



Oxidative stress in the cyanobacterium *Microcystis aeruginosa* PCC 7813: Comparison of different analytical cell stress detection assays

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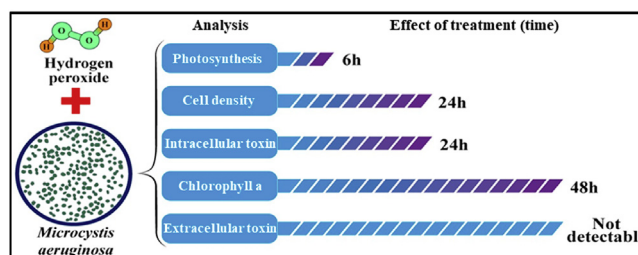
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HIGHLIGHTS

- Time lag observed between cell stress occurring and its detection by most methods.
- Photosynthetic activity analysis was the fastest method for cell stress detection.
- Advantages and drawbacks of five different cell stress detection assays elucidated.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyanobacterial blooms are observed when high cell densities occur and are often dangerous to human and animal health due to the presence of cyanotoxins. Conventional drinking water treatment technology struggles to efficiently remove cyanobacterial cells and their metabolites during blooms, increasing costs and decreasing water quality. Although field applications of hydrogen peroxide have been shown to successfully suppress cyanobacterial growth, a rapid and accurate measure of the effect of oxidative stress on cyanobacterial cells is required. In the current study, H_2O_2 (5 and 20 $mg\ L^{-1}$) was used to induce oxidative stress in *Microcystis aeruginosa* PCC 7813. Cell density, quantum yield of photosystem II, minimal fluorescence and microcystin (MC-LR, -LY, -LW, -LF) concentrations were compared when evaluating *M. aeruginosa* cellular stress. Chlorophyll content (determined by minimal fluorescence) decreased by 10% after 48 h while cell density was reduced by 97% after 24 h in samples treated with 20 $mg\ L^{-1}$ H_2O_2 . Photosystem II quantum yield (photosynthetic activity) indicated cyanobacteria cell stress within 6 h, which was considerably faster than the other methods. Intracellular microcystins (MC-LR, -LY, -LW and -LF) were reduced by at least 96% after 24 h of H_2O_2 treatment. No increase in extracellular microcystin concentration was detected, which suggests that the intracellular microcystins released into the surrounding water were completely removed by the hydrogen peroxide. Thus, photosynthetic activity was deemed the most suitable and rapid method for oxidative cell stress

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detection in cyanobacteria, however, an approach using combined methods is recommended for efficient water treatment management.

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1. Introduction

Climate change, and eutrophication contribute to cyanobacterial blooms in freshwater reservoirs (Weenink et al., 2015). Cyanobacteria can be a threat to drinking water quality since they are potential producers of a wide variety of toxins. These toxins are a hazard to both human and animal health (Falconer et al., 1983; Jochimsen et al., 1998; Pinho et al., 2015) and high cell densities may complicate the potable water treatment process by reducing filter run times and increasing the use of chemicals (e.g., coagulants and disinfectants), which raises the cost of water treatment (De Julio et al., 2010).

Microcystins (MC) are one of the most commonly reported cyanotoxins found in freshwater (Pinho et al., 2015). These toxins are cyclic heptapeptides that share a common structure with two amino acid domains which vary in positions 2 and 4 of the structure (Rinehart et al., 1994; Harke et al., 2016). Currently, there are at least 247 identified microcystin analogues (Spoof and Catherine, 2017). Microcystins are normally localized inside the cells and are mostly released to the water after cell membrane lysis and death (Tsai, 2015).

Conventional water treatment processes (coagulation, flocculation, sedimentation and filtration) can be ineffective in removing high quantities of cyanobacteria and their metabolites (Fan et al., 2013a; Zhou et al., 2014). Chemical treatments using conventional oxidants may be used to help remove cyanobacteria (e.g., ozone, chlorine, potassium permanganate and chlorine dioxide) but can cause cell damage and the release of cyanotoxins (Zamyadi et al., 2011; Chang et al., 2018). Due to the inefficiency of conventional water treatment for the removal of cyanobacteria and their toxins, it is necessary to evaluate complementary technologies that can be applied in freshwater reservoirs, i.e., eliminating cyanobacteria and their toxins prior to them entering the treatment plant.

Hydrogen peroxide (H_2O_2) has been applied as an algicide to control cyanobacterial blooms. H_2O_2 can generate reactive oxidative species (ROS) that have high oxidative strength and are capable of compromising the cell wall. Due to this, the detection of cell stress and/or damage is important because it indicates the efficacy of the method and potential toxin release (Chow et al., 1998).

Studies have suggested that H_2O_2 is an effective algicide for cyanobacterial treatment in-reservoir, however, it is necessary to compare and verify the most suitable and rapid method to analyse the effects of H_2O_2 on cyanobacteria.

Cyanobacteria are particularly susceptible to H_2O_2 due to their physiology. One of the factors promoting the use of hydrogen

peroxide is that it can be used directly in freshwater reservoirs as an algicide to oxidize cyanobacterial cells and their toxins (Fan et al., 2013b, 2019) without producing oxidant residuals, as it decomposes into water and oxygen (Barroian and Feuillade, 1986).

Cell integrity and evaluation of cell numbers can be used to determine the effects of treatment technologies on cyanobacteria. Analytical methods to determine the effect of treatments on cyanobacteria, such as fluorescence detection of photosynthetic activity, can be used as a measure of cell stress providing an indirect measure of photoinhibition in photosynthesis (Campbell et al., 1998; Yang et al., 2013; Schuurmans et al., 2015; Weenink et al., 2015; Ogawa et al., 2017).

In this study, a range of methods for identifying cell stress in the cyanobacterium *Microcystis aeruginosa* PCC 7813 under the effect of different concentrations of H_2O_2 were compared to identify which analytical method provides the most accurate and rapid response. Further, although several studies have analyzed the effects of H_2O_2 on MC-LR (Qian et al., 2010; Matthijs et al., 2012; Papadimitriou et al., 2016; Kansole and Lin, 2017; Chang et al., 2018; Fan et al., 2019; Wang et al., 2015, 2018, 2019), here, for the first time, the degradation of intra- and extracellular concentration of four different microcystin analogues (MC-LR, MC-LF, MC-LY, and MC-LW) was evaluated under different concentrations of H_2O_2 .

