

## Article

# Differential Effects of Hydrogen Peroxide and L-Lysine Treatments on the Growth of Freshwater Cyanophyta and Chlorophyta

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**Abstract:** Harmful cyanobacterial blooms of the toxin-producing *Microcystis* have become a growing problem for Southwest Florida freshwater bodies. Recently, a 2016 bloom in Lake Okeechobee and a 2018 bloom in the Caloosahatchee River both led to the declaration of a state of emergency for the state of Florida. Fast-acting suppression methods are needed to protect residents and wildlife. Hydrogen peroxide and L-lysine have shown promising results in selectively inhibiting the growth of *Microcystis aeruginosa* and are more ecologically friendly due to fast degradation in water or the biological enhancement of nontarget organisms, respectively. We further explored the use of hydrogen peroxide, L-lysine, and combined treatments of both chemicals, which have never been tested before, for the rapid suppression of *Microcystis*. We assessed the susceptibility of seven *M. aeruginosa* strains and six other phytoplankton (*Cyanobium* spp., *Synechococcus* sp., *Dolichospermum planctonica*, *Mychonastes homosphaera*, and *Chromochloris zofingiensis*) commonly found in Florida, and revealed that susceptibility was diverse. All three treatments were effective at inhibiting the growth of *M. aeruginosa*, mixed treatments (16.7 mg/L hydrogen peroxide: 8 mg/L L-lysine) were most effective with a median growth inhibition ratio of 94.2% on the last day of the experiment, while hydrogen peroxide (16.7 mg/L) (83.8%) and L-lysine (8 mg/L) (78.5%) were less so. We found axenic *M. aeruginosa* to be significantly more sensitive to hydrogen peroxide when compared with nonaxenic strains ( $p < 0.01$ ,  $n = 18$ ). L-lysine was found to be significantly more toxic to *M. aeruginosa* than other examined cyanobacteria and chlorophyte strains at the end of the experiment ( $p < 0.001$ ,  $n = 33$ ), demonstrating its specificity to this cyanobacterium, while hydrogen peroxide and mixed treatments had varying effects on the other tested phytoplankton.



**Citation:** Dahedl, E.K.; Urakawa, H. Differential Effects of Hydrogen Peroxide and L-Lysine Treatments on the Growth of Freshwater Cyanophyta and Chlorophyta. *Ecologies* **2023**, *4*, 355–370. <https://doi.org/10.3390/ecologies4020023>

Academic Editors: José Ramón Arévalo Sierra and Volker Lüdertitz

Received: 25 April 2023

Revised: 24 May 2023

Accepted: 31 May 2023

Published: 3 June 2023



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**Keywords:** hydrogen peroxide; L-lysine; algacide; *Microcystis aeruginosa*; cyanobacteria

## 1. Introduction

*Microcystis* is one of the most common and widely distributed toxic bloom-forming cyanobacteria in freshwater and brackish ecosystems [1]. These organisms can produce microcystin toxins, which threaten human health and wildlife in aquatic systems [2]. In recent years, cyanobacterial harmful algal blooms (cyanoHABs) have become a growing problem for Southwest Florida (SWFL). Lake Okeechobee, the largest lake in the Southern United States, and its connecting waterways (the Caloosahatchee River and Saint Lucie River) frequently experience *Microcystis* blooms [3,4]. Recent cyanoHABs in Lake Okeechobee and both waterways led to the declaration of a state of emergency in 2016 and 2018 [4–7]. Nutrient reduction is currently considered the best strategy to reduce bloom events; however, these strategies can take many years to show positive results [7]. The characteristically shallow water depth of these subtropical freshwater systems and reduced stratification periods also leave them more vulnerable to nutrient loading, making nutrient reduction strategies more difficult [8,9]. Many short-term reduction methods have focused

on chemical spraying methods, such as copper sulfate and aluminum; however, these chemicals can become concentrated in the environment and often are nonselective in targeting cyanobacteria [10]. Rapid bloom-suppression methods are desperately needed to manage cyanohABs to reduce the risk to human health and wildlife [7].

Hydrogen peroxide has gained popularity as an environmentally safe algaecide that targets cyanobacteria over other eukaryotic algae due to differential coping mechanisms when dealing with oxidative stress [11–13]. This algaecide is considered environmentally safe due to fast degradation rates in water and ecologically friendly due to positive succession from toxic cyanobacteria to nontoxic eukaryotic algae [11]. L-lysine, an amino acid, has been found to selectively inhibit the growth of *Microcystis* over other eukaryotic algae and cyanobacteria [14]. Mechanisms targeting *Microcystis* specifically are still not well understood [15,16]. Pond experiments have had success in the removal of *Microcystis* blooms leading to positive succession by eukaryotic phytoplankton [15,17]. This biologically derived substance is also considered environmentally friendly; as an essential amino acid for many fish and aquatic organisms, it can be easily metabolized in these systems and is also freely soluble [15].

In this study, we assessed the use of L-lysine, hydrogen peroxide, and the novel use of both together for inhibiting the growth of *Microcystis*. The novel use of both L-lysine and hydrogen peroxide for the treatment of *Microcystis* has never been examined to our knowledge, and we hypothesized that the synergetic effects of both algaecides acting on different growth inhibition mechanisms toward *Microcystis* will be highly effective in their removal. For this study, we assessed both axenic and unialgal *Microcystis* strains, with different morphological characteristics to gain an overall understanding of treatment sensitivity. We also used three *Microcystis* strains isolated from the Caloosahatchee River, Southwest Florida to better understand suitable treatment options for regional water bodies, as well as six other commonly found phytoplankton taxa isolated in local areas, to gain insight into potential phytoplankton community succession patterns that may occur after treatment.

## 2. Materials and Methods

### 2.1. Organisms and Cultured Conditions

*Microcystis aeruginosa* strains used in this study are listed in Table 1 along with isolation location, ecological characteristics, as well as axenic or nonaxenic conditions. *Microcystis* strains FD4, HC1, and AL2 were isolated from the Caloosahatchee River and its branching waterways in the 2018 bloom event [6]. *Microcystis* were identified based on microscopic observation using a compound microscope (OMAX MD82ES10) and genomic characterization. NIES-88, 90, 102, 843, and 4325 were obtained from the Microbial Culture Collection, National Institute for Environmental Studies (NIES), Tsukuba, Japan (Table 1). Genomes of five of the *M. aeruginosa* strains used in this study (FD4, NIES-843, NIES-88, NIES-4325, and NIES-102) have been sequenced and the reported genome size ranged from 3.88 Mb (NIES-4325) to 5.87 Mb (NIES-102) [6,18–21]. NIES-102 was originally reported as *Microcystis viridis*, one of five commonly reported morphospecies of *Microcystis* (*M. aeruginosa*, *M. viridis*, *M. novacekii*, *M. ichthyoblabe*, and *M. wesenbergii*) [22]. Morphospecies of *Microcystis* have been recognized primarily by colony morphology which is highly variable, and DNA-DNA hybridization reports have shown in the testing of multiple *Microcystis* strains, including NIES-102, the genus cannot be distinguished by species because the DNA reassociation values reach 70% [23–25]. Kondo et al. [24], for example, found NIES-102 had high DNA relatedness to *M. aeruginosa* strains NIES-87 (90.5%), NIES-89 (91.2%), and NIES-289 (78.5%). More recent genome comparisons have recognized NIES-102 as *M. aeruginosa* [20,21] and, therefore, in this study, it will be referred to as *M. aeruginosa*. *M. aeruginosa* strains NIES-102, NIES-843, NIES-88, AL2, and HC1 all produce microcystins, while *M. aeruginosa* strains FD4 and NIES-4325 are nontoxic, lacking complete microcystin synthetase gene clusters [6,25]. Other phytoplankton taxa assessed in this study are listed in Table 2. These phytoplankton strains were also locally isolated and identified using microscopy

and either 16S rRNA or 23S rRNA gene sequences. Accession numbers for deposited 23S rRNA gene sequences are available in GenBank: FGCU3 (OL772022), FGCU52 (OL772026), FGCU54 (OL772027), FGCU15 (OL772003), and FGCU59 (OL772005).

