Impacts of Hydrogen Peroxide and Copper Sulfate on the Control of *Microcystis aeruginosa* and MC-LR and the Inhibition of MC-LR Degrading Bacterium *Bacillus* sp.

Michelline M. R. Kansole 1 and Tsair-Fuh Lin 1,2,*

1 Department of Environmental Engineering, National Cheng Kung University, Tainan 70101, Taiwan; dieutrinity@hotmail.fr
2 Global Water Quality Research Center, National Cheng Kung University, Tainan 70955, Taiwan
* Correspondence: tflin@mail.ncku.edu.tw; Tel.: +886-6-236 4455

Academic Editor: Wilhelm Püttmann
Received: 3 February 2017; Accepted: 1 April 2017; Published: 5 April 2017

Abstract: Laboratory batch experiments were carried out to evaluate the impacts of H$_2$O$_2$ and copper sulfate on *M. aeruginosa* PCC7820, microcystin-LR (MC-LR) and its degrading bacteria *Bacillus* sp., previously isolated from Hulupi Lake in Taiwan. The study shows that 3 mg·L$^{-1}$ hydrogen peroxide removed only 9% *M. aeruginosa* within seven days of exposure, from an initial cell concentration of 2 × 10$^6$ cells/mL. With copper sulfate, a concentration of 2 mg·L$^{-1}$ removed 99% *M. aeruginosa* cells, but showed negligible efficacy in removing 0.05 mg·L$^{-1}$ MC-LR. At a higher dosage, 20 mg·L$^{-1}$ H$_2$O$_2$ led to 40% and 95% removal, respectively for MC-LR and *M. aeruginosa* cells. Copper sulfate and H$_2$O$_2$ were both lethal to *Bacillus* sp. population, with mortality rate constants of k = 0.04 h$^{-1}$ and 0.03 h$^{-1}$ under 1 mg·L$^{-1}$ copper sulfate and 5 mg·L$^{-1}$ H$_2$O$_2$, respectively. H$_2$O$_2$ is competitive in terms of cost, with a capability of degrading organic compounds with the assistance of ultraviolet (UV) light, and it may be considered as an alternative algaecide to copper sulfate in reservoirs for algae growth control.

Keywords: algaecides; *Bacillus* sp.; biodegradation; copper sulfate; hydrogen peroxide; hydroxyl radicals; microcystin; *Microcystis*

1. Introduction

Harmful cyanobacteria blooms are becoming a significant threat all over the world with this issue growing due to climate change [1]. The decay of such cyanobacteria may release cyanotoxins, with microcystins (MCs), especially known to be harmful to animal, human and aquatic life [2]. microcystin-LR (MC-LR) is the most toxic microcystin amongst approximately 100 congeners of the known microcystins [3]. The heptapeptide is reported to be hepatotoxic [4] and a potential human carcinogen [5]. Therefore, a guideline of 1 µg·L$^{-1}$ has been set for this toxin by the World Health Organization (WHO) for drinking water quality [6]. Effective MC removal in water treatment plants (WTPs) is usually carried out via nanofiltration [7], oxidation [8], activated carbon [9] and biodegradation [10]. Among these processes, biodegradation usually takes place in sand filters in WTPs [11], and may also happen in natural water environments involving a variety of bacteria [12,13]. Among the MC degrading bacteria, *Bacillus* sp. [12,14] has been reported to be especially effective, with more than 80% removal of 15 mg·L$^{-1}$ MC-LR reached within nine days’ incubation [14].

Algaecides, and particularly cyanocides can be used to control algae blooms by killing cyanobacteria [15]. The main authorized algaecides include hydrogen peroxide (H$_2$O$_2$), copper-based algaecides, such as copper sulfate and copper citrate, potassium permanganate, chlorine, and lime
The use of algaecides in water may cause a few issues, including increasing resistance of the targeted cyanobacteria to algaecides, and suppressing the growth of non-targeted microorganisms. Some algaecides may also break cyanobacteria cell walls, causing the release of intracellular toxins and/or taste and odor (T&O) compounds into the water [18], thus complicating the treatment in the following water treatment processes. One way to avoid the excessive release of toxins and/or T&O compounds is to apply the algaecides in the early stage of the harmful algae blooms (HABs) [17], in which the cell concentrations and toxins are relatively low.

Cyanobacteria may also develop tolerance to algaecides [19]. For instance, in one study in California, USA, repetitive uses of copper sulfate led to a copper-tolerance Phormidium sp. [20]. In addition, long-term and high-dose application of algaecides could cause a decrease in non-targeted microorganisms which may include natural degraders of cyanotoxins [21]. Therefore, the Australian guidelines for water utilities do not recommend the use of copper sulfate because of its ecological effects [17].

Copper sulfate is a commercial algaecide that is widely used because cyanobacteria are not tolerant to high doses of copper [22]. For example, 0.16 mg L\(^{-1}\) of copper has been reported to control phytoplanktonic communities in reservoirs [22]. Chemical treatment with copper derived algaecides is the most common technique used to control algae blooms in drinking water reservoirs [23], lakes and ponds [24]. The use of copper sulfate generally leads to the formation of copper residues in sediment [25]. As much as 50 mg Cu/kg-sediment has been estimated to accumulate in sediment after five applications of copper sulfate [26], which is much higher than the 35.7 mg Cu/kg-sediment set by the Canadian interim sediment quality guidelines (ISQGs) standards for freshwater [27]. Besides being used as algaecides, copper based chemicals have also been used as bactericides since ancient times [28]. Although copper is a micronutrient, the chemical is one of the most toxic metal ions to all water living organisms [29] and its limit in drinking water is 2.0 mg L\(^{-1}\), according to the WHO [30]. When copper is in excess, it may destroy the membranes of bacteria cells via interactions with lipids [31], leading to the death of the cells [32]. Lin et al. (1996) [33] reported that 0.1 mg L\(^{-1}\) copper completely inactivated bacterium L. pneumophila serogroup 1 within 2.5 h exposure. Although copper sulfate has been used for more than 100 years, studies about its impact on cyanobacteria and toxin degrading bacteria together are very limited.

