Evaluating the effectiveness of various control and water treatment processes on the membrane integrity and toxin fate of cyanobacteria

Jiajia Fan

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School of Earth and Environmental Sciences

The University of Adelaide

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List of Abbreviations

AWQC	Australian Water Quality Centre
DAFF	Dissolved Air Flotation and Filtration
DOC	Dissolved Organic Carbon
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
EOM	Extracellular Organic Matters
Fe	Iron
HPLC	High Performance Liquid Chromatography
MCs	Microcystins
Mn	Manganese
NOM	Natural Organic Matters
THM	Trihalomethanes
WHO	World Health Organisation
WSP	Waste Stabilization Ponds
WTPs	Water Treatment Plants
WWTP	Waste Water Treatment Plants

Declaration

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Abstract

Cyanobacterial blooms could reduce the available volume of source water for use as drinking, sanitation and irrigation due to the associated toxins which could be severely harmful to humans and animals. Generally, the majority of cyanotoxins are intracellular in healthy populations but they could be released into the surrounding waters when the membranes are compromised by aging or chemical stress. However, conventional water treatment processes are not able to remove the dissolved toxins but only intracellular toxins in the intact cells. Although various chemical compounds have trialled for cyanobacterial bloom control or cyanobacterial cells/metabolites removal in water treatment processes, the effect of these treatments on the membrane integrity and toxin fate of cyanobacterial cells have not been systematically studied and compared. This study evaluated the effectiveness of copper sulphate ($CuSO_4$), chlorine, potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2) and ozone on the cell integrity, densities, toxin release and degradation of *Microcystis aeruginosa* cultured with ASM-1 medium. All of these technologies can compromise the cell membrane of cyanobacteria to varying degrees. Chlorine showed the strongest ability to impair the cell integrity with a majority ($\geq$ 88%) of the cells compromised within the first minute. Ozone dose of 6 mg L^{-1} also could induce 90% lysis of the cyanobacterial cells in 5 minutes and the cell lysis rate of $KMnO_4$ (10 mg L^{-1}) was 0.829 h^{-1}. $CuSO_4$ and H_2O_2 could not only destroy the viability of cyanobacterial cells but also showed algistatic potential over the 7 day treatment. All the chemicals expect $CuSO_4$ could remove the total toxins and chlorine was the most effective one with the fastest rate up to 2161 $M^{-1}s^{-1}$. Although the intracellular toxins were liberated due to cell lysis, there was no build-up of dissolved toxins detected during chlorine and H_2O_2 exposure which may due to the faster toxin oxidation rates than release rates. 1 and 3 mg L^{-1} $KMnO_4$ degraded both the intracellular and extracellular toxins with the cyanobacterial cells remaining intact while ozone induced significant increase of dissolved toxins. Wastewater reuse is important for irrigation; however, cyanobacterial blooms occurred frequently in the wastewater treatment systems with the ideal conditions for cyanobacterial growth. Tertiary treated effluent water was applied to investigate the cell lysis and toxin kinetics based on culture medium study. Similar impacts on the cyanobacterial cells were found using wastewater and medium but higher oxidant demand may be needed for wastewater treatment due to the higher concentrations of dissolved organic materials. In addition, the advantages and drawbacks of these chemicals on the downstream water quality

were assessed to suggest the water authorities to choose the suitable option against cyanobacterial issues.

Statement of Authorship

Journal paper 1

Evaluating the effectiveness of copper sulphate, chlorine, potassium permanganate, hydrogen peroxide and ozone on cyanobacterial cell integrity

Jiajia Fan[1], Lionel Ho[1,2], Peter Hobson[2], Justin Brookes[1,**]

[1]*School of Earth and Environmental Sciences, The University of Adelaide, South Australia, 5005, Australia*
[2]*Australian Water Quality Centre, SA Water Corporation, GPO Box 1751, SA 5001, Australia*

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Jiajia Fan
Performed the laboratory experiments and data analysis, wrote the manuscript.
Signature: Date: 28/8/13

Lionel Ho
Helped to evaluate and edit the manuscript.
Signature: Date: 28/8/13

Peter Hobson
Helped in experimental design and evaluated the manuscript.
Signature: Date: 28/8/13

Justin Brookes
Supervised development of work, helped in manuscript evaluation and acted as corresponding author.
Signature: Date: 28/08/13

Statement of Authorship
Journal paper 2

The effects of various control and water treatment processes on the membrane integrity and toxin fate of cyanobacteria

Jiajia Fan[1*], Peter Hobson[2], Lionel Ho[1,2], Robert Daly[2], Justin Brookes[1,**]

[1]*School of Earth and Environmental Sciences, The University of Adelaide, South Australia, 5005, Australia*
[2]*Australian Water Quality Centre, SA Water Corporation, GPO Box 1751, SA 5001, Australia*

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Jiajia Fan
Performed the laboratory experiments and data analysis, wrote the manuscript and acted as corresponding author.
Signature: Date: 28/8/13

Peter Hobson
Helped in experimental design and evaluated the manuscript.
Signature: Date: 28/8/13

Lionel Ho
Helped to evaluate and edit the manuscript.
Signature: Date: 28/8/13

Robert Daly
Helped in data analysis and evaluated the manuscript.
Signature: Date: 28/8/13

Justin Brookes
Supervised development of work, helped in manuscript evaluation and acted as corresponding author.

28/08/13.

Statement of Authorship
Journal paper 3

Application of various chemicals for cyanobacteria control and cyanotoxin removal in wastewater treatment

Jiajia Fan[1*], Lionel Ho[1,2], Peter Hobson[2], Robert Daly[2], Justin Brookes[1,**]

[1]*School of Earth and Environmental Sciences, The University of Adelaide, South Australia, 5005, Australia*
[2]*Australian Water Quality Centre, SA Water Corporation, GPO Box 1751, SA 5001, Australia*

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Jiajia Fan
Performed the laboratory experiments and data analysis, wrote the manuscript and acted as corresponding author.
Signature: Date: 28/8/13

Lionel Ho
Helped to evaluate and edit the manuscript.
Signature: Date: 28/8/13

Peter Hobson
Helped in experimental design and evaluated the manuscript.
Signature: Date: 28/8/13

Robert Daly
Helped in data analysis and evaluated the manuscript.
Signature: Date: 28/8/13

Justin Brookes
Supervised development of work, helped in manuscript evaluation and acted as corresponding author.
Signature: Date: 28/08/13.

Statement of Authorship
Journal paper 4

Impact of potassium permanganate on cyanobacterial cell integrity and toxin release and degradation

Jiajia Fan[1*], Robert Daly[2], Peter Hobson[2], Lionel Ho[1,2], Justin Brookes[1,**]

[1]*School of Earth and Environmental Sciences, The University of Adelaide, South Australia, 5005, Australia*
[2]*Australian Water Quality Centre, SA Water Corporation, GPO Box 1751, SA 5001, Australia*

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Jiajia Fan
Performed the laboratory experiments and data analysis, wrote the manuscript .
Signature: Date: 28/8/13

Robert Daly
Helped in data analysis and evaluated the manuscript.
Signature: Date: 28/8/13

Peter Hobson
Helped in experimental design and evaluated the manuscript.
Signature: Date: 28/8/13

Lionel Ho
Helped to evaluate and edit the manuscript.
Signature: Date: 28/8/13

Justin Brookes
Supervised development of work, helped in manuscript evaluation and acted as corresponding author.
Signature: Date: 28/08/13.

Chapter 1. Introduction

1.1 Background

Cyanobacteria, also known as blue-green algae, are one of the most common aquatic organisms frequently occurring in many water bodies including surface freshwaters, marine ecosystems and wastewater stabilisation lagoons/ponds. Cyanobacterial blooms tend to occur in water bodies when environmental conditions become favourable, including excessive nutrients, warm temperatures and appropriate light intensity (Correll, 1998; Chorus and Bartram, 1999; Paerl and Huisman). The increasing intensity and frequency of cyanobacterial blooms are one of the major environmental issues affecting aquatic ecosystems worldwide. The presence of cyanobacteria can reduce the availability of source water for drinking, sanitation and irrigation by increasing turbidity and through the production of taste, odours and toxins. Cyanotoxins pose a threat to humans and animals by causing skin irritation, liver cancer and some are severely neurotoxic (Carmichael, 1992; Ueno et al., 1996; Codd et al., 1999).

The cyanobacterial genera *Microcystis*, *Nodularia*, *Cylindrospermopsis*, *Anabaena*, and *Aphanizomenon* are the most common problem species as they can produce toxins including microcystins (MCs), nodularins, cylindrospermopsins, anatoxins and saxitoxins (Chorus and Bartram, 1999). MCs are one of the most frequently occurring hepatotoxins (liver toxins) worldwide with a group of cyclic heptapeptides (a seven amino acid ring) that share a common structure and can occur in both fresh and marine waters. MCs are produced by *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*), *Nostoc*, *Hapalosiphon* and *Anabaenopsis* species (Chorus and Bartram, 1999). Over 75 variants of MCs have been characterised with microcystin-leucine arginine (MC-LR) emerging as the most commonly occurring (Chorus and Bartram, 1999, Svrcek and Smith, 2004). Generally, most MCs are intracellular in healthy populations of cyanobacteria and are released into the surrounding water when cell membranes are compromised by aging or through chemical and physical stresses. *Cylindrospermopsis raciborskii* appears to be the exception to this with approximately 50% of toxin found intracellularly (Smith et al., 2008).

Climate change, population growth and changes in land use practices have exaggerated the proliferation of cyanobacteria causing increased problems in drinking water reservoirs and wastewater treatment systems (Brookes and Carey, 2011; Martins et al., 2011; Carey et al., 2012; Zamyadi et al., 2012). Therefore, the search for methods and technologies to control cyanobacteria and reduce their negative impacts on water quality is of great importance to water authorities. Up-stream treatment using various cyanobacterial growth control technologies in reservoirs/lakes and downstream treatment technologies such as conventional water treatment processes (coagulation, flocculation and filtration) are currently being used by water utilities. However, most of these technologies have their own limitations which need further investigations to optimize their use.

1.2 Problem statement

Generally, control methods can be divided into a number of groups including nutrient control, physical, biological and chemical strategies with each of them having advantages and drawbacks. Lowering nutrient (nitrogen and phosphorus) inputs has been suggested as a way of reducing cyanobacteria growth (Conley et al, 2009; Brookes and Carey, 2011). However, this process can take many years and may not be a viable option due to political and economic issues associated with changing activities within the water catchment area (Jancula and Marsalek, 2011). Physical techniques like artificial mixing of water bodies are usually costly; however, there are notable examples of success (e.g. Visser et al., 1996). Biological options such as bio-manipulation in theory have some merits but its effectiveness is dependent on phosphorus availability and it cannot provide an immediate solution (Benndorf et al., 2002). Furthermore, there are doubts as to whether bio-manipulation is suitable in specific circumstances (Boon et al., 1994). Chemical control methods, such as algicides and algistats, offer a short term option which provides immediate results. However, such chemical treatments are not without issue and a comparative evaluation of all the technologies is required.

Copper sulphate ($CuSO_4$) has been used as an algicide for many years and has provided a low cost and easy method to control cyanobacterial growth (Elder and Horne, 1978; Hobson et al., 2012). However, environmental concerns about its activity as a broad spectrum biocide and

accumulation of copper in sediments have seen its use diminish. Hydrogen peroxide (H_2O_2) has been suggested as an alternative to copper sulphate due to its selectivity to cyanobacteria over other organisms (Kay et al., 1982; Matthijs et al., 2012). However, investigations of the use of H_2O_2 have generally been limited and further work is needed to ensure its effectiveness and applicability for large scale use.

Conventional water treatment technologies provide another barrier to reduce the negative impacts of cyanobacterial blooms. These processes have been shown to be effective for removal of whole, intact cells, but inefficient in removing dissolved toxins (Chow et al., 1998; 1999). Oxidants such as chlorine, potassium permanganate ($KMnO_4$) and ozone are widely used as disinfectants in water/wastewater treatment systems. In addition, they can be applied as pre-oxidants which can improve the removal of cyanobacterial cells by conventional water treatment processes (Plummer and Edzwald, 2002; Chen and Yeh, 2006; Chen et al., 2009). Some studies have demonstrated that chlorine, $KMnO_4$ and ozone can also lyse cyanobacterial cells which can result in the release of toxins into the surrounding water (Daly et al., 2007; Chen and Yeh, 2006). This could then become a problem due to the inability of conventional water treatment processes to remove dissolved toxins. However, studies have shown that these chemicals can also oxidise dissolved toxins if applied correctly (Rositano, 1998; Hall et al., 2000; Zamyadi et al., 2013). To optimise the use of these oxidants in a treatment plant it is important to understand their activity in terms of cell rupture kinetics, toxin release and toxin degradation. This information will then allow an appropriate choice of oxidant for a particular set of conditions.

1.3 Aims

To aid water authorities in the application of methods to control cyanobacterial blooms and associated negative impacts, this study provided a novel evaluation of the impacts of five popular treatment chemicals $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone on cyanobacterial cells. The overall objectives were to (1) systematically assess and compare these chemicals for effective cyanobacteria control with an emphasis on the assessment of membrane integrity of cyanobacterial cells during such treatment, (2) further investigate the effect of $CuSO_4$, chlorine, $KMnO_4$, H_2O_2 and ozone on toxin release and degradation using a culture of

Microcysits aeruginosa grown in ASM-1 medium, (3) investigate the impact of $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone on membrane integrity and concomitant toxin release and degradation using cyanobacterial cultures in tertiary treated wastewater.

1.4 Approach

The work detailed in this thesis evaluated the impact of $CuSO_4$, chlorine, $KMnO_4$, H_2O_2 and ozone on *M. aeruginosa* in terms of cell integrity, cell densities, toxin release and degradation. These tests were conducted in both culture medium and tertiary treated wastewater. The major research outcomes are presented as four journal publications/manuscripts in Chapters 2 through to 5 of this thesis. The effect of $CuSO_4$, chlorine, $KMnO_4$, H_2O_2 and ozone on the cell integrity and density of *M. aeruginosa* grown in culture medium (ASM-1) is systematically evaluated in Chapter 2. In Chapter 3, the work is expanded to couple both cell lysis and toxin fate of cyanobacteria treated with a range of chemicals. In this chapter the effect of $CuSO_4$, chlorine, $KMnO_4$, H_2O_2 and ozone on toxin release and degradation was investigated using a culture of *M. aeruginosa* grown in ASM-1 media. Chapter 4 investigates what impact the water matrix has on the kinetics of toxin release and degradation. $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone were all applied to cells to assess membrane integrity concomitant toxin release and degradation of cyanobacterial cultures in tertiary treated wastewater. Chapter 5 provides a more detailed investigation into the use of $KMnO_4$ as a pre-treatment chemical and its impact on cyanobacterial cell integrity and the concomitant toxin release and toxin degradation. More specifically, Chapter 5 presents a toxin kinetics model which was developed and tested using different realistic scenarios involving cyanobacteria in the presence of manganese and iron.

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Chapter 2. Evaluating the effectiveness of copper sulphate, chlorine, potassium permanganate, hydrogen peroxide and ozone on cyanobacterial cell integrity

Abstract

Cyanobacterial blooms are continuously critical challenges in drinking water systems which can have various negative impacts such as production of taste, odour and toxic compounds. Furthermore, the intracellular metabolites could be released into surrounding waters when the cyanobacterial membranes are destroyed. Although a variety of techniques have been developed to control cyanobacterial blooms and remove cyanobacterial cells or metabolites in water treatment processes, the effect of these treatments on the membrane integrity of cyanobacterial cells have not been systematically studied and compared. This study evaluated the effectiveness of copper sulphate ($CuSO_4$), chlorine, potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2) and ozone on the cell integrity and densities of *Microcystis aeruginosa*. All of these technologies can compromise the cell membrane of cyanobacteria to varying degrees. Chlorine showed the strongest ability to impair the cell integrity with a majority ($\geq$ 88%) of the cells compromised within the first minute and with the cell lysis rates ranging of 0.640-3.82 h^{-1} during 1 to 60 minutes. Ozone dose of 6 mg L^{-1} also could induce 90% lysis of the cyanobacterial cells in 5 minutes and the cell lysis rate of $KMnO_4$ (10 mg L^{-1}) was 0.829 h^{-1}. $CuSO_4$ and H_2O_2 could not only destroy the viability of cyanobacterial cells but also showed algistatic potential over the 7 day treatment. The potential of all the oxidants (chlorine, $KMnO_4$, H_2O_2 and ozone) considered as algicides were discussed in this study. The benefits and drawbacks of these control and water treatment options were assessed as well.

2.1 Introduction

Cyanobacterial blooms continue to be one of the most serious global issues facing water supply and the maintenance of healthy water ways. The aesthetics of potable water can be compromised by taste and odour compounds produced by cyanobacteria such as geosmin and

2-methylisoborneol. In addition, cyanobacteria may pose a threat to human health because they can produce several types of toxic compounds (Carmichael, 1992; Codd et al., 1999). Various toxins can cause skin rashes, lead to liver cancer and some of them are strong neurotoxins (Ueno et al., 1996; Rapala et al., 2005). It is generally the case that most of the cyanotoxins are intracellular in healthy populations (Chorus and Bartram, 1999) except for *Cylindrospermopsis* sp. which can have a significant proportion of the toxin extracellularly (Chiswell et al., 1999; Smith et al., 2008). However, the intracellular toxins, taste and odour compounds could be released into surrounding waters when the cell integrity of cyanobacteria is disrupted. This may present additional challenges for water treatment authorities as it is harder to remove extracellular toxins than intracellular toxins which are readily removed with coagulation (Chow et al., 1999).

Several chemical compounds have been trialled for use as algicides (any chemical added to water which is toxic to, and kills algae and/or cyanobacteria) or algistats (any chemical or additive, added to water that inhibits or retards the growth of algae, either directly, or by chemical modification of the water column). Copper sulphate ($CuSO_4$) is the most commonly applied algicide since it is economical, effective, and easy to apply (Elder and Horne, 1978; Padovesi-Fonseca and Philomeno, 2004). However, copper addition to lakes and reservoirs raises concerns about heavy metal accumulation and toxicity. The search for alternatives to copper based algicides is necessary as it is unlikely cyanobacteria occurrence will decline given future climate and land use practices (Brookes and Carey, 2011; Carey et al., 2012). Hydrogen peroxide (H_2O_2) is an environmentally friendly oxidant dissociating to water and oxygen with no other chemical residual. It is proposed that H_2O_2 could be a potential algicide in reservoirs as it may attack the cyanobacteria cells and restrain the photosynthesis activity by inhibiting photosynthetic electron transfer (Kay et al., 1982; Drabkova et al., 2007a).

Conventional water treatment technologies including coagulation, flocculation and filtration are effective at removing intracellular toxins with intact cells without causing additional release of intracellular toxins (Lam et al., 1995; Chow et al., 1999). In contrast, these treatment processes are inefficient at removing dissolved toxins, taste and odour compounds (Himberg et al., 1989; Svrcek and Smith, 2004). It is common practice to add oxidants prior to conventional treatment to improve coagulation or to oxidise iron (Fe) and manganese (Mn). Common pre-treatment oxidants include chlorine (Plummer and Edzwald, 2002; Ma et al.,

2012), ozone (Miao and Tao, 2009; Chen et al., 2009), and potassium permanganate ($KMnO_4$) (Ellis et al., 2000; Chen and Yeh, 2006). Oxidation can alter the surface characteristics and charge of cyanobacteria which result in enhancing the effectiveness of coagulation and flocculation for their subsequent removal. Nevertheless, some studies have shown that chlorine pre-oxidation could induce cell lysis and concomitant toxin release (Peterson et al., 1995). Other oxidants may share similar trade-offs, which warrants a systematic study of whether they also cause cell lysis, in particular, which concentrations are required for effective pre-oxidation.

Flow cytometry has not only become a powerful tool in biomedical sciences but also gradually been introduced in biological oceanography and limnology (Balfoort et al., 1992; Brookes et al., 2000; Regel et al., 2004). The combination of flow cytometry with fluorescent molecular probes provides the opportunity to assess the membrane integrity of microorganism cells. Furthermore, this technique is more rapid compared with conventional enumeration methods (Vives-Rego et al., 2000) and shows promise as a comparative tool for evaluating cell integrity of cyanobacteria.

To date, no studies have systematically assessed and compared a wide range of algicides/oxidants for effective cyanobacteria control with an emphasis on the assessment of membrane integrity of cyanobacterial cells during such treatment. Therefore, the aims of this study were to evaluate the effect of various algicides and oxidants on the cell integrity of cyanobacteria and to assess the advantages and disadvantages of these control and pre-treatment options.

2.2 Materials and Methods

2.2.1 Materials and reagents

All chemicals and reagents used were analytical grade and solutions were made using ultra-pure water purified to a resistivity of 18 MΩ cm by a Milli-Q water purification system (Millipore Pty Ltd, USA). All experiments were performed at room temperature 20 ± 2 ℃. A toxic strain of *Microcystis aeruginosa* (338) Kutz. emend Elenkin (from the Australian Water Quality Centre Culture Collection) was grown in ASM-1 medium (Gorham et al., 1964) and

routinely subcultured to maintain growth in logarithmic phase for experiments. This culture remained unicellular or as double cells. All cultures were incubated in an incubation cabinet (in the absence of UV light) under constant cool-fluorescent light intensity 50 μmol m^{-2} s^{-1} (measured with a scalar quantum sensor (LI-COR LI-190)) on a 12h:12h light-dark cycle at a constant temperature of 20 ± 1 ℃, and bubbled with air. Prior to use in experiments, the algal cultures were adjusted to pH 7.5 ± 0.1 using either 0.1M sterile filtered hydrochloric acid or sodium hydroxide. Cells were exposed to the algicides or oxidants at varying concentrations in time course experiments. Samples were taken regularly for cell counts and analysis for evidence of cell lysis.

Samples for cell counts by microscopy were treated with Lugol's iodine, pressurized to 900 kPa for 2 minutes to collapse gas vesicles and then counted at 400 × magnification using methods described by Brookes et al. (1994). Cultures having an initial cell density of 7.0 × 10^5 cells mL^{-1} were used in all experiments.

2.2.2 Membrane integrity determination

Cell integrity was determined for individual cells using flow cytometry in combination with fluorescent probes. A FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with an air cooled 15 mW argon laser emitting at a fixed wavelength of 488 nm was used for all fluorescence measurements and optically expressed cell features. Fluorescent filters and detectors were all standard with green fluorescence collected in channel FL1 (530 nm) and red fluorescence collected in channel FL3 (670 nm). Probe fluorescence, chlorophyll *a* fluorescence, forward scatter - FSC (cell size) and side scatter - SSC (cell granularity) (Brookes et al., 2000) measurements were collected and analysed using CellQuest software (Becton Dickinson, USA) and Cyflogic (CyFlo Ltd., Finland). FSC and FL3 were used to identify the cyanobacterial cells. FL1 was used to determine the fluorescent intensity of SYTOX Green.

