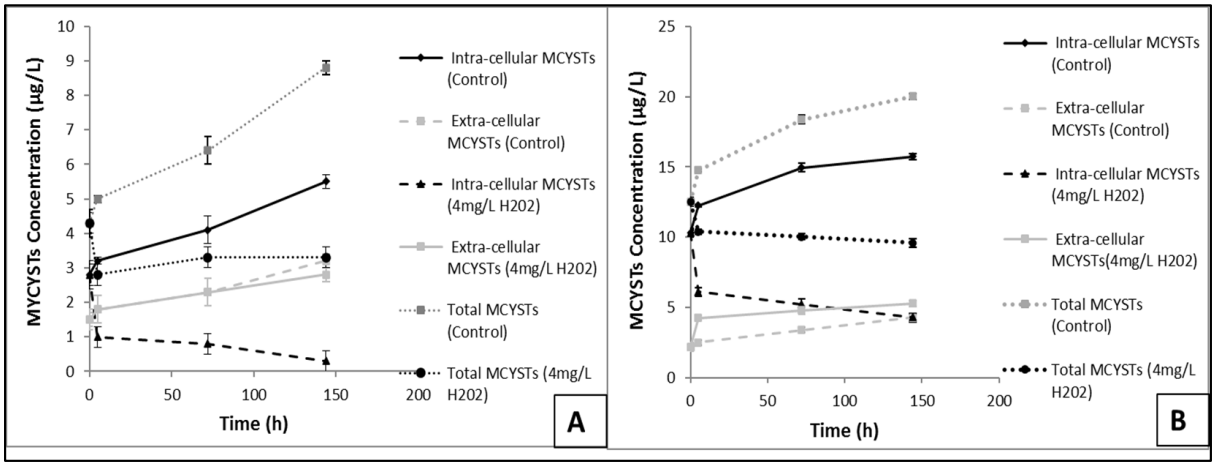

Figure 10. Variation of intra-cellular, extra-cellular, and total MCYSTs concentrations in: (**A**) the Karla Reservoir and (**B**) Lake Pamvotis in the control and treated samples (4 mg/L H_2O_2) over time (hour).

4. Discussion

In the current study, the effects of H_2O_2 on phytoplankton biomass, cyanobacteria species, and MCYSTs concentrations of water samples originated from eutrophic—hypertrophic waterbodies, one lake and one reservoir, were evaluated. Both waterbodies have a history of toxic cyanobacterial blooms [39,40,46], making them suitable as study areas for collecting samples for our experiment. The two investigated waterbodies were characterized by similar physicochemical parameters (Temperature, Dissolved Oxygen, pH, Nitrate, Nitrite, Ammonium, SRP) apart from electrical conductivity. Water from the Karla Reservoir had higher conductivity than Lake Pamvotis water, which may be attributed to the geological profile of the Karla Reservoir's whole catchment area [57], but also to runoff from the surrounding agricultural area [58]. Regarding biotic parameters, in the Karla Reservoir, almost 100% of phytoplankton consisted of cyanobacteria. In Lake Pamvotis, cyanobacteria were also contributing 98% of the total phytoplankton. Nevertheless, other phytoplankton groups (chlorophytes, diatoms, cryptophytes) were also observed in Lake Pamvotis at countable biomass levels. An important difference between the two waterbodies is related to the species composition and biomass of cyanobacteria: in the Karla Reservoir, filamentous cyanobacterial species were dominant (*R. raciborski*, *A. favaloroi*, *P. limnetica*), while in Lake Pamvotis colonial species were the dominant cyanobacterial species (*M. aeruginosa*, *M. wesenbergii*, *M. panniformis*). Furthermore, Lake Pamvotis was characterized by three times higher cyanobacterial biomass than the Karla Reservoir. These differences between the environments possibly contributed to the difference in cyanobacterial biomass reduction in samples from the two waterbodies. According to our results, the initial cyanobacterial biomass in Karla Reservoir samples treated with H_2O_2 decreased by 93%, and in Lake Pamvotis a cyanobacterial biomass decrease of 71% was shown. This difference in cyanobacterial reduction could be explained by differences in H_2O_2 degradation rates shown in the two waterbodies. The biological and chemical degradation rate in Lake Pamvotis was significantly higher than the corresponding rate in the Karla Reservoir, while the chemical H_2O_2 degradation rate was similar in both areas. Additionally, H_2O_2 degradation rates in filtered water samples from both studied areas was significantly lower than in unfiltered water samples, suggesting that a large part of H_2O_2 degradation is due to biological processes. Additionally, H_2O_2 in Lake Pamvotis was dissipated completely in 3 h, while in the Karla Reservoir H_2O_2 was dissipated completely in 5 h. The efficiency of H_2O_2 to control phytoplankton biomass depends on H_2O_2 decomposition and its residence time in water [32,59,60], which can be affected either by biotic factors, such as phytoplankton density and composition, or abiotic factors, such as water reductive power (affected by organic matter) [59,61]. Weenink et al. [59] used three phytoplankton densities (2:1:0.5 ratio) to calculate the rate of degradation of added H_2O_2. They showed that H_2O_2 degradation