H\(_2\)O\(_2\), a powerful oxidant, has recently been used as an alternative algaecide [34]. It is also said to be selective to cyanobacteria, eco-friendly due to the benign final products, and easy to apply because of its solution form [35]. It is especially effective to remove both cells and cyanotoxins if assisted by ultraviolet (UV) light, cuprous or ferrous ions [36]. H\(_2\)O\(_2\) inhibits the growth of microorganisms, but can enhance microbial growth for the bioremediation of contaminated water bodies, because it is an oxygen released compound (ORC) [37]. H\(_2\)O\(_2\) is also commonly used for control of odor, removal of organic matters, oxidation of organic and inorganic contaminants, and biocontrol in water treatment plants [38]. Although copper sulfate and H\(_2\)O\(_2\) both kill cyanobacteria by breaking their cells, the impact of these algaecides on cyanobacteria and bacteria were studied for limited species [21,39]. In addition, their capability of degradation and impact on the biodegradation for the released cyanotoxins are not well understood, limiting the application of the algaecides in the management of harmful cyanobacteria.

The objective of this study is thus to investigate the impact of copper sulfate (in the form of copper sulfate pentahydrate) and H\(_2\)O\(_2\) on M. aeruginosa, MC-LR and its degrading bacteria, Bacillus sp. Such a study is needed to give information on MC-LR and the fate of its degrading bacteria during water treatment with algaecide, as there is a crucial lack of such information in the research domain.

2. Materials and Methods

2.1. Microcystis Aeruginosa PCC7820 Culture and Cell Count

M. aeruginosa PCC7820 (Pasteur Culture Collection of Cyanobacteria, Paris, France) was cultured with an artificial sputum medium (ASM) [40] (composition of the medium described in
the Supplementary Information) in 250 mL polystyrene cell culture flasks (Nest Biotechnology Co.,
Ltd., Wuxi, China). The culture was incubated under a 12 h/12 h dark and light cycle in a 25 °C
incubator (Hipton MD-302F, Jiuh Hsing Instrument Co. Ltd., Kaohsiung, Taiwan). The solar radiance
equivalent in the incubator under light was 2.3 W·m⁻², with the radiance shown in Figure S1 of the
Supplementary Information, and measured using a fiber optic spectrometer (StellarNet, Inc., Tampa,
FL, USA). M. aeruginosa PCC7820 were cultured to reach a cell concentration of 3 × 10⁶ cells/mL, and
the cells were counted using a haemacytometer (Hausser Scientific, Horsham, PA, USA), under an
optical microscope (Olympus BX51, Olympus Optical Co. Ltd., Tokyo, Japan) at 100× magnification
(Graticules Ltd., Kent, UK).

2.2. Bacillus sp. Culture, Cells Preparation and Heterotrophic Plate Count (HPC)

Bacillus sp. bacteria were obtained from a previous study [12] for MC-LR degrading bacteria
isolation [41]. An amount of 100 mL Luria-Bertani (LB) broth (Sigma-Aldrich, Steinheim, Germany)
was used to grow the isolated colonies of Bacillus sp. in a 150 mL polystyrene cell culture flask
(Nest Biotechnology Co., Ltd., Wuxi, China) at 37 °C for three days under constant shaking at 170 rpm
in an incubator (Cheng Sang Scientific Co. Ltd., Puhsin Hsiang, Taiwan). An amount of 20 mL of the
exponentially grown Bacillus sp. was then added to 200 mL of a fresh LB broth and incubated at 37 °C
for three days, then used as a Bacillus sp. stock solution.

The bacteria cells were harvested from 50 mL of the stock culture then centrifuged at 5000× g for
3 min (Hermle Labortechnik GmbH, Model Z206A, Wehingen, Germany), and the centrifuged bacterial
pellets were collected. The bacterial pellets were further washed with a 10 mL sterile phosphate
buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA), centrifuged at 5000× g for 3 min, and this
was repeated three times. The bacterial pellet laden solution was then re-suspended in 50 mL of fresh
Mineral Salt Medium (MSM) [42] and inoculated with MC-LR (extraction described in Section 2.4)
of 0.02 mg·L⁻¹, and 0.05 mg·L⁻¹, and incubated at 25 °C to monitor the MC-LR degradation by
the bacteria.

To test the impact of algaecides on Bacillus sp., the Bacillus sp. pellets were suspended in 50 mL of
fresh Mineral Salt Medium, with MC-LR added to part of the inoculum to reach a MC-LR concentration
of 0.2 mg·L⁻¹. Algaecides were then added to the Bacillus sp. laden solutions as shown in Section 2.3,
incubated at 25 °C, and monitored for the impact of copper sulfate and H₂O₂ on the bacteria.

The heterotrophic plate count (HPC) method was used to count the bacteria, according to the
instructions in Bartram et al. (2003) [43]. Bacillus sp. concentration (in colonies formed per unit
CFU/mL) was determined by plating the inoculum in a bacteria growth agar (plate count agar
HiMedia Ref M091, Bombay, India). The mixed solution Bacillus sp.-MC-LR was filtered through
a 0.45-µm then 0.22-µm-pore-size membranes (Sigma-Aldrich, Darmstadt, Germany) before plating.

2.3. Exposure of MC-LR, M. aeruginosa, and Bacillus sp. to Copper Sulfate and H₂O₂

Copper sulfate (CuSO₄·5H₂O; Thermo Fisher Scientific, Waltham, MA, USA) and H₂O₂ (30% w/w,
Sigma-Aldrich, Saint Louis, MO, USA) were used as the main algaecides in this study. MC-LR,
M. aeruginosa, and Bacillus sp. were exposed to different doses of copper sulfate and H₂O₂, with
0.00 mg·L⁻¹, 0.05 mg·L⁻¹, 0.1 mg·L⁻¹, 0.5 mg·L⁻¹, 1 mg·L⁻¹, 1.5 mg·L⁻¹ and 2 mg·L⁻¹ for copper
sulfate, and 0.00 mg·L⁻¹, 1 mg·L⁻¹, 2 mg·L⁻¹, 3 mg·L⁻¹, 5 mg·L⁻¹, 10 mg·L⁻¹ and 20 mg·L⁻¹ for
H₂O₂ (Table S1). The samples were incubated at 25 °C, monitored for the impacts of algaecides on the
studied cyanobacteria, bacteria, and MC-LR. Stock solution of H₂O₂ was renewed every three days
because of its chemical instability.