SYTOX Green nucleic acid stain (Molecular Probes, USA) was used to determine the percentage of viable (SYTOX negative) to nonviable (SYTOX positive) cells in a sample as described by Regel et al. (2004) and Daly et al. (2007), with slightly modifications. A development incubation time of 10 min was used with a final SYTOX concentration of 0.2

μM. An unstained control sample was measured to provide the baseline cell fluorescence at 530 nm with no SYTOX addition. Approximately 5000 events (cells) were recorded by the flow cytometty in the viable and nonviable regions.

2.2.3 $CuSO_4$

$CuSO_4$ was used in this study as a bench mark to compare with other water treatment technologies for cyanobacteria control. Appropriate amounts of copper stock solution (0.119 g L^{-1}) were added to 1 L Erlenmeyer flasks containing cyanobacterial culture to achieve desired copper concentrations of 0.0 (control), 0.5, 1.0 and 1.5 mg L^{-1}. Three replicates for each concentration were conducted. The experiments were carried out in the incubation cabinet. Samples were taken after copper incubation on days 1, 2, 3 and 7 for analyses.

2.2.4 Chlorine

A chlorine stock solution was prepared by bubbling gaseous chlorine through Milli Q water in an amber Schott bottle. The bottle was then sealed and stored at 4 ℃ in darkness. Chlorine stock concentrations and free chlorine residuals were determined using the DPD-FAS titration method described in Standard Methods (APHA et al., 1998). Typical chlorine stock solution concentrations ranged from 4000–5000 mg L^{-1} as free chlorine. A chlorine decay experiment was conducted prior to each experiment. For chlorine oxidation experiments, cyanobacterial samples were dosed with chlorine to achieve the desired chlorine concentrations (3, 4 and 5 mg L^{-1}) in triplicate and samples taken at specified contact times (0, 1, 2, 5, 10, 20, 30 and 60 min) for analyses. All chlorination experiments were conducted in amber glass bottles and incubated in darkness. Samples from each sampling time were quenched with sodium thiosulfate at a stoichiometric ratio specified in Standard Methods (APHA et al., 1998).

2.2.5 $KMnO_4$

$KMnO_4$ stock solution (1.0 g L^{-1}) was prepared by dissolving crystal $KMnO_4$ in Milli-Q water. It was then standardized by titration with sodium oxalate and kept under darkness at 4 ℃. Sodium thiosulfate stock solution (4.0 g L^{-1}) was prepared by dissolving $Na_2S_2O_3$ $5H_2O$ in Milli-Q water and was added to samples upon sampling to quench the reaction of permanganate and arrest further oxidation of cyanobacterial cells. $KMnO_4$ concentrations in the following experiments were measured after filtering the sample through 0.2 μm nylon syringe filters (Whatman, UK) to remove manganese oxide. Specific concentrations diluted by the stock solution were analysed by measuring the absorbance at 525 nm in a 10 mm cuvette in a UV/VIS spectrophotometer (Thermo Scientific, UK) and compared with a standard curve which described a linear relationship between absorbance at 525 nm and $KMnO_4$ concentrations ranging between 0 - 20 mg L^{-1}. The oxidation experiments were conducted in 2 L Schott bottles. Cyanobacterial samples were dosed with the desired $KMnO_4$ concentrations (1, 3, 5 and 10 mg L^{-1}) and mixed with magnetic stirrer at a low speed. Samples were withdrawn at different reaction times up to 7 hours and immediately analysed to determine $KMnO_4$ residual.

2.2.6 H_2O_2

Appropriate amounts of Stabilised Hydrogen Peroxide (Sigma Aldrich 30%) were added to 1 L Erlenmeyer flasks containing cyanobacterial culture to achieve desired H_2O_2 concentrations of 0.0 (control), 10.2, 51 and 102 mg L^{-1}. Three replicates for each concentration were conducted. The H_2O_2 experiments were carried out in the incubation cabinet. Samples were taken after H_2O_2 incubation on days 1, 2, 3 and 7 to determine the H_2O_2 residual and cyanobacterial cell integrity (immediately quenched with sodium thiosulfate).

H_2O_2 concentrations were measured using a spectrophotometrical method described by Drabkova et al. (2007a) with slight modifications. Buffer stock solutions were prepared by mixing 0.5 M Na_2HPO_4 and 0.5 M NaH_2PO_4 in order to achieve pH 6. This buffer was added outside of the cultivation system as it was only added to the samples for testing H_2O_2 residual and the contact time of phosphate buffer with experimental organisms was one minute. 0.1

mL of the buffer solution was added to 0.9 mL of Milli Q water and 0.04 mL sample. The buffered sample (1.04 mL) was complemented with 40 µL of DPD reagent (0.1 g of N, N-diethyl-1, 4-phenlenediammonium sulfate, p.a., Fluka, diluted in 10 mL of 0.1 N H_2SO_4) and 10 µL of HRP reagent (10 mg of horseradish peroxidase, Sigma, Type II, 181 purpurogallin units mg^{-1}, diluted in 10 mL of deionized water) followed by continual stirring. The developed colour was measured using a UV/VIS spectrophotometer (Thermo Scientific, UK) at a wavelength of 551 nm in a 1 cm path-length optical cuvette. Absorbance of the whole mixture without HRP addition was measured as a blank. A standard curve was made which described a linear relationship between the absorbance and H_2O_2 concentrations ranging from 0 to 150 mg L^{-1}.

2.2.7 Ozone

The ozone experiments were conducted in the fume hood and no extra light was involved, so the influence by light should be minimal. Ozone was prepared by feeding high grade oxygen into a CFS-1A ozone generator (Ozonia, Switzerland; see Ho et al., 2002 for details). The ozone experiments were conducted in 250 mL volumetric flasks containing 180 mL of test samples. Milli-Q water was added to each flask such that after the addition of ozone the final volume of samples was 200 mL. Ozone was introduced by adding aliquots of the ozone stock solution (up to 20 mL) to obtain the desired ozone concentrations. The flasks were shaken vigorously for 0.5, 1, 2, 3, 4 and 5 minutes separately. Then 50 mL of indigo solution was added to the flasks and shaken for a few seconds then residual was determined. The ozonation of *M. aeruginosa* cultures was conducted in 250 mL Schott bottles. Cyanobacterial samples were dosed with the desired ozone concentrations (0, 2, 4 and 6 mg L^{-1}) in triplicate and shaken vigorously by hand. Samples were withdrawn after 5 minutes and immediately quenched with high purity nitrogen for 5 minutes to remove any residual ozone.

2.2.8 Statistics

Data (cell lysis rates) were processed using Prism 5.0 software (GraphPad Software, USA). Three replicated rates for each dose of every treatment were calculated and then these values were averaged and their standard deviation calculated. One-way ANOVA was performed to determine whether there was a statistically significant difference between every dose. If the

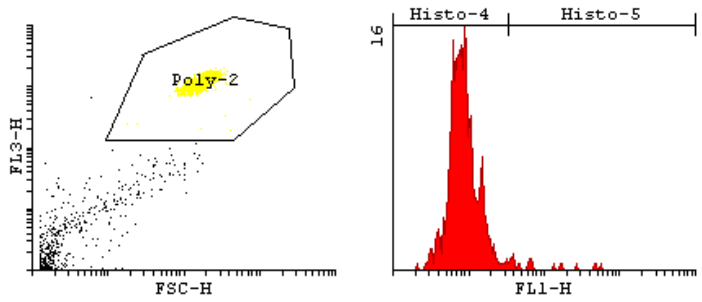
rates could not be calculated (e.g. ozone), one-way ANOVA was performed to determine if there was a statistically significant difference between initial and final proportions of intact cyanobacterial cells.

Specific dose of every algicide/oxidant was chosen to compare the impact on cyanobacterial cells. For $CuSO_4$ and H_2O_2, 0.5 and 10.2 mg L^{-1} were selected because they were regarded as recommend doses in water bodies for cyanobacterial removal (Hobson et al., 2012). The applied doses of chlorine, $KMnO_4$ and ozone in water treatment plants (WTPs) are more dependent on water quality parameters such as dissolved organic carbon (DOC), Fe and Mn. 3 mg L^{-1} chlorine, 3 mg L^{-1} $KMnO_4$ and 2 mg L^{-1} ozone were chosen for comparison as effective doses for pre-treatment (Chen and Yeh, 2005; Ma et al., 2006; Ma et al., 2012).

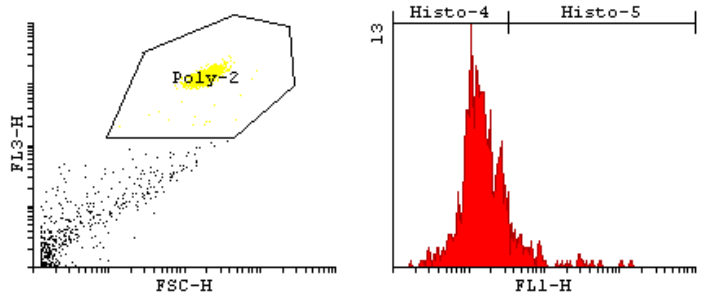
2.3 Results

2.3.1 $CuSO_4$

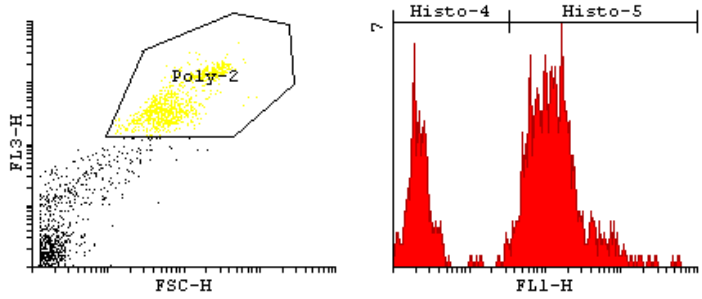
The optical properties of cells shift as their physiology changes in response to chemical treatment. The group of *M. aeruginosa* cells shifted after 1 day $CuSO_4$ treatment with reduced chlorophyll auto fluorescence and full scatter as shown in the flow cytometric dot-plots in Figure 2.1. Addition of $CuSO_4$ to the *M. aeruginosa* culture gave rise to a second population of cyanobacterial cells (Histo-5) with increased green fluorescence relative to intact cells (Histo-4). The population of lysed cells increased with increasing $CuSO_4$ concentration. The percentage of compromised cyanobacterial cells almost achieved 100% with highest $CuSO_4$ concentration (1.5 mg L^{-1}) treatment (Figure 2.1d). Other oxidants presented similar population shifting in the flow cytometric dot-plots and histogram when the cells lost membrane integrity (e.g. Figure 2.2 for H_2O_2, data not shown for other oxidants).



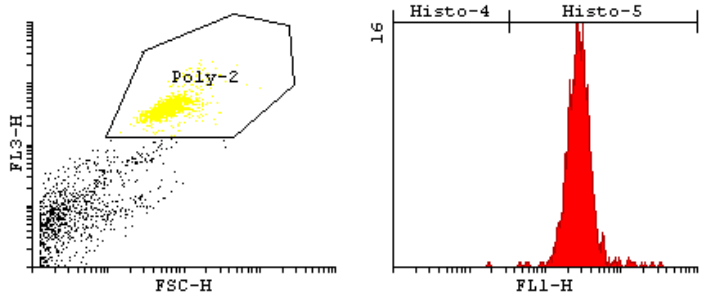
(a)



(b)

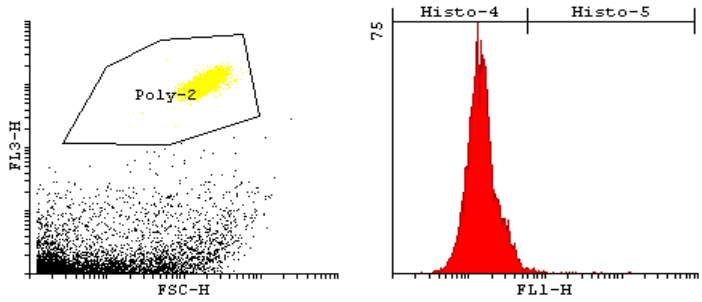


(c)

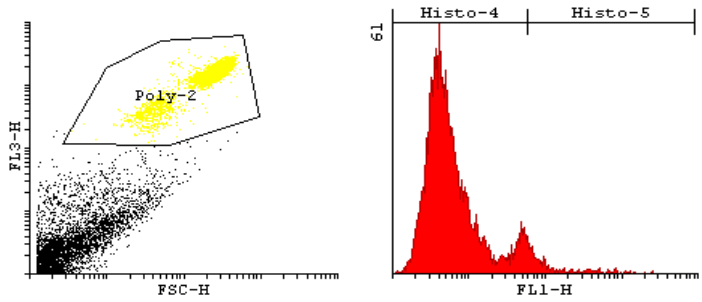


(d)

Figure 2.1 Flow cytometry results following $CuSO_4$ addition to Microcystis aeruginosa at concentrations of (a) 0 mg L^{-1}, (b) 0.5 mg L^{-1}, (c) 1.0 mg L^{-1} and (d) 1.5 mg L^{-1} after 1 day treatment. Poly-2 is region used to define populations as M. aeruginosa cell group. Histo-4 and Histo-5 were used to define populations as intact and lysed cells, respectively. Axes: FL1-Green corresponds to SYTOX Green stain fluorescence detected at 530 nm, FL3-Red corresponds to chlorophyll autofluorescence detected at 670 nm. FSC corresponds to full scatter detected at 488nm.



(a)



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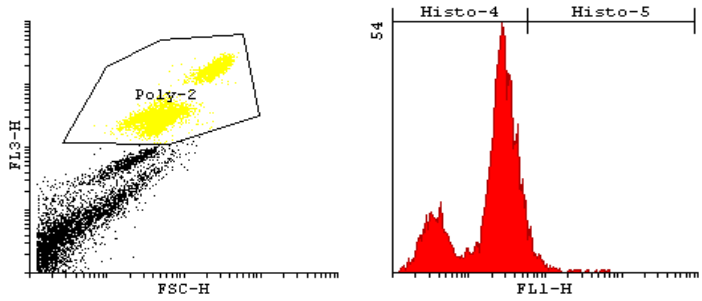
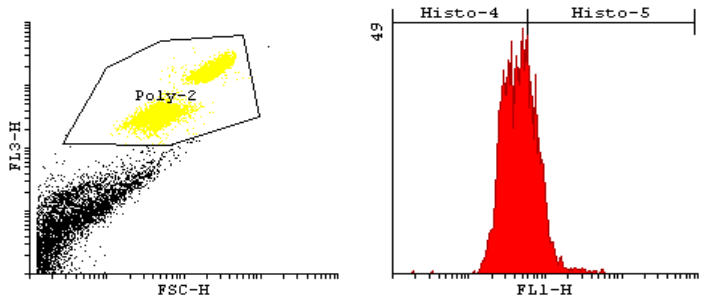


(c)



(d)

Figure 2.2 Flow cytometry results following H_2O_2 addition to Microcystis aeruginosa at concentrations of (a) 0 mg L^{-1}, (b) 10.2 mg L^{-1}, (c) 51 mg L^{-1} and (d) 102 mg L^{-1} after 1 day treatment. Poly-2 is region used to define populations as M. aeruginosa cell group. Histo-4 and Histo-5 were used to define populations as intact and lysed cells, respectively. Axes: FL1-Green corresponds to SYTOX Green stain fluorescence detected at 530 nm, FL3-Red corresponds to chlorophyll autofluorescence detected at 670 nm. FSC corresponds to full scatter detected at 488nm.

13% of the cyanobacterial cells lost membrane integrity with copper concentration of 0.5 mg L^{-1} treatment after 1 day. The percentage of compromised cells increased with time during the first 3 days (Figure 2.3a). Approximately 70% of cells exposed to 1.0 mg L^{-1} $CuSO_4$ lost membrane integrity after 1 day with 98% of the cells impaired after 2 days treatment.

However, the cell population in the intact cell region increased (a decrease was detected in SYTOX fluorescence) after 3 days with a concomitant decrease in the proportion of lysed cells (Figure 2.3a). The highest concentration of $CuSO_4$ (1.5 mg L^{-1}) resulted in more than 95% of compromised cells during the treatment. The percentage of intact cells dropped progressively to 90% on day 7 for control (0 mg L^{-1} copper) samples.

The cell density of the control samples increased with time with up to 2.1×10^6 cells mL^{-1} detected after 7 days. Cell counts decreased about 22% after 3 days treatment but did not change too much during the first two days using the copper concentration of 1.0 and 1.5 mg L^{-1}. As a relatively lower concentration of 0.5 mg L^{-1} as copper, the cell density increased slightly comparing to the initial control samples (Figure 2.3b).

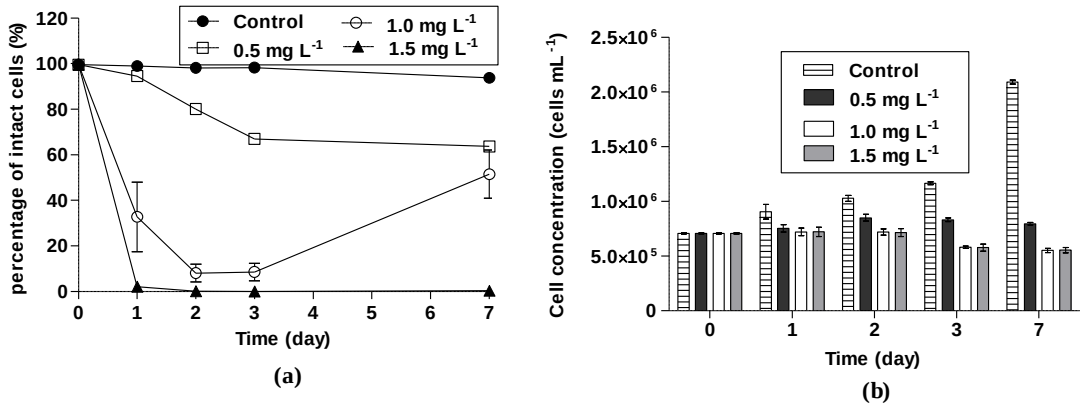


Figure 2.3 The change of Microcystis aeruginosa cells treated with $CuSO_4$ at concentrations of 0, 0.5, 1.0, and 1.5 (mg L^{-1} as copper) measured after 1, 2, 3 and 7 days treatment. (a) Percentage of intact cells; (b) Cell densities. Error bars represent standard deviation from triplicate analyses.

2.3.2 Chlorine

The chlorine concentrations decreased rapidly within the first minute of contact followed by a more gradual decay over the remaining time with initial chlorine doses of 3, 4 and 5 mg L^{-1} (Table 2.1). There was no chlorine residual detected with the chlorine dose of 3 mg L^{-1} after 30 min whist a residual concentration of 0.1 mg L^{-1} and 1.0 mg L^{-1} remained using the initial doses of 4 and 5 mg L^{-1}, respectively. A chlorine residual of 0.2 mg L^{-1} was present after a contact time of 60 min using a 5 mg L^{-1} chlorine dose but no residual was detected where 4 mg L^{-1} chlorine was added initially.

Chlorine dosing caused loss of membrane integrity; 3 mg L^{-1} chlorine treatment resulted in 97% *M. aeruginosa* cell lysis within the first minute. 4 and 5 mg L^{-1} of chlorine caused similarly high rates; 95% and 88% of cells lysed within one minute, respectively .The SYTOX Green stain worked unstably in the first five minutes since there was an increase of intact cells shown during this period as initial chlorine concentration of 3 and 4 mg L^{-1}. More than 95% of the cyanobacterial cells were membrane-comprised within 5 min of contact for all concentrations (3, 4 and 5 mg L^{-1}) after chlorine exposure (Figure 2.4a). There were no significant differences in cyanobacterial cell densities observed during the chlorination in 60 minutes (Figure 2.4b).

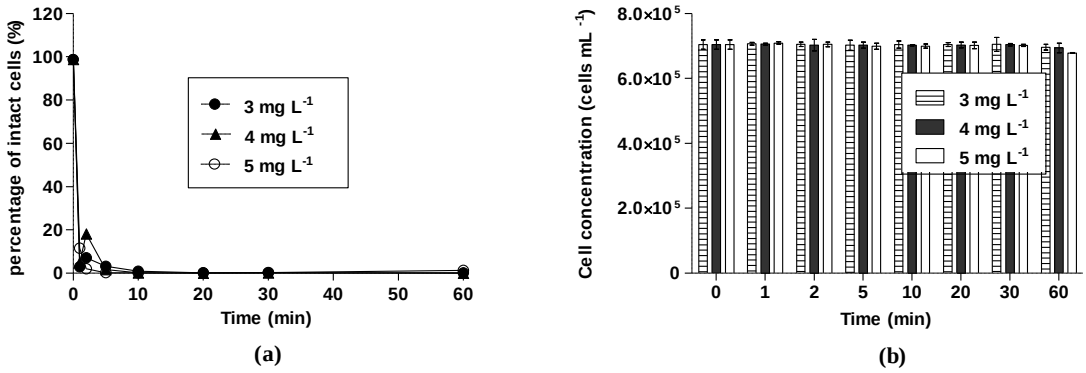


Figure 2.4 The change of Microcystis aeruginosa cells treated with chlorine at concentrations of 3, 4, and 5 (mg L^{-1}) measured at 0, 1, 2, 5, 10, 20, 30 and 60 minute reaction time. (a) Percentage of intact cells; (b) Cell densities. Error bars represent standard deviation from triplicate analyses.

2.3.3 $KMnO_4$

A $KMnO_4$ residual of 0.2 mg L^{-1} was present after 1 hour in the 1 mg L^{-1} $KMnO_4$ treatment but no residual was detected after 2 hours. The $KMnO_4$ concentrations decreased progressively from 3, 5 and 10 mg L^{-1} to 1.0, 2.2 and 3.8 mg L^{-1} respectively after 7 hours treatment (Table 2.1). $KMnO_4$ concentrations were measured in a cell-free ASM-1 media with an initial $KMnO_4$ dose of 10 mg L^{-1} which induce a slightly decrease of 0.4 mg L^{-1} after 7 hours.