was faster in concentrated samples than in diluted samples. However, in our study, this effect could not be evaluated since the chemical composition of dissolved organic matter in the studied environments remains to be investigated.

In contrast to cyanobacteria, chlorophytes in Lake Pamvotis were positively affected by the application of H_2O_2. Biomass of diatoms and cryptophytes, in total, consisted of <2% of the phytoplankton biomass in Lake Pamvotis. However, diatoms and cryptophytes significantly increased their biomass after treatment with H_2O_2 at the end of the experiment. Positive effects of H_2O_2 on biomass of diatoms have been shown in cultures and in natural samples [27,59]. Alloxanthin (a carotenoid primarily found in cryptophytes) increased in phytoplankton samples treated with natural concentrations of H_2O_2 according to studies of Pflaumer [62]. Additionally, Barrington et al. [31] showed that cryptophytes Chl a concentrations increased 33% compared with the initial values. The positive effect of H_2O_2 on chlorophytes could be explained by the suggestion that these species benefited from the collapse of cyanobacteria and utilized the available nutrients [63].

Several laboratory studies have indicated that cyanobacteria are more sensitive to hydrogen peroxide (H_2O_2) than green algae and diatoms. Barroin and Feuillade [64] showed that as little as 1.75 ppm (corresponding to 1.75 mg/L) of H_2O_2 had a deleterious effect on laboratory cultures of the cyanobacterium *Planktothrix rubescens* (formerly known as *Oscillatoria rubescens*), while a 10 times higher concentration proved totally harmless to the green alga *Pandorina morum*. Subsequently, Dra'bkova' et al. [65] investigated several more species and showed that H_2O_2 had generally a much stronger inhibitory effect on the photosynthesis of cyanobacteria than of eukaryotic phytoplankton. Matthijs et al. [32] introduced 2 mg/L of H_2O_2 homogeneously into the entire water volume of Lake Koetshuis (The Netherlands) and they showed that the cyanobacterial population collapsed by 99% within a few days. Furthermore, they concluded that eukaryotic phytoplankton (including green algae, cryptophytes, chrysophytes, and diatoms), zooplankton, and macrofauna remained largely unaffected. The higher sensitivity of cyanobacteria to H_2O_2 compared with eukaryotic phototrophs can be explained by the absence of compartmentalized organelles protected by membranes, as well as the lack of Mehler reaction as found in higher plants and algae [66–68]. This reaction consists of O_2 reduction to superoxide anion (O_2-) by electrons from photosystem I (PSI). Superoxide is then converted to H_2O_2 by superoxide dismutase and finally to water by peroxidase. In cyanobacteria, flavoproteins suppress O_2- generation and the electrons from PSI are donated to O_2 producing H_2O without the formation of reactive oxygen species (ROS) [66,67]. Thus, cyanobacteria do not possess an anti-ROS system