# Mode of action of hydrogen peroxide, peroxymonosulfate and persulfate on *Microcystis aeruginosa* strain PCC 7806

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**Abstract** Eutrophication of surface water has increased globally the past few decades because of human activities, such as land fertilization, disposal of inadequately processed domestic and industrial wastes, in combination with climate change. Excessive concentrations of phosphorus, a key ingredient to eutrophication, are now detected in freshwater lakes, artificially made reservoirs, and streams. Eutrophic ecosystems host a large range of microorganisms including, the harmful strains of cyanobacteria, such as Microcystis aeruginosa. The latter ones produce bioactive metabolites such as microcystins a group of hepatotoxins that affects both humans and wild life. Investigations on the formation of harmful cyanobacteria in eutrophic lakes and ways to reduced their photosynthetic activity, in order to inhibit their blooming are currently being conducted. This study investigated the application of three environmentally friendly oxidants, hydrogen peroxide (HP), persulfate (PS) and peroxymonosulfate (PMS) for the treatment of Microcystis aeruginosa PCC7806. The results have shown that HP acts like a cyanocide, PMS inhibits the growth of cyanobacteria while PS has no effect on the cells.

**Keywords**: *Microcystis aeruginosa*, hydrogen peroxide, persulfate, peroxymonosulfate

## 1. Introduction

Cyanobacterial nuisance in freshwater lakes is among the most concerning issues in science today as it causes tremendous consequences not only on the system's ecology but also in the social economy. Eutrophication, can lead to cyanobacteria harmful blooms (Cyano-HABs) formation causing conspicuous deterioration of the aquatic system. Biodiversity in such dominated-cyanobacteria systems decreases considerably with only few species of cyanobacteria presented affecting the other living organisms such as zooplankton and fish populations (Sigee, 2005; Reynolds, 2006). Cyanobacteria, known as blue-green algae, belong to the kingdom of bacteria but they are photoautotrophic, oxygen-producing species (Hamilton *et al*., 2015). Aquatic cyanobacteria are known for their wide-ranging and particularly perceptible blooms that can form in both freshwater and marine environments, discoloring the water or forming scum and producing undesirable odor and taste (Paerl & Paul, 2011). Under favorable conditions of nutrients and light, some species of cyanobacteria produce toxic secondary metabolites, known as cyanotoxins. The most distinguished cyanotoxins are the peptide toxins called microcystins and anatoxins, mainly produced by Microcystis species (USEPA, 2014) and frequently lead to the closure of recreational waters when noted because they can cause irritations of vital organs (liver), the nerves, and dermis of mammals (Antoniou *et al*, 2014) Based on WHO, the concentration of microcystin-LR, which is considered as the most toxic one, should not exist the 1.0 μg/L in drinking waters; a limit that shows the importance of their occurrence.

Cyanobacteria outgrow the remaining phytoplankton (green algae and diatoms) due to their different ecological and functional traits (Mantzouki *et al*, 2015). For example buoyant cyanobacterial species exploit the incoming light and they proliferate on the surface forming blooms. Hence, the light absorption of the other phytoplankton species is blocked and their population is reduced significantly.

Due to the inherent diversity of Cyano-HABs, different strategies have been applied for their prevention and treatment in drinking water facilities (Verspagen *et al*. 2006). Treatment of cyanobacterial contaminated water drastically increases the cost of treatment as the cells can easily clog filters, while pilot scale studies have shown that the mechanical force applied on the cells can cause them to lyse and increase the toxin content of the treated water (Schmidt *et al*. 2002). For these reasons, recent studies are currently focusing on treating Cyano-HABs at source (in-lake treatment), as a primary mitigation strategy. Cyanobacteria have been found to be more sensitive to hydrogen peroxide (HP) treatment than the rest of phytoplankton. Previous studies have shown that there is a damage to the photosynthetic apparatus when cyanobacteria are being exposed to HP (Drabkova *et al*., 2007). A difference in the electron flow routing at the stromal side of photosystem I gives rise to the so-called Mehler reaction in the chloroplasts of plants and eukaryotic microalgae. Hence, excess photosynthetic electron flow results in use of oxygen as electron acceptor and formation of superoxide. This Reactive Oxygen

Species (ROS) is converted into hydrogen peroxide and water plus oxygen by anti-ROS enzymes lacking in cyanobacteria. Furthermore, the presence of flavoproteins flv 1 and flv2 in cyanobacteria excludes formation of the superoxide anion radical, thus excess electrons make water directly. These differences have been utilized in the selective suppression of harmful cyanobacteria in an entire lake with homogenously injected hydrogen peroxide (Mattjis *et al*., 2012). The fact that hydrogen peroxide ($H_2O_2$, HP) completely dissociates into $H_2O$ and $O_2$ within 24 hours while leaving other ecological valuable phyto- and zoo- plankton species unaffected, makes this treatment option highly desirable.