2.4. Extraction of Crude Microcystin-LR

An amount of 5 L of 3 × 10⁶ cells·mL⁻¹ M. aeruginosa PCC7820 (producer of MC-LR [44]) was
centrifuged at 3000× g (Hermle Labortechnik GmbH, type Z206A, Darmstadt, Germany) for 2 min
using 50 mL tubes (Labcon, SuperClear™, Petaluma, CA, USA). The concentrated Microcystis cells
were dried in a Lyophilizer (FD3-12P, Kingmech, Taiwan) for 48 h. The powder was then utilized for microcystin extraction following the procedures described by Hu et al. (2009) [45]. After extraction, a concentration of 10 mg L\(^{-1}\) for 0.2 pg cell\(^{-1}\) of MC-LR was obtained [46]. The biodegradation of MC-LR was fitted in a first order degradation reaction using Equation (1).

\[
\frac{C}{C_o} = e^{-kt}
\]  

where C is the MC-LR concentration, \(C_o\) is the MC-LR concentration at time \(t = 0\), \(k\) is the reaction rate constant, and \(t\) is the time of degradation.

2.5. Quantification of Microcystin-LR

Quantification of MC-LR (chemical structure represented in Figure S2 of Supplementary Information) was determined using the Enzyme-Linked Immuno-Sorbent Assay (ELISA) with specificity to microcystins (commercial kit No. ALX-850-319-KI01, Enzo Life Sciences Inc., Farmingdale, NY, USA). The measurements were made according to the method described by Fischer et al. (2001) [47], and the absorbance read at 450 nm (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer 357, Vantaa, Finland) against a calibration curve described in a previous study [12] with a MC-LR detection limit of 0.1 \(\mu g\) L\(^{-1}\).

2.6. Quantification of Residual H\(_2\)O\(_2\)

H\(_2\)O\(_2\) was quantified using potassium titanium oxide oxalate dihydrate (K\(_2\)TiO(C\(_2\)O\(_4\))\(_2\)·2H\(_2\)O ≥ 98%, Sigma-Aldrich, Saint Louis, MO, USA), following the procedures described by Sellers (1980) [48]. An amount of 5 mL of titanium reagent, prepared as described by Sellers (1980) [48]), was added to 10 mL of the H\(_2\)O\(_2\) containing sample, then the ensemble was adjusted to 25 mL with deionized (DI) water. An amount of 0.2 \(\mu m\) pore size cellulose membrane (Sartorius Stedim Biotech GmbH, Germany) was used to filter the solution before measuring light absorbance at 400 nm (Hach DR-3900, Loveland, CO, USA). The calibration curve of H\(_2\)O\(_2\) and light absorbance shows a linear relation with a high coefficient of determination (\(R^2 = 0.99\)), as shown in Figure S3 of the Supplementary Information. Control was also measured with H\(_2\)O\(_2\) and without addition of cyanobacteria, and bacteria cells.

2.7. Quantification of Residual Hydroxyl Radicals

Measurement of residual OH radicals was performed using a spectrophotometric method with Rhodamine B (RhB, Sigma-Aldrich Chemie GmbH, Riedstr, Germany) as the probe chemical, according to the protocol described by Kwon et al. (2014) [49]. Different concentrations of RhB (10\(^{-3}\), 0.5 10\(^{-3}\), 10\(^{-4}\), 0.5 10\(^{-4}\), 10\(^{-5}\), 10\(^{-6}\) M) were read for their absorbance at 554 nm (Hach DR-3900, Loveland, CO, USA). An amount of 5 mL of the bacterial suspension (preparation described in Section 2.2) was added to the samples spiked with 5 mg L\(^{-1}\) H\(_2\)O\(_2\) and 10\(^{-3}\) M RhB, to reach 10 × 10\(^9\) CFU/mL of Bacillus sp. In another set of experiments, 0.2 mg L\(^{-1}\) MC-LR was also spiked. The ensemble was incubated under 2.3 W m\(^{-2}\) at 25 °C, and the residual OH radicals’ concentrations were estimated against a calibration curve plotted in Figure S4 of the Supplementary Information for 8100 s. Equation (2), as presented by Kwon et al. (2014) [49], was used for the calculation of residual [-OH].

\[
R_{\text{OH,UV}} = \frac{\int_0^t [\text{OH}] \, dt}{E_{0.t}} = \frac{k_T^{\text{Dapp}} - k_d^{\text{Dapp}}}{k_{\text{OH,RhB}}^{\text{app}}} 
\]  

where \(R_{\text{OH,UV}}\) is the OH radical exposure per UV dose, \(t\) is the time (s), [\text{OH}] is the concentration of OH radicals (M), \(k_T^{\text{Dapp}}\) is the fluence-based appearance rate constant for direct UV photolysis, \(k_d^{\text{Dapp}}\) is the fluence-based appearance rate constant for the H\(_2\)O\(_2\) added conditions, \(k_{\text{OH,RhB}}^{\text{app}}\) is the
appearance rate constant of RhB decolorization (3.75 ± 0.15 × 10⁹ M⁻¹·s⁻¹), and E₀ is the incident photon irradiance (MW·cm⁻²).

2.8. Measurement of Total Alkalinity and pH

The pH was measured according to the method described by Galster (1991) [50]. The water samples containing MC-LR, M. aeruginosa and/or Bacillus sp. were filtered using a 0.45 μm syringe (Whatman® GD/X syringe filters sterile, Sigma-Aldrich, GmbH, Essen, Germany) followed by a second filtration with 0.2 μm pore size cellulose membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The obtained filtrates were measured in triplicates for their pH values at different times, using a pH meter (Sp-701, Suntex Instruments Inc., Taipei, Taiwan).

Total alkalinity was measured for the water containing 3 × 10⁶ cells·mL⁻¹ M. aeruginosa using titration [51] with H₂SO₄ (0.1 N, E. Merck AG, Darmstadt, Germany), phenolphthalein (E. Merck AG, Darmstadt, Germany) and methyl orange (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Four drops of 0.1% phenolphthalein, previously prepared in ethanol/water (v/v, 20:80), were added to 100 mL of the filtrated (by 0.2 μm) Microcystis culture solution, then titrated with 1/50 N of H₂SO₄ until the pink color faded out. The second titration was then done by adding one drop of 0.5% methyl orange (previously prepared in ethanol/water (v/v, 50:50)), and the ensemble was then titrated with 1/50 N H₂SO₄ until the orange color turned light orange. The total alkalinity was calculated using Equation (3).

\[
\text{Total alkalinity (mg/L)} = \frac{\text{T} \times 1000}{\text{Volume of sample}}
\] 

where T is the total volume of H₂SO₄ used for the total titration (phenolphthalein and methyl orange) in mL.