2. Materials and methods

2.1. Cyanobacteria

The cyanobacterium *M. aeruginosa* PCC 7813 (Pasteur Culture Collection, Paris) was cultured in BG-11 medium (Stanier et al., 1971) at 21 ± 1 °C on a 12/12 h light/dark cycle illuminated by cool white fluorescent lights (correlated color temperature 1400K–5000K) with an average illumination of $10.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ without agitation. This particular strain of *M. aeruginosa* produces four main microcystin analogues (MC-LR, MC-LY, MC-LW, and MC-LF).

2.2. *M. aeruginosa* PCC 7813 cell enumeration

Cell counting by Multisizer for *M. aeruginosa* numbers and determination of average cell diameter has been previously demonstrated (Wojtasiewicz and Stoń-Egiert, 2016; Kim et al., 2020). A Multisizer 3 (Beckman Coulter, USA) was used to enumerate *M. aeruginosa* PCC 7813 cell density, to evaluate bio-volume and average cell diameter. A $50 \mu\text{m}$ aperture was used, which allows particle size detection from 1 to $30 \mu\text{m}$. Samples were diluted 100 to 600-fold in Isoton carrier liquid (Beckman Coulter, USA), depending on the sample density.

2.3. Effect of H_2O_2 on cyanobacterium *M. aeruginosa* PCC 7813

A 100 mL cell suspension of *M. aeruginosa* PCC 7813 (in 250 mL conical flasks) with a final concentration of 5×10^6 cells mL^{-1} in BG-11 was prepared and cultured for three days. Hydrogen peroxide (5 and 20 mg L^{-1}) was added to the conical flasks containing *M. aeruginosa* PCC 7813. Aliquots of 3 mL were removed at known intervals (0, 6, 12, 24, 30, 36, 48, 60, 72, 84 and 96 h) over 4 days.

Samples were incubated under the same conditions as the strain was initially cultured. Treatments were performed in triplicates. Aliquots were removed for analysis of H_2O_2 concentration (100 μL) and cell enumeration (900 μL), intra/extracellular microcystin determination (1 mL) and photosynthetic activity measurements (1 mL). The aliquots for toxin analysis were centrifuged for 10 min at $13000\times g$ and the supernatant was transferred to a fresh microcentrifuge tube (1.5 mL) and stored at -20°C , with the cell pellet also stored at -20°C . The aliquots for all other analyses were used immediately. A negative control (no H_2O_2 addition) was also prepared in triplicate.

2.4. Analysis

2.4.1. H_2O_2 analysis

To determine the H_2O_2 concentration, a method by Drábková et al. (2007a) with modifications by Fan et al. (2013b) was used. A phosphate buffer solution was prepared with 0.5 M sodium phosphate dibasic (Na_2HPO_4) solution and 0.5 M sodium phosphate monobasic (NaH_2PO_4) solution with a final pH of 6 (all Sigma-Aldrich, UK). A solution with 0.1 g of N,N-diethyl-1,4-phenyldiammoniumsulphate (Sigma-Aldrich, UK) in 10 mL of 0.1 N sulfuric acid (H_2SO_4 , Fisher, UK) was prepared (DPD solution). Further, a horseradish peroxidase (HRP) (Sigma-Aldrich, UK) solution 1 mg L^{-1} in ultrapure water was prepared.

For analysis, 900 μL of ultrapure water and 100 μL of phosphate buffer solution were transferred into a 1 mL cuvette. Aliquots of the cell suspension were centrifuged and 40 μL of the supernatant were added to the cuvette, followed by 40 μL of DPD solution and 10 μL of HRP solution. A blank was prepared by adding 900 μL of ultrapure water followed by 100 μL of buffer solution, 40 μL of the supernatant from the control and 40 μL of DPD solution into a cuvette. All the samples were measured using a UV/VIS spectrophotometer (WPA Lightwave II, UK) at a wavelength of 551 nm H_2O_2 (30%, Fisher, UK) was used for the H_2O_2 degradation assay. Sodium Thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3\cdot 5\text{H}_2\text{O}$, Fisher, UK) was added to excess into the supernatant after the H_2O_2 determination to quench the sample.

2.4.2. High-performance liquid chromatography (HPLC) analysis of extra- and intracellular microcystin concentrations

The supernatant was removed and freeze-dried. Aliquots were resuspended in methanol (1 mL), vortexed and centrifuged for 10 min at 13000 G. Following this, 950 μL were transferred to a fresh microcentrifuge tube (1.5 mL) and dried in a Genevac (EZ-II evaporator, UK), resuspended in 80% methanol (100 μL) and analyzed. To the cell pellet, 80% aqueous methanol (250 μL) was added and sample tubes placed in a dispersive extractor for 5 min at 2500 rpm followed by centrifugation for 5 min at 13000 G and if not analyzed immediately, samples were stored at -20°C until analysis.

The concentrations of four microcystin analogues (MC-LR, MC-LY, MC-LW, and MC-LF) were quantified using a 2965 separation module and a 2996 photodiode array (PDA) detector (Waters, Elstree, UK). Separation of analytes was achieved on a Symmetry C18 column (5 μm particle size, 2.1 mm ID \times 150 mm long; Waters, Elstree, UK). The mobile phases used for analysis were A: ultrapure water (18.2 M Ω) and B: acetonitrile each with 0.05% trifluoroacetic acid at a flow rate of 0.3 mL min $^{-1}$, an injection volume of 35 μL and a column temperature of 40°C . Initial condition was set to 80% A and 20% B, increasing to 70% B over 25 min followed by an organic wash and a return to the initial condition. All chromatograms were analyzed at 238 nm and quantified using standards (as per Enzo Life Sciences) for calibration between 0.05 and 25 $\mu\text{g mL}^{-1}$ in the Empower software (V3). The limit of quantification was

0.05 $\mu\text{g mL}^{-1}$.

2.4.3. Determination of photosynthetic activity

A Mini-PAM system (Walz, Germany) was used at room temperature to determine the effect of H_2O_2 on the photosynthetic activity. This instrument evaluates the photosynthetic activity by measuring the maximal values of quantum yield of photosystem II (PSII) (F_V/F_M), where F_V is the difference between the true maximal fluorescence (F_M) and the minimal fluorescence (F_0).