**Table 1.** *Microcystis aeruginosa* strains used in this study.

Strain	Microcystin Production	Axenic	Isolation Location
NIES-88	X		Lake Kawaguchi, Japan
NIES-102	X	X	Lake Kasumigaura, Japan
NIES-843	X	X	Lake Kasumigaura, Japan
NIES-4325		X	Lake Abashiri, Japan
FD4			Caloosahatchee River, Fort Denaud, FL, USA
HC1	X		Hickey Creek, Alva, FL, USA
AL2	X		Caloosahatchee River, Alva, FL, USA

**Table 2.** Cyanobacteria and Chlorophyta taxa examined in this study.

Strain	Species	Taxon	Isolation Location
FGCU3	<i>Synechococcus</i> sp.	Cyanophyta	Hickey Creek Alva, FL, USA
FGCU52	<i>Cyanobium</i> sp.	Cyanophyta	Caloosahatchee River, Moore Haven, FL, USA
FGCU54	<i>Cyanobium</i> sp.	Cyanophyta	Caloosahatchee River, Fort Denaud, FL, USA
FGCU122	<i>Dolichospermum planctonica</i>	Cyanophyta	Lake Okeechobee, FL, USA
FGCU15	<i>Mychonastes homosphaera</i>	Chlorophyta	Lake Okeechobee, FL, USA
FGCU59	<i>Chromochloris zofingiensis</i>	Chlorophyta	Lake Okeechobee, FL, USA

Prior to the treatment experiments, phytoplankton were precultured to exponential phase, harvested, and then transferred to fresh BG-11. For the initial examination using *M. aeruginosa* strain FD4 and *Cyanobium* sp. strain FGCU54, 3 mL of culture was transferred to 27 mL of diluted BG-11 (10%). For the proceeding treatment experiments, 1 mL of culture was transferred to 9 mL of diluted BG-11. Cultures were incubated at 25 °C in a Precision Plant Growth Chamber at an irradiance of 27.8  $\mu\text{mol}/\text{m}^2/\text{s}$  and a light/dark cycle of 12/12 h. All tests were carried out in triplicate, where the same exponential phase batch culture was used for each strain's examination.

*M. aeruginosa* FD4 and *Cyanobium* sp. FGCU54 were initially used to find the optimal concentrations of each treatment for the further testing of other *M. aeruginosa* and phytoplankton strains. L-lysine was added to a final concentration of 3 mg/L and 8 mg/L and compared to a control. Hydrogen peroxide was added to a final concentration of 16.7 mg/L and 33.3 mg/L and compared to a control. The initial low- and high-dose concentrations of L-lysine and hydrogen peroxide treatments were then combined for the assessment of mixed treatments of both chemicals (low dose—3 mg/L L-lysine: 16.7 mg/L hydrogen peroxide; high dose—8 mg/L L-lysine: 33.3 mg/L hydrogen peroxide). Treatment experiments ran for an 8-day period. After the initial examination, it was found that a single application of high-dose hydrogen peroxide greatly suppressed the growth of *Cyanobium* sp. FGCU54, leading to the decision to use low-dose concentrations (16.7 mg/L) of hydrogen peroxide for further testing. The minimal impact of high-dose L-lysine concentration (8 mg/L) on *Cyanobium* sp. FGCU54 led to the decision to move forward with testing this concentration, and the final mixed treatment concentrations of 16.7 mg/L hydrogen peroxide: 8 mg/L L-lysine. The proceeding treatment experiments examining other *M. aeruginosa* strains and phytoplankton ran for a period of 7 days, with optical density (OD) measurements taken at the start of the experiment and on days 3 and 7.

## 2.2. Growth Measurements of Phytoplankton

Phytoplankton growth was examined spectrophotometrically at an OD of 750 nm using a HACH DR/2400 spectrophotometer. Growth was examined over a 7- to 8-day

period. Prior to absorbance measurements, cultures were vortexed to disperse colonies and attain a homogeneous mixture. Three replicate readings were used for each treatment culture to average the optical density readings for each recording day. Relative growth inhibition (GI) ratios were calculated using the formula:

$$(GI) = [(OD_c - OD_t) / OD_c] \times 100$$

where OD<sub>c</sub> and OD<sub>t</sub> are the OD growth at 750 nm of the control and treated samples, respectively. The OD<sub>c</sub> readings for control samples were averaged ( $n = 3$ ) for comparison to treatment replicates, which are displayed as  $\pm$  standard deviation.

### 2.3. Fluorescence Microscopy

Formalin-fixed cells (20  $\mu$ L) were diluted to 1 mL with ultrapure water and fluorescently stained with 1  $\mu$ L of SYBR Green I (Lonza Bioscience, Walkersville, MD, USA) for 5 min in the dark. The stained cells were collected onto a 0.2  $\mu$ m pore size Isopore membrane filter (25 mm, MilliporeSigma, Burlington, MA, USA) set in a vacuum filtration unit with a hand-held pump. After filtration, the filter was mounted on a microscope slide with an antibleaching agent (AF1: Citifluor, London, UK). The cells were viewed with an inverted fluorescence microscope IX51 (Olympus, Center Valley, PA, USA).