The *M. aeruginosa* cells membrane integrity was relatively unaffected using $KMnO_4$ doses of 1 and 3 mg L^{-1} (Figure 2.5a). However, the percentage of intact cells decreased to 74% when using 5 mg L^{-1} $KMnO_4$ after 6 hours treatment. The highest concentration of $KnMO_4$ (10 mg L^{-1}) resulted in a greater loss of membrane integrity with no intact cells remaining after 6 hours. The cell densities remained fairly constant after 6 hours of treatment using the initial KMnO4 doses of 1, 3 and 5 mg L^{-1}, while about 15% decrease of cell density was evident in the 10 mg L^{-1} $KMnO_4$ treatment (Figure 2.5b).

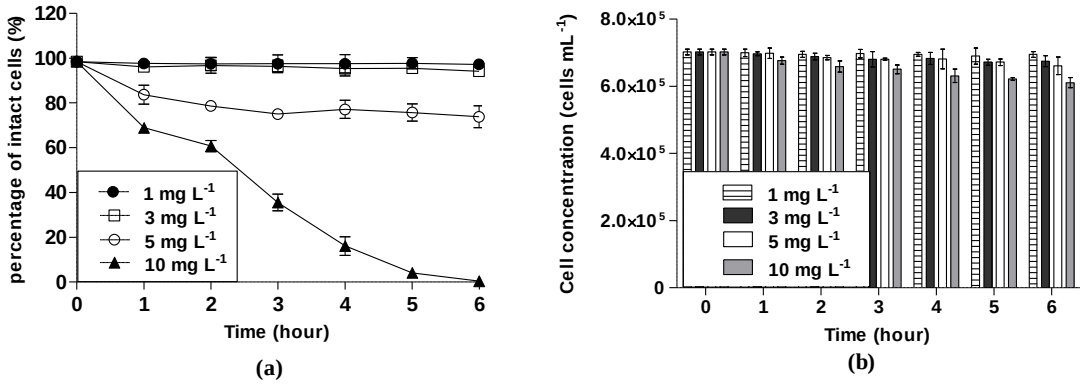


Figure 2.5 The change of Microcystis aeruginosa cells treated with $KMnO_4$ at concentrations of 1, 3, 5 and 10 (mg L^{-1}) measured hourly for six hours. (a) Percentage of intact cells; (b) Cell densities. Error bars represent standard deviation from triplicate analyses.

2.3.4 H_2O_2

H_2O_2 decomposition kinetics were measured in a cell-free ASM-1 medium (control) with an initial H_2O_2 dose of 102 mg L^{-1} as well as *M. aeruginosa* cultures where initial H_2O_2 doses of 10.2, 51 and 102 mg L^{-1} were applied. The concentration of H_2O_2 remained relatively stable (a final residual of 96 mg L^{-1}) in the control samples over the 8 day period. The cyanobacterial cells exerted an oxidant demand and the H_2O_2 residual decreased steadily from 10.2, 51 and 102 mg L^{-1} to 0.0, 30.8 and 83.0 mg L^{-1} after 8 days, respectively (Table 2.1).

Approximately 86%, 46% and 51% of the cyanobacterial cells maintained membrane integrity with H_2O_2 doses of 10.2, 51 and 102 mg L^{-1}, respectively after 1 day. The percentage of intact cells decreased to 16%, 31% and 7% on day 2 with the initial H_2O_2 doses of 10.2, 51 and 102 mg L^{-1}, respectively (Figure 2.6a). However, the cell population in intact cell region increased (decrease in SYTOX fluorescence) after 3 days which showed a decrease of lysed cells. A H_2O_2 concentration of 51 mg L^{-1} even presented 82% of viable cells in the day 7. The percentage of intact cells dropped progressively to 91% as a control (Figure 2.6a). The cell density of control samples increased with time with up to 2.1×10^6 cells mL^{-1} detected on day 7. There were no significant differences in cyanobacterial cell densities observed when H_2O_2 was applied, irrespective of the initial dose (Figure 2.6b).

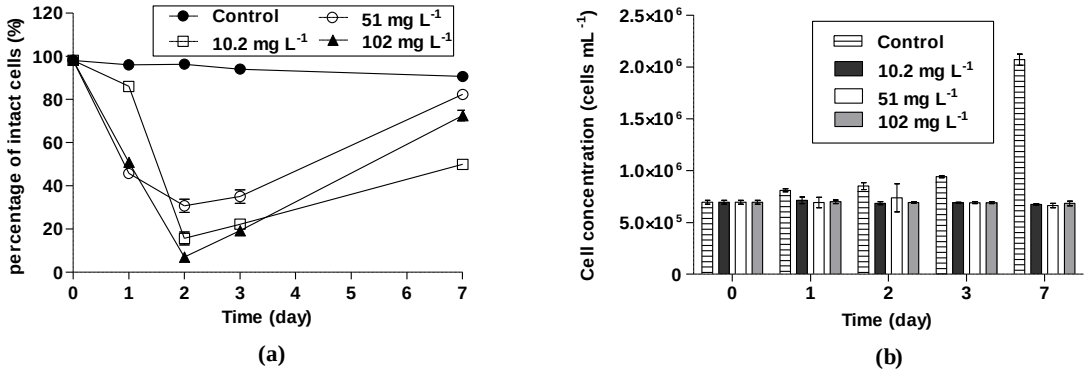


Figure 2.6 The change of Microcystis aeruginosa cells treated with H_2O_2 at concentrations of 0, 10.2, 51, and 102 (mg L^{-1}) measured after 1, 2, 3 and 7 days treatment. (a) Percentage of intact cells; (b) Cell densities. Error bars represent standard deviation from triplicate analyses.

2.3.5 Ozone

There were no ozone residual detected within the first 30 s using an initial ozone concentration of 2 mg L^{-1}. Even for the higher doses (4 and 6 mg L^{-1}), ozone was consumed rapidly within the first 30 s but much slower during the following reaction time. There was no ozone residual observed after 5 min even for the highest ozone concentration (6 mg L^{-1}) (Table 2.1).

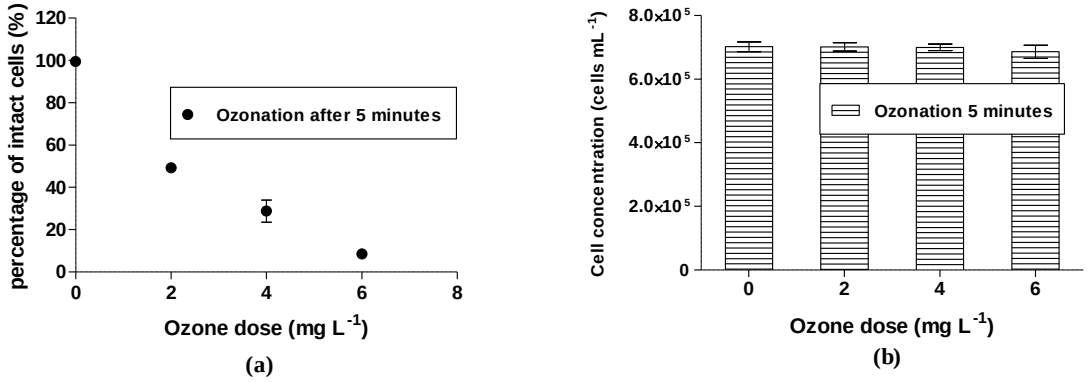


Figure 2.7 The change of Microcystis aeruginosa cells treated with ozone at concentrations of 0, 2, 4, and 6 (mg L^{-1}) measured after 5 minutes. (a) Percentage of intact cells; (b) Cell densities. Error bars represent standard deviation from triplicate analyses.

The percentage of intact cells remained around 50% with initial ozone dose of 2 mg L^{-1} after 5 min contact time. The 4 and 6 mg/L of ozone doses induced membrane loss in 70% and 90% cyanobacterial cells (Figure 2.7a). There were no significant differences in cyanobacterial cell densities observed between the applied ozone doses (Figure 2.7b).

2.3.6 Cell lysis rates

Reactions between the oxidants and *M. aeruginosa* cultures are second order but $CuSO_4$ is not an oxidant and has a different mode of activity. The concentration of $CuSO_4$ remained constant and therefore a second order reaction is not suitable. In addition, cell lysis rates of $CuSO_4$ and H_2O_2 were calculated using the data of day 0, 1 and 2; the [H_2O_2 residual] >>

[H_2O_2 consumption] during this period, therefore, the H_2O_2 concentration can be considered constant and first order is in nature. The results of cell lysis by chlorine oxidation can be described by two stages: fast and slow. The fast stage represents the rapid destruction of membrane integrity of cyanobacterial cells observed within the first minute of contact while the slow stage represents subsequent membrane rupture. The results suggest the majority of the cells (≥ 88%) exposed to chlorine were compromised during the fast stage at a much faster rate than the other chemicals tested. Chlorine reaction rates could only be calculated for the slow stage. The aim of calculating the reaction rates for chlorine using first order kinetics was to show that it had a greater impact on cell integrity than the other chemicals tested. Therefore, a first order model used to estimate the cell lysis rates for all the chemical treatments is shown in the following equation (Equation 1):

$$N_t/N_0 = e^{-k_{lysis}t} \qquad \text{Equation 1}$$

Where t = the contact time; N_t = the number of intact cells after a given treatment; N_0 = the number of intact cells at t = 0; and k_{lysis} = the rate at which lysis occurs.

The lysis rates of $CuSO_4$ with *M. aeruginosa* cells were in the range of 0.005-0.135 h^{-1}. The reaction rates of H_2O_2 with the membrane are in a narrower range and slower than copper treatment (0.024-0.055 h^{-1}). The rates of $KMnO_4$ are from 0.002 to 0.829 h^{-1} (the compromising ability increased with the growing initial $KMnO_4$ concentration) (Table 2.2). For ozone and the first stage of chlorine oxidation, there were inadequate data points to calculate the rates due to rapid reaction. Ozone was relatively fast in damaging membrane (≥ 50% of the cyanobacterial cells were compromised) in 5 minutes. Chlorine had the strongest ability to impair the cell integrity with a majority proportion (≥ 88%) of the cells compromised within the first minute and with the rates ranging of 0.640-3.82 h^{-1} in the slow stage. The rate of cell lysis was concentration dependent and there were significant differences (ANOVA, $p < 0.05$) between all concentrations for each of the oxidants/algicides The rates for 0.5 mg L^{-1} copper, 10.2 mg L^{-1} H_2O_2, and 3 mg L^{-1} $KMnO_4$ are 0.005, 0.039,

0.007 h^{-1}, respectively (ANOVA, $p < 0.05$). 2 mg L^{-1} ozone induced 50% cell lysis and 3 mg L^{-1} chlorine caused more than 90% cell lysis in the first min and a rate of 3.82 h^{-1} in the following time.

Table 2.1 The concentrations of chlorine, $KMnO_4$, H_2O_2 and ozone during the oxidation with the *Microcystis aeruginosa* cultures.

time (min)	chlorine concentration ($mg\ L^{-1}$)			time (hour)	$KMnO_4$ concentration ($mg\ L^{-1}$)					time (day)	H_2O_2 concentration ($mg\ L^{-1}$)				time (min)	ozone concentration ($mg\ L^{-1}$)		
0	5	4	3	0	10.0[a]	10.0	5.0	3.0	1.0	0	102[a]	102	51	10.2	0	2	4	6
1	3.3	2.0	1.3	0.25	9.9[a]	9.4	4.3	2.5	0.6	1	101.6[a]	98.5	49.3	7.3	0.5	0.0	0.2	0.4
2	3.0	1.6	1.1	0.5	9.8[a]	9.2	4.2	2.4	0.4	2	99.7[a]	96.2	46.0	6.4	1	0.0	0.2	0.3
5	2	1.2	0.6	1	9.7[a]	8.5	3.9	2.2	0.2	3	97.9[a]	92.9	41.5	4.2	2	0.0	0.1	0.2
10	1.8	0.7	0.4	2	9.7[a]	8.0	3.4	1.8	0.0	4	97.8[a]	88.5	38.8	1.3	3	0.0	0.1	0.1
20	1.4	0.4	0.2	3	9.7[a]	7.5	3.2	1.6		5	97.1[a]	85.6	34.2	1.1	4	0.0	0.0	0.1
30	1.0	0.1	0.0	4	9.6[a]	6.8	2.9	1.4		8	96.0[a]	83.0	30.8	0.0	5	0.0	0.0	0.1
60	0.2	0.0	0.0	5	9.6[a]	6.0	2.7	1.3										
				6	9.6[a]	4.6	2.4	1.1										
				7	9.6[a]	3.8	2.2	1.0										

[a] The concentrations of oxidants in cell-free ASM-1 media.

2.4 Discussion

All of the oxidants and algicidal agents trialled had a detrimental impact on cyanobacterial cell integrity. $CuSO_4$ is a potent algicide (Chorus and Bartram, 1999) and even the lowest $CuSO_4$ concentration of 0.5 mg L^{-1} led to lysis in 33 % of the population after 3 days copper treatment (Figure 2.3a). This is in agreement with the study by Kenefick et al., (1993) which demonstrated that 0.5 mg L^{-1} copper resulted in loss of membrane integrity in *M. aeruginosa* cells. Of the compounds developed as alternatives to copper based algicides, H_2O_2 shows considerable promise as it can produce hydroxyl radicals which cause damage to cyanobacterial cells by the oxidation of proteins, lipids and DNA (Latifi et al., 2009). It has been reported that cyanobacteria appear more sensitive to H_2O_2 than other phytoplankton species under laboratory and waste water situations (Drabkova et al., 2007b; Barrington and Ghadouani, 2008) and in a whole lake trial in the Netherlands (Matthijs et al., 2012). H_2O_2 could induce cell lysis (Figure 6a; Mikula et al., 2012; Hobson et al., 2012) and the lysis rate is faster at the recommend dose of 10.2 mg L^{-1} than 0.5 mg L^{-1} $CuSO_4$.

It is evident that chlorination has a strong action on membrane disruption (Figure 2.4a; Daly et al., 2007). The reaction of chlorine with cyanobacterial culture in ASM-1 media had decomposition kinetics similar to studies conducted in reservoir water (Daly et al., 2007; Zamyadi et al., 2010). The cell lysis rates of chlorine were much faster than all other treatments (even in the slow reaction stage with the rate range of 0.640-3.82 h^{-1}). The action of $KMnO_4$ at concentrations $\leq$ 3 mg L^{-1} did not compromise the membrane integrity but the higher concentrations (5 and 10 mg L^{-1}) induced significant lysis of cyanobacterial cells. Although this study has shown that exposure to either chlorine and $KMnO_4$ can lead to cell lysis and some literature reported that chlorine and $KMnO_4$ have been used for the control of algae as well (Fitzgerald, 1964; Kemp et al., 1966; Holden, 1970), these two oxidants have not been widely applied as algicides in reservoirs even though the application of these two chemicals is similar to $CuSO_4$ by spraying a solution or by dragging sacks (Holden, 1970). As a powerful oxidant, ozone induced significant lysis of cyanobacterial cells after a 5 min contact time, which is comparable to other research (Chen and Yeh, 2006; Chen et al., 2009). However, this study suggests ozone has a weaker action than chlorine on cell membranes. Chlorine (3, 4 and 5 mg L^{-1}) resulted greater than 95% cell lysis in 5 minutes while 90% lysis was detected even with the highest ozone dose (6 mg L^{-1}) after 5 minutes. It is possible that

rapid consumption of ozone by dissolved organic compounds left lower residual oxidant available to lyse cells more rapidly. Although ozone shows promise as an alternative to copper based algicide, its application in reservoirs is limited due to the inflexibility of operation.

Table 2.2 The cell lysis rates for algicides/oxidants in *Microcystis aeruginosa* cultures.

$CuSO_4$		H_2O_2		Chlorine		$KMnO_4$	
$[CuSO4]_0$	k_{lysis} (h^{-1})	$[H_2O_2]_0$	k_{lysis} (h^{-1})	$[Chlorine]_0$	k_{lysis} (h^{-1})	$[KMnO_4]_0$	k_{lysis} (h^{-1})
0.5	0.005	10.2	0.039	3	3.82	1	0.002
1.0	0.055	51	0.024	4	3.80	3	0.007
1.5	0.135	102	0.055	5	0.640	5	0.038
						10	0.829

Considered as potential algicides, the oxidants assessed in this study (chlorine, $KMnO_4$, H_2O_2, and ozone) also employed to treat other issues in the drinking water system (e.g. chlorine is a popular disinfectant). The standard oxidation potential of chlorine, $KMnO_4$, H_2O_2 and ozone under acidic conditions are 1.4, 1.7, 1.8 and 2.1 V, respectively, but this varies with pH. The effectiveness of H_2O_2 oxidation can be enhanced with UV light; however, the wavelength of the cool fluorescent light in this study is in the range of 400-700 nm which determined as visible light. UV-H_2O_2 process has been introduced as advanced oxidation processes to improve water purification (Zhou and Smith, 2001). The decomposition of H_2O_2 was relatively slow in this study (Table 2.1), which may be a function of having no UV light during the incubation period. Mikula et al. (2012) also found a residual of 29 mg L^{-1} remained after 3 days H_2O_2 exposure to *M. aeruginosa* cells exposed at initial dose of 40 mg L^{-1} in darkness. However, H_2O_2 should react more quickly in the reservoir because natural light comprises a wide spectrum of wavelengths including UV, at least in the surface (Brookes et al., 2004; Hipsey et al., 2004). Chlorine, $KMnO_4$ and ozone are better suited to

pre-treatment or applied as oxidants or disinfectants in WTPs. Since chlorine is low-cost, effective and easy to apply, it still may be used for conditioning water in the presence of cyanobacteria if concentrations are sufficiently high and contact times sufficiently long to lyse cells and oxidise exuded toxins (Daly et al., 2007). It is apparent that $KMnO_4$ ($\leq$ 3 mg L^{-1}) may also be used for pre-oxidation in the presence of cyanobacteria without impacting cell membranes which is a benefit over other oxidants. The reaction of ozone is highly pH-dependent. Ozone can react as direct molecular ozone and indirect hydroxyl free radicals (·OH). Under alkaline conditions ozone reacts with OH^- to generate ·OH. A recent study indicated that ·OH had larger impacts on cyanobacterial cells due to greater loss of cell integrity at higher pH (8.0) compared with lower pH (6.0) (Coral et al., 2013). Furthermore, Li et al. (2011) also demonstrated the effect of inactivation of *M. aeruginosa* followed the trend: pH 9.0 > pH 11.0 > pH 7.0 > pH 3.0. The greater production of HO_2^- could scavenge ·OH, which provides evidence for the higher inactivation efficiency at pH 0.9 compared with pH 11.0. Ozone has been regarded as the most consistently efficient oxidant for destruction of microcystins, nodularin and anatoxin-a (Himberg et al,, 1989; Rositano and Nicholson, 1994).

This study demonstrated that $CuSO_4$ can prevent the growth of cyanobacteria as an algistat as well as an algicide (Figure 2.3b), which agrees with previous studies (Padovesi-Fonseca and Philomeno, 2004; Hobson et al., 2012). H_2O_2 could also be a potential algistat to control the growth of cyanobacteria (Figure 2.6b). Although the densities of cyanobacterial cells exposed to H_2O_2 did not decrease as obviously as $CuSO_4$ treatment, population growth was stalled and the cells were getting smaller and collapsed after H_2O_2 treatment. A similar finding was documented by Hobson et al. (2012). The reactions of chlorine, $KMnO_4$ and ozone with cyanobacteria culture were much faster than $CuSO_4$ and H_2O_2, cell densities did not change obviously and it is unclear whether these oxidants could be potential algistats or not. Although it was reported by Miao and Tao (2009) that ozonation could control the growth of cyanobacteria, there is limited information to draw conclusion on the algistatic potential of ozone, chlorine and $KMnO_4$.

An increase in the percentage of intact cells was observed using a copper concentration of 1.0 mg L^{-1} on day 7 based on SYTOX Green fluorescence (Figure 2.3a) which coincided with a decrease in the cell densities. This may be attributed to the DNA changing after long-term copper addition to the cyanobacterial cells since SYTOX Green exhibits fluorescence upon

binding DNA. Similar results were observed in the H_2O_2 experiments, especially for the highest concentration of H_2O_2 (102 mg L^{-1}) (Figure 2.6a) which due to its stronger oxidation ability may cause more severe damage of DNA. The chlorophyll *a* fluorescence data (data no shown) collected from flow cytometry showed the fluorescence dropped from day 1 for both $CuSO_4$ and H_2O_2 treatments, and the chlorophyll *a* fluorescence did not increase from day 3 to 7, suggesting there was no regeneration of the *Microcystis* cultures. These results suggested that concerns should be taken when SYTOX Green is applied to assess the cell viability after long time algicide treatment.

From a practical perspective, the application of $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ or ozone as algicides/algistats should be applied at the early stages of cyanobacterial blooms when cell densities are low to decrease the release of intracellular metabolites. If there are high concentrations of healthy cyanobacterial cells in WTPs, it may be judicious to cease pre-oxidation and remove the intact cells with physical treatments. Furthermore, each of the algicides/oxidants has drawbacks that limit their application in reservoirs or WTPs. The major difficulty with the use of $CuSO_4$ is that it is a heavy metal that accumulates in sediment making it unsuitable for use in natural lakes because of its persistence and indiscriminate toxicity. Literature also indicated that copper could cause fish kills, although it is not apparent whether this is caused by copper toxicity or oxygen depletion (Hanson and Stefan, 1984). Furthermore, it may be inefficient in the long-term eliminating cyanobacterial blooms because of the development of copper-resistant cyanobacterial species/strains (Shavyrina et al., 2001; Garcia-Villada et al., 2004). In addition, copper reacts with dissolved organic carbon and so is not toxic to cyanobacteria for a short period of time (Burch et al., 2002) and its removal during coagulation and flocculation renders the alum sludge toxic making disposal more costly.

The most notable disadvantage with the use of chorine is that chlorine can react with organics forming by-products toxic to mammals (Svrcek and Smith, 2004) including carcinogenic trihalomethanes (THMs) (El-Shafy and Grunwald, 2000). Similarly, ozonation can lead to bromate formation which is a potential carcinogen (Bouland et al, 2004). Moreover, the cost of using ozone is relatively high and it has inherent complexity in operation both in reservoirs and WTPs. In contrast, little is known about the possibility of hazardous by-products caused by $KMnO_4$ whilst over dosing must be avoided since a residual $KMnO_4$ of 0.05 mg L^{-1} or

greater will result in a pink taint to the drinking water (Welch, 1963). H_2O_2 may not have few chemical issues while care must be taken using high concentrations of H_2O_2 since it is a strong oxidant and difficult to handle in its pure form.