F_0 is determined by emitting a low intensity measuring light for 20 s, followed by a saturating pulse, which yields the maximal fluorescence (F_M). After 40 s, actinic light is activated (actinic light intensity at specified level 3), which allows the determination of the steady-state fluorescence (F_S) (Ogawa et al., 2017). This is true for higher plants and green algae, however, the photosynthetic complex in cyanobacteria functions differently due to an effect called state transition. This means that there is a change in energy allocation between the two photosystems (PSI and PSII) in the cells, resulting in more energy in PSI (Schuurmans et al., 2015; Ogawa et al., 2017). Due to this, it is necessary to add diuron (Sigma-Aldrich, UK), an algaecide capable of inhibiting photosynthesis, under actinic light to detect the true maximal fluorescence in cyanobacteria (F_M') by a saturating pulse (Ogawa et al., 2017).

A sample of cells (400 μL) was added into a cuvette containing a small stirrer bar for agitation. After measuring F_0 and F_M readings, Diuron (0.5 M) was added to photoquench the sample and measure F_M' (Campbell et al., 1998).

2.5. Statistical analyses

The values shown are the results of the mean of triplicates and all results were analyzed using one-way ANOVA. A significance level of $p < 0.05$ was used to identify significant differences between the results.

3. Results and discussion

3.1. Cell enumeration and characterization

When analyzing *M. aeruginosa* PCC 7813 cell density, the distribution was observed for the particle diameter range of 2.8–6.9 μm (Fig. 1A and B) for all the samples at time T_0 which were considered cells. Particles in the range of 1.3–2.7 μm were considered cell fragments. The decision to consider particles in the range of 2.8 and 6.9 μm cells was based on the size distribution of the initial sample (Fig. 1) and published data. For example, Komárek et al. (2002) report average cell sizes from 4 to 6 μm for *M. aeruginosa* and Harke et al. (2016) report cell sizes from 1 to 9 μm for the genus *Microcystis*. A decrease in the average particle diameter and an increase in the distribution maxima caused by a rise in cell density were observed in the control (Fig. 1A) and the 5 mg L $^{-1}$ (Fig. 1B) samples from 0 to 96 h. The particle diameter decrease is likely due to cyanobacterial reproduction by binary fission, which leads to a decrease in individual cell diameter due to cell division (Cassier-Chauvat and Chauvat, 2014).

The 20 mg L $^{-1}$ of H_2O_2 caused extensive cell damage to *M. aeruginosa* PCC 7813, increasing the number of fragments (particles in the range of 1.3–2.8 μm) (Fig. 1C). The effect of 20 mg L $^{-1}$ H_2O_2 was immediate with cells rapidly fragmenting by 97%. Over the next 24 h the cell density increased slightly again from about 45 h onwards (Fig. 2), which could represent post-treatment recovery. Although cells did not show immediate removal in the 5 mg L $^{-1}$ H_2O_2 samples, there is evidence of growth inhibition with cell density significantly lower ($p = 1 \times 10^{-4}$) than that of the

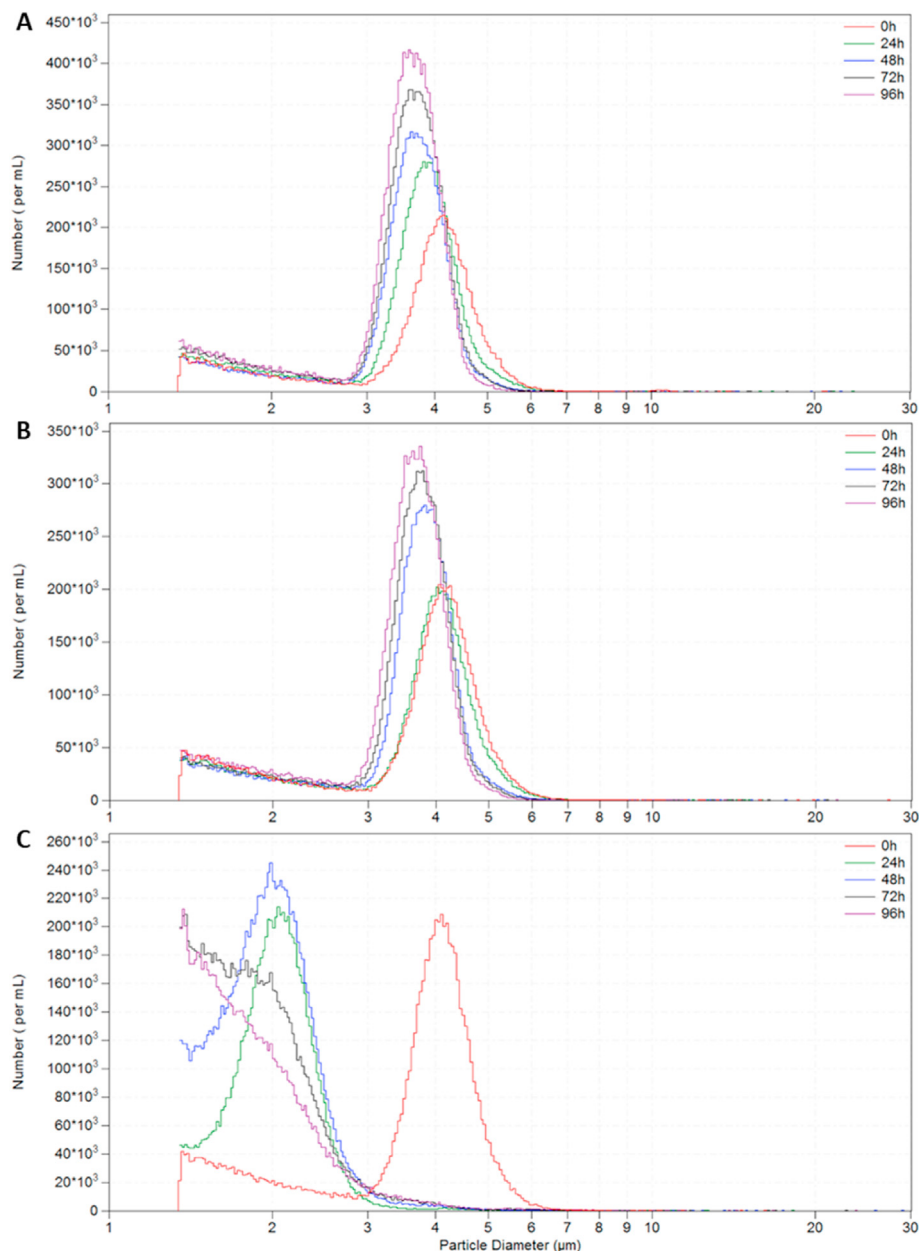


Fig. 1. *Microcystis aeruginosa* PCC 7813 cell density (A) control, (B) 5 and (C) 20 mg L⁻¹ hydrogen peroxide over 96 h under cool white fluorescent lights of 10.5 μmol photons m⁻² s⁻¹.

control (Fig. 2).