### 2.4. Statistical Analysis

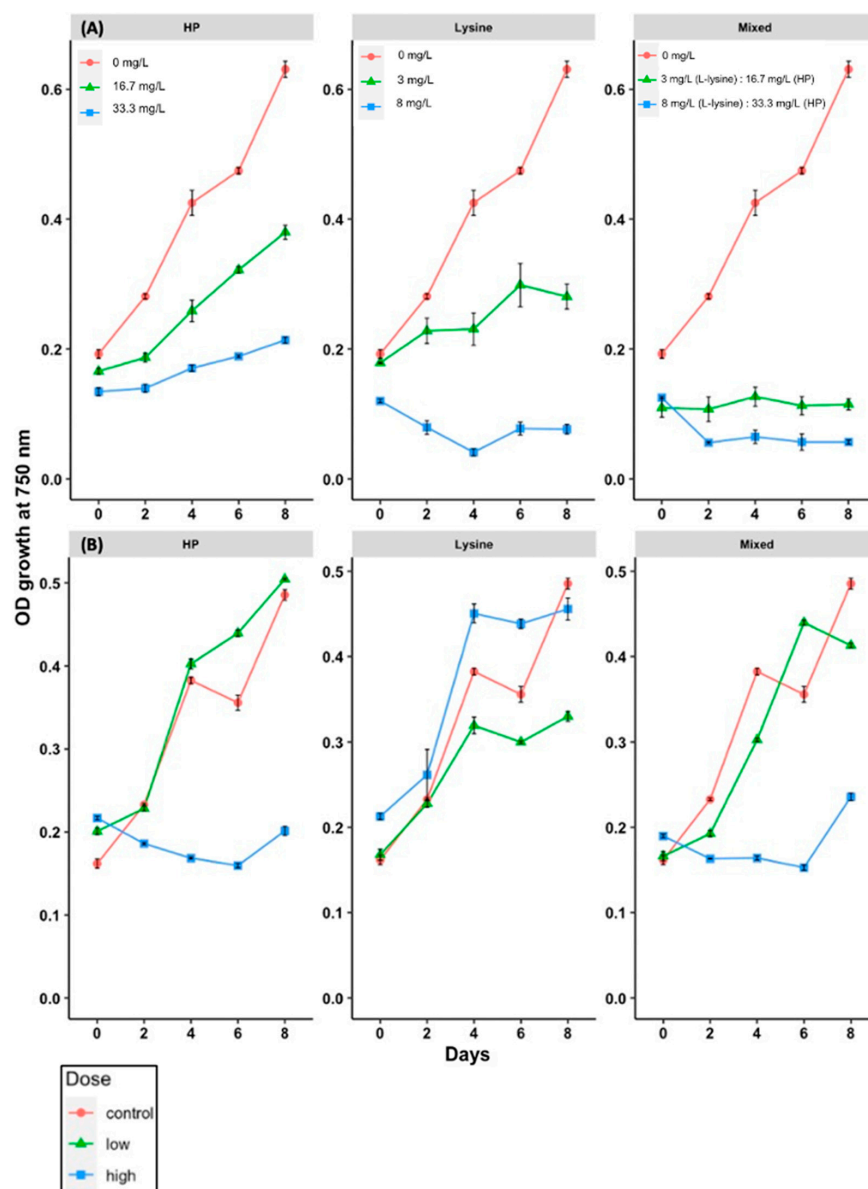
The Shapiro–Wilk test was used to analyze the normality of growth inhibition ratio data for the tested phytoplankton groups. Since some data were nonparametric, a two-tailed Wilcoxon rank sum test was used to analyze the effects of hydrogen peroxide, L-lysine, and mixed treatments on the growth of examined phytoplankton in R [26]. The growth inhibition ratios from each replicate phytoplankton strain ( $n = 3$ ) were grouped based on described characteristics (axenic, nonaxenic, *M. aeruginosa*, cyanobacteria, chlorophytes, etc.), for each treatment application on individual sampling days (3 and 7), to assess differences in treatment sensitivity among the tested phytoplankton groups.

## 3. Results and Discussion

### 3.1. Dose Sensitivity of *Microcystis aeruginosa* and *Cyanobium* sp.

*M. aeruginosa* strain FD4 and *Cyanobium* sp. FGCU54 were initially used to find the optimal concentrations of each treatment for further testing of the susceptibility of other algal strains to hydrogen peroxide, L-lysine, and mixed treatments of both chemicals. All treatment application concentrations showed an inhibitory effect on the growth of *M. aeruginosa* FD4 after two days; this was not found for *Cyanobium* sp. FGCU54 (Figure 1). The inhibitory effect on *M. aeruginosa* varied after this period by treatment application and concentration. Low-dose L-lysine (3 mg/L) and hydrogen peroxide (16.7 mg/L) treatments showed signs of recovery on the last day of the experiment, while mixed low-dose treatments of both chemicals (3 mg/L L-lysine: 16.7 mg/L hydrogen peroxide) showed synergetic effects in deterring recovery, with a growth inhibition ratio of  $81.6 \pm 0.7\%$  ( $n = 3$ ) on the last day (Figure 1). *M. aeruginosa* strain FD4 was found to be highly sensitive to 8 mg/L L-lysine treatment, with growth inhibition reaching 90% just 48 h after treatment application, while *Cyanobium* sp. FGCU54 did not show signs of sensitivity to any of the L-lysine treatments; however, it was found to be more sensitive to high-dose hydrogen peroxide (33.3 mg/L) (Figure 1). High-dose mixed treatments of both chemicals were the most effective at inhibiting the growth of *M. aeruginosa*.

To better understand strain sensitivity and safe application for the natural environment, it was decided to proceed with the low-dose hydrogen peroxide (16.7 mg/L) for a single application and mixed treatments (16.7 mg/L hydrogen peroxide and 8 mg/L L-lysine), and high-dose L-lysine (8 mg/L).