2.9. Statistical Analyses

Data were statistically analyzed via a one-way ANOVA (p < 0.05), and a Normality Test (Shapiro–Wilk) (p ≥ 0.05 normal, and p < 0.05 not normal) (SigmaPlot v11.0, Systat Software, Inc., Chicago, IL, USA) to find the significant differences in MC-LR/Bacillus sp. degradation, M. aeruginosa PCC7820 removal, H₂O₂ degradation, and for hydroxyl radical production, under copper sulfate or H₂O₂.

3. Results and Discussion

3.1. Toxicity of Copper Sulfate and H₂O₂

3.1.1. Toxicity to M. aeruginosa PCC7028

Figure 1 shows the change in concentration of H₂O₂ for the studied water with and without 10⁶ cells/mL of M. aeruginosa. In the experiments, 20 mg·L⁻¹ of H₂O₂ was dosed into the reactor, with a light intensity of 2.3 W·m⁻² and the spectrum shown in Figure S1 of the Supplementary Information. The figure shows that after 72 h, the amount of H₂O₂ was reduced by 75% and 24.4% for the cases of with and without Microcystis addition, respectively. Clearly, the presence of cyanobacteria led to a faster decay of H₂O₂. Mikula et al. (2012) [52] observed that light (140 μmol·m⁻²·s⁻¹~30.4 W·m⁻²) is a sine qua non-condition for H₂O₂ decomposition and for its toxicity to M. aeruginosa. They also reported that in dark conditions, H₂O₂ decomposes very slowly over 72 h. Zepp et al. (1987) [53] reported that algae might have a role in natural waters for the production of H₂O₂. They also suggested that H₂O₂ degradation follows a second order reaction in the dark. However, when exposed to sunlight, the algae may both produce and degrade H₂O₂ [53]. In the current study, the oxidant degraded for more than 72 h, and led to the long-term low toxicity of H₂O₂ with regard to the algae. The present study shows that under the condition of light intensity = 2.3 W·m⁻² and H₂O₂ dose = 20 mg·L⁻¹, degradation of H₂O₂ was three times faster in the sample with M. aeruginosa (2.3 × 10⁶ cells/mL) than that in deionized water (Figure 1). Huo et al. (2015) [54] reported that H₂O₂ remained stable for up
to 3.5 h when 60 mg·L⁻¹ H₂O₂ was incubated in the dark along with Microcystis cells. In addition, the degradation of H₂O₂ is known to follow a pseudo-first order reaction when incubated with UV light [55], and this supports the importance of light in H₂O₂ degradation.

![Figure 1](image_url)  
**Figure 1.** Hydrogen peroxide degradation in deionized water (control) and in the sample with *M. aeruginosa* (2.3 × 10⁶ cells/mL), under 2.3 watt·m⁻², at 25 °C. The error bars represent one standard deviation for three measurements.

Figure 2 shows the effect of copper sulfate and H₂O₂ on the growth of *M. aeruginosa*. Within the exposure time (14 days), copper sulfate efficiently inhibited the growth of Microcystis cells at doses greater than 1 mg·L⁻¹. Compared with the controlled sample, at 14 days the copper sulfate suppressed cell growth by 99%, 97% and 90%, respectively at the doses of 2, 1.5, and 1 mg·L⁻¹ for *M. aeruginosa* with an initial concentration of 3 × 10⁶ cells/mL (Figure 2a). The cell concentrations at all the applied copper doses showed statistically significant differences (*p* < 0.05) if compared with the controlled case. Tsai (2015) [56] reported that 0.16 mg·copper·L⁻¹ (≈ 0.62 mg copper sulfate pentahydrate·L⁻¹ in this study) may cause a 90% reduction in *M. aeruginosa* cells (initial concentration = 10⁶ cells/mL) within eight days. McKnight et al. (1983) [57] reported a general copper dose ranging from 0.025 to 1 mg·L⁻¹ that can be used to achieve control of algae blooms. With lower copper sulfate doses, although slight inhibition was observed if compared with controlled samples, cells still grew within 14 days of the experiments (Figure 2a). Gibson (1972) [58] observed that 0.25 mg·L⁻¹ copper only led to a growth depression followed by a recovery of nine days for an aged *Anabaena flos-aquae* culture. However, the same dosage killed a freshly cultured *Anabaena flos-aquae*. It has been reported that some cyanobacteria might develop a resistance to algaecides, and can therefore colonize the lake environment. For instance, García-Villada et al. (2004) [59] reported a copper resistant *M. aeruginosa* mutants, with Cu²⁺ resistance to concentrations greater than 5.8 µM (1.44 mg·L⁻¹ copper sulfate pentahydrate in this study). Erickson et al. (1994) [60] reported that high values of pH affect both adsorption and absorption of the metal-based algaecides (toxic chemicals) by the cell, thus reducing their toxicity. In this study, with the addition of copper sulfate, the pH increased from 9.1 to 10.6 after eight days of incubation when 1 mg·L⁻¹ copper sulfate was dosed. However, the pH decreased again until pH ~8 at the 12th day of culture for 2 mg·L⁻¹ copper sulfate (Figure S5a of the Supplementary Information), and the calculated alkalinity was 130 mg·L⁻¹ leading to a safe maximum copper sulfate dose of 1 mg·L⁻¹ [61] for algae growth control.