Particles in the range from 1.3 to 2.8 μm were classified as cell debris (Fig. 3), which included damaged cells and cell fragments. No significant change ($p = 9 \times 10^{-2}$) was observed between 0 mg L⁻¹ and 5 mg L⁻¹ H₂O₂. However, the amount of cell debris in the 20 mg L⁻¹ sample significantly increased ($p = 9.2 \times 10^{-6}$) over 60 h of exposure as cells continued to fragment. There was an increase of cell fragment density over the first 60 h which then decreased as the cell debris continued to break down into smaller fragments to the point where fragments had decreased in size to below 1.3 μm and were undetectable (Fig. 1C). A similar observation was made by Fan et al. (2013b) where the effects of hydrogen peroxide doses (10.2, 51 and 102 mg L⁻¹) on *M. aeruginosa* (strain 338) membrane integrity were evaluated over 7 days. There were no significant differences in cyanobacterial cell density after 7 days of exposure to

H₂O₂, although it was possible to observe a decrease in cell density in the present study when using 20 mg L⁻¹. However, despite the differences in cell density results between the two studies, in Fan et al. (2013b), the cells decreased in size and fragmented under all the H₂O₂ concentrations which suggests a similar response as indicated by the cell debris observed in the current study.

The cyanobacterial degradation processes by H₂O₂ occur by the production of ROS that attack and destroy the cyanobacterial cell membrane. After that, ROS enter the cell resulting in photo-inhibition while the cyanobacterial intracellular material is released into the extracellular matrix. Finally, ROS facilitate the oxidation of pigments (e.g., chlorophyll *a*) (Wang et al., 2017).

Comparing the current study with the study of Fan et al. (2013b), there were differences in the study design that could explain the results observed: the growth light intensity and the light intensity

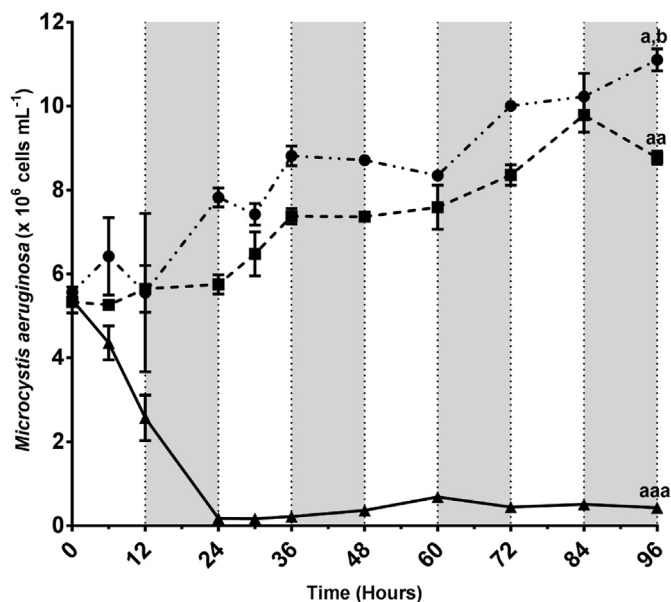


Fig. 2. *Microcystis aeruginosa* cell density for particle size range 2.8–6.9 μm after exposure to 0 (\bullet), 5 (\blacksquare) and 20 (\blacktriangle) mg L^{-1} H_2O_2 over 96 h under cool white fluorescent lights of $10.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Grey sections on the graph denote dark time. a, aa, aaa = significantly different from time T_0 ; b = significantly different from 96 h 5 mg L^{-1} ($n = 3$, error bars = σ_{-1}).

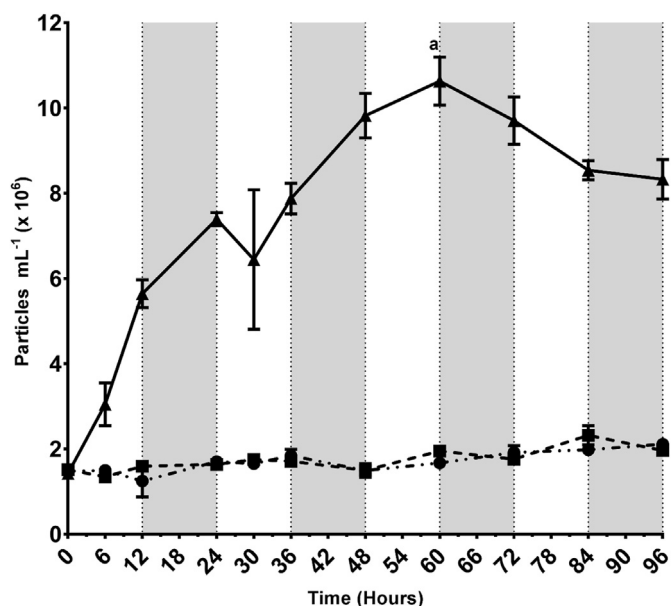


Fig. 3. *Microcystis aeruginosa* PCC 7813 particle density for particle size range 1.3–2.8 μm after exposure to 0 (\bullet), 5 (\blacksquare) and 20 (\blacktriangle) mg L^{-1} H_2O_2 over 96 h under cool white fluorescent lights of $10.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Grey sections on the graph denote dark time. a = significantly different from time T_0 . ($n = 3$, error bars = σ_{-1}).

used during the current experiment were almost five times lower compared to the Fan et al. (2013b) study. Further, the growth media used in the current study (BG-11) had a higher iron content than the one used (ASM-1) in the study by Fan et al. (2013b). This higher iron content may lead to photo-fenton reactions, intensifying cell disruption in the present study. The toxicity of H_2O_2 on cyanobacteria depends on several factors such as light intensity, pre-adaptation to growth at a higher light intensity and the generation of hydroxyl radicals by the photo-fenton reaction of H_2O_2 with

Fe^{2+} ions present in the medium (Drábková et al., 2007a; Chen et al., 2016). Further, a different strain of *M. aeruginosa* was used in the current investigation which might cause further differences in the results.