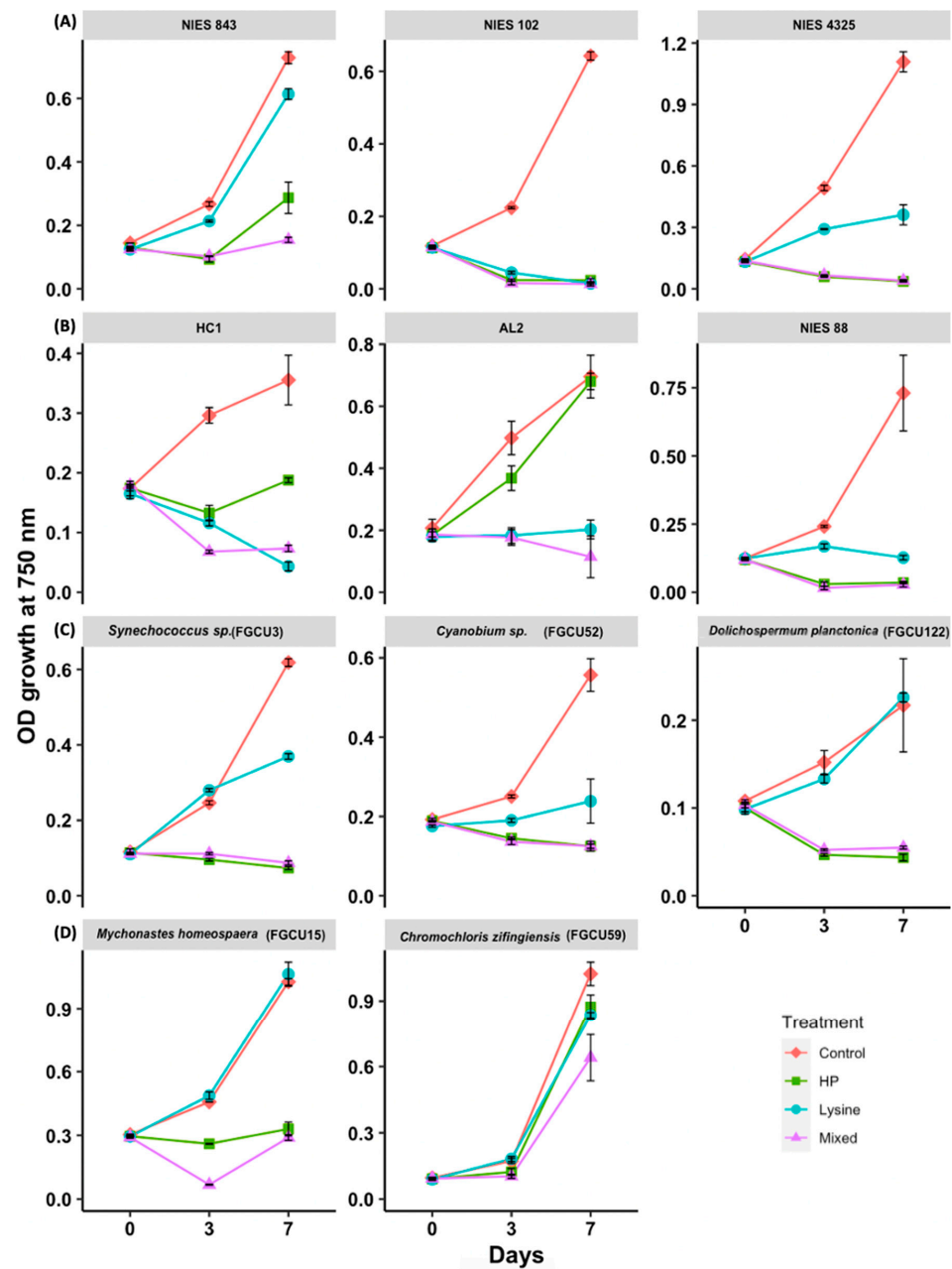


**Figure 1.** Susceptibility of *Microcystis aeruginosa* FD4 (A) and *Cyanobium* sp. FGCU54 (B) to low- and high-dose hydrogen peroxide (16.7 mg/L, 33.3 mg/L), L-lysine (3 mg/L, 8 mg/L), and mixed treatments (3 mg/L L-lysine: 16.7 mg/L hydrogen peroxide, 8 mg/L L-lysine: 33.3 mg/L hydrogen peroxide). Growth was measured by optical density (OD) at 750 nm.

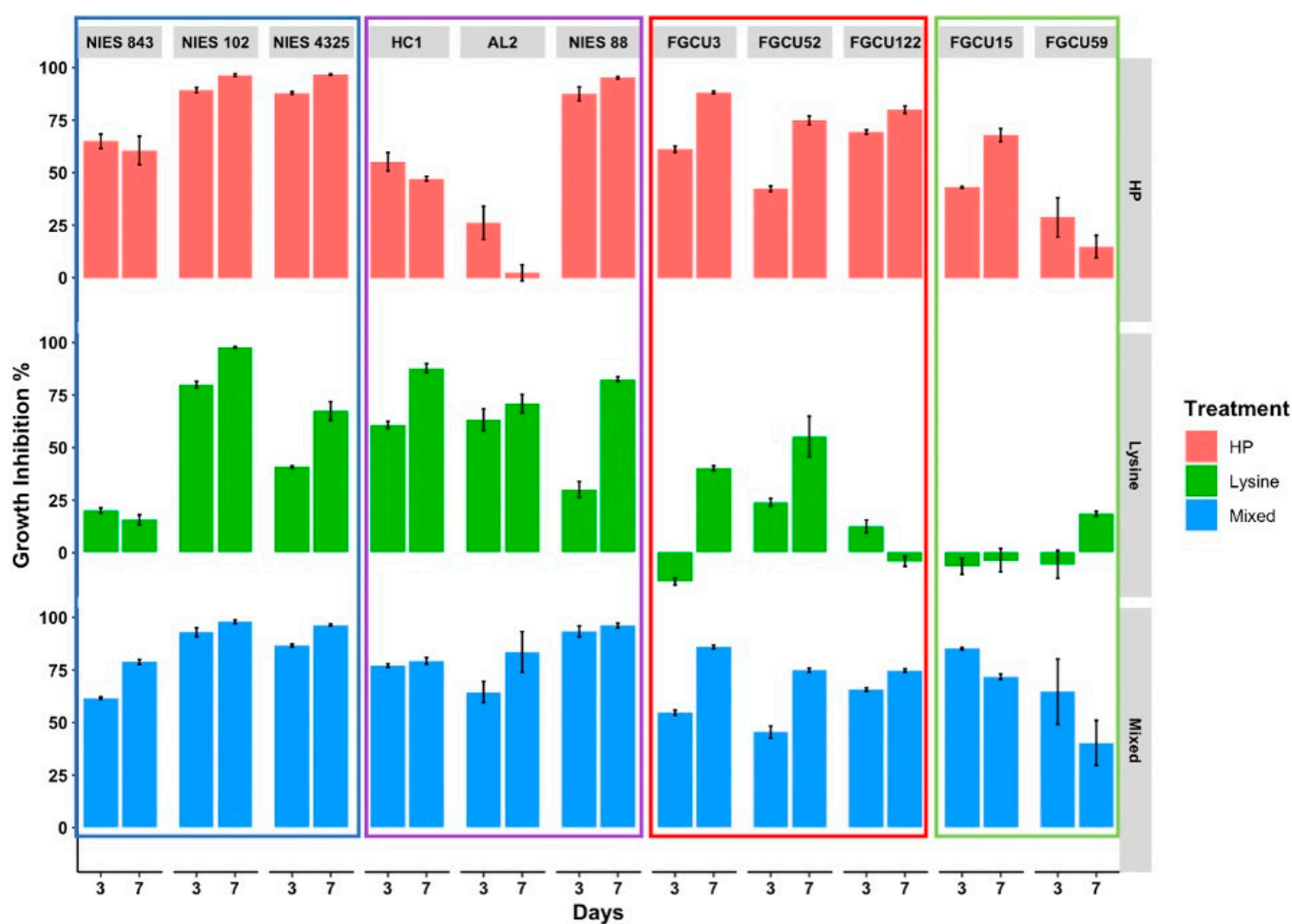
### 3.2. Differences in Sensitivity of *Microcystis aeruginosa* Strains to Hydrogen Peroxide and L-Lysine Treatments

To examine the variable susceptibility of *M. aeruginosa* strains to hydrogen peroxide, L-lysine, and mixed treatments, three axenic strains (NIES-843, NIES-4325, and NIES-102) and three nonaxenic strains (NIES-88, HC1, AL2) were assessed in a 7-day treatment experiment (Table 1). *M. aeruginosa* strains were found to differ in sensitivity to treatments. Mixed treatments (16.7 mg/L hydrogen peroxide and 8 mg/L L-lysine) were found to be most effective at inhibiting the growth of *M. aeruginosa* (Figure 2). By day 3 of the experiment, growth at OD 750 nm for all the mixed treatment samples had dropped to below 50% of the measured OD 750 nm growth for the control samples. The mixed treatments resulted in the near-complete growth inhibition of the tested *M. aeruginosa* strains by day 7, with a median relative growth inhibition of 94.2% (interquartile range (IQR): 79.4–96.3%,  $n = 18$ ). *M. aeruginosa* strains had variable sensitivity to hydrogen peroxide (16.7 mg/L) treatments, displaying near-total growth inhibition on *M. aeruginosa* strains NIES-102, NIES-4325, and

NIES-88, ranging from 95 to 97% of growth inhibition on day 7 (Figure 3), while AL2 was found to grow under hydrogen peroxide treatments (Figures 2 and 3). Hydrogen peroxide treatment application was found to have faster growth-inhibitory effects on *M. aeruginosa* than L-lysine applications, with a median growth inhibition of 76.4% (IQR: 57.6–88.6%) on day 3 of the experiment compared to 47.6% for L-lysine (IQR: 33.2–64.8%); by the end of the experiment, however, the median growth inhibition for L-lysine-treated *M. aeruginosa* reached 78.5% by day 7 (Figure 3).



**Figure 2.** Susceptibility of axenic *Microcystis aeruginosa* strains: NIES-843, NIES-102, and NIES-4325 (A), nonaxenic *Microcystis* strains: HC1, AL2, and NIES 88 (B), Southwest Florida isolated cyanobacterial strains: *Synechococcus* sp. FGCU3, *Cyanobium* sp. FGCU52, and *Dolichospermum planctonica* FGCU122 (C), and chlorophyte strains: *Mychonastes homosphaera* FGCU15 and *Chromochloris zofingiensis* FGCU59 (D), to hydrogen peroxide (16.7 mg/L), L-lysine (8 mg/L), and mixed treatments of both chemicals (16.7 mg/L hydrogen peroxide: 8 mg/L L-lysine).