2.5 Conclusions

Each of the water treatment technologies evaluated ($CuSO_4$, chlorine, $KMnO_4$, H_2O_2 and ozone) could impair cell integrity of *M. aeruginosa*. It is apparent that chlorine has a high oxidation rate and rapidly leads to lysis of most of the population. Ozone showed similarly high rates of cell lysis within a rapid time frame. $CuSO_4$ and H_2O_2 also showed algistatic potential for *M. aeruginosa* control over the 7 day treatment. The application of these technologies depends on the different situations in reservoirs or WTPs and how the benefits and disadvantages of each fits within the risk profile the water utility is willing to accept. The benefits and negative impacts of these processes discussed in this study may inform these decisions and guide future discussion on cyanobacterial control.

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Chapter 3. The effects of various control and water treatment processes on the membrane integrity and toxin fate of cyanobacteria

Abstract

Cyanobacterial blooms are one of the main contaminants that can degrade drinking water quality with the associated taste, odour and toxic compounds. Although a wide range of techniques have shown promise for cyanobacterial bloom control and cyanobacterial cell/metabolite removal in reservoirs and water treatment plants (WTPs), these treatments may have negative consequences through release of intracellular metabolites into the surrounding water. This study assessed the impact of copper sulphate ($CuSO_4$), chlorine, potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2) and ozone on *Microcystis aeruginosa* culture and the toxins it produced. All of these agents induced the loss of cyanobacterial membrane integrity. However, no associated increase in dissolved toxins was detected during chlorine and H_2O_2 treatments which may be due to faster toxin oxidation rates than release rates. $KMnO_4$ doses of 1 and 3 mg L^{-1} degraded dissolved toxins while having no impact on cyanobacterial membrane integrity. In contrast, ozone induced a significant increase in extracellular toxins but it was unable to degrade these toxins to the same degree as the other oxidants which may due to the lack of residual. All chemicals, except $CuSO_4$, were able to reduce cyanotoxins and chlorine was the most effective with a rate up to 2161 $M^{-1}s^{-1}$.

3.1 Introduction

Cyanobacteria blooms continue to present aesthetic and water quality challenges for drinking water authorities (Brookes and Carey, 2011) by increasing turbidity and producing taste and odour compounds. Furthermore, the occurrence of cyanotoxins (e.g. microcystins, cylindrospermopsin and saxitoxins) produced by many bloom-forming cyanobacteria species

are not only harmful to fish and livestock, but also to humans (Carmichael, 1992; Codd et al., 1999).

Microcystins (MCs) are the most frequently occurring hepatotoxins (liver toxins) produced by a diverse range of cyanobacteria including *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*), *Nostoc*, *Hapalosiphon* and *Anabaenopsis* species (Chorus and Bartram, 1999). Severe issues caused by MCs have been well documented, for example, a lethal outbreak attributed to the occurrence of MCs in the water used for dialysis induced 50 deaths of haemodialysis patients in Brazil (Jochimsen et al., 1998). MCs are monocyclic heptapeptides that contain seven amino acids and structure variations have been reported in all of them. Over 75 variants of MCs have been characterised from cyanobacteria with microcystin-leucine arginine (MC-LR) emerging as the most commonly occurring and toxic variant of the MCs family (Chorus and Bartram, 1999; Svrcek and Smith, 2004). The water quality guideline value for MC-LR recommended by the World Health Organization (WHO) and Australian Drinking Water Guidelines is 1.0 and 1.3 $\mu g\ L^{-1}$, respectively.

MCs are considered relatively stable compounds possibly because of their cyclic structure (Lawton and Robertson, 1999). They can remain potent even after boiling and may persevere for many years when stored dry at room temperature. MCs undergo slow photochemical breakdown and isomerisation in full sunlight (Tsuji et al., 1994). In spite of their chemical stability, MCs are biodegradable by aquatic bacteria (Jones et al., 1994; Ho et al., 2012). MCs usually show persistence and stability in intact cyanobacterial cells but typically degrade upon release. However, the concentration of extracellular MCs could be much higher in ageing blooms or enhanced by chemical treatments (e.g. algicides) which can liberate the intracellular toxins but inhibit the activity of degrading organisms.

Although conventional water treatment processes (coagulation, flocculation, sedimentation and filtration) in drinking water treatment plants (WTPs) have been recognized as effective technologies to remove intracellular toxins with intact cells (Chow et al., 1998; 1999), recent studies have observed breakthrough and accumulation of cyanobacterial cells and associated toxins in these processes (Zamyadi et al., 2012). Further these technologies are ineffective at removing dissolved metabolites (Rositano and Nicholson, 1994; Chow et al., 1998). Up-stream treatments like algicides can be applied in reservoirs to control cyanobacterial population and prevent both off-flavours and toxins associated with extended blooms. These

treatments are likely to remain in use as climate and nutrient inputs continue to favour cyanobacterial populations (Carey et al., 2011). Copper sulphate ($CuSO_4$) is regarded as an effective algicide (Elder and Horne, 1978, McKnight et al., 1983) and hydrogen peroxide (H_2O_2) is emerging as a potential, environmentally friendly algicide (Drabkova et al., 2007). Another popular process is pre-treatment ahead of conventional treatment processes with chlorine, potassium permanganate ($KMnO_4$) or ozone which could enhance the overall removal of cyanobacterial cells followed by coagulation and flocculation (Chen and Yeh, 2006; Chen et al., 2009; Ma et al., 2012).

Although the control and water treatment options mentioned above have performed well in cyanobacteria control/removal, concerns are raised because some of them have the unwanted consequence of releasing intracellular metabolites into the surrounding water. For instance, chlorination of water prior to conventional treatment can result in cell lysis and concomitant toxin release (Peterson et al., 1995; Daly et al., 2007). However, algicides and pre-treatment may be necessary in specific circumstances. Consequently, algicides could be applied at the early stages of a cyanobacterial bloom to reduce the potential for release of intracellular metabolites. Optimizing contact time and chlorine concentrations could reduce the negative impacts of chlorine pre-oxidation (Daly et al., 2007). Therefore, it is very important to consider the effects of treatments on the membrane integrity of cyanobacteria cells and cyanotoxin fate during algicide/oxidant treatment.

To date, minimal studies have evaluated various control and water treatment processes on cyanobacterial cell integrity and toxin degradation kinetics. A previous study which systematically evaluated the impact of $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone on cyanobacterial membrane integrity revealed that all these treatments could induce loss of cell integrity (Fan et al., 2013b). The aim of the current study was to investigate whether treatment of *Microcystis aeruginosa* cultures with these chemicals could increase dissolved MCs which could impair water quality and treatment processes in downstream water supply.

3.2 Materials and Methods

3.2.1 Materials and reagents

A toxic strain of *Microcystis aeruginosa* Kutz. emend Elenkin which has a toxin profile >90% MC-LR plus < 10% MC-LA (from the Australian Water Quality Centre Culture Collection strain 338) was cultured in ASM-1 medium (Gorham et al., 1964) and routinely subcultured to maintain growth in logarithmic phase. This culture remained unicellular or as double cells. Cultures were incubated under constant cool-fluorescent light intensity 50 μmol m^{-2} s^{-1} (measured with a scalar quantum sensor, LI-COR LI-190) on a 12h:12h light-dark cycle at a constant temperature of 20 ± 1 ℃, and bubbled with air. The initial cell density of 7.0×10^5 cells mL^{-1} and pH 7.5 ± 0.1 were adjusted prior to chemical treatment. The pH adjustment was achieved using 0.1M sterile filtered hydrochloric acid or sodium hydroxide.

3.2.2 Experimental methods

All experiments were performed at room temperature 20 ± 2 ℃. All chemicals and reagents used were analytical grade and solutions were made using ultra-pure water (Millipore Pty Ltd, USA). *M. aeruginosa* cells were exposed to the algicides or oxidants at varying doses in time course experiments. Specific amounts of stock solutions were spiked into the prepared cultures to achieve the desired initial chemical doses (0, 0.5, 1.0 and 1.5 mg L^{-1} $CuSO_4$; 0, 10.2, 51 and 102 mg L^{-1} H_2O_2; 3, 4 and 5 mg L^{-1} chlorine; 1, 3, 5 and 10 mg L^{-1} $KMnO_4$; 2, 4 and 6 mg L^{-1} ozone) followed by mixing. Details on chemical solution preparation are provided in Fan et al. (2013b). For oxidant decay experiments, samples were withdrawn at pre-determined intervals, and immediately analysed to determine oxidant residual. Analyses methodologies for the concentrations of each chemical are provided in Fan et al. (2013b). Each treatment experiment was conducted in triplicate, and samples were taken regularly at specific reaction times to determine incidence of cell lysis and toxin concentration. The residual oxidants were quenched with sodium thiosulfate addition (for H_2O_2, chlorine and $KMnO_4$) and high purity nitrogen (for ozone) to arrest further oxidation of cyanobacterial cells and toxins. Cell integrity was evaluated immediately using flow cytometry and the fluorescent probe SYTOX Green (Regel et al., 2004; Daly et al., 2007; Fan et al., 2013b).

High-performance liquid chromatography (HPLC) was used to determine the concentrations of intracellular and total MCs (Ho et al., 2006). Methods for pre-extraction are detailed in Fan et al. (2013a) and toxin extraction procedures are provided in Nicholson et al. (1994).

3.2.3 Modelling

A model was developed to integrate the rates of cell lysis, toxin release and toxin degradation and used to predict toxin concentration under different oxidant exposure scenarios (CT). CT was calculated by integrating the residual curve for each oxidant. For comparison, it was assumed that the copper residual remained the same as the initial copper concentration throughout the exposure period when calculating CT of $CuSO_4$. Ozone is a strong oxidant reacting with cyanobacterial cells very quickly and significant toxin release was evident in 5 min. Due to the rapid rate of reaction it was difficult to collect data that was suitable for developing a model.

The relationship between contact time, cell inactivation (by inference toxin release) and toxin degradation can be described as a first order processes. If these assumptions are valid then the process of toxin release and degradation may be considered as consecutive reactions (Equation 1), where the concentration of intracellular (A) and extracellular (B) toxin are described by Equations 2 and 3 (Jones, 1970; Fan et al., 2013a; Zamyadi et al., 2013). Since $CuSO_4$ is not an oxidant, there was no degradation of toxin and so only Equation 2 was used to develop the model to describe the kinetics of intracellular toxin release.

$$A \overset{k_i}{\rightarrow} B \overset{k_e}{\rightarrow} D \qquad \text{(Equation 1)}$$

$$A = A_0 e^{-k_i CT} \qquad \text{(Equation 2)}$$

$$B = B_0\, e^{-k_e CT} + A_0 (e^{-k_e CT} - e^{-k_i CT})/(1-k_e/k_i) \quad \text{(Equation 3)}$$

Where CT = chemical exposure; A_0 = the initial intracellular MCs concentration; A = the concentration of intracellular MCs at a specified CT; B_0 = the initial dissolved MCs concentration; B = the concentration of dissolved MCs at a specified CT; k_i = the rate of intracellular MCs release; k_e = the rate of extracellular MCs degradation. D = the concentration of degraded MCs at specified CT. Model parameters were estimated by fitting the Equation 2 and 3 to experimental data using the Solver add-in for Microsoft Excel and a least squares objective function based on the intracellular and extracellular toxin concentrations. Nash–Sutcliffe model efficiency coefficient was calculated as a performance metric for the model (Mayer and Butler, 1993) and describes the amount of the variance in the data that is explained by the model (Fan et al., 2013a).

3.3 Results

3.3.1 Copper sulphate

The lowest copper dose (0.5 mg L^{-1}) caused lysis in 33% of the *M. aeruginosa* population after 3 days treatment and cells were increasingly compromised as the initial copper doses increased. For the highest copper concentration (1.5 mg L^{-1}), almost all the cells lost membrane integrity after 1 day of exposure (Appendix A). The rupture of cell integrity was also evident by the increase of extracellular MCs observed during copper treatment. The initial *M. aeruginosa* culture contained 63 µg L^{-1} MCs with the majority proportion (76%) determined to be intracellular. Reduction of the intracellular MCs was observed on day 1 with complete release of toxin by day 3 resulting in 63 and 60 µg L^{-1} of extracellular MCs after the 1.0 and 1.5 mg L^{-1} copper treatment, respectively (Figure 3.1). The MC concentrations (intracellular and extracellular) remained relatively stable during the 0.5 mg L^{-1} copper treatment. For the control samples, the concentration of total MCs increased to 134 µg L^{-1} with 75% of the toxin shown to be intracellular on day 7.

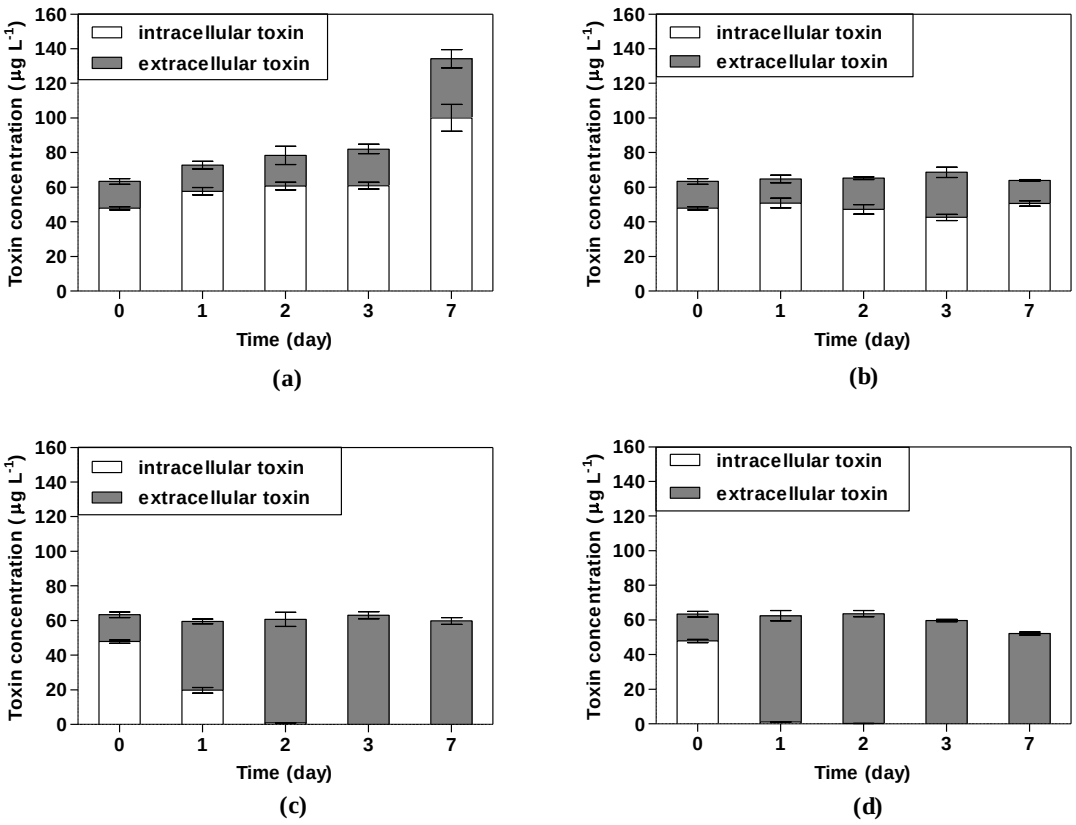


Figure 3.1 Intracellular and extracellular MCs concentrations after treatment with $CuSO_4$ (up to 7 days contact time) at concentrations of (a) 0, (b) 0.5, (c) 1.0 and (d) 1.5 mg L^{-1}. Error bars represent standard deviation from triplicate analyses.

3.3.2 Hydrogen peroxide

As an alternative algicide to $CuSO_4$, H_2O_2 showed promise in compromising membrane integrity of cyanobacterial cells. Approximately 16%, 31% and 7% of the cells maintained membrane integrity on day 2 with initial H_2O_2 doses of 10.2, 51 and 102 mg L^{-1}, respectively (Appendix A). The concentrations of intracellular and extracellular MCs before exposure to H_2O_2 were 36 and 10 µg L^{-1}, respectively. In the control samples MCs increased to a total of 90 µg L^{-1} by day 5 with 78% of the concentration shown to be intracellular (Figure 3.2). Consistent decreases of the intracellular MCs were observed when dosing H_2O_2 (10.2-102 mg L^{-1}) with removal to below analytical detection by day 5. However, there was no increase in dissolved MCs observed during the H_2O_2 treatments (10.2-102 mg L^{-1}); rather these toxins

were progressively degraded to below analytical detection during the course of the experiment. The degradation of total MCs increased with the growth of CT values. The MCs were oxidised to below analytical detection at CT values of 35700, 314000 and 676000 mg min L^{-1} for 10.2, 51 and 102 mg L^{-1} H_2O_2 treatment, respectively.

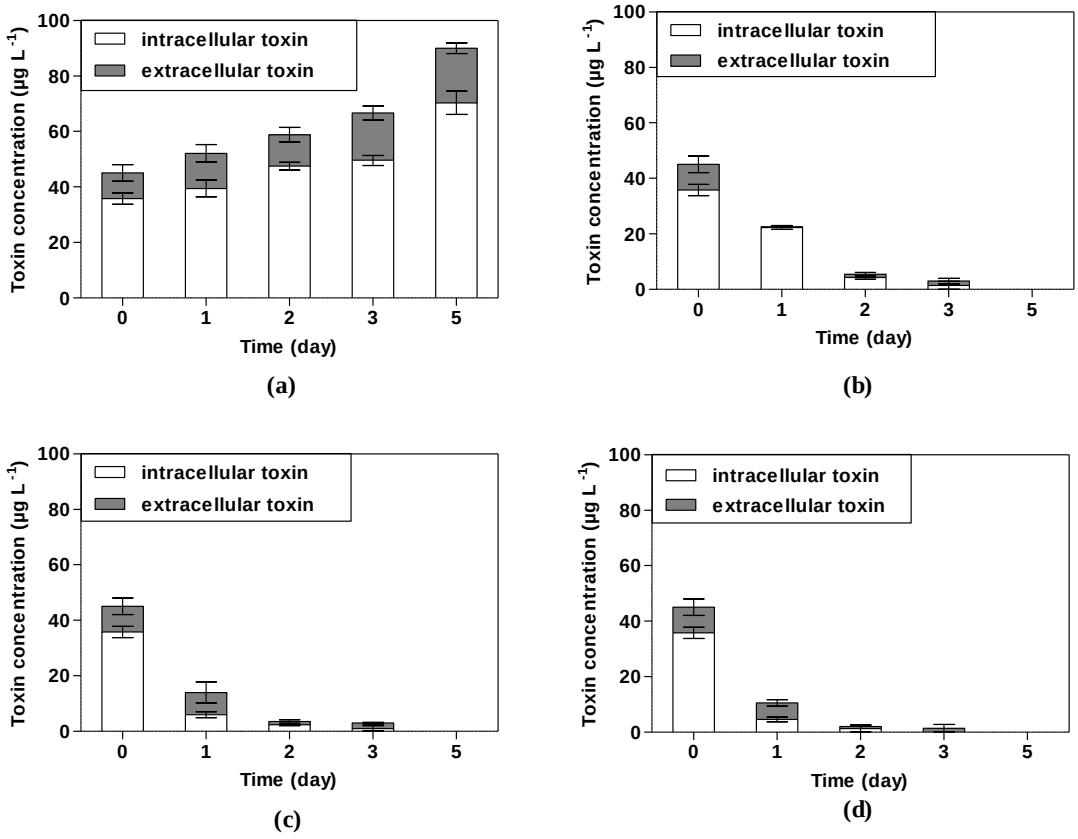


Figure 3.2 Intracellular and extracellular MCs concentrations after treatment with H_2O_2 (up to 5 days contact time) at concentrations of (a) 0, (b) 10.2, (c) 51 and (d) 102 mg L^{-1}. Error bars represent standard deviation from triplicate analyses.

3.3.3 Chlorine

More than 95% of the cyanobacterial cells lost membrane integrity within 5 min of contact with all chlorine doses (3-5 mg L^{-1}) (Appendix A). The intracellular MCs were released consistently and subsequently oxidised (Figure 3.3). However, the rate of toxin release is slower than the cell lysis rates in a similar study by Fan et al. (2013b). Similar to the H_2O_2

treatments, the extracellular MCs were oxidised to below the initial concentration (10.8 µg L^{-1}) during the treatment with chlorine doses of 3, 4 and 5 mg L^{-1}. The total toxin (58 µg L^{-1}) was oxidised to below analytical detection by 5 mg L^{-1} chlorine while there was 5 and 4 µg L^{-1} of MCs remaining when applying 3 and 4 mg L^{-1} chlorine respectively, after 60 min (Figure 3.3). For all chlorine doses (3-5 mg L^{-1}), the degradation of total MCs occurred within CT values from 0 to 71 mg min L^{-1} and progressed with increasing CT values. The lowest chlorine dose (3 mg L^{-1}) oxidised 90% of the total MCs at a CT of 13 mg min L^{-1}.

3.3.4 Potassium permanganate

$KMnO_4$ doses of 5 and 10 mg L^{-1} caused loss in membrane integrity to 26% and 100% of cells, respectively within 6 hours. The proportion of intact cells remained constant for 1 and 3 mg L^{-1} $KMnO_4$ treatments (Appendix A) with no considerable increase of extracellular MCs even though the intracellular MCs decreased consistently (Figure 3.4). Although total MCs decreased with time for all cultures (initially 36 and 8 µg L^{-1} of intracellular and extracellular MCs, respectively) when treated with $KMnO_4$ between 1 and 10 mg L^{-1}, there was up to an 86% and 120% increase of extracellular MCs due to the leakage of intracellular MCs following 5 and 10 mg L^{-1} $KMnO_4$ oxidation, respectively. Extracellular MCs were oxidised by $KMnO_4$ to a final concentration of 9 µg L^{-1} following the addition of 5 mg L^{-1} $KMnO_4$ and below analytical detection after adding 10 mg L^{-1} $KMnO_4$ after 7 hours. The toxin degradation progressed consistently with increasing CT values for all $KMnO_4$ doses (0-2892 mg min L^{-1}).