3.2. *M. aeruginosa* photosynthetic activity assay

A factor that can represent the level of stress in a cyanobacterial cell is photosynthetic activity as expressed as the F_v/F_m ratio (Yang et al., 2013). Photosynthesis is the primary production in cyanobacteria/algae. Energy in the form of light is captured and drives the synthesis of sugar while consuming carbon dioxide and generating oxygen. The addition of H_2O_2 can generate the production of intracellular ROS that are mainly created in cyanobacteria when the absorption of light energy by chlorophyll α is higher than the amount of energy that can be used by the photosynthetic apparatus of the cell. These ROS cause damage in cyanobacteria by blocking the electron transport of PSII thus decreasing the photosynthetic activity in a process known as photoinhibition (Lupínková and Komenda, 2004; Wang et al., 2019). Photoinhibition in cyanobacteria causes a decrease in F_v/F_m and, when F_v/F_m is close to or zero, the cells are so damaged or stressed that photosynthetic activity is absent. F_v/F_m was not significantly different for the control and the 5 mg L^{-1} samples of the experiment (for all samples $p > 0.05$; Fig. 4). F_v/F_m decreased in the 20 mg L^{-1} samples and the photosynthetic activity was inhibited from 6 to 48 h indicating that the photosynthetic system of *M. aeruginosa* cells was inhibited (Fig. 4). It must be noted that the observed increase in F_v/F_m after 48 h is likely to be an artifact as one of the triplicate samples started showing signs of recovery.

The immediate decrease of F_v/F_m was detected following 6 h of exposure to 20 mg L^{-1} H_2O_2 . It is possible that F_v/F_m was affected before 6 h of treatment but was not detected earlier because the first sampling was only performed after 6 h of exposure to H_2O_2 .

The difference between replicates in the 20 mg L^{-1} dose from 60 h onwards can be explained by the fact that the differences after the photoinhibition were very small (Fig. 4), therefore even small variance can cause large error. The F_0 varied between 7 and 15 and the F_m varied between 7 and 24. Further, one of the samples showed signs of recovery with a F_v/F_m increasing from 0.06 to 0.44 between 48 and 60 h.

The H_2O_2 concentration was analyzed over the 96 h of treatment. After 30 h, the H_2O_2 was completely consumed in the 5 and 20 mg L^{-1} samples (Fig. 5). The fast decrease of the H_2O_2 concentration in the 20 mg L^{-1} samples could have made recovery possible for the *M. aeruginosa* PCC 7813 cells, which, in turn, would have prevented longer-term suppression.

Several investigations described the behavior of *M. aeruginosa* photosynthetic yield when treated with different H_2O_2 concentrations. In a study by Wang et al. (2019), *M. aeruginosa* FACHB-905 was treated with different concentrations of H_2O_2 (0, 2, 5, 8 and 10 mg L^{-1}) for 2 h. A decrease in the F_v/F_m was observed for every dosage used in the study by Wang et al. (2019), even for lower dosages than the H_2O_2 concentration used in the present investigation (20 mg L^{-1}). This decrease could be because the photosystem II of the cells was not completely quenched, so part of the energy was allocated to photosystem I. This reallocation of light energy from one photosystem to another in cyanobacteria is called state transition (Ogawa et al., 2017). To suppress state transition, diuron was added in the current study, allowing a determination of the true maximal fluorescence F_m value in cyanobacteria. Further, in the Wang et al. (2019) study the cells were centrifuged before analysis, which could have further affected the cell stress response.

Wang et al. (2018) demonstrated the effects of H_2O_2 at different

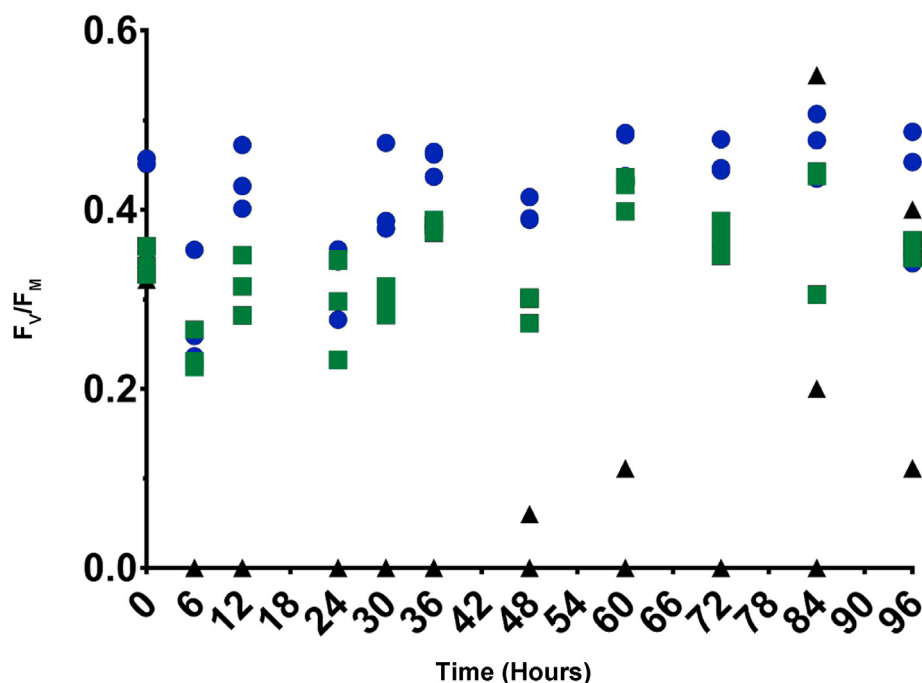


Fig. 4. *Microcystis aeruginosa* PCC 7813 maximal quantum yield results after being treated with 0 (●), 5 (■) and 20 (▲) mg L⁻¹ H₂O₂ over 96 h under cool white fluorescent lights of 10.5 μmol photons m⁻² s⁻¹. Each point represents the individual replicates.