Based on the above results, it was decided to test other oxidants, which produce additional types of radicals to hydroxyl, and have been successfully used for the treatment of cyanotoxins in water (Antoniou *et al*., 2010). Particularly, persulfate ($S_2O_8^{2-}$, PS) and peroxymonosulfate ($KHSO_5^-$, PMS) were utilized because they can produce sulfate radicals ($SO_4^{\bullet-}$) upon activation. The latter ones have higher redox potential for electron abstraction than hydroxyl radicals and can cause selective oxidation.

Specifically, this study examined the response of a toxic cyanobacterium species *Microcystis aeruginosa* PCC7806 in bench-scale experiments. The effectiveness of HP, PS, and PMS was determined by measuring the phytoplankton density of treated samples for 3 days following oxidant addition. In addition the minimum inhibitory concentration (MIC) was estimated. To the best of our knowledge, this is the first study investigating the mode of action of these environmentally friendly oxidants on a Microcystis toxic strain and gives a first insight on their efficiency on cyanobacterial deduction in freshwater systems.

## 2. Materials and Methods

Batch culture of wild type strain of Microcystis aeruginosa PCC 7806 was maintained in a chemostat at 25 °C and light intensity of 30 μmol photons $m^{-2}s^{-1}$ with a continuous flow of media (BG-11). Culture had reached steady states. Sample from the chemostat was taken to be used for the experiment with the HP, PS and PMS treatment. All cultures were inoculated into BG-11 medium (Rippka *et al*., 1979) comprising 10 mM $NO_3^-$ and 175μM of $PO_4^{3-}$. Experiments were performed in six-well plates (COSTAR) with an initial total culture volume of 6 mL in triplicates and initial density of $3*10^6$ cells per mL. Cultures were exposed to lower light intensity averaging 15 μmol photons $m^{-2}s^{-1}$ and ambient $CO_2$ concentration (~400 mg/L). Growth was measured by optical density at 750 nm. Samples of 200μL were taken from each culture and transferred into a 96 well plate. The absorption was measured using a Versamax 96 well plate reader (Molecular Devices, Sunnyvale, USA). Samples from PMS and PS treatment were taken right after the addition of the oxidants at 0, 1, 2, 3, 4, 5, 20, 22, 24, 48 and 72 hours while samples from HP treatment were taken at 0, 2 , 4, 24, 48 and 72 hours because it was carried out in a different day.

Hydrogen peroxide remaining concentration was quantified colorimetrically using 5mM p-nitrophenylboronic acid (p-NPBA) reagent pH 8.9 (Lu *et al*., 2011). In a 96-well microtiterplate, culture supernatant and p-NPBA reagent were mixed 1:1 (v/v) with a total volume of 300 μL. Supernatant was obtained by centrifugation (14000 rpm, 10 min, and 4°C) where 200 μL was taken each time from each culture. Incubation took place for 3 to 20 hours at room temperature. Absorption was measured at 405 nm, were yellow nitrophenolates maximally absorb, using the same plate reader.

In addition, a side experiment was conducted to determine the minimum inhibitory concentration (MIC) of HP by exposing different cell density cultures (3.75, 7.00, 15.00 and 30.00 million cells per mL) to a range of HP concentration (1, 2, 5, 10 and 20 mg/L). Experiments were conducted in 250 mL erlenmeyer flasks with a final volume of 50 mL keeping the same conditions and timeframe as on the previously descripted experiments.

## 3. Results and Discussion

Earlier studies have shown that HP treatment is an effective method for decreasing cyanobacteria population in aqueous solutions (Drabkova *et al*. 2007 & Matthijs *et al*. 2012). Cyanobacteria express high sensitivity to HP because their lack of defensive enzymes to the ROS production. The production of hydroxyl radicals ($HO^{\bullet}$) by the activation of HP can damage the photosynthetic system of the cells and thus are impotent to survive. To the best of our knowledge, HP is the most efficient and environmentally friendly oxidant so far used in aquatic systems for eliminating cyanobacteria. In this study two additional to HP oxidants, were used persulfate (PS) and peroxymonosulfate (PMS). These two oxidants have been mainly used in studies for the effective degradation of recalcitrant substances, such as phenols (industrial toxins) and microcystin-LR (cyanotoxin), because of their ability to form sulfate radicals ($SO_4^{\bullet-}$) in addition to $^{\bullet}OH$. Peroxymonosulfate can be found as a triple salt (Oxone®) and is often used as a non-chlorinated bleaching agent. Both oxidants are part of Advanced Oxidation Processes (AOPs) used in wastewater tertiary treatment to further improve the effluent quality (Antoniou *et al*. 2010 & Anipsitakis, 2004).