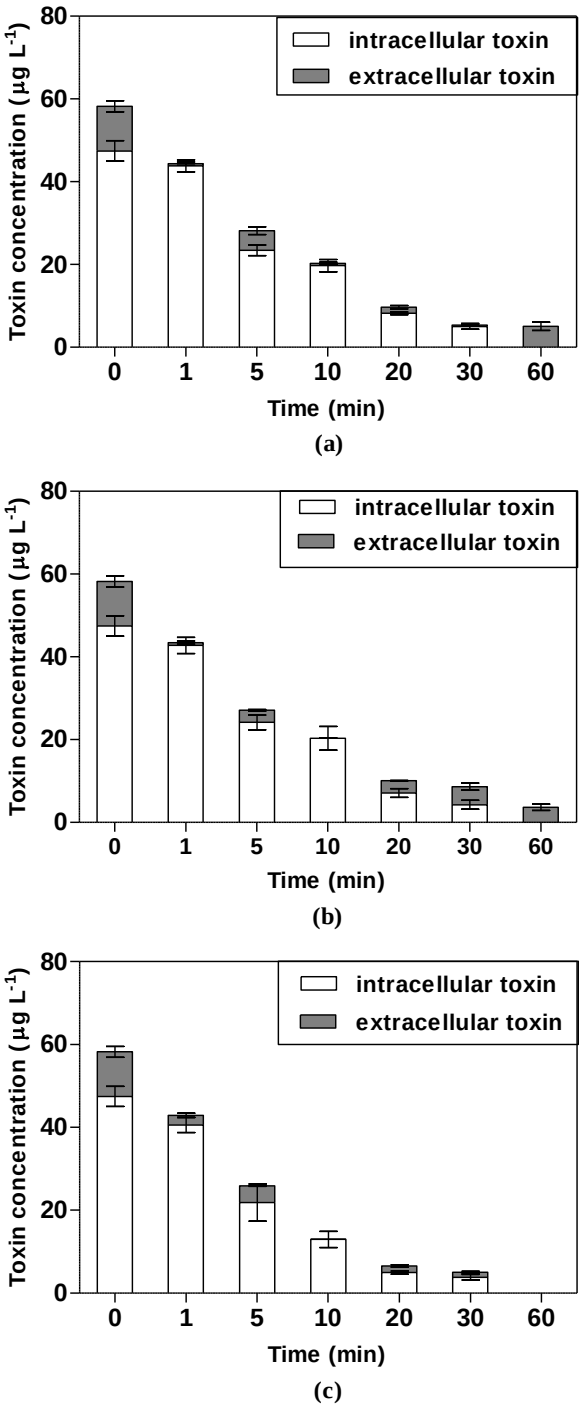


Figure 3.3 Intracellular and extracellular MCs concentrations after treatment with chlorine (up to 60 min contact time) at concentrations of (a) 3, (b) 4, and (c) 5 mg L^{-1}. Error bars represent standard deviation from triplicate analyses.

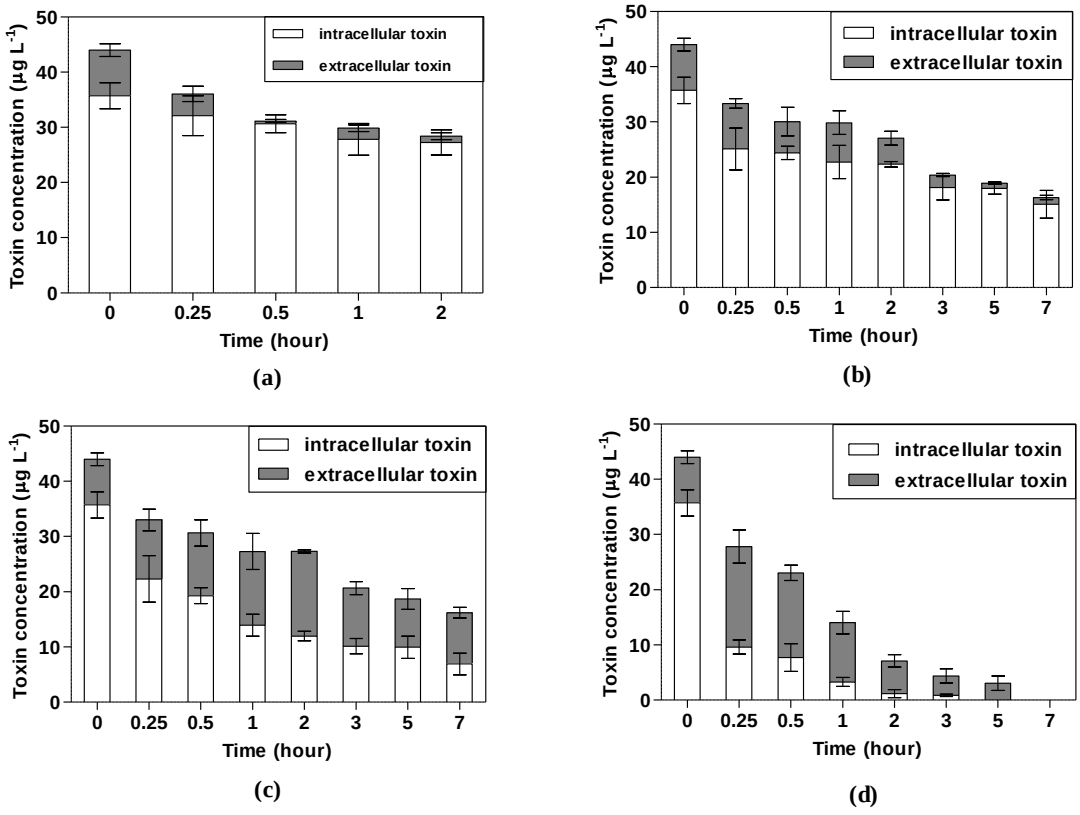


Figure 3.4 Intracellular and extracellular MCs concentrations after treatment with $KMnO_4$ (up to 7 hours contact time) at concentrations of (a) 1, (b) 3, (c) 5 and (d) 10 mg L^{-1} presented by Fan et al. (2013a). Error bars represent standard deviation from triplicate analyses.

3.3.5 Ozone

Ozone treatment (2, 4 and 6 mg L^{-1}) induced significant cell lysis which resulted in 50%, 70% and 90% of the cyanobacterial cells losing membrane integrity within 5 min of contact (Appendix A). The *M. aeruginosa* culture initially contained 44 µg L^{-1} of total MCs with 74% determined to be intracellular. Substantial release of intracellular MCs was observed after 5 min which resulted in a 110%, 140% and 110% increase in the concentrations of extracellular MCs after dosing with 2, 4 and 6 mg L^{-1} ozone respectively. This corresponded to reductions of 70%, 90% and 95% of the intracellular MCs (Figure 3.5). Ozone induced a decline of 23%, 30% and 42% of the total toxin within 5 min of contact.

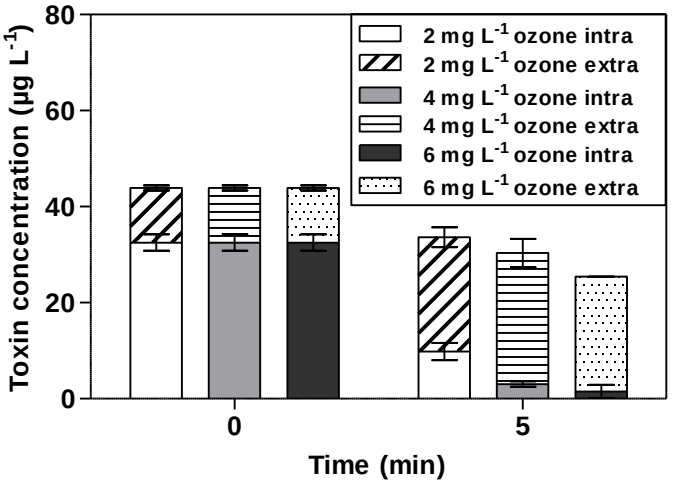


Figure 3.5 Intracellular and extracellular MCs concentrations after treatment with ozone (5 min contact time) at concentrations of 2, 4, and 6 mg L^{-1}. Error bars represent standard deviation from triplicate analyses.

3.3.6 Modelling

The kinetics of MC release and degradation was modeled by fitting the rate of intracellular MC release (k_i) and the rate of extracellular MC degradation (k_e) with CT using a first-order model (Figure 3.6; Figure 3.7; Figure 3.8; Figure 3.9). The kinetics of MC release and degradation varied between each of the chemicals and all were dose dependent. The fitted k_i values for the $CuSO_4$ doses of 0.5, 1.0 and 1.5 mg L^{-1} were 0.003, 0.808 and 1.85 $M^{-1}s^{-1}$, respectively. Values of k_i (0.038, 0.013 and 0.008 $M^{-1}s^{-1}$) and k_e (0.621, 0.021 and 0.013 $M^{-1}s^{-1}$) were obtained using 10.2, 51 and 102 mg L^{-1} H_2O_2, respectively. The toxin release rates for $KMnO_4$ ranged from 4.51 to 21.5 $M^{-1}s^{-1}$, while the fitted k_e was in the range of 6.59 to 412 $M^{-1}s^{-1}$. The kinetics of MCs was much faster for chlorine with fitted k_i ranging from 61.7 to 155 $M^{-1}s^{-1}$ and k_e between 864 to 2161 $M^{-1}s^{-1}$. The derived Nash–Sutcliffe efficiency coefficients verified the applicability of the model to describe the data (Table 3.1). For those experiments where the extracellular toxin did not increase (i.e. chlorine and H_2O_2), it is confirmed that the rate of toxin release is the rate limiting step. Under these conditions the fitted values for k_e have a high degree of uncertainty.

Table 3.1 The rate constants and evaluation of model performance.

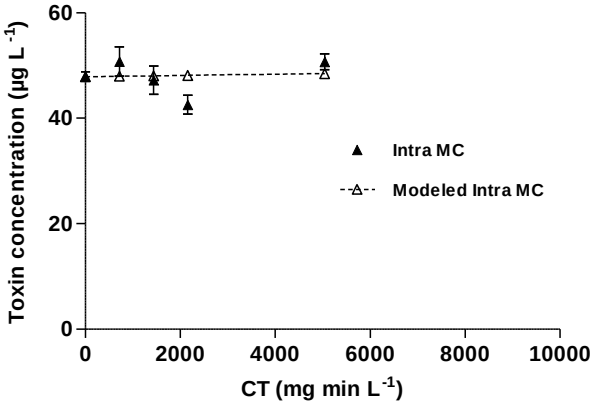
Chemical	$[Chemical]_0$ (mg L^{-1})	k_i ($M^{-1}s^{-1}$)	k_e ($M^{-1}s^{-1}$)	Nash–Sutcliffe Coefficient	
				Intra toxin	Extra toxin
$CuSO_4$	0.5	0.003		0.01	
	1.0	0.808		0.98	
	1.5	1.85		1.00	
H_2O_2	10.2	0.038	0.621*	0.92	0.96
	51	0.013	0.021*	1.00	0.96
	102	0.008	0.013*	1.00	0.97
Chlorine	3	155	2161*	0.92	0.59
	4	94.8	1502*	0.94	0.49
	5	61.7	864*	1.00	0.89
$KMnO_4$	1	21.5	412	0.99	0.90
	3	4.51	32.0	0.40	0.83
	5	6.05	6.59	0.63	0.09
	10	16.3	10.3	0.97	0.81

*These fitted values are only estimates as explained in the result section

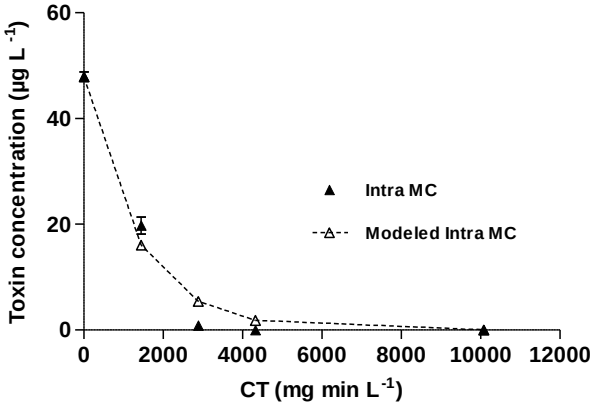
3.4 Discussion

$CuSO_4$ (1.0 and 1.5 mg L^{-1}) has been shown to be toxic to cyanobacteria inducing both a loss of membrane integrity and inhibition of the growth (Kenefick et al., 1993; Hobson et al., 2012; Fan et al., 2013b). However, in this study the 0.5 mg L^{-1} copper dose lysed less than 40% of the *M. aeruginosa* cells, the numbers of which did not increase. It is possible that while cells were stressed (and were unable to reproduce) during $CuSO_4$ treatment, they did not reach the end point of death (membrane loss) after such treatment. Alternatively, copper could complex with ethylene-diamine-tetra-acetic acid (EDTA) in ASM-1 media resulting in reduced algicidal activity against *M. aeruginosa* (Hobson et al., 2012).

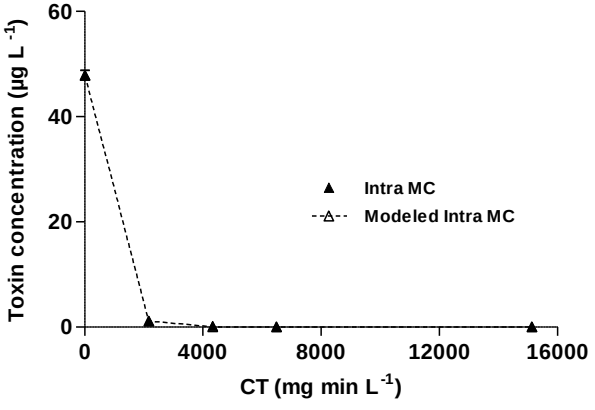
The total MC concentrations did not increase after copper treatments of 0.5-1.5 mg L^{-1} over the full 7 day period (Figure 3.1). This is most likely due to copper inhibiting the growth of cyanobacteria and preventing any further production of MCs (Hobson et al., 2012; Fan et al., 2013b). The observed increase in extracellular MC concentrations following treatment with $CuSO_4$ would be a concern for drinking water safety (Figure 3.1). It is generally understood that indigenous microbial flora would biodegrade extracellular cyanobacterial toxins (Ho et al., 2010; Smith et al., 2008); however, algicides can also be active against such MC-degrading bacteria (Smith et al., 2008). Kenefick et al. (1993) found a maximum MC-LR release from cyanobacteria in lake water after 2 to 4 days copper treatment and suggested approximately 3 weeks would be necessary for 99% removal of any resultant extracellular MC-LR. Jones and Orr (1994) showed that extracellular MC-LR increased rapidly and persisted at high levels (1300-1800 μg L^{-1}) for 9 days by biodegradation following copper treatment of a *M. aeruginosa* bloom in Lake Centenary (Australia). Therefore, the use of $CuSO_4$ as an algaecide when cyanobacterial numbers are high is not recommended unless an extended period can be guaranteed to allow biodegradation of the toxins in the treated water before consumption by humans or animals.



(a)



(b)



(c)

Figure 3.6 Comparison of experimental and model predicted results of MCs concentrations after treatment $CuSO_4$ at initial concentrations of (a) 0.5 mg L^{-1}, (b) 1.0 mg L^{-1} and (c) 1.5 mg L^{-1}.

The toxin release rates during H_2O_2 treatment was slower (0.008-0.038 $M^{-1}s^{-1}$) than $CuSO_4$ (0.003-1.85 $M^{-1}s^{-1}$). Considered as an alternative algicide, H_2O_2 did not only impair the membrane integrity of cyanobacterial cells (Appendix A), but it also effectively degraded MCs (k_e up to 0.621 $M^{-1}s^{-1}$) (Figure 3.7). While the use of H_2O_2 combined with ozone, UV radiation or iron salts (Fenton reagent) to remove MC-LR in water treatment processes is well documented (Rositano et al., 1998; Bandala et al., 2004; He et al., 2012), examples of H_2O_2 being used on its own are somewhat limited. Furthermore, previous studies have shown that H_2O_2 alone is relatively ineffective in decomposing purified MC-LR solutions (in the absence of cyanobacterial cells) (Rositano et al., 1998; Pyo and Yoo, 2008). This could be due to the shorter contact times between H_2O_2 and cyanobacteria (< 1 hour) used in these studies. Few studies have evaluated the dynamics of cyanotoxins in the presence of cyanobacterial cells using H_2O_2.

Although H_2O_2 may be considered as a potential chemical to control cyanobacteria and cyanotoxins, the reasonable contact time and dose in field application should be carefully assessed. Fan et al. (2013b) observed residual H_2O_2 was available after 5 days in cyanobacteria cultures for all H_2O_2 doses which suggested the action of H_2O_2 on cyanobacteria under laboratory condition without UV radiation was quite slow. The action of H_2O_2 would be faster and the effectiveness should be enhanced in reservoirs since the natural sunlight includes a wide spectrum of wavelengths including UV radiation. This study included relatively high doses of H_2O_2 (51 and 102 mg L^{-1}) which would be inappropriate for application in reservoirs as concentrations exceeding 2.5 mg L^{-1} H_2O_2 have been shown to harm zooplankton (Matthijs, et al., 2012). Barrington et al. (2011; 2013) indicated that the required dose of H_2O_2 with cyanobacteria could be reduced by an order of magnitude when UV radiation was applied. Therefore, a lower dose may be sufficient for cyanobacteria control and cyanotoxin degradation under natural light. Nevertheless, the UV radiation is usually high in the surface and attenuated with depth (Brookes et al., 2004). Therefore, in the case of a large cyanobacteria bloom, light penetration would be very limited, H_2O_2 would still be applied as an algicide and would also act to reduce toxin concentrations provided sufficient contact time occurs.

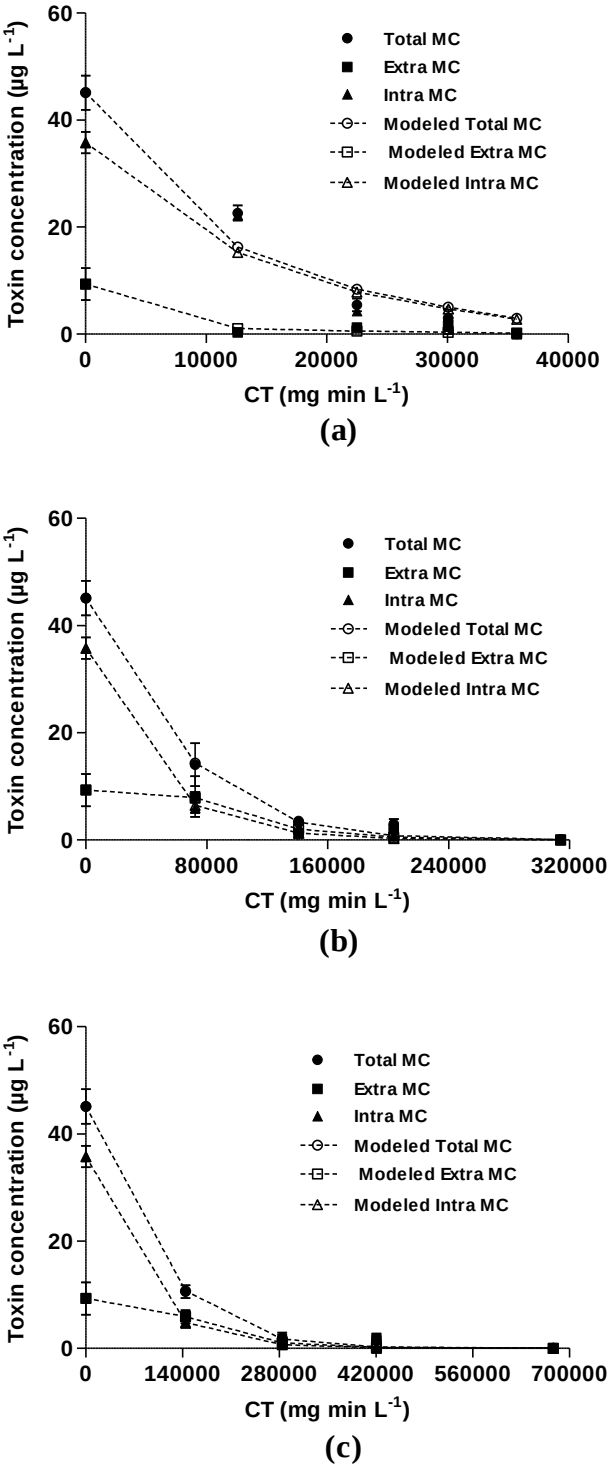
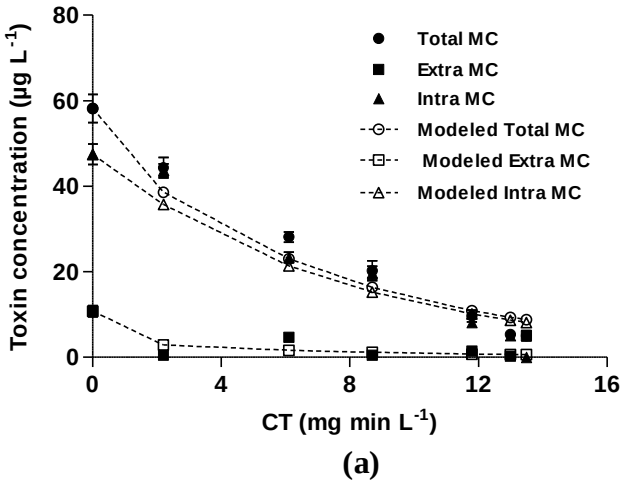
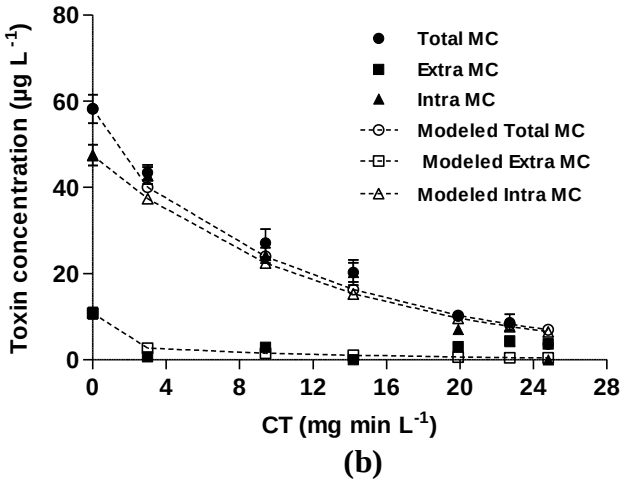


Figure 3.7 Comparison of experimental and model predicted results of MCs concentrations after treatment with H_2O_2 at initial concentrations of (a) 10.2 mg L^{-1}, (b) 51 mg L^{-1} and (c) 102 mg L^{-1}.



(a)



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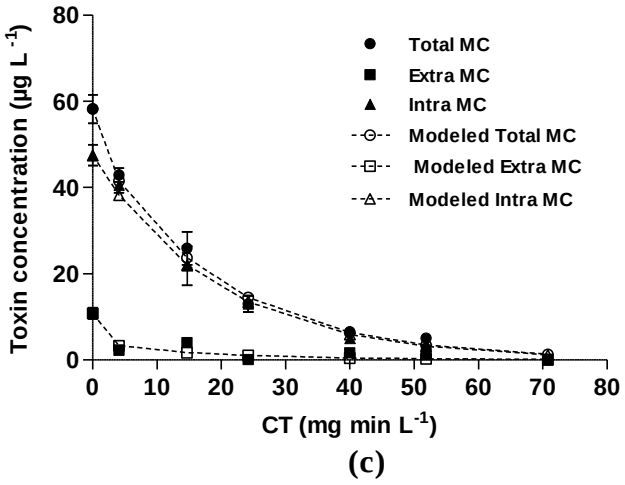

(c)

Figure 3.8 Comparison of experimental and model predicted results of MCs concentrations after treatment with chlorine at initial concentrations of (a) 3 mg L^{-1}, (b) 4 mg L^{-1} and (c) 5 mg L^{-1}.