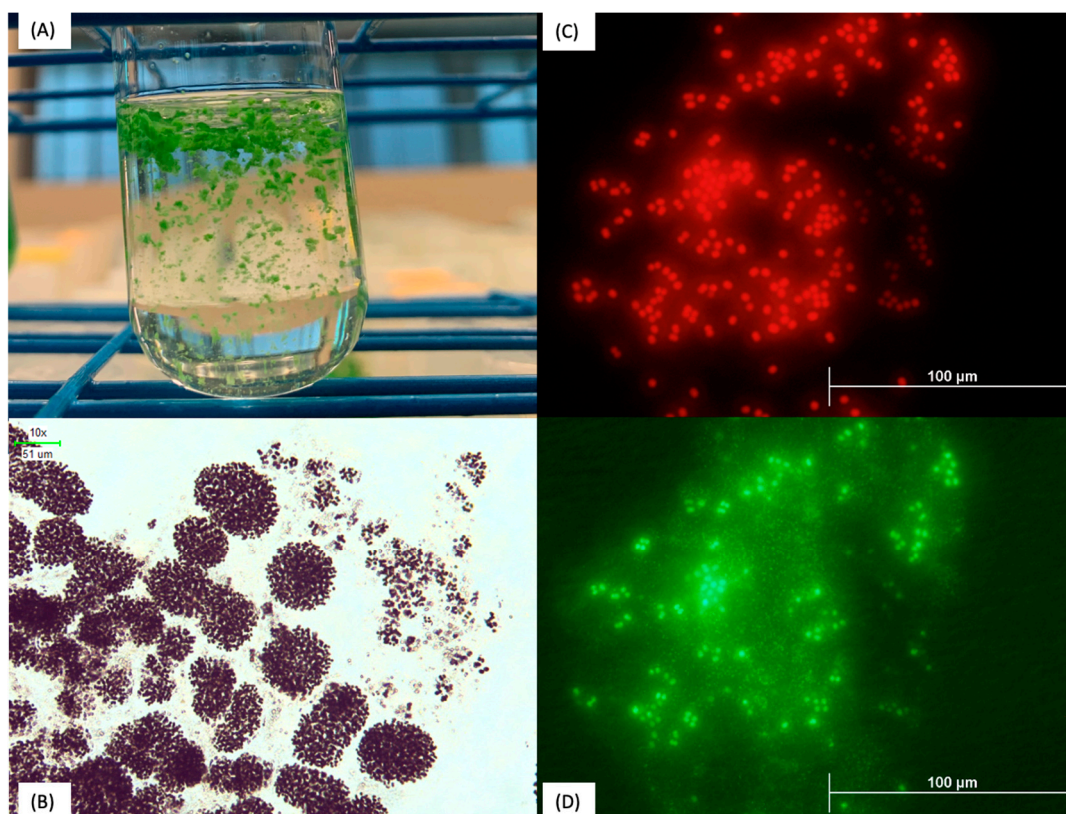
**Figure 3.** Relative growth inhibition (%) for axenic *Microcystis* strains: NIES-843, NIES-102, and NIES-4325 (blue box), nonaxenic *Microcystis* strains: HC1, AL2, and NIES-88 (purple box), FGCU cyanobacteria strains: *Synechococcus* sp. FGCU3, *Cyanobium* sp. FGCU52, and *Dolichospermum planctonica* FGCU122 (red box), and FGCU chlorophyte strains: *Mychonastes homosphaera* FGCU15 and *Chromochloris zofingiensis* FGCU59 (green box) under hydrogen peroxide, L-lysine, and mixed treatments.

The growth of axenic *M. aeruginosa* (NIES-102, NIES-843, and NIES-4325) was found to be more inhibited by hydrogen peroxide application during the experiment; when compared with nonaxenic *M. aeruginosa* (AL2, HC1, and NIES-88) on day 7, growth was found to be significantly different ( $p < 0.01$ ,  $n = 18$ ). This finding was more significant when comparing the growth of only SWFL *M. aeruginosa* strains (HC1 and AL2) with the growth of axenic strains on day 7 ( $p < 0.001$ ,  $n = 15$ ); resilience to hydrogen peroxide treatments was also seen for the SWFL strain FD4 (Figure 1) and may be attributed to colony formation and the high bacterial presence found in these SWFL nonaxenic strains. *M. aeruginosa* lacks the genes for catalase which are used for reactive oxygen species (ROS) scavenging under high hydrogen peroxide conditions [27]. Kim et al. [28] found that *M. aeruginosa* cocultured with *Rhizobium* bacteria containing catalase genes had significantly higher growth rates than axenic *M. aeruginosa* cultures under 500  $\mu\text{M}$  hydrogen peroxide treatments. Kim et al. [29] further confirmed this bacterial ROS remediation activity using additional high light stress along with hydrogen peroxide treatments, where *M. aeruginosa* cocultured with high-catalase-functioning bacterial groups were less sensitive to both ROS conditions compared to *M. aeruginosa* cocultured with low-catalase-functioning bacterial groups; hydrogen peroxide was also degraded significantly faster in high-catalase bacterial communities. This may have been demonstrated in our results, where axenic *M. aeruginosa* strains (NIES-843,

NIES-102, and NIES-4325) were more sensitive to hydrogen peroxide than nonaxenic SWFL strains (FD4, HC1, and AL2) (Figures 1–4). Another difference that may have lessened SWFL strain hydrogen peroxide sensitivity compared with unicellular axenic *M. aeruginosa* is these strains' phenotypic characteristic of colony formation, which may be an adaptive advantage for *M. aeruginosa* species found in this subtropical region. Subtropical regions experience greater solar ultraviolet (UV) radiation, where algal cells are at an enhanced risk of UV damage and photoinhibition at the water surface [30]. *M. aeruginosa* colonies have been found to be better adapted to these high-light conditions compared with unicells due to self-shading mechanisms and increased extracellular polysaccharide content, which can facilitate the attachment of UV-B screening compounds [31,32]. Colony formation may also be favored due to environmental chemical stressors [33]. Ndungu et al. [5] found that along the Caloosahatchee River, where SWFL *M. aeruginosa* strains were isolated, hydrogen peroxide surface water concentrations were measured as high as 5.07  $\mu\text{M}$  during bloom and rain events may increase the surface hydrogen peroxide concentrations, suggesting phytoplankton from this region may be exposed to high levels of environmental oxidative stress. Extracellular polymeric substances (EPS) are essential components of *M. aeruginosa* colony formation [34] and have been shown to have a strong scavenging ability toward hydrogen peroxide. Gao et al. [35] found in the hydrogen peroxide treatment of *M. aeruginosa* that 50% of the applied hydrogen peroxide could be consumed by EPS, for which *M. aeruginosa* cells with EPS were able to recover much quicker than those without, highlighting the potential reduced sensitivity of these colony-forming strains to hydrogen peroxide in comparison with unicellular forms. Colonies may also be better protected from these conditions due to the reduced surface area interaction of inner cells with chemical stressors [33] as well as increased antioxidative enzyme activity, such as superoxide dismutase (SOD), which has been reported in observations of colonies and unicellular cells under oxidative stress [36]. These unique conditions may promote the survival of *M. aeruginosa* morphospecies with phenotypic plasticity toward colony formation, allowing better protection from these localized environmental stressors, as well as heterotrophic bacterial communities capable of remediating this environmental ROS stress, together making them less sensitive to hydrogen peroxide algicidal treatments [28,35,36]. This idea is supported by the dense colony morphology and high bacterial presence found in the mucilage of SWFL strain AL2 (Figure 4), which was least sensitive to hydrogen peroxide of the tested *M. aeruginosa* strains (Figures 2 and 3). Liu et al. [37] reported that a dosage of 20 mg/L was necessary to efficiently control *Microcystis* blooms whose colony size exceeds 25  $\mu\text{m}$ , which the colony size of the examined *M. aeruginosa* AL2 far surpasses (>100  $\mu\text{m}$ ) (Figure 4). These findings may hold an important insight for biological controls of *M. aeruginosa* blooms in which higher concentrations of hydrogen peroxide are necessary to more effectively remove the bloom than the simplified laboratory experimental conditions.