Drabkova *et al* (2007) reported that a 2.5mg/L HP concentration is sufficient to damage cyanobacteria and their intracellular toxins, leaving other species unaffected. Also 5 mg/L (0.150 mM) revealed similar effects. To enhance potential degradation it was decided to treat cyanobacteria with a concentration of 0.150 mM (equivalent of 5 mg/L HP). Herein, results are represented as molarities (mM) of the active ingredient of each oxidant. The tested concentration range was 0.015 - 3.500 mM for PS and PMS and 0.03 - 1.50mM for HP.

Control experiments were performed, first in the absence of oxidants, where the growth rate of the cultures was estimated to be $0.0245 \pm 0.003$ $h^{-1}$. In Figure 1, data from the PS, HP and PMS 0.150 mM after 4, 24, 48 and 72 hours treatment are presented as percentage of control for each oxidant and time point individually. The cell density of the cultures was monitored by recording the optical density at a wavelenght of 750 nm (near Infra-red), where

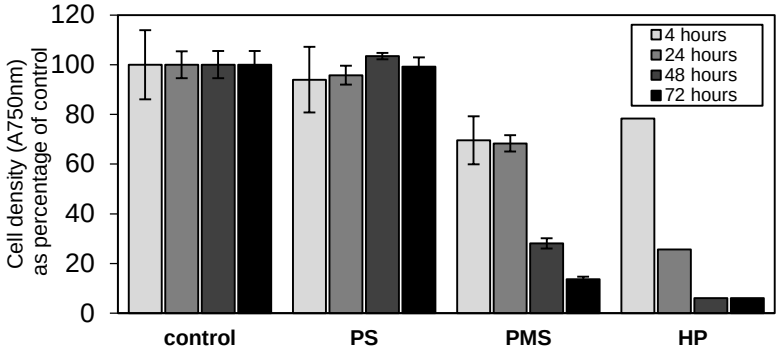


**Figure 1:** Reduction of cell density of *Microcystis aeruginosa* PCC7806 in laboratory incubations enclosures exposed to different oxidants (PS, PMS and HP) at similar concentration of 0.150mM. The cell density is expressed as percentage of the control (no oxidant addition), and was determined 4, 24, 48 and 72h after oxidants had been added to the cultures. Data show the mean of three replicates per treatment.

pigments do not absorb. Degradation of cyanobacterium *M. aeruginosa* PCC7806 was only observed in the presence of HP and PMS. The biomass degradation 4 hours after of oxidants addition had only a small decrease of ~25%. However, after 24 hours of contact time, the obtained results indicated that HP treatment is the most effective, PMS is less effective and PS has no observed effects on cyanobacteria. Specifically, optical densities reduced to 25.62% ± 0.01, 68.32% ± 3.30 and 95.80% ± 3.75 for HP, PMS, and PS, respectively. With HP addition, a continuous degradation of the tested cyanobacterium's cell density is observed, while PMS showed an initial drop after 4 hours of treatment, followed by a significant reduction at 48 hours of contact time, while PS gave had effects on cell density.

The ability of HP to degrade cyanobacteria is also due to the properties of $H_2O_2$ molecule which can passively diffuse in the internal stromal of the cells through cell membrane. On the other hand, PS and PMS are "bigger" charged molecules (salts) and cannot pass through passive diffusion into the cell. However, all oxidants can be activated by the matrix components to give a mixture of ROS (primarily composed of $HO^{\bullet}$ and in the case of PS and PMS, $SO_4^{\bullet-}$). These ROS can either react with the remaining matrix components, the cyanobacterium cells, or each other to form new radicals (Antoniou *et al*. 2010 & Anipsitakis, 2004). Since PS, even at higher tested concentrations (up to 3.500 mM) allows cells to proliferate unaffectedly it means that most of the radicals formed are used up by the matrix components or converted into less reactive radicals. PMS which is (a better e- acceptor than PS) (Antoniou *et al*. 2010) can produce a combination of $HO^{\bullet}$ and $SO_4^{\bullet-}$ at concentrations that can inhibit cyanobacterial growth. The fact that PMS requires higher contact times to achieve the same degree of cell density reduction as HP, it means that its mode of action is that of an inhibitor rather than a cyanocide.

Based on the findings above, a second batch of experiment was conducted to estimate the MIC of HP on *Microcystis aeruginosa* PCC7806 because it was the most efficient treatment. For an initial cell density of $30x10^6$ cells/mL, $15x10^6$ cells/mL, $7.5x10^6$ cells/mL and $3.75x10^6$ cells/mL $MIC_{HP}$ was found 20 mg/L, 10 mg/L, 5 mg/L and 2 mg/L respectively. This indicates that HP dosing depends on the cells numbers that are present in the solution.

To conclude, the obtained data showed that HP acts as a cyanocide, PMS has the characteristics of an inhibitor, and PS has no observed effects on toxic *Microcystis aeruginosa* species.

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