as efficient as that of green algae and higher plants [68]. Although there is enough information concerning sensitivity of cyanobacteria to H_2O_2, there is a lack of knowledge concerning the differential effects of H_2O_2 on cyanobacterrial genera or species, with few exceptions. According to our results, although *R. raciboskii* and *A. favaloroi* occurred in the Karla Reservoir with similar biomass (21.72 and 23.88 mg/L, respectively), *R. raciboskii* was the one most affected after treatment with 4 mg/L H_2O_2. *R. raciboskii* decreased its biomass to undetectable levels 72 h after the experiment started. *A. favaloroi* also decreased its biomass, but even after the end of the experiment remained in water samples in lower concentrations. According to these results, *R. raciboskii* seems to be more vulnerable to H_2O_2 than *A. favaloroi*, which shifted in filaments of smaller dimensions (both length and width). It has been shown that filamentous cyanobacteria are more vulnerable to H_2O_2 effects than colonial cyanobacteria [29,33]; however, little is known about the effects of H_2O_2 on different species of filamentous cyanobacteria. Yang et al. [33] showed that the H_2O_2 half-maximal effective concentration (EC50) values were similar for *R. raciborskii* and *Anabaena flos-aquae* when the same concentration of H_2O_2 was applied to cultured strains of these species. However, the results are not comparable to ours, since experimental conditions (cultures vs. natural samples) and species used (*A. flos-aquae* vs. *A. favaloroi*) were different. *P. limnetica*, another filamentous species of *Oscillatoriales*, was not affected by the application of 4 mg/L H_2O_2. In contrast, *Chroococcus* sp.'s biomass decreased significantly. The growth as multicellular filamentous clumps or colonies confers cyanobacteria with

several advantages including resistance to oxidative stress [54]. Wu et al. [69] showed that colonial *Microcystis* was more resistant to the application of copper sulfate (copper sulfate is known as a chemical causing oxidative stress in microorganisms) compared with unicellular *Microcystis*. Therefore, low resistance of *Chroococcus* sp. to H_2O_2 treatment could be attributed to its unicellular form [70]. On the other hand, Lusty and Gobler [37] showed that the addition of 4 mg/L H_2O_2 in environmental lake samples resulted in the reduction of cyanobacteria and they also showed that *Planktothrix* was highly sensitive, *Microcystis* was moderately sensitive, and *Raphidiopsis* (*Cylindrospermopsis*) was the most resistant cyanobacterial species. However, they concluded that the effects of H_2O_2 are somewhat conditional upon the original cyanobacterial community composition. It seems that the dominant cyanobacterial genus is most reduced by H_2O_2, perhaps by providing larger organic surface area for the H_2O_2 to react with, allowing other genera at lower relative abundances replace the most H_2O_2-affected taxa.