As demonstrated, the *M. aeruginosa* strains were highly sensitive to L-lysine treatment compared with the other examined phytoplankton (Figures 2 and 3). Our results support the previous finding that L-lysine can be selectively toxic toward *M. aeruginosa* over other phytoplankton taxa (Figures 2 and 3) [14,38]. *M. aeruginosa*'s sensitivity to 8 mg/L L-lysine addition was diverse, with NIES-102 showing 79.1% growth inhibition 3 days after addition, and near-total growth inhibition at the end of the experiment, while NIES-843 was largely found to be nonsensitive (Figure 3). *M. aeruginosa* strains NIES-88, AL2, and HC1 were also found to be highly sensitive to L-lysine (growth inhibition: >74%) (Figure 3). Unlike hydrogen-peroxide-treated *M. aeruginosa*, significant differences between the growth of axenic and nonaxenic strains were not found under L-lysine application. L-lysine toxicity mechanisms on *M. aeruginosa*, over other phytoplankton and cyanobacterial genera, are still not well understood [14]. Takamura et al. [15] suggested meso-diaminopimelic acid may be replaced by L-lysine in cell wall peptidoglycan, and differentiation in cyanobacterial sensitivity may be the result of carbon sources used for growth or varied metabolization rates of amino acids. Zimba et al. [38] examined *M. aeruginosa* sensitivity to L-arginine and L-leucine which had more and fewer nitrogen groups than L-lysine, respectively,

and found these basic amino acids were not inhibitory to growth, postulating that L-lysine may increase enzyme active sites, causing feedback inhibition relating to Chl-*a* accumulation. Tian et al. [16] found that *M. aeruginosa* cells treated with L-lysine had significant increases in malondialdehyde (MDA) content, likely causing lipid peroxidation cell membrane damage, and oxidative stress relief mechanisms (e.g., SOD) were induced. These mechanisms inhibiting *M. aeruginosa* growth warrant further study as L-lysine could act as an environmentally safe *M. aeruginosa*-specific bloom mitigation method, where the essential amino acids can be easily metabolized and degraded in freshwater ecosystems [15,16]. The two most recent high-resolution studies also supported these ideas and concluded that L-lysine incorporated into the leaky peptide glycan structure caused irreversible damage to the photosynthetic system and membrane integrity [39], and affected arginine metabolism and the ornithine–ammonia cycle to inhibit the growth of *M. aeruginosa* [40]. Although our results support previous findings of *M. aeruginosa* sensitivity to L-lysine, longer duration laboratory and field application studies are needed to fully evaluate the effective use of L-lysine for the removal of nuisance *M. aeruginosa* blooms. For example, Lüring and Van Oosterhout [41] found L-lysine additions above 4.3 mg/L were sufficient for a complete growth inhibition of *M. aeruginosa*; however, photosystem II efficiency showed recovery after 6 days.



**Figure 4.** *Microcystis aeruginosa* AL2 growing in BG-11 medium. (A) Surface scum, (B) colony, (C) autofluorescence of *M. aeruginosa* cells, and (D) *M. aeruginosa* and phycosphere bacteria. Large coccoid cells are *M. aeruginosa* and smaller cells are heterotrophs embedded in mucilage. It should be noted that some *M. aeruginosa* cells are not stained by SYBR Green I, possibly due to the incomplete penetration of the dye through mucilage.

The novel use of combining both hydrogen peroxide and L-lysine for cyanoHAB treatment was found to be highly successful at inhibiting the growth of the examined *M. aeruginosa* strains (Figures 2 and 3). Mixed treatments resulted in the near-complete growth inhibition of all the tested *M. aeruginosa* strains, showing no variation in toxicity based on the presence of bacteria, or unicellular and colony morphological characteristics

(Figures 2 and 3). Past algaecide combination treatments have largely focused on combinations of chemical and coagulant algaecides; however, coagulants are not suitable for shallow lakes where resuspension is prevalent and have been noted to cause potential ecological harm through the nonselective targeting of phytoplankton [42], growth reductions of aquatic plants [43], acute toxicity to fish and benthic invertebrates [44], and an accumulation of coagulant in sediments [45]. Given the efficacy of the fast degradation rates found for both L-lysine and hydrogen peroxide, while also providing more selective targeting of cyanobacteria over eukaryotic algae [11,15], we suggest this chemical and biological combination warrants further exploration for the water management of *M. aeruginosa* where synergetic effects are achieved, similar to other algicidal combinations, without causing enhanced ecological harm. Although hydrogen peroxide is recognized as being a safe and effective cyanoHAB mitigation method due to its fast degradation rates and reduced sensitivity to beneficial eukaryotic plankton, low-dose application to achieve these benefits may not be sufficient to eliminate dense *M. aeruginosa* blooms with the environmental and physiological characteristics discussed previously [11,28,35,46]. This can lead to the need for the costly reapplication of chemicals, where residents and wildlife have prolonged risks of exposure to cyanotoxins. Our results demonstrate that mixed treatments of hydrogen peroxide and L-lysine chemicals were highly effective at inhibiting the growth of *M. aeruginosa* in just 3 days (median growth inhibition: 82.1%) (Figure 3).