Chlorine was shown to have the greatest impact in disrupting the membrane of the cyanobacterial cells in previous study (Fan et al., 2013b). The lowest dose (3 mg L^{-1}) was the most efficient dose and compromised over 95% of the cyanobacterial membranes with a CT of 2.2 mg min L^{-1} (Figure 3.8). This was considerably faster than previous studies (Daly et al., 2007; Zamyadi et al., 2013). This phenomenon could be attributed to the variation between cyanobacterial species, cell densities, water matrix and the parameters of chlorination which could influence on the cell rupture (Lin et al., 2009; Zamyadi et al., 2010).

Chlorine has not only been widely applied as an effective pre-treatment oxidant, but it has also been shown to be highly efficient in oxidising cyanobacterial toxins under various water quality conditions in the presence and absence of cyanobacterial cells (Acero et al., 2005; Ho et al., 2006; Zamyadi et al., 2013). The highest chlorine dose applied in this study (5 mg L^{-1}) degraded the total MC concentration to below analytical detection, which corresponded to a CT value of 71 mg min L^{-1} (Figure 3.8). This agrees with Ding et al. (2010) who documented that 60 mg min L^{-1} chlorine exposure removed > 90% of the MC-LR (without cells). No increase in dissolved MC concentration was observed over the exposure period and most MCs (both intracellular and extracellular) were oxidised after 30 min for 3-5 mg L^{-1} chlorine (CT value of 13-52 mg min L^{-1}) (Figure 3.3). Ding et al. (2010) reported that 1-2 mg L^{-1} chlorine (20-25 ℃, pH 7.5) inactivated all *M. aeruginosa* cells at a CT value of over 150 mg min L^{-1} and no significant build-up of dissolved MC-LR was observed during the treatment. However, other researchers have shown significant increases in dissolved toxins after chlorination of cyanobacterial cells though the dissolved toxins may subsequently oxidised when the residual chlorine and contact time was sufficient (Daly et al., 2007; Zamyadi et al., 2010; 2013). The toxin degradation rate constant for a 3 mg L^{-1} chlorine dose (Table 3.1) was 2161 $M^{-1}s^{-1}$ which is much higher than that reported by other researchers using spiked toxins in ultrapure and natural waters (Acero et al., 2005; Daly et al., 2007; Zamyadi et al., 2013). The oxidation of cyanobacterial toxins has been shown to be more efficient at lower pH (below 8) (Hall et al., 2000; Acero et al., 2005). The pH of cultures used in the current study was 7.5 and this could partly explain the larger rate constant compared to those presented by other researchers. In addition, the faster rate of cell lysis would also release more organic matters to the cultures and the increasing extracellular organic matters (EOM) may attribute more reactive toward MCs than chlorine alone (Ho et al., 2006). Therefore, this study indicated that chlorine was the most effective chemical for both *M. aeruginosa* inactivation

and MCs oxidation. Especially, the decrease of dissolved MCs suggested that chlorine is feasibly used as a pre-treatment oxidant even when cyanobacteria density is relatively high provided sufficient contact time to degrade release toxins.

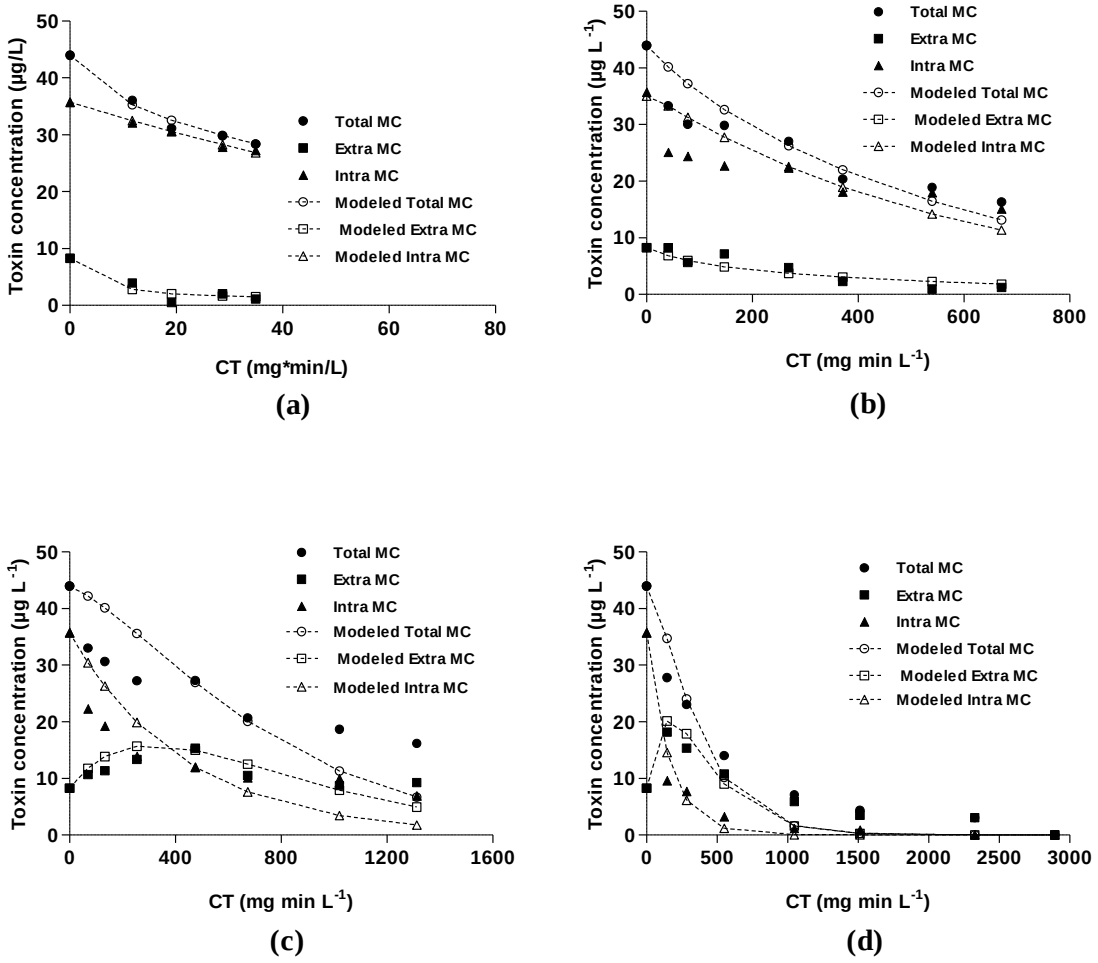

Figure 3.9 Comparison of experimental and model predicted results of MCs concentrations after treatment with $KMnO_4$ at initial concentrations of (a) 1 mg L^{-1}, (b) 3 mg L^{-1}, (c) 5 mg L^{-1} and (d) 10 mg L^{-1} presented by Fan et al. (2013a).

$KMnO_4$ is the only chemical tested in this study that had minimal impact on membrane integrity of *M. aeruginosa* cells using lower doses ($\leq$ 3 mg L^{-1} with CT ranging from 0 to 671 mg min L^{-1}). The decrease of both intracellular and extracellular MC concentrations at the lower doses was similar to those observed for H_2O_2 and chlorine (Figure 3.4). However, in

contrast to H_2O_2 and chlorine, $KMnO_4$ had minimal impact on membrane integrity (Appendix A) suggesting a different mode of activity. In contrast, work by Ding et al. (2010) indicated 1-2 mg L^{-1} $KMnO_4$ with CT values ranging from 100-200 mg min L^{-1} did not increase extracellular MC-LR but a 60-99% inactivation of *M. aeruginosa* cells was achieved. However, this study demonstrated the increase of extracellular MCs were consistent with the lysis of cyanobacterial cells; e.g. k_i (16.3 $M^{-1}s^{-1}$) was faster than k_e (10.3 $M^{-1}s^{-1}$) for 10 mg L^{-1} $KMnO_4$ with complete rupture of the cells in 6 hours. Overall, it may be feasible to use $KMnO_4$ as pre-treatment at the lower dose ($\leq$ 3 mg L^{-1}) which could also reduce dissolved cyanotoxin concentration with minimal disruption to cell integrity.

Ozone inactivated over 50% of the *M. aeruginosa* cells in 5 min of contact with lysis increasing with larger ozone doses (Appendix A). Although there were too few sampling intervals (quick reaction) to adequately model the toxin release and decay, considerable increase of extracellular MCs were observed with a corresponding reduction in intracellular MCs (Figure 3.5). Although ozone could degrade the total MCs (Figure 3.5), the oxidizing process was not as efficient as previous studies (Miao and Tao, 2009; Rositano et al., 1998; Hall et al., 2000; Onstad et al., 2007). For instance, Rositano et al. (1998) indicated that pure MC-LR (166 µg L^{-1}) was completely oxidized by less than 0.2 mg L^{-1} of ozone in 4 min. Hall et al. (2000) found that ozone was effective at removing pure MC-LR and MCs contained by *M. aeruginosa* with an ozone dose above 2 mg L^{-1} but the concentration of MC-LR was apparent lower than this study. The reduced efficiency observed in the current study could be attributed to differences in water quality parameters including alkalinity, dissolved organic carbon (DOC), pH and natural organic matters (NOM) character (Rositano et al., 1998; 2001). Miao and Tao (2009) showed ozone doses of between 1-5 mg L^{-1} increased the amount of DOC derived from cyanobacteria which could inhibit the oxidation of MCs through competitive and possibly preferential reactions with ozone. In addition, no ozone residual was observed after 5 min for all ozone doses applied in the current study (2-6 mg L^{-1}). This is particularly relevant since the presence of ozone residual has been documented to be essential for the effective destruction of cyanotoxins (Rositano et al., 1998; 2001).

The investigations in this study were laboratory based, and this should be taken into account when assessing the efficacy and risks of full-scale treatment in reservoirs or WTPs. Although H_2O_2, chlorine, $KMnO_4$ ($\leq$ 3 mg L^{-1}) decreased the dissolved toxins during the treatments,

whether there is an increase of other dissolved metabolites (e.g. taste and odour) are unsure due to the cell lysis. Thus, it is better to evaluate the kinetics of other cyanobacterial metabolites during chemical treatment before the application under real situations. In addition, each of the chemicals ($CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone) has advantages and disadvantages which need to be assessed against particular water conditions (Fan et al., 2013b).

3.5 Conclusions

The total concentration of MCs did not increase following treatment with $CuSO_4$ due to inhibition of cyanobacterial growth however there was considerable increase in the extracellular component. In contrast, cultures treated with H_2O_2 showed a decrease in extracellular MC concentration due to faster toxin oxidation rates compared to release rates. Similarly, chlorine resulted in decrease of extracellular MCs during the treatment and was shown the most effective oxidant to degrade MCs. $KMnO_4$ doses of 1 and 3 mg L^{-1} degraded intracellular and extracellular MCs with cyanobacterial membrane relatively unaffected. However, ozone induced a significant increase in extracellular MCs in 5 min. Consequently, water authorities may consider H_2O_2 as a potential algicide owing to its effectiveness on cyanobacteria control and cyanotoxin degradation. Chlorine and $KMnO_4$ are recommended as feasible chemicals applied as pre-treatment oxidants in WTPs.

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Chapter 4. Application of various chemicals for cyanobacteria control and cyanotoxin removal in wastewater treatment

Abstract

Cyanobacteria blooms are an increasing issue in wastewater treatment systems with the associated toxins that could induce health risk to humans and animals by access to the irrigation. This study investigated the impacts of copper sulphate ($CuSO_4$), hydrogen peroxide (H_2O_2), chlorine, potassium permanganate ($KMnO_4$) and ozone on *Microcystis aeruginosa* cell viability and the concomitant toxin release and degradation in wastewater. All of these treatments could inactivate *M. aeruginosa* cells to varying degrees in wastewater with chemically challenging matrix present. Chlorine is a feasible option used as the last barrier in wastewater treatment plants due to its strong activity with complete cell inactivation and the degradation of majority cyanotoxins in 30 min. However, there is an increase of extracellular toxins during the treatment though they are subsequently oxidised for 5 mg L^{-1} chlorine but inadequate residual for 3 and 4 mg L^{-1} chlorine to degrade the resultant extracellular toxins. $KMnO_4$ is also an appropriate chemical when a high dose like 10 mg L^{-1} was applied as a post oxidation step to reduce the negative impacts caused by cyanobacteria in wastewater. H_2O_2 was found to degrade the majority of intracellular and extracellular toxins after 2 day treatment which is an additional benefit as an environmentally friendly algicide.

4.1 Introduction

Water-related issues such as water scarcity and deterioration of water quality are recognized as some of the most immediate and serious environmental threats globally. To meet the future water needs of growing population and industry, alternative source water options are being explored, particularly in dry countries such as Australia. Wastewater reclamation and reuse has been considered as a sustainable alternative water source but to date has been applied mainly in agriculture and to specific domestic uses (e.g. toilet flushing, building air

conditioning). This has the dual benefits of reusing wastewater to reduce contamination of receiving waters and conserves freshwater for higher quality uses (Bixio et al., 2006; Meneses et al., 2010).

Wastewater treatment plants (WWTP) have been developed to reduce nutrient and pathogen pollutants in sewage before discharging into the receiving water bodies. The treatment systems usually include three stages: preliminary treatment (e.g., screening; sedimentation); secondary treatment such as activated sludge ponds and waste stabilization ponds (WSP); and where it is destined for reuse further treatment (e.g., coagulation, disinfection) (Meneses et al., 2010; Martins et al., 2011). Unfortunately, WSP offer an ideal place for cyanobacterial growth due to the nutrient richness, warm temperature and high light availability. Cyanobacteria are one of most common aquatic organisms and they can produce toxic blooms under favourable conditions, which could impair source water quality. Since the first scientific literature documented in 1878 that cyanobacteria bloom induced animal deaths (Francis, 1878), scientists and water authorities have focused on them as contaminants in source water. Recent studies have also demonstrated that cyanobacterial blooms could be additional challenges for wastewater treatment. A review by Martins et al. (2011) highlighted the emerging issue of high levels of undesirable cyanobacteria and associated cyanotoxins in WWTP. It was also revealed that microcystins (MCs), which can exhibit toxic effects to the liver (Carmichael, 1992), are the most frequent toxins detected in WWTP. In addition to inhibiting the growth of crops, MCs have the propensity to accumulate in crops which also poses a potential health risk for human and animal via consumption of crop-based foods (Codd et al., 1999; Chen et al., 2004).

Additionally, the occurrence of cyanobacteria may reduce the efficiency of secondary treatment systems (maintaining a large community of heterotrophic bacteria and phytoplankton) to breakdown organic pollutants. A large density and diverse cyanobacterial community could increase biological oxygen demand (BOD) and result in competition with bacteria for nutrients like nitrate and phosphorus (Kirkwood et al., 2001). Furthermore, cyanotoxins might have negative impacts on protozoan communities (Ward and Codd, 1999) playing an important role for aquatic toxicity assessment (Twagilimana et al., 1998). Therefore, the management of wastewater treatment processes to maintain the quality of outlet effluents needs to consider the impacts of cyanobacterial cells and their resultant toxins.

Water authorities have searched for effective technologies to control/remove cyanobacterial cells and metabolites in drinking water systems for decades. Although there are variations between drinking water and wastewater, it may be possible to apply similar techniques like algicides or oxidants to reduce the negative consequences caused by cyanobacteria blooms in WWTP. It is generally understood that the majority of cyanotoxins are intracellular until age or stress (e.g. algicides) releases them into surrounding waters (Chorus and Bartram, 1999). Thus, it is favourable to remove the intact cells to avoid releasing intracellular toxins during treatment. However, it may be necessary to apply algicides like copper sulphate ($CuSO_4$) in WSP to avoid growing population and toxins produced by extensive cyanobacterial blooms. Barrington and Ghadouani (2008) also found hydrogen peroxide (H_2O_2) was a potential algicide in wastewater treatment. On the other hand, breakthrough and accumulation of cyanobacterial cells and toxins were observed through conventional water treatment processes including coagulation, clarification and filtration (Zamyadi et al., 2012). Hence an advanced oxidation step may be necessary to reduce any remaining cyanobacteria and dissolved metabolites in tertiary treatment systems.

Ozone, chlorine and potassium permanganate ($KMnO_4$) have been widely used as the final barrier of wastewater reclamation to remove micro-pollutants (Macauley et al., 2006; Fayad et al., 2013; Margot et al., 2013). These oxidants are also common pre-treatment oxidants applied in drinking water systems to enhance the overall removal of cyanobacteria cells by coagulation/flocculation (Chen and Yeh, 2006; Chen et al., 2009; Ma et al., 2012). The preferred pre-treatment should ensure that cyanobacterial membrane integrity is relatively unaffected during treatment such as using $KMnO_4$ doses below 3 mg L^{-1} (Fan et al., 2013a). However, for post-treatment in WWTP, it may be favourable to apply oxidants to inactivate the majority of cells and with a subsequent toxin removal. The information about these oxidants on the inactivation of cyanobacteria cells and subsequent cyanotoxin removal in wastewater is limited. There is a need to ascertain the effectiveness of the aforementioned algicides/oxidants for the control/removal of cyanobacteria cells during wastewater treatment whilst avoiding increase of dissolved toxins in the finished wastewater.

The objectives of this study were to investigate the impacts of $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone on the membrane integrity and densities of cyanobacterial cells and to study the concomitant toxin release and degradation in wastewater. Such systematic studies

are needed to provide information on cyanobacterial cell viability and toxin kinetics during wastewater treatment particularly as there is a lack of such information in the published domain.

4.2 Materials and Methods

4.2.1 Water source and quality

Tertiary treated effluent water was collected from the outlet of Bolivar WWTP (cyanobacterial blooms occurred historically) in Adelaide, South Australia after dissolved air flotation and filtration (DAFF) but before post chlorination. The upstream treatment processes of DAFF water include preliminary grit removal, primary sedimentation, secondary activated sludge treatment and detention in WSP. The DAFF water had a dissolved organic carbon (DOC) concentration of 8.1 mg L^{-1}, soluble and total iron (Fe) concentrations of 0.0006 and 0.0108 mg/L, soluble and total manganese (Mn) concentrations of 0.0057 and 0.0067 mg/L, pH of 7.1, true colour of 3 Hazen Units, turbidity of 0.89 NTU, and an alkalinity of 100 mg/L as $CaCO_3$. The water was filtered through the following steps to remove the organisms present in the water: GFC filter (Whatman, UK), 0.45 µm and then 0.2 µm membrane filter (Advantec MFS Inc., USA). The filtered DAFF water was stored in fridge (4 ℃) for experiments with a final DOC of 6.0 mg L^{-1}.

4.2.2 Materials and reagents

Microcystis aeruginosa (strain 338) Kutz. emend Elenkin which has a toxin profile > 90% MC-LR plus < 10% MC-LA from the Australian Water Quality Centre Culture Collection was cultured in ASM-1 medium (Gorham et al., 1964) and routinely subcultured to maintain growth in logarithmic phase. This strain remained as single or double cells in media. Cultures were incubated under constant light flux with the wavelength in the range of 400-700 nm (in the absence of UV light) on a 12h:12h light-dark cycle at 20 ± 1 ℃, and bubbled with air to achieve healthy culture with high cell density (> 10^6 cells mL^{-1}). The initial cell density of 7.0

$\times 10^5$ cells mL^{-1} for following experiments was adjusted by DAFF water and ASM-1 medium (with final components of 50% DAFF water and 50% ASM-1 medium each). 0.1M sterile filtered hydrochloric acid or sodium hydroxide was applied to achieve pH 7.5 $\pm$ 0.1 prior to use in chemical treatments. All experiments were performed at room temperature 20 $\pm$ 2 ℃. All chemicals and reagents used in this study were analytical grade and solutions were made using ultra-pure water (Millipore Pty Ltd, USA).

4.2.3 Chemical treatment experiments

M. aeruginosa cells were treated with a range of chemical doses and contact times. Specific amounts of stock solutions were spiked into the prepared cyanobacterial samples to achieve the desired initial chemical doses: 0.0, 0.5 and 1.0 mg L^{-1} for $CuSO_4$; 0.0, 10.2, 30.6 and 51 mg L^{-1} for H_2O_2; 3, 4, 5 and 6 mg L^{-1} chlorine; 1, 3, 5 and 10 mg L^{-1} for $KMnO_4$; 4 and 6 mg L^{-1} for ozone (see Fan et al., 2013b for chemical preparation details). For the oxidant decay experiments, samples were withdrawn at intervals, over different periods of time, and immediately analysed to determine oxidant residual (see Fan et al., 2013b for full procedures). A 50% ASM-1 and 50% DAFF water mixture without cells was used as the control. Each of the treatment experiments was conducted in triplicate, and samples were taken at specific reaction times to determine cell lysis and toxin concentration (time course experiments were only conducted for 5 mg L^{-1} chlorine and 10 mg L^{-1} $KMnO_4$). The residual oxidants were removed with sodium thiosulfate addition (for H_2O_2, chlorine and $KMnO_4$) and high purity nitrogen (for ozone) to arrest further oxidation of cyanobacterial cells and toxins. The cyanobacterial cell integrity was evaluated immediately upon sampling using flow cytometry combined with SYTOX Green stain (Regel et al., 2004; Daly et al., 2007; Fan et al., 2013b). High-performance liquid chromatography (HPLC) system was used to determine the concentrations of intracellular and total MCs (Ho et al., 2006) after sample preparation (Fan et al., 2013a) and extraction (Nicholson et al., 1994).