Figure 2b demonstrates that exposure of Microcystis may inhibit cell growth by 9%, 46%, 58%, and 95%, respectively at day 7 of exposure to 3, 5, 10, and 20 mg·L⁻¹ doses, with statistically significant differences (*p* < 0.05) between the samples of all the H₂O₂ dosed samples and the controlled sample.
After seven days, Microcystis cells regrew and increased to 197%, 174%, 141%, and 125% of their initial concentrations, respectively for 3, 5, 10, and 20 mg·L⁻¹ of H₂O₂ doses. For the cases of lower H₂O₂ doses (1 and 2 mg·L⁻¹), although lower inhibitions were observed compared to those for the controlled samples, cells continued to grow. This kind of inhibition followed by regrowth of cyanobacteria during the application of H₂O₂ has been reported by Qian et al. (2010) [62], where M. aeruginosa grew after 96 h of exposure to a dose of 100 µM (3.4 mg·L⁻¹) H₂O₂. In addition, Huo et al., (2015) [54] reported a two-stage in M. aeruginosa cell integrity change when exposed to H₂O₂ under light illumination, with cell rupturing following the Delayed Chick–Watson Model, where before the lag time all cells remained integrated and after the lag time the cells started to be ruptured. Although the experiments in that study were only conducted for 6 h, much less than in the current work, their results demonstrated that Microcystis cells are not resistant to H₂O₂ exposure with 99% of the Microcystis cells damaged within 3 h when exposed to 22.34 W·m⁻² (solar irradiance at the surface of the water). In the present study, attempts were made to obtain the rate constants for Microcystis cells degradation using the commonly known degradation models, but they did not fit the degradation pattern. pH is a very important parameter to consider for photo-degradation because it causes differences for the chemical adsorption by the cell. In the present study, it was observed that with H₂O₂ the pH increased to reach 11.4 at day 8 under 5 mg·L⁻¹ H₂O₂. The increase in the pH is due to the depletion in CO₂ through the high photosynthesis by Microcystis cells, but it may also be due to the production of hydroxyl anions.

The sudden change in pH has been reported to be lethal to some aquatic animals, such as the catfish, which cannot tolerate a rapid pH change of 1 unit. As the growth period of the cells increased, the pH decreased to reach a value of 9.8 on the 12th day (Figure S5b of the Supplementary Information).

![Figure 2](image)

**Figure 2.** (a) Effect of copper sulfate on M. aeruginosa growth; (b) Effect of H₂O₂ on M. aeruginosa growth (for initial Microcystis of 2 × 10⁶ cells/mL) at 25 °C. The error bars represent one standard deviation for three measurements.

3.1.2. Toxicity to Bacillus sp.

Figure 3 shows the impact of copper sulfate on Bacillus sp. growth under different doses. It was observed that copper sulfate ≥1 mg·L⁻¹ was enough to kill Bacillus sp. The mortality of Bacillus sp. under exposure to copper sulfate followed a first-order reaction, with rate constants = 0.07 h⁻¹, 0.05 h⁻¹ and 0.04 h⁻¹, respectively, for 2, 1.5 and 1 mg·L⁻¹ of copper sulfate doses when incubated with only Bacillus sp., and = 0.05 h⁻¹, 0.05 h⁻¹ and 0.04 h⁻¹, respectively, when the bacteria were incubated together with MC-LR, under the same copper sulfate doses. The results show that the mortality rates were not influenced by the presence of crude MC-LR in the water. However, higher copper doses led to larger bacteria mortality rates (p = 0.001). For the conditions of copper sulfate doses <0.5 mg·L⁻¹, the mortality rate constants were all less than 10⁻³ h⁻¹, suggesting that the effect on the studied bacterium is negligible. Sani et al. (2001) [63] reported an IC₅₀ of 13.3 µM copper (3.3 mg copper sulfate pentahydrate·L⁻¹) to sulfate-reducing bacteria (SRB) Desulfovibrio desulfuricans G20.
When a higher dose was applied, 30 μM copper (7.4 mg copper sulfate pentahydrate·L⁻¹), 100% of the SRB were killed in 25 h and no bacteria were detected after 384 h of incubation. In addition, Zevenhuizen et al. (1979) [64] observed a Pseudomonas bacterium very tolerant to cupric ions Cu²⁺ for up to 10⁻³ M (250 mg·L⁻¹ copper sulfate pentahydrate). Our study showed 100% mortality for Bacillus sp. at 1 mg copper sulfate pentahydrate·L⁻¹, and this is lower than the concentrations for the SRB and Pseudomonas, and may suggest that different bacteria may have different resistances to copper.

Figure 3. Growth of Bacillus sp. (initial bacteria concentration for 8.4 × 10⁶ CFU/mL) under different concentrations of Copper Sulfate at 25 ℃. The error bars represent one standard deviation for three measurements.

The change in pH was monitored for the experiments at copper sulfate pentahydrate doses of 1 mg·L⁻¹, 1.5 mg·L⁻¹, and 2 mg·L⁻¹, and the results are shown in Figure S6 of the Supplementary Information. The pHs were found to reduce from an initial 7.4 to 6.2 at the end of the experiments for all the studied cases. Yu-Sen et al. (2002) [65] observed that at pH 9 cupric ions led to only a 10-fold reduction of Legionella sp. in 24 h while, a million-fold decrease was observed for pH 7.0, with the precipitation of insoluble copper complexes observed at pH > 6.0, suggesting that pH is an important factor in determining the efficiency of copper ionization for killing bacteria.
factor in determining the efficiency of copper ionization for killing Legionella species in water. Water chemistry varies with many parameters, such as pH, and a decrease in copper toxicity has been reported with an increase in pH [66]. In addition, numerous studies have been conducted to assess copper toxicity in water environments [67], and it was found to be due to free cupric ion Cu²⁺ in Sunda (1975) [68]. The chemical speciation of copper may thus enable us to estimate the toxicity of the metal. To estimate the species of copper in the solution, a water chemistry software package, Visual MINTEQ V3.1 [69], was used to predict the speciation. Table 1 summarizes the model’s results for the copper species in the experimental solution at different pHs. It is clear that at pH = 7.4 the copper was initially in the form of 50.9% Cu²⁺ and 39.7% CuOH⁺, and at the end of the experiment (pH = 6.2) Cu²⁺ was the predominant copper species (94.54%) in the solution. Yu-sen et al. (2002) [65] reported that at pH 9 in the form of 50.9% Cu²⁺ and 39.7% CuOH⁺, and at the end of the experiment (pH = 6.2) Cu²⁺ was the predominant copper species (94.54%) in the solution. Yu-sen et al. (2002) [65] reported that at pH 9 in the form of 50.9% Cu²⁺ and 39.7% CuOH⁺, and at the end of the experiment (pH = 6.2) Cu²⁺ was the predominant copper species (94.54%) in the solution.

Table 1. Copper sulfate speciation (%) at 2 mg L⁻¹ predicted via Visual MINTEQ.