Apart from the above-mentioned differences on the effects of H_2O_2 on different genera of cyanobacteria, this is the first time, to our best knowledge, that the differential effect of H_2O_2 was observed on a phtoplankton community including different cyanobacterial species of the same genus. According to our results, the treatment with 4 mg/L H_2O_2 differently affected the biomass of the three species of *Microcystis* found in Lake Pamvotis. The effect of H_2O_2 on biomass of *M. aeruginosa*, *M. wesenbergii*, and *M. panniformis* were compared by the rate of biomass reduction observed for each of *Microcystis* species. *M. wesenbergii* and *M. panniformis* biomass decreased by 100%, with *M. panniformis* affected only 72 h after the start of the experiment. *M. wesenbergii* was affected 144 h after the start of the experiment. *M. aeruginosa* was the least-affected species since its biomass only decreased by 53%. Furthermore, the largest reduction in biomass of *M. aeruginosa* occurred in 72 h after the start of the experiment and after this time its biomass began to increase. To date, there are no other studies about species-dependent variation in sensitivity of *Microcystis* species to H_2O_2. However, Wu et al. [69] showed species-dependent variation in sensitivity of *Microcystis* species to copper sulfate. Copper sulfate has been also used as a chemical reagent for blooms control [71] and according to the results of Wu et al. [69], *M. wesenbergii* and *M. flos-aquae* were the most vulnerable species to copper sulfate compared with *M. aeruginosa* and *M. viridis*. They also concluded that the species-dependent variation in the sensitivity of Microcystis species to copper sulfate may have resulted from variations in extracellular polysaccharide content in different *Microcystis* species. Gao et al. [72] showed that extracellular polymeric substances buffer against the biocidal effect of H_2O_2 on *M. aeruginosa*. Furthermore, roles of loosely-bound extracellular polymeric substances and tightly bound extracellular polymeric substances in the sensitivity of cyanobacterial species in H_2O_2 is still unknown, but they may contribute to species-dependent variation in the sensitivity of *Microcystis* species found in our study. *M. wesenbergi* is characterized by tightly-bound extracellular polymeric substances which makes this species more sensitive to cell rupture and maybe more vulnerable to H_2O_2 effect. In contrast, the loosely-bound extracellular polymeric substances of *M. aeruginosa* may protect this species against cell rupture and maybe also against the negative effect of H_2O_2.

The effect of H_2O_2 on cyanobacteria seems to be even more variable since different strains of the same species were found to have different sensitivities to H_2O_2 addition. Schuurmans et al. [73] showed that microcystin-producing strains are less prepared for high levels of oxidative stress and are therefore hit harder by H_2O_2 addition than non-toxic strains. In addition, some strains of *Microcystis* lack typical cyanobacterial catalases, thus being more sensitive to H_2O_2 effect [74]. Further research is needed to explain the mechanisms which are responsible for the species- and even the strain-dependent variation in sensitivity of cyanobacteria to H_2O_2.

The effects of H_2O_2 on cyanobactrerial biomass are in accordance with results related to effects of H_2O_2 on pigment concentrations in the studied waterbodies. Both concentrations of Chl a and phycocyanin reduced in treated samples originated from the Karla Reservoir and Lake Pamvotis. Phycocyanin is the characteristic pigment of cyanobacteria and a

reduction in phycocyanin is justified due to the reduction in cyanobacterial biomass in both waterbodies. Chl a is a significant pigment found in cyanobacteria and in other phytoplanktonic organisms. Since phytoplankton of Karla Reservoir and Lake Pamvotis consists of >99% and >98% cyanobacteria, respectively, a decrease in cyanobacteria would result in a subsequent reduction of Chl a. Our results agree with studies of Spoof et al. [75], who showed a reduction of Chl a concentration in water samples from Lake Köyliönjärvi after treatment with 5 mg/L H_2O_2. Cyanobacteria in Lake Köyliönjärvi also consisted in the largest part of phytoplankton.