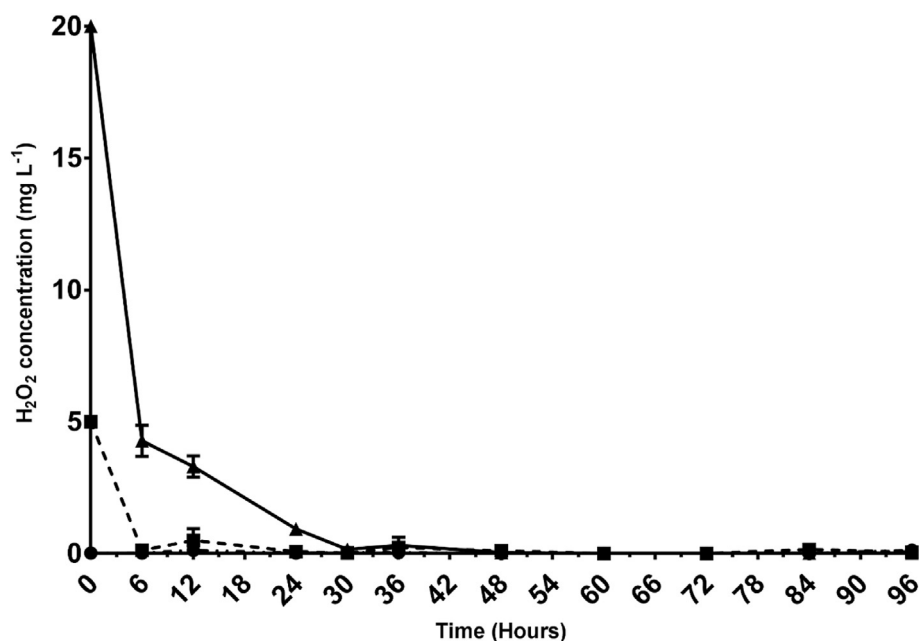


Fig. 5. Decrease in hydrogen peroxide concentration of different dosages 0 (●), 5 (■) and 20 (▲) mg L⁻¹ over 96 h in the presence of *Microcystis aeruginosa* PCC 7813 under cool white fluorescent lights of 10.5 μmol photons m⁻² s⁻¹ ($n = 3$, error bars = σ_{-1}).

dosages (0, 2, 5 and 10 mg L⁻¹) on *M. aeruginosa* FACHB-905 cells using the same initial cell density analyzed in the present study. In the Wang et al. (2018) study, a significant decrease in the F_v/F_m ratio after 72 h was observed even when using lower dosages of H₂O₂ (5 mg L⁻¹) compared to the present study (20 mg L⁻¹). Again, this could have happened due to the reallocation of energy to PSI, decreasing F_v/F_m value as no addition of state transition-suppressing chemicals was reported. The higher illumination and temperature used by Wang et al. (2018), 40 μmol m⁻² s⁻¹ at 25 °C

compared to 10.5 μmol m⁻² s⁻¹ and 21 °C, could be another factor that influenced the results.

Other studies evaluated different cyanobacterial species. Weenink et al. (2015) studied the effects of different concentrations of H₂O₂ (2.5, 5, 10, 20 and 50 mg L⁻¹) treating *Planktothrix*-dominated lake samples containing three phytoplankton groups (cyanobacteria, green algae, and diatoms). In the concentrated sample, the fluorescence value increased after 4 days of treatment in all H₂O₂ concentrations, showing similar signs of recovery from the

ones found in the current study. In another large-scale study using different cyanobacterial species, Matthijs et al. (2012) verified a similar decrease in the photosynthetic viability when analyzing *Planktothrix agardhii*-dominated lake samples under the effects of lower dosages of H_2O_2 (0, 0.5, 1, 2 and 4 mg L^{-1}).

In the present study, the F_0 was used as an indirect indicator of chlorophyll α concentration which plays a central role in cyanobacterial photosynthesis (Qian et al., 2010). After 6 h, the impact on photosynthetic activity (decrease in F_v/F_m) was already obvious in the 20 mg L^{-1} samples (Fig. 4) but a decrease in F_0 (i.e., chlorophyll α) was only noticed after 48 h of H_2O_2 exposure (Fig. 6).

Increase in the fluorescence in 20 mg L^{-1} samples when compared to the control between 6 and 36 h (Fig. 6) was also observed by Chen et al. (2016). When analyzing the effects of 0, 1, 5, and 20 mg L^{-1} on lake samples dominated by *Microcystis* sp. and *Anabaena* sp. over 72 h, they also found a chlorophyll α increase between 12 and 24 h for 5 and 20 mg L^{-1} H_2O_2 samples. An increase in F_0 values is connected to cell damage after stress (Drábková et al., 2007b) so it is possible that the increase in F_0 is a fast chemical response due to an increase in measurable chlorophyll α as a cell protection mechanism or a physical change in the cells which allow more chlorophyll α to be detected.

3.3. Effect of H_2O_2 on intracellular and extracellular microcystins

The removal efficiency of microcystins by H_2O_2 depended on the dosage of H_2O_2 (Fig. 7). All four intracellular microcystin analogues were completely degraded after 24 h of treatment when using 20 mg L^{-1} H_2O_2 , i.e., as cells were lysed and microcystins released, toxin was rapidly degraded. However, a significant increase of intracellular MC-LW and -LF after 60 h, and in MC-LY after 72 h (for all the samples tested $p < 0.05$) was observed. There was an increase ($p < 0.05$) of intracellular MC-LR, -LY, -LW and -LF after 96 h of exposure when using 0 and 5 mg L^{-1} of H_2O_2 which corresponds

to the increase in *M. aeruginosa* PCC 7813 cell density (Fig. 2) for the same dosages due to cell growth. The increase for MC-LR was more marked than for the other microcystin analogues produced by *M. aeruginosa* PCC 7813 which can be explained by the fact that MC-LR is the main microcystin produced by this organisms and has been observed previously (Pestana et al., 2020).

Microcystins are normally contained within the cells, and are released into the surrounding water along with other intracellular content when cell integrity is compromised. H_2O_2 is capable of lysing cells and releasing the microcystins. After the liberation of intracellular microcystins in all the 20 mg L^{-1} samples (Fig. 7), no extracellular microcystin was detected. This suggests that the intracellular microcystins that were released into the surrounding water were rapidly removed by H_2O_2 .

A few studies have investigated the removal of MC-LR by H_2O_2 , but this is the first time that the effect of H_2O_2 on these four microcystin analogues, both intra- and extracellular, has been evaluated (MC-LR, -LY, -LW and -LF). Papadimitriou et al. (2016) showed that both intra- and extracellular MC-LR decreased over 4 h in a study with naturally occurring MC-LR and 4 mg L^{-1} H_2O_2 . The study by Papadimitriou et al. (2016) observed that the use of H_2O_2 caused cyanobacterial cell lysis followed by a release of intracellular microcystin which became extracellular microcystin and then was removed by H_2O_2 . The successful removal of MC-LR even at lower H_2O_2 dosages was most likely due to a lower initial concentration of dissolved toxins used by Papadimitriou et al. (2016), intracellular and extracellular microcystins were below $2.5 \mu\text{g L}^{-1}$ and $0.5 \mu\text{g L}^{-1}$, respectively.