<table>
<thead>
<tr>
<th>Species Name</th>
<th>pH 6.2</th>
<th>pH 7</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO₄²⁻</td>
<td>99.4</td>
<td>99.6</td>
<td>99.9</td>
</tr>
<tr>
<td>CuSO₃(aq)</td>
<td>1.0</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>94.5</td>
<td>68.4</td>
<td>50.9</td>
</tr>
<tr>
<td>CuOH⁺</td>
<td>4.6</td>
<td>21.1</td>
<td>39.7</td>
</tr>
<tr>
<td>Cu(OH)₂(aq)</td>
<td>0.01</td>
<td>0.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Cu₂(OH)₃²⁺</td>
<td>0.4</td>
<td>8.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Cu₃(OH)₄²⁻</td>
<td>-</td>
<td>1.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Figure 4 shows the effects of six different H₂O₂ doses on Bacillus sp. viability. It was observed that H₂O₂ at doses ≥5 mg L⁻¹ was lethal to the bacterium, with the mortality rate following the first order reaction and with rate constants of 0.03 h⁻¹, 0.1 h⁻¹ and 0.14 h⁻¹, respectively, for H₂O₂ doses of 5, 10 and 20 mg L⁻¹. For lower H₂O₂ doses, negligible inhibition of the bacterium was observed, with the rate constants all less than 2 × 10⁻³ h⁻¹. The effect of H₂O₂ on the Bacillus sp. viability became lessened when crude MC-LR was added into the experimental water matrix. Lower H₂O₂ doses and the presence of crude MC-LR in the water may lead to slower mortality rates for the studied bacterium (p < 0.05). During the experimental period, 288 h, the Bacillus sp. population decreased by 90%, 75%, and 5% when exposed to 10, 5 and 3 mg L⁻¹ H₂O₂, respectively. Figure S7 of the Supplementary Information shows the concentration of residual OH radicals over 2.25 h (8100 s). The hydroxyl radical concentrations were very low, at 0.58 × 10⁻¹⁹ M, 1.86 × 10⁻¹⁹ M, and 0.27 × 10⁻¹⁹ M, respectively, with MC-LR, Bacillus sp., and for the control (without bacteria nor MC-LR), and the statistical analysis indicates no significant difference (p = 0.069) among the three tested cases. The low concentration of OH radicals is reasonable since the irradiance used was very low (2.3 W m⁻²). Huo et al. (2015) [54] reported 1.54 × 10⁻¹⁵ M of OH radical concentration in their experimental system, when M. aeruginosa PCC7820 was incubated with 10 mg L⁻¹ H₂O₂ under 22.34 W m⁻² (9.7 times higher than in this study) solar irradiance. In addition, under dark conditions, no hydroxyl radical production was detected. Thomas et al. (1994) [70] showed that both doses of and exposure time to H₂O₂ were essential parameters for H₂O₂ to kill Streptococcus mutans, serotype c (Strain GS-5), in which 6, 10, 0.3 and 7 × 10⁻³ g L⁻¹ H₂O₂ were required when the exposure time was 15 s, 2 min, 1 h and 24 h, respectively. The organic matters present in the water, including cells and associated metabolites in this studied
system, may react with hydrogen peroxide [71], decreasing the efficiency with which H$_2$O$_2$ oxidizes the cyanotoxins, which is similar to our observation that slower mortality rates were found for the cases with crude MC-LR addition. Additionally, with 5, 10 and 20 mg·L$^{-1}$ H$_2$O$_2$ doses, the pH generally increased from 6.8 to 7.6 (Figure S6 of the Supplementary Information). Variations were statistically significant between crude MC-LR, Bacillus sp. and MC-LR/Bacillus sp. solutions ($p = 0.013$), but there was no statistical difference for different H$_2$O$_2$ concentrations ($p = 0.271$). Jung et al. (2009) [72] observed that with 5% (50 g·L$^{-1}$) H$_2$O$_2$, the pH increased from 9.0 to 9.8 within 88 h, and the increase in pH is due to H$_2$O$_2$ decomposition, since there is consumption of H$^+$ or production of OH$^-$ [73], and the change in the pH value may affect the adsorption or the effect of H$_2$O$_2$ (via hydroxyl radicals) on the targeted cells.

![Growth of Bacillus sp. (C$_0$ = 8 × 10$^6$ CFU/mL) under different doses of H$_2$O$_2$ at 25 °C. The error bars represent one standard deviation for three measurements.](image)

**Figure 4.** Growth of Bacillus sp. (C$_0$ = 8 × 10$^6$ CFU/mL) under different doses of H$_2$O$_2$ at 25 °C. The error bars represent one standard deviation for three measurements.

Figure 5 summarizes the degradation of H$_2$O$_2$ under 2.3 W·m$^{-2}$ visible light illumination, at 25 °C during 12 days (288 h) of experiments. H$_2$O$_2$ was observed to degrade and reached a non-detectable
limit within 50 h. The H\textsubscript{2}O\textsubscript{2} degradation rate constants were 0.97 h\textsuperscript{-1}, 0.88 h\textsuperscript{-1} and 0.22 h\textsuperscript{-1}, when 10 mg·L\textsuperscript{-1} was incubated with MC-LR-Bacillus sp., Bacillus sp., and MC-LR, respectively, according to a first order degradation reaction simulation. Schmidt et al. (2006) [74] reported that in eutrophic to somewhat oligotrophic fresh water, the half-life of naturally occurring H\textsubscript{2}O\textsubscript{2} is around 2–8 h, although this could be up to several days in natural water without microorganisms. H\textsubscript{2}O\textsubscript{2} degrades quickly when it is inoculated with organic compounds in natural waters [75], and such degradation is enhanced mainly by bacteria, UV-light, pigments and humic substances.

![Figure 5. H\textsubscript{2}O\textsubscript{2} degradation with Bacillus sp. (8.7 × 10\textsuperscript{6} CFU/mL), and microcystin-LR (MC-LR) (0.2 mg·L\textsuperscript{-1}) under 2.3 watt·m\textsuperscript{-2}, at 25 °C. The error bars represent one standard deviation for three measurements.]