4.2.4 Modelling

Kinetic models have previously been applied (Fan et al., 2013a; Zamyadi et al., 2013) to integrate the rates of cell lysis, toxin release and toxin degradation of *M. aeruginosa* cells exposed to chlorine and $KMnO_4$ treatment. 5 mg L^{-1} chlorine and 10 mg L^{-1} $KMnO_4$ were chosen in this study to investigate the toxin kinetics in wastewater. Oxidant exposure (CT) was calculated by integrating the residual curve from each oxidant. The relationship between contact time, cell inactivation (by inference toxin release) and toxin degradation can be described as first order processes. If this assumption is valid then the process of toxin release and degradation may be considered as consecutive reactions (Equation 1), where the concentration of intracellular (A) and extracellular (B) toxin are described by Equations 2 and 3 (Jones, 1970; Fan et al., 2013a; Zamyadi et al., 2013).

$$A \xrightarrow{k_i} B \xrightarrow{k_e} D \qquad \text{(Equation 1)}$$

$$A = A_0 e^{-k_i CT} \qquad \text{(Equation 2)}$$

$$B = B_0\, e^{-k_e CT} + A_0 (e^{-k_e CT} - e^{-k_i CT})/(1-k_e/k_i) \qquad \text{(Equation 3)}$$

Where CT = oxidant exposure; A_0 = the initial intracellular MCs concentration; A = the concentration of intracellular MCs at a specified CT; B_0 = the initial dissolved MCs concentration; B = the concentration of dissolved MCs at a specified CT; k_i = the rate of intracellular MCs release; k_e = the rate of extracellular MCs degradation. D = the concentration of degraded MCs at specified CT. Model parameters were estimated by fitting the equation to experimental data using the Solver add-in for Excel and a least squares objective function based on the intracellular and extracellular toxin concentrations. Nash–

Sutcliffe model efficiency coefficient (NS) was calculated as performance metric for the model (Mayer and Butler, 1993; Fan et al., 2013a).

4.3 Results

4.3.1 Oxidant degradation kinetics

Oxidant decay in the presence of cells was measured to determine the oxidant demand of the wastewater. Chlorine decreased rapidly in the first min sharing similar trends for initial chlorine doses of 3, 4 and 5 and 6 mg L^{-1} (Figure 4.1a). There was no chlorine residual detected with the initial chlorine doses of 3 and 4 mg L^{-1} after 30 min whilst a residual concentration of 0.1 and 0.4 mg L^{-1} remained with the doses of 5 and 6 mg L-1, respectively. However, no residual was detected after 60 min for either 5 or 6 mg L^{-1} chlorine.

Assays were performed to determine the baseline degradation of $KMnO_4$ (10 mg L^{-1}) and H_2O_2 (51 mg L^{-1}) with a cell-free ASM-1 and DAFF water solution (control) to determine the oxidant demand (Figure 4.1b; 4.1c). The concentration of $KMnO_4$ declined progressively with a residual of 7.0 mg L^{-1} detected after 6 hours while the concentration of H_2O_2 remained relatively stable (a final residual of 45.7 mg L^{-1}) over the 6 day period. In the *M. aeruginosa* samples, no residual was detected after 2 h where 1 mg L^{-1} $KMnO_4$ was added initially and $KMnO_4$ concentrations decreased steadily from 3, 5 and 10 mg L^{-1} to 0.2, 1.2 and 1.8 mg L^{-1} respectively after 6 h treatments (Figure 4.1b). H_2O_2 decomposed relatively slowly even in the presence of cyanobacterial cells and the H_2O_2 residual decreased steadily from 10.2, 30.6 and 51 mg L^{-1} to 0.0, 17.4 and 30.7 mg L^{-1} respectively by day 6 (Figure 4.1c). Ozone decayed fastest of all the oxidants and with the majority consumed within the first 30 s followed by slower decay. No ozone residual was detected after 5 min for both 4 and 6 mg L^{-1} ozone doses (Figure 4.1d).

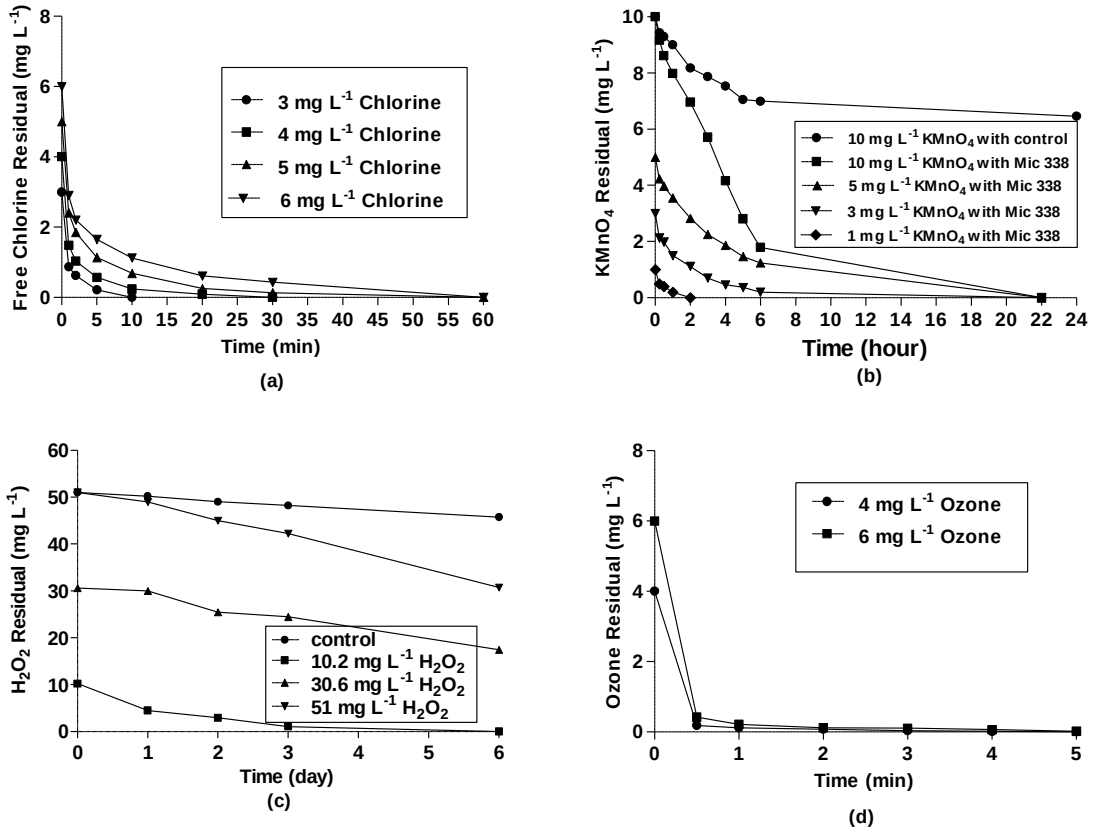

Figure 4.1 Oxidant residual of Microcystis aeruginosa cultures treated with (a) chlorine at 3, 4, 5 and 6 mg L^{-1}; (b) $KMnO_4$ at 1, 3, 5 and 10 mg L^{-1}; (c) H2O2 at 10.2, 30.6 and 51 mg L^{-1}; and (d) ozone at 4 and 6 mg L^{-1}. $KMnO_4$ and H_2O_2 added to ASM-1 Medium and DAFF water mixture are included as control.

Pseudo-first-order rate constants (k_t) were calculated to represent the degradation of each oxidant in *M. aeruginosa* samples using data from the current study and published work (Fan et al., 2013b). Chlorine and ozone were consumed rapidly during the first min and much slower in the subsequent time period, consequently the decay could be considered as two pathways (fast and slow). However, due to the difficulties in collecting sufficient data during the fast period, the results of chlorine and ozone decay were presented as one even pathway (Table 4.1). Results show that the fastest degradation rate was recorded for ozone (61.4 h^{-1}).

4.3.2 Cell integrity

The cell integrity results were analysed using Cyflogic flow cytometry software (CyFlo Ltd., Finland) (see Fan et al., 2013b for full details). Analysis showed that 97% of the cyanobacterial cells were healthy before $CuSO_4$ and H_2O_2 treatments and 80% of the populations were intact after two days for the control samples (Table 4.2). Approximately 24% of the cyanobacterial cells maintained membrane integrity when exposed to 0.5 mg L^{-1} $CuSO_4$ and all the cells were compromised following exposure to 1.0 mg L^{-1} $CuSO_4$ after 2 days. H_2O_2 also showed significant impact on compromising the cyanobacterial cell membranes with the proportion of intact cells decreasing to 6%, 6% and 4% on day 2 using H_2O_2 doses of 10.2, 30.6 and 51mg L^{-1}, respectively.

All of the *M. aeruginosa* cells were membrane compromised within 30 min following chlorine additions of 3, 4 and 5 mg L^{-1} (Table 4.2). The percentage of intact *M. aeruginosa* cells was still greater than 95% after dosing with 1 and 3 mg L^{-1} $KMnO_4$ for 180 min (Table 4.2). In contrast, higher doses of $KMnO_4$ resulted in a greater loss of membrane integrity with 75% (5 mg L^{-1} $KMnO_4$) and 8% (10 mg L^{-1} $KMnO_4$) of the cells remaining intact after 180 min. The percentage of intact cells remained around 35 % and 22% with initial ozone doses of 4 and 6 mg L^{-1} respectively after 5 min contact time. There were no significant differences in cyanobacterial cell densities observed after all the chemical treatments (data not shown).

Table 4.1 Pseudo-first-order rate constants (k) for the degradation of oxidants in *Microcystis aeruginosa* samples. Linear regression coefficient (R^2) values for the first-order fits presented in parentheses.

K (h^{-1})									
chlorine			$KMnO_4$			H_2O_2			ozone		
$[chlorine]_0$ ($mg\ L^{-1}$)	A+D	A	$[KMnO_4]_0$ ($mg\ L^{-1}$)	A+D	A	$[H_2O_2]_0$ ($mg\ L^{-1}$)	A+D	A	$[ozone]_0$ ($mg\ L^{-1}$)	A+D	A
3	28.3 (R^2 0.89)	7.75 (R^2 0.94)	1	1.51 (R^2 0.96)	1.51 (R^2 0.98)	10.2	0.03 (R^2 0.98)	0.02 (R^2 0.92)		61.4 (R^2 0.73)	56.4 (R^2 0.74)
4	10.0 (R^2 0.89)	5.67 (R^2 0.94)	3	0.409 (R^2 0.99)	0.151 (R^2 0.98)	51	0.004 (R^2 0.98)	0.003 (R^2 0.97)		51.1 (R^2 0.78)	39.0 (R^2 0.67)
5	6.58 (R^2 0.93)	2.67 (R^2 0.95)	5	0.223 (R^2 0.99)	0.104 (R^2 0.97)						
			10	0.263 (R^2 0.96)	0.125 (R^2 0.95)						

A+D: *Microcystis aeruginosa* in ASM-1 and DAFF water mixture in this study.

A: *Microcystis aeruginosa* in ASM-1 medium, calculated using the data presented by Fan et al. (2013a).

Table 4.2. The cell integrity data for for algicides/oxidants in *Microcystis aeruginosa* samples at time 0 and specific contact time.

$[CuSO_4]_0$ (mg L^{-1})	intact cells (%)		$[chlorine]_0$ (mg L^{-1})	intact cells (%)		$[KMnO_4]_0$ (mg L^{-1})	intact cells (%)		$[H_2O_2]_0$ (mg L^{-1})	intact cells (%)		$[ozone]_0$ (mg L^{-1})	intact cells (%)	
	0	2 (d)		0	30 (min)		0	180 (min)		0	2 (d)		0	5 (min)
0	97	80	3	97	0	1	99	98	0	97	80	4	96	35
0.5	97	24	4	97	0	3	99	95	10.2	97	6	6	96	22
1	97	0	5	97	0	5	99	75	30.6	97	6			
						10	99	8	51	97	4			

4.3.3 Toxin release and degradation

The concentrations of intracellular and extracellular MCs before exposure to $CuSO_4$ and H_2O_2 were 44 and 8 μg L^{-1}, respectively. The concentration of total MCs increased to 63 μg L^{-1} with 76% of the toxins found to be intracellular on day 2 for control samples (Figure 4.2). A considerable increase of extracellular MCs was observed due to cell lysis resulting from copper doses of 0.5 and 1.0 mg L^{-1}. A 43% and 90% reduction of intracellular MCs was observed resulting in 28 and 50 μg L^{-1} of MCs determined to be extracellular 2 days after copper treatments of 0.5 and 1.0 mg L^{-1} respectively (Figure 4.2a). Although H_2O_2 induced loss of cyanobacterial membrane integrity, there was no increase in extracellular MCs observed 2 days after H_2O_2 doses of 10.2-51 mg L^{-1} (Figure 4.2b). The total concentration of MCs dropped to 10, 5 and 5 μg L^{-1} after 10.2, 30.6 and 51 mg L^{-1} H_2O_2 treatment, respectively.

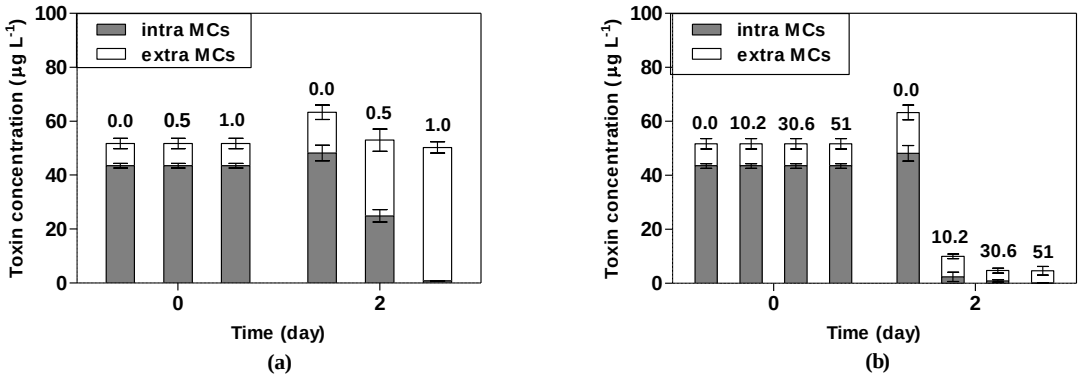

Figure 4.2 Concentrations of intracellular and extracellular MCs after 2 days treatments with (a) $CuSO_4$ (0.0, 0.5 and 1.0 mg L^{-1}) and (b) H_2O_2 (0.0, 10.2, 30.6 and 51 mg L^{-1}). Error bars represent standard deviation from triplicate analyses.

For chlorine and ozone treatments, the initial *M. aeruginosa* culture contained 53 μg L^{-1} MCs with the major proportion (83%) being intracellular (Figure 4.3a; 4.3c). The intracellular MCs decreased to 3, 3 and 2 μg L^{-1} with chlorine doses of 3, 4 and 5 mg L^{-1} after 30 min. Meanwhile, 63% and 10% increases in extracellular MC concentrations were observed for 3 and 4 mg L^{-1} chlorine treatments respectively. In contrast, the extracellular MCs decreased to 2 μg L^{-1} (75% reduction) and total MCs to 4 μg L^{-1} after a 5 mg L^{-1} chlorine exposure for 30 min (Figure 4.3a). Ozone also showed a strong ability to oxidise MCs with 40 and 32 μg L^{-1}

of total MCs remaining after 4 and 6 mg L^{-1} ozone treatment respectively within 5 min (Figure 4.3c). At the same time an apparent increase in extracellular MCs was detected using 4 (206% increase) and 6 mg L^{-1} (181% increase) ozone which was accompanied by a rapid decrease in intracellular MCs.

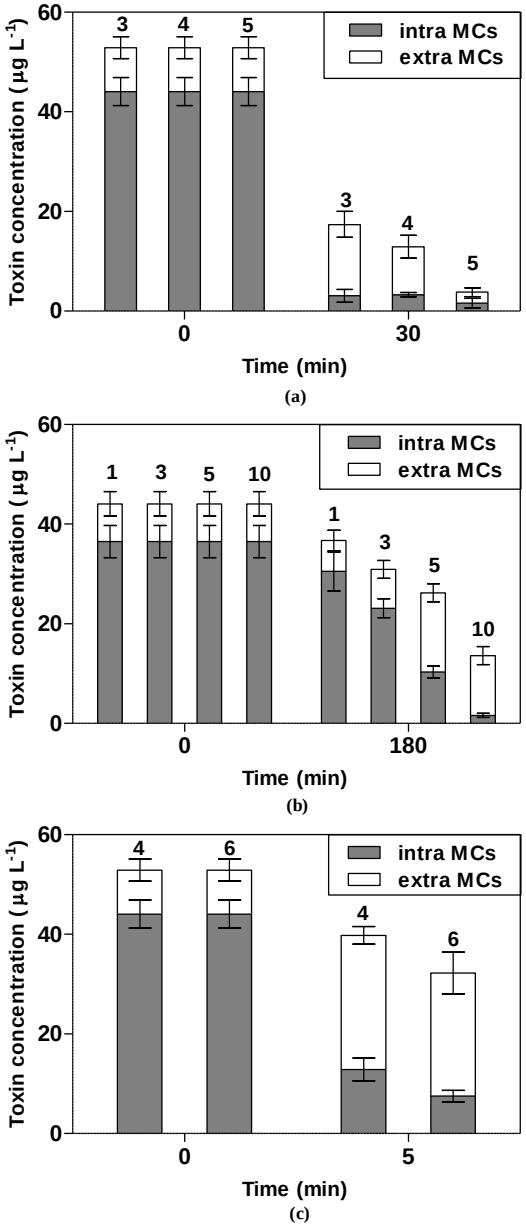

Figure 4.3 Concentrations of intracellular and extracellular MCs after treatments with (a) chlorine (3, 4 and 5 mg L^{-1}) at 30 min contact time, (b) $KMnO_4$ (1, 3, 5 and 10 mg L^{-1}) at 180 min contact time, and (c) ozone (4 and 6 mg L^{-1}) at 5 min contact time. Error bars represent standard deviation from triplicate analyses.

The *M. aeruginosa* samples contained 44 µg L^{-1} MCs with the majority (83%) identified as intracellular before $KMnO_4$ treatment. An 18% reduction of extracellular MCs was observed after a 1 mg L^{-1} $KMnO_4$ treatment with a concomitant decrease in intracellular MCs to 31 µg L^{-1} (Figure 4.3b). A higher $KMnO_4$ dose of 3 mg L^{-1} achieved an approximately 37% reduction in intracellular MCs while dissolved MCs remained unchanged compared with the initial concentration. Although there was an obvious decrease in total MCs following addition of $KMnO_4$ at 5 and 10 mg L^{-1}, there was an increase of 109% and 58% respectively in dissolved MCs after 180 min.

4.3.4 Toxin kinetics for chlorine and $KMnO_4$ treatment

For 5 mg L^{-1} chlorine dose, the degradation of total MCs occurred between CT values from 0 to 21.4 mg min L^{-1} with 72% of total MCs oxidised at a CT value of 21.4 mg min L^{-1} (Figure 4.4a). A larger CT value (1866 mg min L^{-1}) was needed for 10 mg L^{-1} $KMnO_4$ to achieve a comparable degradation (74%) of the total MCs (Figure 4b). The fitted k_i and k_e for chlorine oxidation is 133 (NS: 0.91) and 227 $M^{-1}s^{-1}$ (NS: 0.53), respectively. The toxin release rate of $KMnO_4$ is 12.8 $M^{-1}s^{-1}$ (NS: 0.93) and the fitted k_e is 3.30 $M^{-1}s^{-1}$ (NS: 0.77).

4.4 Discussion

The DAFF water from Bolivar WWTP used in this study was sampled before post chlorination. For experiments a mixture of 50% wastewater and 50% ASM-1 medium was used rather than 100% wastewater to avoid osmotic shock and possible death of cyanobacterial cells due to alteration of the water matrix. A test was conducted (data not shown) to culture *M. aeruginosa* cells in the 50% wastewater without chemical treatments and almost all of the cell membranes remained intact during 24 h. There were higher oxidant demands in wastewater than ASM-1 medium under equivalent conditions (Table 4.1). The varying physical-chemical properties could contribute to the competition to cyanobacterial cells/toxins towards oxidants. Particularly, the DOC concentration (6 mg L^{-1}) of filtered wastewater was higher than ASM-1 medium (2.5 mg L^{-1}) which increased the oxidant demands (Daly et al., 2007; Liu et al., 2010; Coral et al., 2013). Historical data has shown

breakthrough of cyanobacterial cells through the DAFF plant with cell numbers of up to 5.0 × 10^5 cells mL^{-1} after DAFF treatment. Therefore, to accomplish cyanobacteria inactivation and toxin removal from wastewater, it is important that the contact time and oxidant residual levels are appropriate with respect of the water chemistry and cell densities.

Chlorine induced greatest membrane loss of *M. aeruginosa* cells in wastewater amongst all of the investigated chemicals (Table 4.2) which is consistent with a study by Fan et al. (2013b) using ASM-1. A 93% reduction of intracellular MCs was observed due to the rapid cell lysis for both 3 and 4 mg L^{-1} chlorine doses (Figure 4.3a). The released MCs were partially oxidised resulting in concentrations of 14 and 10 µg L^{-1} remaining (for 3 and 4 mg L^{-1} chlorine doses, respectively) which were above the initial level of extracellular MCs. For the higher chlorine dose (5 mg L^{-1}), an increase of extracellular MCs also occurred at CT values ranging from 0 to 10.3 mg min L^{-1}; however, the toxins were subsequently degraded to 2 µg L^{-1} with a CT of 21.4 mg min L^{-1} (Figure 4.4a). In contrast, Fan et al. (2014) have reported that the extracellular MCs were constantly below the initial values during 3-5 mg L^{-1} chlorine treatments and greater amounts of total MCs were oxidised after 30 min in ASM-1 medium with considerably higher toxin degradation rates (k_e = 864 $M^{-1}s^{-1}$ for 5 mg L^{-1} chlorine). This may be attributed to the chlorine residual being inadequate in the current study which induced weaker activity on toxin degradation (Lin et al., 2009).

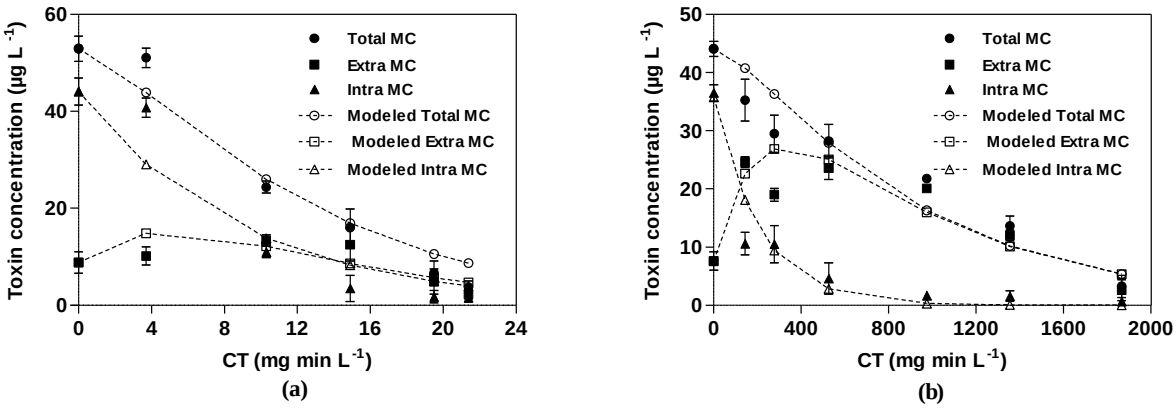

Figure 4.4 Comparison of experimental and model predicted results of MCs concentrations after treatments with (a) chlorine at initial concentration of 5 mg L^{-1} and (b) $KMnO_4$ at initial concentration of 10 mg L^{-1}. Error bars represent standard deviation from triplicate analyses.