### 3.3. Differences in Sensitivity of Other Phytoplankton to Hydrogen Peroxide and L-Lysine Treatments

Other Cyanophyta and Chlorophyta isolated from SWFL water bodies were used to assess sensitivity and potential succession that may occur if hydrogen peroxide, L-lysine, and mixed treatments were applied to treat local *M. aeruginosa* blooms. Similar to *Cyanobium* sp. FGCU54 (Figure 1), two other picocyanobacterial strains (FGCU3 and FGCU52) were found to be less sensitive to L-lysine than the examined *M. aeruginosa* strains on the last day of the experiment ( $p < 0.05$ ,  $n = 24$ ) (Figures 2 and 3). These strains were found, however, to be sensitive to hydrogen peroxide, with the average growth inhibition ratios on the last day of the experiment being 88.1% for *Synechococcus* sp. FGCU3 and 74.9% for *Cyanobium* sp. FGCU52 (Figure 3). The growth of *Dolichospermum planctonica* strain FGCU122 was also not susceptible to L-lysine addition (Figures 2 and 3). This finding agrees with past studies showing *Anabaena*, also a member of the order Nostocales, was not sensitive to L-lysine concentrations up to 10 mg/L [14]. FGCU122 was highly sensitive to hydrogen peroxide and the growth never recovered after the initial crash on day 3 (Figure 2), supporting previous findings of a 2 mg/L hydrogen peroxide dose being required to completely remove *Dolichospermum circinale* [47]. *Dolichospermum* are another toxin-producing cyanobacterium known to frequently form nuisance blooms in SWFL [48], and this result highlights the effectiveness of hydrogen peroxide on *Dolichospermum* blooms [49]. All the aforementioned cyanobacterial strains (FGCU3, FGCU52, and FGCU122) were found to be highly sensitive to mixed treatments, with the growth inhibition ranging from 73.2 to 87.5% ( $n = 9$ ) on the last day of the experiment (Figure 3). The examined SWFL chlorophytes had varying sensitivity to hydrogen peroxide, L-lysine, and mixed treatments (Figure 2). Both chlorophyte *Chromochloris zofingiensis* strain FGCU59 and *Mychonastes homosphaera* strain FGCU15 were not sensitive to L-lysine (Figure 3). This result is supported by previous findings indicating eukaryotes are less susceptible to L-lysine; Zimba et al. [38] found the Chl-*a* accumulation for the chlorophyte *Scenedesmus dimorphus* was not inhibited by L-lysine concentrations  $< 400$  mg/L, and the diatom *Cyclotella meneghiniana* was not sensitive up to concentrations of 500 mg/L. *C. zofingiensis* growth was not found to be inhibited by hydrogen peroxide and was the least susceptible of the tested taxa to mixed-treatment applications (Figures 3 and 4). *M. homosphaera* strain FGCU15, a small picoeukaryote, was susceptible to these treatments; however, it showed signs of recovery on the last day (Figure 2).

The specificity of L-lysine toxicity to *M. aeruginosa* was clearly demonstrated in this study, where *M. aeruginosa* growth was found to be significantly more inhibited when compared with other examined cyanobacteria and chlorophytes (FGCU3, FGCU15, FGCU52, FGCU59, and FGCU122) on days 3 ( $p < 0.001$ ,  $n = 33$ ) and 7 ( $p < 0.001$ ,  $n = 33$ ) (Figure 3). These results demonstrate L-lysine may be a viable option for targeting *M. aeruginosa* blooms specifically, leading potentially to succession by eukaryotic algae. Takamura et al. [15] found in a pond experiment that L-lysine application of 50  $\mu\text{M}$  was able to completely remove *M. aeruginosa* colonies after 3 days, where the eukaryote *Euglena* then became dominant and *M. aeruginosa* remained absent for the proceeding 11 days of the experiment. Kaya et al. [17] had different findings in pond experiments, where, after L-lysine application, *M. aeruginosa* dominance was succeeded by diatoms, but only lasted 7 days before *M. aeruginosa* returned. Our laboratory results, therefore, are cautionary findings, and the further investigation of L-lysine toxicity on *M. aeruginosa* growth suppression should be assessed for further field applications as well as other potential environmental impacts. Given the high nitrogen content in L-lysine, nutrient regimes and further eutrophication should be evaluated when potentially using this algaecide. Our concentration of 8 mg/L would be equivalent to 1.5 mg/L as nitrogen when applied [15,50], which is negligible because the expected dose could be extremely small in comparison with the size of a water body. The severe cell lysing of *M. aeruginosa* cells could also influence dissolved oxygen (DO); 6.7 mg/L application in a pond experiment led to a 70% decline in DO after application. However, levels eventually recovered to those of controls, but low DO periods could impact other biota [17]. In the aforementioned experiment, cladoceran zooplankton as well as two macrophytes were found to increase in comparison with controls. Being an essential amino acid, this algaecide likely will not cause direct harm to nontarget biota; however, these benefits should be balanced with the potential impacts to ecosystem functioning also described. The sensitivity of other cyanobacteria (FGCU3, FGCU52, and FGCU122) to L-lysine was significantly less than *M. aeruginosa* on the last day of the experiment ( $p < 0.01$ ,  $n = 27$ ) (Figure 3), showing this treatment option does not have broad application potential for the removal of other CyanoHAB-forming taxa as is found with hydrogen-peroxide-based algaecides [14]. From our understanding, picocyanobacterial sensitivity to L-lysine application is limited, and our finding that the tested strains (FGCU3, FGCU52, and FGCU54) were less sensitive than most examined *M. aeruginosa* strains is beneficial as these organisms are found to have high annual abundance in this subtropical region [48,51], where they contribute significantly to carbon fixation and biogeochemical cycling [52].

Although highlighted for selective toxicity toward cyanobacteria, at high concentrations, hydrogen peroxide can be harmful to nontarget phytoplankton and biota [11,53]. Growth inhibition toward the chlorophyte *M. homosphaera* strain FGCU15 was observed with hydrogen peroxide treatments (16.7 mg/L) (Figures 2 and 3). However, its small cell size (1.5–4.5  $\mu\text{m}$ ) may be why it was found to be more sensitive than chlorophyte *C. zofingiensis* strain FGCU59. The growth inhibition of hydrogen-peroxide-treated FGCU59 ( $14.8\% \pm 9.3\%$ ) was significantly less than that of the tested cyanobacteria taxa (Tables 1 and 2) on the last day of the experiment ( $p < 0.02$ ,  $n = 30$ ), which had a median growth inhibition of 79.6% ( $n = 27$ ). This finding reinforces the commonly reported characteristic of hydrogen-peroxide-based algaecide's specific targeting of cyanobacteria while having a lesser impact on eukaryotic algae [11,12,46]. For instance, in a study by Weenink et al. [54], it was observed that the photosynthetic yield of Chlorophyte strains, including *Chlorella*, *Desmodesmus*, *Kirchneriella*, *Ankistrodesmus*, and *Monophoridium*, was significantly inhibited only when the concentration of hydrogen peroxide exceeded 35 mg/L. On the other hand, *Chlamydomonas* showed no sensitivity until the dosage reached 75 mg/L. Although our 16.7 mg/L exceeds the recommended environmental dosage of  $>5$  mg/L [11], the high concentration may be necessary for the removal of *M. aeruginosa*. Yang et al. [12] found the EC<sub>50</sub> concentration of hydrogen peroxide necessary for *Microcystis* to be ten times higher (5.06 mg/L) than that of *Anabaena* (0.50 mg/L), *Cylindrospermopsis* (0.32 mg/L), and *Planktothrix* (0.42 mg/L); this,

along with the previously described resilience of colony-forming *M. aeruginosa* [35,37], was the reason for our comparatively high hydrogen peroxide dosage selection. This dosage could impact nontarget zooplankton and macroinvertebrates and, therefore, the consideration of nontarget impacts should be weighed with ecological benefits of cyanoHAB removal. Yang et al. [12] found little impact on zooplankton at a hydrogen peroxide concentration of 6.7 mg/L in mesocosm experiments, while 20 mg/L dosage caused significant declines in comparison with controls. In an aquaculture pond experiment comparing algaecides, liquid 10.2 mg/L hydrogen peroxide application was found to significantly reduce zooplankton one day after application; however, biomass was found to steadily increase over 35 days, where at the termination of the experiment, the zooplankton biomass was similar to that of controls [55]. The two copper-based products were found to have the lowest zooplankton biomass at the end of the experiment, while another sodium carbonate peroxyhydrate product was also found to have significantly lower zooplankton biomass. Concerns over nontarget organism impacts in using high-dose hydrogen peroxide, therefore, are valid. However, a shortfall in mesocosm studies as well as laboratory toxicity trials is the removal of active avoidance behaviors these organisms may exhibit in the natural environment when exposed to surface-layer hydrogen peroxide [56], affecting exposure time. Exposure is also impacted by the residence time and degradation rates of hydrogen peroxide in water bodies of varying physiochemical and biological characteristics [46,57]. The removal of nontarget organisms is of critical concern and should be assessed based on distinct water body characteristics, where the potential for the removal of nontarget organisms should be avoided, or weighed with the ecological risk of continued persistence of *M. aeruginosa*.