3.2. MC-LR Degradation in the Systems with Copper Sulfate and Hydrogen Peroxide

Figure 6 presents the degradation of MC-LR at 25 °C under different copper sulfate and H\textsubscript{2}O\textsubscript{2} doses. MC-LR degradation was very low for both algaecides, ~10%–40% for all the tested cases. At a copper sulfate dose of 1–2 mg·L\textsuperscript{-1}, MC-LR degradation was negligible (Figure 6a), with only less than 10% of 0.05 mg·L\textsuperscript{-1} MC-LR being degraded after 12 days of incubation. In addition, under the three doses, the MC-LR degradation was not statistically significant (p > 0.05). Jones and Orr (1994) [18] observed that for Microcystis aeruginosa and MC-LR laden water treated with copper sulfate, MC-LR could persist in the water for 21 days.

Figure 6b shows that H\textsubscript{2}O\textsubscript{2}, used at doses of 5, 10 and 20 mg·L\textsuperscript{-1}, was more effective in removing MC-LR than copper sulfate. About 40% MC-LR was oxidized after 288 h of reaction for 20 mg·L\textsuperscript{-1} H\textsubscript{2}O\textsubscript{2} dose and for an initial MC-LR concentration of 0.05 mg·L\textsuperscript{-1}. MC-LR degradation was dependent on H\textsubscript{2}O\textsubscript{2} doses (Figure 6b). The MC-LR degradation was 16%, 38% and 40% for 5, 10, and 20 mg·L\textsuperscript{-1} H\textsubscript{2}O\textsubscript{2} doses after 48 h of exposure under light conditions of 2.3 W·m\textsuperscript{-2}. The three rates were found to be statistically different (p < 0.05). Qiao et al. (2005) [76], reported that up to 94.8% of 0.72 mg·L\textsuperscript{-1} MC-RR were removed only after 60 min of exposure to 1 mmol·L\textsuperscript{-1} H\textsubscript{2}O\textsubscript{2} (34 mg·L\textsuperscript{-1}) under 36.6 W·m\textsuperscript{-2}. Walker (2014) [77] observed that H\textsubscript{2}O\textsubscript{2} was not able to degrade MC-LR without the addition of a UV
light source. Although the light intensity was low in the current study, H$_2$O$_2$ was still able to degrade MC-LR, showing that even a low intensity of light is able to assist H$_2$O$_2$ in the oxidation process.

To compare the degradation capability of these two algaecides on MC-LR with that from bacteria, a biodegradation experiment of MC-LR similar to that in Kansole and Lin (2016) [12] was conducted. In that experiment, MC-LR concentration was also chosen to be 0.05 mg·L$^{-1}$ and 0.02 mg·L$^{-1}$, Bacillus sp. was $8.9 \times 10^6$ CFU/mL, and temperature was 25 °C. Figure S8 shows that the biodegradation of MC-LR followed a first order reaction with a rate constant k = 0.01 h$^{-1}$, and $2 \times 10^{-3}$ h$^{-1}$, respectively, and with 98% and 55% removal after 288 h, which was similar to that reported in Kansole and Lin (2016) [12]. The obtained removals for MC-LR by Bacillus sp., 55 and 98%, are much bigger than those from the two algaecides in this study, ~10%–40%, suggesting that biodegradation is an important removal pathway for MC-LR in water.

![Figure 6](Image)

**Figure 6.** Degradation of MC-LR under different algaecide concentrations. (a) Under copper sulfate; (b) under H$_2$O$_2$ at 25 °C. The error bars represent one standard deviation for three measurements.
3.3. Evaluation of the Effects of H$_2$O$_2$ and Copper Sulfate on the Control of Cyanobacteria and Their Metabolites

H$_2$O$_2$ has been used in both the field and laboratory for the control of cyanobacteria. Barroin and Feuillade (1986) [78] reported that 1.75 mg·L$^{-1}$ H$_2$O$_2$ was enough to kill Planktothrix rubescens (previously known as Oscillatoria rubescens) within 24 h and under 9 W·m$^{-2}$ of light intensity. However, Wang et al. (2012) [79] observed that a dose of 60 mg·L$^{-1}$ was necessary to destroy colonial Microcystis aeruginosa cells for 2 h with a light intensity of 400 µmol photons·s$^{-1}$·m$^{-2}$ (~87 W·m$^{-2}$). In the current study, 3 mg·L$^{-1}$ H$_2$O$_2$ could only remove 9% of Microcystis cells (3 × 10$^6$ cells/mL) within seven days of incubation at 25 °C and under 2.3 W·m$^{-2}$ of light (Figure 2b), which may be attributed to both the low H$_2$O$_2$ dose and low light intensity used in this work. H$_2$O$_2$ kills cyanobacteria with the assistance of hydroxyl radicals produced under the presence of UV light and ferrous ions [54]. In this study, the low UV intensity led to the low production of hydroxyl radicals and low removal of cyanobacteria cells.

H$_2$O$_2$ has been reported to be an effective bactericide to Gram-positive bacteria [80], such as Bacillus sp. The bactericidal effect may be through the production of short-lived free radicals that break down the cell walls of the bacteria [81]. Although bacteria generally cannot tolerate H$_2$O$_2$ exposure, the degradation rates are not reported in the literature due to a lack of data [74]. H$_2$O$_2$ toxicity to microorganisms is nevertheless mitigated through the short exposure time due to the fast decay of H$_2$O$_2$, the acclimation of the bacteria, and the capacity of the bacteria to repopulate the water environment [74]. This study showed that 3 mg·L$^{-1}$ H$_2$O$_2$ led to negligible Bacillus sp. mortality with a rate constant k = 2 × 10$^{-3}$ h$^{-1}$ (Figure 4), thus indicating that 3 mg·L$^{-1}$ H$_2$O$_2$ should be a good dosage to avoid killing non-targeted microorganisms, such as Bacillus sp. Although with this dose the removal of M. aeruginosa is also low, the growth of the cyanobacteria is inhibited for seven days. The advantage of H$_2$O$_2$ for algae growth control is its sustainability compared to most of the other known algaeicides [82], as it decomposes to oxygen and water in water.