Kansole and Lin (2017) showed similar results to the present study when evaluating cyanobacterium *M. aeruginosa* PCC 7820 with a similar initial cell density ($2 \times 10^6 \text{ cells mL}^{-1}$) and under the effects of several H_2O_2 dosages (0, 1, 2, 3, 5, 10 and 20 mg L^{-1}). The highest dosage of H_2O_2 used was able to reduce both MC-LR and *M. aeruginosa* PCC 7820 cells by 40% and 95%, respectively. It should

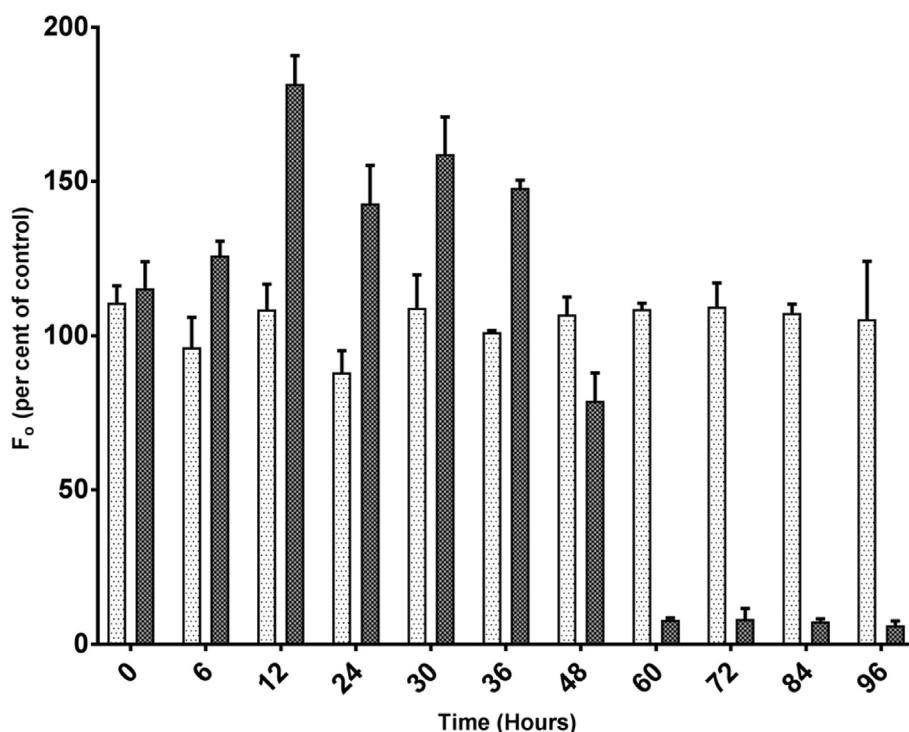


Fig. 6. *Microcystis aeruginosa* PCC 7813 minimal fluorescence (indicative of chlorophyll α) results (percentage of the control) after being treated for 96 h with different H_2O_2 dosages (\square) 5 and (\blacksquare) 20 mg L^{-1} under cool white fluorescent lights of $10.5 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with different dosages of H_2O_2 ($n = 3$, error bars = σ_{-1}).

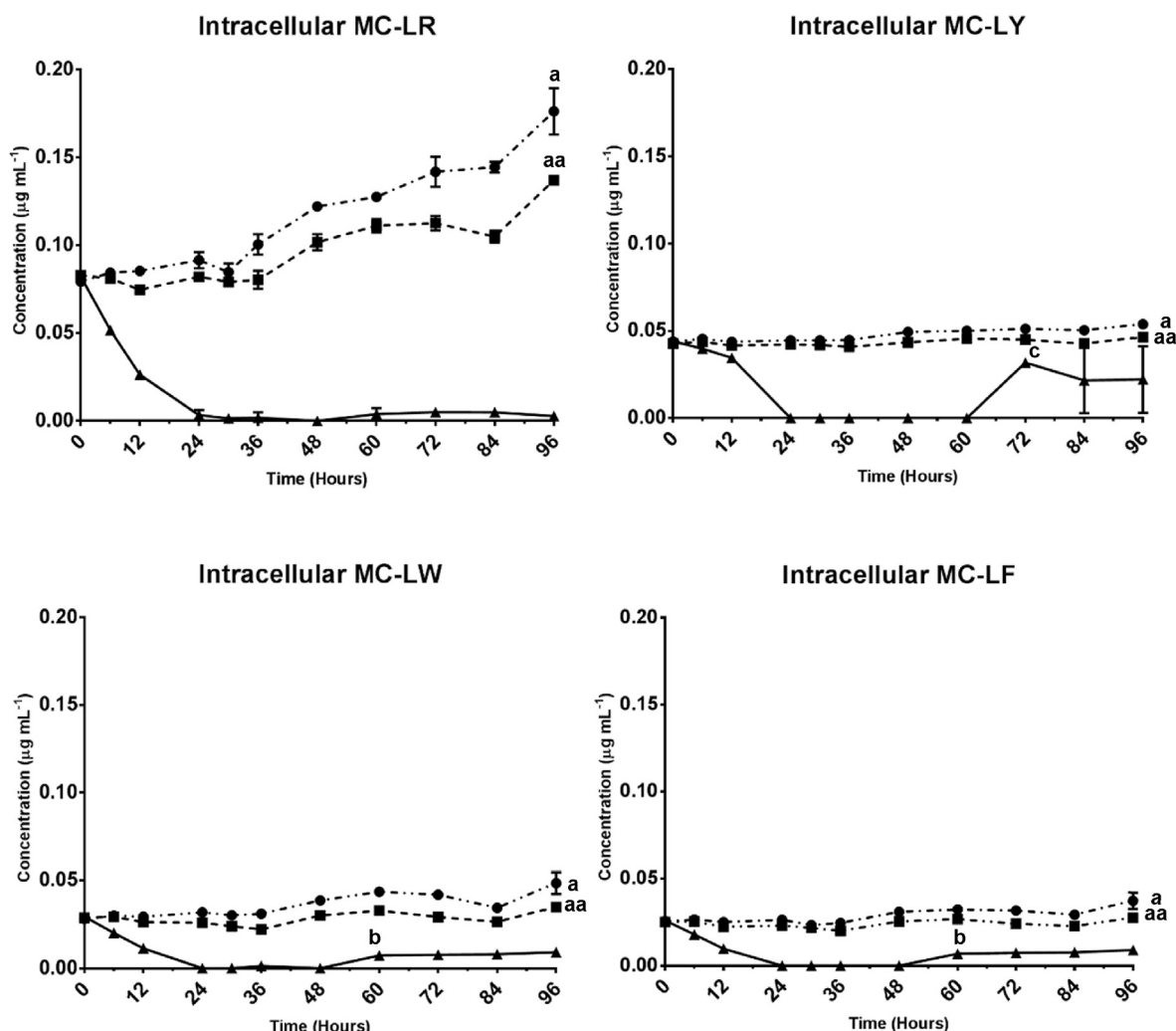


Fig. 7. Removal of intracellular MC-LR, -LY, -LW and -LF after exposure to 0 (●), 5 (■) and 20 (▲) mg L⁻¹ hydrogen peroxide over 96 h under cool white fluorescent lights of 10.5 μmol photons m⁻² s⁻¹. a, aa = significantly different from T₀; b = significantly different from 48 h; c = significantly different from 60 h (n = 3, error bars = σ₋₁).