The other SWFL isolated cyanobacteria and chlorophyte strains were also found to be highly sensitive to mixed-treatment applications. Chlorophyte strain FGCU59 was again found to have reduced sensitivity, with the growth inhibition ( $40.3\% \pm 18.6\%$ ) on the last day of the experiment being significantly less than that of all examined cyanobacteria strains (Tables 1 and 2) ( $p < 0.001$ ,  $n = 30$ ), which had a median growth inhibition of 84.5% (Figure 3). These results suggest the combination of hydrogen peroxide and L-lysine treatments may be a viable option for targeting *M. aeruginosa* blooms which could lead to positive succession by eukaryotic algae. Residence time and other environmental factors could greatly influence the degradation of hydrogen peroxide as well as L-lysine in the natural environment and, therefore, further examination through field studies is needed to assess the appropriate dosage for safe and effective application [46,50]. Our result of *M. aeruginosa* FD4 as sensitive to mixed combinations of low-dose hydrogen peroxide (16.7 mg/L) and L-lysine (3 mg/L) suggests that lowered concentrations to achieve ecologically beneficial results may still be effective for the removal of *M. aeruginosa* blooms. Although not examined in this study, L-lysine combinations with lower-dose hydrogen peroxide could be used for the improved protection of nontarget organisms while still attaining the benefits of *M. aeruginosa* removal, and should be explored further in laboratory and field studies. Wang et al. [46] found that a 10.2 mg/L single-application hydrogen peroxide dose was sufficient to completely remove *Microcystis*, and conceivably, given the examined *M. aeruginosa* sensitivity to L-lysine, a lower hydrogen peroxide dose application along with L-lysine may be sufficient to remove *Microcystis*.

### 3.4. Other Application Considerations

Although not examined in the present study, the potential for microcystin exposure after the application of the described algaecides and *M. aeruginosa* cell lysis, releasing intracellular microcystin, is an important consideration in the application of any algaecide. From our understanding, extracellular measurements of microcystin after *Microcystis* exposure to L-lysine have not been examined. In a pond treatment of 9.1 mg/L L-lysine, Kaya et al. [17] found that the total microcystin concentration dropped by half two days after application in the treated pond and further decreased in concentration up to day 7, until reaching the starting value on day 28 after *Microcystis* had reappeared. The total microcystin was significantly less than the controls, however, throughout the 28-day experiment.

In another experiment, the exposure of *Microcystis* to 100  $\mu\text{mol/L}$  (14.62 mg/L) L-lysine after no nitrogen incubation for 7 days was reported to result in continually decreasing microcystin production; even when compared with controls that continued N-starvation, microcystin production was always less in L-lysine treated *Microcystis* [58]. These results suggest L-lysine can reduce intracellular microcystin content, and application may result in an overall reduction in microcystin.

The release of intracellular microcystin after hydrogen peroxide application has been reported. Yang et al. [12] observed an increase in measured extracellular microcystin 24 h after hydrogen peroxide applications (1.3 mg/L, 6.7 mg/L, and 20 mg/L); however, after 7 days, these levels returned to those seen in the control mesocosms, and the intracellular levels for higher doses (6.7 mg/L and 20 mg/L) were less than that in the controls. Chen et al. [59] similarly observed a sharp increase in extracellular microcystin 12 h after 10 and 20 mg/L hydrogen peroxide application; however, microcystin concentration returned to similar starting values one day later. The risk of microcystin exposure after hydrogen peroxide application, therefore, is warranted; however, other beneficial findings have also shown reduced transcription of microcystin synthetase genes after hydrogen peroxide exposure [60], as well as the potential capabilities of oxidized hydrogen peroxide to degrade extracellular microcystin in the water column [59,61], which—in comparison with other algaecides—could also reduce the potential risk of toxin exposure. The intracellular release of microcystin under combined hydrogen peroxide and L-lysine application, therefore, is a concern for the use of this treatment and should be further examined in laboratory studies to assess the risk.

The estimated market price of L-lysine per ton has been previously calculated to range from USD 1000–2000 [41,62], which is similar to the described cost of hydrogen peroxide per ton (USD 500–2000) [59]. Without doubt, the combined use of both algaecides would be costly; however, given the extreme sensitivity of the examined *M. aeruginosa* to this combined treatment, a one-time application to completely remove *M. aeruginosa* may be more achievable, reducing the potential costs of reapplication in comparison with singular chemical treatments. Although more expensive than some metal-based algaecides and coagulants [62], many of the ecological benefits, such as fast degradation rates and low toxicity for nontarget organisms, may outweigh these costs. Copper sulfate, for instance, is a cheap, widely used algaecide; however, it has proven to be nonselectively toxic to other organisms such as fish as well as accumulate in sediments, further impairing ecosystem functioning and biota [10,42,63].

#### 4. Conclusions

In summary, our laboratory-scale experiment provided valuable information on the application concentrations and comparative toxicity of *M. aeruginosa* and other phytoplankton to L-lysine, hydrogen peroxide, and mixed treatments for future implementation at larger scales. We found that *M. aeruginosa* sensitivity to L-lysine and hydrogen peroxide treatments varied by strain; axenic *M. aeruginosa* strains were significantly more sensitive to hydrogen peroxide than nonaxenic strains, while the growth of axenic and nonaxenic *M. aeruginosa* did not differ for L-lysine. All the tested *M. aeruginosa* strains were highly sensitive to mixed treatments of L-lysine and hydrogen peroxide, showing this combination can be an effective mitigation method. L-lysine was found to selectively inhibit the growth of *M. aeruginosa*, while the other tested phytoplankton had reduced or no sensitivity. Some of these strains were sensitive to hydrogen peroxide and mixed treatments; however, chlorophyte *C. zofingiensis* was found to be more resilient to both treatments. Given the growing concern over increased eutrophication and incidences of *M. aeruginosa* blooms worldwide, information such as this on environmentally friendly algaecide options can greatly assist water managers, taking into consideration the environmental and ecological characteristics of applied water bodies.

**Author Contributions:** Conceptualization, E.K.D. and H.U.; methodology, E.K.D. and H.U.; validation, E.K.D.; formal analysis, E.K.D.; investigation, E.K.D.; data curation, E.K.D. and H.U.; writing—original draft, E.K.D.; writing—review and editing, E.K.D. and H.U.; supervision, H.U.; project administration, H.U.; funding acquisition, H.U. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was partially supported by the Florida Department of Environmental Protection Innovative Technology Grant INV\_007, and the NSF Division of Environmental Biology grant DEB-1664052. Elizabeth Dahedl and Hidetoshi Urakawa were also supported by Seidler Student/Faculty Scholarly Collaboration Fellowship (2020 summer research) at Florida Gulf Coast University.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Acknowledgments:** We thank Julia Davis, Michael Kratz, and Taylor Hancock, Haruka Urakawa at Florida Gulf Coast University for their assistance.

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

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