Copper sulfate is a very well-known algaeicide that has been used in algae growth control for centuries. For instance, copper sulfate was the sole algaeicide officially used by the Illinois State, USA, until 1986 [83]. Generally, copper dosages of around 0.26 mg·Cu$^{2+}$·L$^{-1}$ (1 mg·L$^{-1}$ of copper sulfate pentahydrate) are suggested in field applications [83] due to the strong inhibition of Cu$^{2+}$ to photosynthesis of cyanobacteria [84]. The use of copper sulfate for cyanobacteria control is strongly influenced by the water matrix, including total alkalinity and pH, with higher doses needed for higher alkalinity and pH. For field applications, the dose of copper sulfate should be less than 2.0 mg·L$^{-1}$ in order to avoid any toxicity to aquatic living organisms, such as fish, when the water alkalinity is ≥200 mg·L$^{-1}$ [61]. Our experiments show that with total alkalinity = 130 mg·L$^{-1}$ in the water matrix, 0.5 mg·L$^{-1}$ of copper sulfate is not enough to control either the studied cyanobacterium or bacterium (Figures 2a and 3). Only when the copper sulfate doses were ≥1 mg·L$^{-1}$, the algaeicide was lethal to both M. aeruginosa and Bacillus sp. (Figures 2a and 3). However, for total alkalinity of 90–200 mg·L$^{-1}$, copper sulfate doses ≥1 mg·L$^{-1}$ may cause damage to aquatic organisms, as suggested by Clayton (2009) [61]. Although copper might be beneficial to the growth of some living organisms [83], it is not environmentally friendly because copper itself is a metal that is not biodegraded once in the environment [85]. Moreover, in the last few years, the price of copper has been rising, practically doubling from January 2004 to January 2017 [86], and this may also prevent the application of copper-based algaeicides to control cyanobacteria in reservoirs. In January 2017, the price of H$_2$O$_2$ was 4.5 times cheaper than that of copper sulfate, with figures of US$2,700/t and US$600/t for copper sulfate and H$_2$O$_2$, respectively [87]. As the maximum algaeicide doses for a lake are 2 and 5 mg·L$^{-1}$ for copper sulfate and H$_2$O$_2$, respectively, from a simple estimation it can be seen that water treatment via copper sulfate is two times more expensive than that with H$_2$O$_2$.

As copper sulfate may kill the cells through cell lysis, cyanobacteria exposed to the algaeicide may release toxins and T&O compounds, such as 2-MIB, geosmin, saxitoxin, cylindrospermopsin, microcystins [88], into the water, complicating water quality issues. It was observed in the current study that even at high doses of copper sulfate, the removal of MC-LR is negligible, at less than...
10%, due to the fact that copper is not a reactive agent with dissolved MC-LR [89]. In addition, as copper sulfate may also kill the studied bacteria, it is possible that the biodegradation effect for algal metabolites in the water will also be suppressed when copper sulfate is applied.

H2O2 is capable of inhibiting the growth of cyanobacteria and has low toxicity end products. In addition, it is competitive in terms of cost, with a capability of degrading organic compounds via the production of hydroxyl radicals with the assistance of UV light. Nonetheless, copper sulfate is not ecofriendly due to the accumulation of non-degradable copper heavy metal which is lethal to aquatic life. As such, when considering the impact on aquatic organisms, accumulation in the environment, cost of application, capability of reaction with toxins, and mortality to toxin degrading bacteria, H2O2 should be seen as a viable alternative to copper sulfate for use as an algaecide in reservoirs.

4. Conclusions

H2O2 and copper sulfate are two algaecides that are commonly used for algal growth control in many water bodies. In the current study, the impacts of both on MC-LR degradation, M. aeruginosa and Bacillus sp. were investigated. H2O2 at a dose of 20 mg L\(^{-1}\) was observed to remove 95% M. aeruginosa cells within seven days, and 2 mg L\(^{-1}\) copper sulfate led to 99% suppression of M. aeruginosa in 14 days’ exposure. One mg L\(^{-1}\) of copper sulfate and 5 mg L\(^{-1}\) of H2O2 were enough to decimate Bacillus sp., with degradation rate constants of 0.04 h\(^{-1}\) and 0.03 h\(^{-1}\), respectively. Copper sulfate is not effective in removing MC-LR, even at a concentration up to 2 mg L\(^{-1}\), with <10% removal. However, H2O2 showed better MC-LR removal, up to 40% under 20 mg L\(^{-1}\) H2O2, even at very low light conditions of 2.3 W·m\(^{-2}\). H2O2 is able to inhibit the growth of cyanobacteria and has low toxicity end products with regard to aquatic living organisms. Moreover, H2O2 is competitive in terms of cost, with the capacity to efficiently degrade organic compounds such as MC-LR under the assistance of UV light. With regard to issues of toxicity for aquatic organisms, cost of application and accumulation in the environment, H2O2 may thus be regarded as a viable alternative to copper sulfate when used as an algaecide to control algae growth in reservoirs. The results of this study may provide useful information about the impact of the two studied algaecides on the targeted cyanobacteria, non-targeted bacteria and MC-LR, and thus may be used for the evaluation of the two algaecides in the control of cyanobacteria in lakes and reservoirs.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4441/9/4/255/s1, Artificial sputum medium (ASM) composition, Figure S1. Solar light spectrum utilized in the present study for the day-like simulation with a total solar wavelength radiance of 2.3 W·m\(^{-2}\); Figure S2. Structure of MC-LR; Figure S3. Hydrogen peroxide concentration calibration curve; Figure S4. Rhodamine B absorbance calibration curve, Figure S5. Variation of the pH during M. aeruginosa growth under different dosages of (a) copper sulfate; (b) H2O2 at 25 °C, Figure S6. pH evolution under different concentrations of copper sulfate pentahydrate speciation and hydrogen peroxide at 25 °C, Figure S7. OH radicals estimation graphs using RhB (10\(^{-3}\) M), with Bacillus sp. (8.7 × 10\(^{9}\) CFU/mL), and MC-LR (0.2 mg L\(^{-1}\)) under 2.293 watt·m\(^{-2}\) at 25 °C, Figure S8. MC-LR degradation under 8–9 × 10\(^{6}\) CFU/mL Bacillus sp. at 25 °C. The error bars represent one standard deviation for three measurements, and Table S1. Copper sulfate pentahydrate and hydrogen peroxide concentrations.

Acknowledgments: This study was supported in part by the National Cheng Kung University, the Government of Taiwan (Ministry of Foreign Affairs) and the UNESCO/Keizo Obuchi fellowship 2015.

Author Contributions: Michelline M. R. Kansole conducted the research and prepared the manuscript; Tsair-Fuh Lin guided the entire research and contributed to preparing the manuscript. Both authors approved the article.

Conflicts of Interest: The authors have no conflicts of interest to declare.

References


© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).