be noted that *M. aeruginosa* PCC 7813 is almost identical to *M. aeruginosa* PCC 7820 (isolated from the same original bloom), producing the same microcystins, with the exception that the PCC 7813 strain does not contain gas vesicles. As would be expected that the absence of gas vesicles would not affect cell inhibition by H₂O₂.

3.4. Comparison of cell stress detection methods

While there are more sophisticated methods that can be used for cell stress determination, such as flow cytometer with nucleoid acid dyes or cellular ROS determination, the methods used in the current study to analyse cyanobacterial cell stress were selected considering operation and availability in a water treatment company laboratory. Each method presented advantages and disadvantages for the determination of H₂O₂ induced oxidative stress in *M. aeruginosa* PCC 7813. It is possible to notice an early stress response from the cells when analyzing the inhibition of photosynthetic activity in cyanobacterial cells compared to other methods (Table 1), such as cell density analysis, chlorophyll *a* and toxin determination. While F₀ (the indicator of chlorophyll *a*) decreased after 48 h (Fig. 6) and cell density indicated complete removal after 24 h (Fig. 2), F_V/F_M provided a more accurate and rapid response demonstrating cyanobacteria cell stress detectable

at 6 h after exposure to H₂O₂ (Fig. 4). Chlorophyll *a* concentration is a poor indicator of cellular stress, as it is initially intracellular and its concentration is only affected a considerable time after cell lysis and intracellular matter leakage into the surrounding water. Detection of chlorophyll *a* only indicates that this pigment is present in the samples. It is not a measure of cell viability, as demonstrated by the fact that a decrease in F₀ was detected only after 48 h but decline cell density was clearly seen after 24 h.

Similar limitations are true for microcystins as an indicator cell lysis and death. After the degradation of the cell membrane, intracellular microcystin is released into the surrounding water which was observable after 24 h, thus, while a reliable proxy measurement for cell integrity, the effects on the intracellular microcystins are less rapid than measurement of the photosynthetic activity. Since it is not possible to detect any differences in extracellular microcystin concentration over 96 h, this method is not suitable to identify cyanobacterial cellular stress responses during oxidation. The measurement of extracellular levels of microcystin as an indicator of cell lysis is confounded by the ongoing destruction of the toxins by the oxidant. As the microcystins increase in concentration in the surrounding media due to leakage from cells they will rapidly be chemically degraded by the H₂O₂. Hence, the detected concentration is a result of the total

Table 1
Comparison of different analytical methods for cell stress determination.

Method	Response time	Advantages	Disadvantages
F_v/F_m photosynthetic activity (photosynthesis)	6 h	<ul style="list-style-type: none"> Fastest response of cell stress Applicable for <i>in-situ</i> and laboratory Rapid analysis (<10 min) 	<ul style="list-style-type: none"> Requires diuron which is hazardous Non-specific (no differentiation between phytoplankton species)
Cell density (by particle counter)	24 h	<ul style="list-style-type: none"> Detects changes in particle distribution Rapid analysis (<10 min) 	<ul style="list-style-type: none"> Laboratory analysis Does not differentiate viable from non-viable cells Less suitable for filamentous organisms
Minimal fluorescence F_0 (chlorophyll α)	48 h	<ul style="list-style-type: none"> Applicable for <i>in-situ</i> and laboratory Rapid analysis (<10 min) 	<ul style="list-style-type: none"> Slowest detection of cellular stress Non-specific (no differentiation between phytoplankton species) Detection of chlorophyll, but not viability
Intracellular toxin	24 h	<ul style="list-style-type: none"> Good proxy for cell integrity 	<ul style="list-style-type: none"> Laboratory analysis Requires long time for sample preparation and analysis (>1 h)
Extracellular toxin	Not detectable	<ul style="list-style-type: none"> Can indicate cell lysis if toxins are not oxidized 	<ul style="list-style-type: none"> Laboratory analysis Requires long time for sample preparation and analysis (>3 h) Unsuitable cell stress detector due to inability to measure dissolved toxins after oxidation

excreted microcystin minus the toxin which has been oxidized.

A further consideration when selecting a suitable cell stress detection method is the applicability of the required equipment. The time from sampling to results with the Mini-PAM fluorescence detection system is less than 10 min and it can be used as a portable instrument. Furthermore, the Mini-PAM can be operated on a boat during in-reservoir treatment, compared to laboratory analysis taking at least 1 h for sample preparation and HPLC analysis of microcystins. While results by a particle counter can be acquired in a similar time frame to the Mini-PAM, the Multisizer can only be used in laboratory, however, both methods using the Mini-PAM (indication of photosynthesis and chlorophyll α) are non-specific, which means that in a natural water sample these would also measure the photosynthesis and chlorophyll α from other organisms, such as diatoms and green algae.

4. Conclusions

Many studies evaluate the effects of H_2O_2 on cyanobacteria, but a reliable and rapid detection method for cell stress caused by oxidative processes is needed to allow inter-study comparison. Several approaches are suitable as a measure of cell stress in cyanobacteria with the present study was able to compare different cell stress assessment methods which could be available to most water treatment laboratories.

The current study clearly demonstrates efficacy of hydrogen peroxide in reducing cyanobacterial cell numbers, viability and microcystin contents. From the five methods investigated, the Mini-PAM fluorometer which measured photosynthetic activity (F_v/F_m) provided the most rapid analysis (<10 min) and presented the fastest response time (i.e., was the method which detected stress first), and is therefore the most suitable method for cyanobacterial cell stress detection. To have a complete understanding of algacide treatment it is desirable to combine photosynthesis, cell number and intracellular toxin detection methods. Therefore, an approach using combined methods is advisable for successful water management and to determine the efficacy of cyanobacterial removal methods.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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