Measurable MCYST concentrations in both waterbodies have already been observed by previous studies [43,46,76]. The high MCYSTs concentrations in the Karla Reservoir and Lake Pamvotis are justified by the presence of toxic cyanobacteria in their water; as shown in the present study concerning Lake Pamvotis, the species *Microcystis aeruginosa* and *Microcystis panniformis* were dominant in the sample and possibly contributed to the production of MCYSTs. *Microcystis wesenbergii* also contributed to the cyanobacterial biomass, although it has been reported as a non-producing MCYST species [77]. Microcystis species were absent from the cyanobacterial biomass of the Karla Reservoir. *Raphidiopsis (Cylindrospermopsis) raciborski*, *Aphanizomenon favaloroi*, and *Planktolyngbya limnetica* were the dominant species in the Karla Reservoir, whereas *Chroococcus* species were present in low numbers. *Cylindrospermopsis* and *Aphanizomenon* sp. have been related to the production of MCYSTs [78], although they are not the main MCYST producers. Microcystin was detected in a coastal lagoon in Spain when *Planktolyngbya contorta* was abundant and *Chroococcus dispersus* was dominant [79]. Our results showed that intra-cellular MCYSTs concentration decreased with time after the application of 4 mg/L H_2O_2, which is consistent with the decrease in cyanobacterial biomass and phycocyanin concentrations. An increase in extra-cellular MCYSTs in treated samples was observed at the end of the experiment in both waterbodies. Lysis of cyanobacterial cells, induced by H_2O_2, probably resulted in the release of intra-cellular MCYSTs into the water. The decrease in intra-cellular MCYSTs concentration was higher than the increase of extra-cellular MCYSTs concentration, resulting in a decrease of Total MCYSTs in the Karla Reservoir and Lake Pamvotis. Spoof et al. [75] also found an increase in extra-cellular MCYSTs concentration and a reduction in intracellular MCYSTs concentration when they applied 5 mg/L H_2O_2 to water samples from Lake Köyliönjärvi. When Barrington et al. [31] applied H_2O_2 (0.1–1 g/L) in a waste-stabilization pond full-scale trial, they showed that both intra-cellular and extra-cellular MCYSTs were reduced. Hydrogen peroxide degrades within hours of addition [27,80], but extra-cellular microcystins will only be oxidized by H_2O_2 whilst it is still present within the water column; hence, degradation by H_2O_2 was not the sole mechanism by which extra-cellular microcystins were decreased in experiments of Barrington et al. [31]. Biodegradation of extra-cellular MCYSTs and adsorption to sediments that naturally occur in lakes and reservoirs may result in the longer-term reduction of extra-cellular MCYSTs compared with experiments under laboratory conditions [81,82].

Although H_2O_2 is an effective option of reducing the biomass of an already formed bloom, further research is needed to investigate its effect on other bentho-pelagic components. Preview studies demonstrated that the implementation of a relatively high concentration of H_2O_2 to control cyanobacterial blooms would lead to the alteration of the sex ratios of zooplankton [83] and result in histopathological changes and brain injuries in fish [84]. Furthermore, H_2O_2 treatment should be applied considering the different characteristics of the waterbodies, since the efficiency of H_2O_2 is influenced by light intensity and nutrient availability [35,85]. It is also dependent on H_2O_2 decomposition and its residence time in water [32,59], which can be affected either by biotic or abiotic factors.

5. Conclusions

Treatment with 4 mg/L H_2O_2 negatively affected the cyanobacteria from the Karla Reservoir and Lake Pamvotis by reducing their abundance. In contrast, other phytoplankton groups like Chlorophytes, Diatoms, and Cryptophytes were positively affected by H_2O_2

treatment. Furthermore, effects of H_2O_2 treatment on cyanobacterial genera or species were different depending on the genus and species. Concentrations of intra-cellular MCYSTs and total MCYSTs of water abstracted from the Karla Reservoir and Lake Pamvotis were also affected negatively, reducing the cyanotoxity of the waterbodies. More research is needed about the efficiency of H_2O_2 as a controller of cyanobacterial blooms and its effects on biotic factors other than phytoplankton.

Author Contributions: Conceptualization, T.P., M.K., N.S., C.S.L., M.M.-G. and K.A.K.; data curation, M.K., N.S., C.S.L. and M.M.-G.; formal analysis, T.P., M.K., N.S., A.P. and V.P.; funding acquisition, C.S.L.; investigation, T.P., A.P. and V.P.; methodology, T.P., A.P., V.P., M.M.-G. and K.A.K.; project administration, C.S.L., M.M.-G. and K.A.K.; resources, C.S.L.; supervision, T.P., C.S.L., M.M.-G. and K.A.K.; validation, T.P., M.K., M.M.-G. and K.A.K.; visualization, T.P., M.K., N.S. and M.M.-G.; writing—original draft, T.P.; Writing—review and editing, M.K., C.S.L., M.M.-G. and K.A.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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