The effect of chlorination on cyanobacterial cells/toxins has been well studied in reservoir water and laboratory medium (Ho et al., 2006; Lin et al., 2009; Ding et al., 2010). Although chlorine has been proved to effectively compromise cyanobacterial cells and degrade toxins, the kinetics can vary depending on the cell densities, species, water matrix, chlorine dose and experimental conditions (Acero et al., 2005; Ma et al., 2012; Zamyadi et al., 2012; 2013). While chlorination could result in the formation of toxic by-products such as carcinogenic trihalomethanes (THMs) when there are high levels of DOC (El-Shafy and Grunwald, 2000, Zamyadi et al., 2013), it is still the most popular oxidant applied in water treatment due to its low cost and ease of operation.

Some wastewater treatment plants apply $KMnO_4$ or ozone as the disinfectant instead of chlorine (Fayad et al., 2013; Margot et al., 2013). The results showed 3 mg L^{-1} was the threshold dose resulting in membrane loss of *M. aeruginosa* cells (Table 4.2) as well as in culture medium (Fan et al., 2013b) and the degree of cell rupture was similar between these two waters for all $KMnO_4$ doses after 180 min. The total MCs were reduced by $KMnO_4$ treatments between 1 to 10 mg L^{-1} (Figure 4.3b) while the oxidising ability was weaker than a previous study using samples in ASM-1 medium (Fan et al., 2014).

Wastewater contains high quantities of metals and as $KMnO_4$ is an effective oxidant for Fe and Mn (Ellis et al., 2000) these processes may exert an additional oxidant demand. Therefore, it is important to measure the concentration of soluble Fe and Mn which can influence the effectiveness of $KMnO_4$ on cyanobacteria oxidation. Soluble Fe concentrations of 0.0006 mg L^{-1} and 0.0057 mg L^{-1} of soluble Mn in the DAFF water consumed 0.012 mg L^{-1} $KMnO_4$ from theoretical equivalents which could be disregarded. As a pre-treatment oxidant in drinking water systems, it is feasible to apply $KMnO_4$ doses below 3 mg L^{-1} to improve the conventional treatment processes of cyanobacteria removal without impacting on the cyanobacteral cell integrity (Chen and Yeh, 2005; Ho et al., 2009; Fan et al., 2013b). However, post-oxidation in the WWTP, requires higher doses of $KMnO_4$ (10 mg L^{-1}) to inactivate all of the cyanobacteria cells and remove the majority of associated toxins with sufficient contact time. Higher concentrations of $KMnO_4$ can cause an undesirable pink colour in drinking water (Fan et al 2013a), however, this was not observed in the current study since all the $KMnO_4$ was consumed within 22 h due to the high oxidant demand (Figure

4.1b). Overall, $KMnO_4$ is a feasible oxidant to treat cyanobacterial cells/toxins with limited possibility of harmful by-product formation and other environmental issues.

Ozone induced loss of membrane integrity in 64%-77% of the *M. aeruginosa* cells after 5 min contact time (Table 4.2) which was a lower percentage than cells in ASM-1 medium (70%-90%) using same ozone doses (Fan et al., 2013b). Coral et al. (2013) reported that ozone caused complete loss of *M. aeruginosa* in 30 seconds even using a low dose (0.5 mg L^{-1}) which showed more effective oxidative ability due to lower ozone demands. This suggests that the immediate ozone demands of DOC and possible metals reduced the amount available to act upon cyanobacterial cells and reveals that these interferences need to be considered when determining dose levels. The total MCs were reduced to 80% and 60% after 4 and 6 mg L^{-1} ozone treatments, respectively however apparent increase of extracellular MCs were observed for both ozone doses. Ozone is a strong oxidant and can remove MCs efficiently (Rositano et al., 1998; Hall et al., 2000), but the ability could be reduced by larger numbers of cyanobacterial cells, a higher concentration of DOC, pH and alkalinity (Rositano et al., 2001; Miao and Tao, 2009). Ozone has less harmful by-products compared to chlorine but it may induce bromate formation which is a potential carcinogen (Bouland et al, 2004). Furthermore, ozone can be an expensive option involving a complicated procedure which needs to be considered before it is chosen as a treatment option. In addition, when cyanobacterial numbers are high the ozone dose will need to be increased to inactivate all the cells and oxidise all cyanotoxins.

Besides post-oxidation, algicides could be applied in waste stabilisation ponds to control cyanobacteria blooms. $CuSO_4$ proved to be an effective algicide in numerous studies and industry applications for many years (Elder and Horne, 1978). $CuSO_4$ can also have a negative impact on beneficial nitrifying and denitrifying bacteria present in stabilisation ponds. Copper can react with DOC to produce less algicidal complexes causing a drop in its activity (Burch et al., 2002). In addition, use of $CuSO_4$ may induce substantial threat since copper (II) caused inhibition of key microbial populations which could remove organic pollutants in waste water treatment systems (Ochoa-Herrera et al., 2011). Even with these issues it is still a popular chemical for control of algae in reservoirs due to ease of application and low cost. Results presented here show that copper inactivated cyanobacterial cells effectively in wastewater (Table 4.2) which was similar with the previous study in pure

ASM-1 medium (Fan et al., 2013b). However, Copper (II) is recognised as one of the most serious heavy metal contaminants in industry wastewater (Heidmann et al., 2008). Hence, cyanobacteria adapted to wastewater environment may be more tolerant to copper treatment due to the long-term adaptation.

The concentration of total MCs did not increase after copper applications of 0.5 and 1.0 mg L^{-1} for 2 days (Figure 4.2a) since the growth of cyanobacteria was inhibited (data not shown). However, because the extracellular toxin released from the lysed cells was not degraded, a further treatment process may be required to ensure the water is safe for irrigation. In spite of these limitations it still provides a cyanobacterial control option for WWTP that requires a low cost immediate response (Jancula and Marsalek, 2011).

Similarly, H_2O_2 showed a strong algicidal ability resulting in a loss of membrane integrity in more than 90% of the cyanobacterial cells after 2 day treatment (Table 4.2). H_2O_2 degrades to water and oxygen and leaves no additional chemical traces in the environment which is a distinct advantage over $CuSO_4$. The DAFF water used in the current study has been filtered to remove microorganisms of greater than 0.2 µm which have been shown to play an important role in H_2O_2 decay in natural seawater (Petasne and Zika, 1997). Therefore, H_2O_2 doses may need to be higher when used in unfiltered raw water. H_2O_2 can react with Fe (II) or UV radiation to produce hydroxyl radicals which have a strong oxidising capacity and hence a stronger algicidal activity (Bandala et al., 2004; Drabkova et al., 2007; Barrington et al., 2011). The total Fe concentration in the DAFF water was 0.0108 mg L^{-1} which has minimal impact on the activity of H_2O_2. However, the current study incubated cultures under light without UV component, it is expected that activity of H_2O_2 would be more effective in WSP which are usually open to sunlight allowing UV radiation at least in the surface (Brookes et al., 2004).

Most of the intracellular and extracellular MCs were oxidised by H_2O_2 after a 2 day treatment (Figure 4.2b) and the H_2O_2 residual was 2.9, 25.5 and 45.0 mg L^{-1} after 2 day H_2O_2 doses of 10.2, 30.6 and 51 mg L^{-1} which would be adequate to oxidise any remaining MCs (Figure 1c). Therefore, the application of H_2O_2 as algicide in WSP would not only control the growth of cyanobacteria but also effectively remove cyanotoxins. One possible limitation of H_2O_2 application is that it may attack other desirable organisms in WSP. It has been shown to be selective to cyanobacteria over eukaryotic phytoplankton (Barroin and Feuillade, 1986;

Drabkova et al., 2007) which provides an advantage over $CuSO_4$. Matthijs et al. (2012) applied 2 mg L^{-1} H_2O_2 in an entire lake with a *planktothrix*-dominated bloom in Netherlands and found the cyanobacteria population collapsed by 99% within a few days while other phytoplankton and zooplankton community were almost unaffected. However, a laboratory study by Reichwaldt et al. (2011) reported that H_2O_2 doses above 3 and 1.5 mg L^{-1} had an adverse effect on two common zooplankton genera, Daphnia and Moina respectively. The H_2O_2 doses applied in the current study are equal to, or above 10.2 mg L^{-1} does reasonable to be applied in WSP. Firstly, more oxidant is required in WSP with complex biological and chemical pollutants and the hydroxyl radicals produce by H_2O_2 could be quickly consumed. Barrington et al. (2013) applied 44 and 95 mg L^{-1} H_2O_2 in field trials depending on the total chl-a concentration in the WSP and suggested the organisms grow in WSP may be able to avoid unfavourable conditions. In addition, H_2O_2 itself or with UV/Fe (II) using a high dose like 1.5 g L^{-1} has been applied in WWTP to reduce the BOD/COD, toxic organic compounds and provide oxygen source which is of benefit for organisms (Wu et al., 2012). Therefore, using H_2O_2 doses above 10.2 mg L^{-1} in WWTP as an algicide should be a feasible option.

4.5 Conclusion

This study demonstrated chlorine, $KMnO_4$ and ozone could all inactivate *M. aeruginosa* cells to varying degrees in wastewater and chlorine (3-5 mg L^{-1}) showed the fastest activity with complete cell rupture achieved in 30 min. These three chemicals all oxidised MCs but the effectiveness was dependent on the availability of oxidant residual. The kinetics of MC release and degradation was investigated using chlorine and $KMnO_4$. There was an increase of extracellular MCs which were subsequently oxidised for both treatments. As a post-oxidation step in wastewater treatment systems, it is important that oxidant doses and contact time are carefully assessed in relation to the physical and chemical properties of the water and the cyanobacterial cell densities. $CuSO_4$ and H_2O_2 induced loss of membrane integrity in the majority of the *M. aeruginosa* cells 2 days after dosing. Both of them showed strong algicidal activity; however, copper showed no ability to degrade MCs resulting in an increase of extracellular MCs which would require further treatment to remove. In contrast, H_2O_2 degraded the majority of MCs while still maintaining significant residual even for the lowest

dose (10.2 mg L^{-1}). All the chemicals evaluated in the current study showed an ability to inactivate cyanobacteria in the more chemically challenging matrix present in wastewater.

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Chapter 5. Impact of potassium permanganate on cyanobacterial cell integrity and toxin release and degradation

Abstract

Potassium permanganate ($KMnO_4$) is commonly used as a pre-treatment oxidant to remove soluble manganese (Mn) and iron (Fe) which can contribute to dirty water in drinking water supplies. Because Mn and Fe problems are commonly associated with thermal stratification in summer and autumn, they frequently coincide with the presence of cyanobacteria. The use of $KMnO_4$ as an oxidant for Mn and Fe control therefore needs to consider the potential impacts on cyanobacterial cell integrity and toxin release. This study aims to assess the effect of $KMnO_4$ on cyanobacteria cell integrity, toxin release and toxin oxidation. A toxic strain of *Microcystis aeruginosa* was exposed to various concentrations of $KMnO_4$ and the cell integrity of cyanobacteria was measured with flow cytometry. Further the intra- and extra-cellular toxin concentrations were quantified and it was apparent that $KMnO_4$ reduced both the intra- and extra-cellular toxins at low initial concentrations of 1 and 3 mg L^{-1} without complete cell lysis. However, the cell integrity of cyanobacteria was compromised at $KMnO_4$ concentrations of 5 mg L^{-1} and 10 mg L^{-1} and led to intracellular toxin release. In the 10 mg L^{-1} $KMnO_4$ treatment, the total toxin was oxidized after 7 hours contact time. A model describing the two step process of release and degradation was developed and may provide a tool to assess the risk water quality posed by toxin release. Consequently, it may be possible to use $KMnO_4$ as a pre-treatment for Mn and Fe at concentrations < 3 mg L^{-1} and short contact time when cyanobacteria are also present.

5.1 Introduction

Cyanobacteria in drinking water supplies can create a water quality hazard because they produce metabolites or toxins that are not readily removed by conventional water treatment processes (Himberg et al., 1989; Chow et al., 1999; Zamyadi et al., 2012). The preferred option for the removal of cyanobacterial toxins, taste and odours involves the removal of

whole, intact cells to avoid release of intracellular metabolites into surrounding waters. However, sometimes other water quality objectives require the use of an oxidant as a pretreatment that can affect the cell integrity of cyanobacterial cells. For example, thermal stratification in reservoirs promotes the growth of cyanobacteria which are able to exploit the relative stability of the water column, these conditions also promote the release of Mn and Fe from sediments which can contribute to dirty water problems (Carlson and Knocke, 1999; Ellis et al., 2000). Potassium permanganate ($KMnO_4$) is a common pre-oxidant used for Mn and Fe control (Ellis et al., 2000; Roccaro et al., 2007). However, $KMnO_4$ can also oxidise cyanobacterial cells and induce leakage of intracellular metabolites which increases the concentration of dissolved metabolites in the surrounding water body (Chen and Yeh 2005, 2006; Chen et al. 2009; Ho et al., 2009) although $KMnO_4$ can also degrade toxins (Rodriguez et al., 2007a; Rodriguez et al., 2007b).

Although the most widespread application is for the oxidation of Mn (Ellis et al., 2000; Carlson and Knocke, 1999; Lee et al., 2003; Roccaro et al., 2007), $KMnO_4$ is also used to fulfil a variety of other objectives including control of algae and associated metabolites (Rodriguez et al., 2007b; Ho et al., 2009; Chen and Yeh, 2005; Chen et al., 2005; Cherry, 1962), antibiotics (Hu et al., 2010), colour (Liu et al., 2011), and disinfection by-products (Chu et al., 2011) (Appendix B). To date, minimal studies have assessed the effects of $KMnO_4$ on cyanobacteria cells and the toxins systematically. Therefore, it is necessary to determine the appropriateness for pre-oxidation with $KMnO_4$ for instances when Mn and Fe and cyanobacteria are simultaneously present. In this study, the effects of $KMnO_4$ pre-treatment on cyanobacterial cell integrity, the concomitant toxin release and toxin degradation was investigated.

5.2 Experimental methods

5.2.1 Materials and Reagents

A toxic strain of *Microcystis aeruginosa* Kutz. emend Elenkin (strain 338 from the Australian Water Quality Centre culture collection) was used in this study and routinely cultured in ASM-1 medium (Gorham et al., 1964) to maintain logarithmic growth. All cultures were

incubated under constant cool-fluorescent light intensity 50 μmol m^{-2} s^{-1} on a 12h:12h light-dark cycle at a constant temperature of 20 ± 1 ℃, and bubbled with air. Prior to use in experiments, the algal cultures were adjusted to pH 7.5 ± 0.1.

Samples for cell counts by microscopy were treated with Lugol's iodine, pressurized to 900 kPa for 2 minutes to collapse gas vesicles and then counted at 400 × magnification (Brookes et al. 1994). Cultures with an initial cell density of 7.0 × 10^5 cells mL^{-1} were used in all experiments. All experiments were performed at room temperature 20 ± 2 ℃. All chemicals and reagents used were analytical grade and solutions were made using ultra-pure water purified to 18 MΩ cm by a Milli-Q water purification system (Millipore Pty Ltd, USA).

Potassium permanganate stock solution (1.0 g L^{-1}) was prepared by dissolving crystal $KMnO_4$ in Milli-Q water. It was then standardized by titration with sodium oxalate and diluted as required for the experiments and kept under darkness at 4 ℃. Sodium thiosulfate stock solution (4.0 g L^{-1}) was prepared by dissolving $Na_2S_2O_3$ $5H_2O$ in Milli-Q water and used to quench the reaction of permanganate and arrest further oxidation of cyanobacterial cells. $KMnO_4$ concentrations in the samples were measured after filtration the sample through 0.2 μm nylon syringe filters (Whatman, UK) to remove manganese oxide. A standard curve was constructed by diluting the stock solution to generate a range of $KMnO_4$ concentrations from 0 to 20 mg L^{-1} and compared to the linear relationship of absorbance at 525 nm in a 10 mm cuvette in a UV/VIS spectrophotometer (Thermo Scientific, UK).

5.2.2 Pre-oxidation experiments

Typically low concentrations of $KMnO_4$ (0.1-1.0 mg L^{-1}) were used in pre-treatment (Carlson and Knocke, 1999; Ho et al., 2009; Liu et al., 2011), however, high concentrations have been applied in some circumstances: e.g. 13 mg L^{-1} (Cherry, 1962); between 5 to 10 mg L^{-1} (Chen et al., 2005). The NSF/ANSI Standard 60 on drinking water treatment chemicals, recommends a maximum $KMnO_4$ concentration of 50 mg L^{-1} be used for disinfection and oxidation (NSF, 2002). Based on the $KMnO_4$ concentrations used to oxidize the suite of contaminants detailed above, concentrations between 1 and 10 mg L^{-1} were considered appropriate for use in this study.

Microcystis aeruginosa samples were treated with the desired $KMnO_4$ concentrations (1, 3, 5 and 10 mg L^{-1}) and mixed with a magnetic stirrer at a low speed. Samples were withdrawn at intervals, over 24 hours, and immediately analysed to determine $KMnO_4$ residual. At each time interval, 1 mL of each sample was taken for cell integrity measurement and 2 mL for cell microscopic counts. Samples with a volume of 200 mL were quenched with $Na_2S_2O_3$ $5H_2O$ and used for microcystin analysis. These samples were divided in two subsamples: 100 mL sample was immediately filtered through a GFC filter (Whatman, UK) to remove extracellular materials and concentrate cells on the filter for intracellular microcystin analysis. The remaining 100 mL sample was freeze-dried for total microcystin determination (both the intracellular and extracellular toxin).

5.2.3 Microcystin Extraction and Analysis

The filter from the 100 mL for intracellular toxin analysis described above and the freeze-dried cells for total toxin measurement were added to a 5 mL solution made with 47.5% of Milli-Q water, 47.5% of Methanol and 5% of formic acid. The solution was kept overnight and then sonicated on ice using Virsonic 475 (VirTis, US) sonicator for 4 min, with 0.5 second bursts punctuated with no pulsation for 0.5 seconds. The solutions were diluted with Milli-Q water to a final volume of 100 mL and filtered with a 0.45 μm membrane filter (Advantec MFS Inc., USA). Microcystins (>90% MC-LR plus < 10% MC-LA) were concentrated from water samples by C18 solid-phase extraction according to the methods described by (Nicholson et al, 1994) prior to toxin analysis. A high-performance liquid chromatography (HPLC) system, consisting of a 600 pump controller, 717plus autosampler, 996 photodiode array detector (Waters Pty Ltd, Australia) and a 150 × 4.6 mm Luna C18 column (Phenomenex, Australia), was used to analyze the toxin samples using procedures and conditions previously documented by Ho et al. (2006).

5.2.4 Flow Cytometry

A FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with an air cooled 15 mW argon laser emitting at a fixed wavelength of 488 nm was used for measurement.

Fluorescent filters and detectors were all standard with green fluorescence collected in channel FL1 (530 nm) and red fluorescence collected in channel FL3 (670 nm). Probe fluorescence, chlorophyll *a* fluorescence, forward scatter- FSC (cell size) and side scatter- SSC (cell granularity) (Brookes et al., 2000) data was collected and analysed using CellQuest software (Becton Dickinson, USA) and Cyflogic (CyFlo Ltd., Finland). FSC and FL3 were used to identify and gate the cyanobacterial cells. FL1 was used to quantify the fluorescent intensity of Sytox Green nucleic acid stain (Molecular Probes, USA). Sytox Green was used to determine the percentage of viable (Sytox negative) to nonviable (Sytox positive) cells in a sample as described by Regel et al., (2004) and Daly et al., (2007). Sytox Green was added to achieve a final concentration of 0.2 µM and measured on the flow cytometer after 10 minutes incubation. Unstained control was also measured to compare with treated samples.

5.2.5 Modelling

The kinetics of dissolved toxin degradation has been shown to follow a first order decay model using CT of $KMnO_4$ (similar to Equation 2) (Rodriguez et al., 2007a). Similarly, the CT concept is also applied to cell inactivation in water treatment using the Chick-Watson equation (AWWA, 1999). If these assumptions are valid then process of toxin release and degradation may be considered as consecutive reactions (Equation 1), where the concentration of intracellular (A) and extracellular (B) toxin are described by Equations 2 and 3 (Jones, 1970; Zamyadi et al., 2013).

$$A \xrightarrow{k_i} B \xrightarrow{k_e} D \qquad \text{(Equation 1)}$$

$$A = A_0 e^{-k_i CT} \qquad \text{(Equation 2)}$$

$$B = B_0\, e^{-k_e CT} + A_0 (e^{-k_e CT} - e^{-k_i CT})/(1-k_e/k_i) \qquad \text{(Equation 3)}$$

Where CT = $KMnO_4$ exposure (calculated by integrating the residual $KMnO_4$ curve); A_0 = the initial intracellular toxin concentration; A = the concentration of intracellular toxin at a specified CT; B_0 = the initial dissolved toxin concentration; B = the concentration of dissolved toxin at a specified ct; k_i = the rate of intracellular toxin release; k_e = the rate of extracellular toxin degradation. D = the concentration of degraded toxin at specified CT. Model parameters were estimated by fitting to experimental data using the Solver add-in for Excel and a least squares objective function based on the intracellular and extracellular toxin concentrations. Nash–Sutcliffe model efficiency coefficient was calculated as performance metric for the model (Mayer and Butler, 1993) and describes the amount of the variance in the data that is explained by the model. For example, 0.90 means 90% of the varience in the data is explained by the model, 1 means 100% of the data explanined by the model. If the number less than 0, it means the data was not explained well by the model.

5.3 Results

An assay was performed to determine the baseline degradation of $KMnO_4$ under light with Milli-Q water and determine if there was oxidant demand from the media. Concentrations of 10 mg L^{-1} of $KMnO_4$ were separately added to Milli-Q water and ASM-1 media with no decay observed for 24 hours, at which time the experiments were terminated (Figure 5.1). In contrast, cyanobacteria exerted an oxidant demand and the $KMnO_4$ residual decreased steadily from 10 mg L^{-1} to 0.76 mg L^{-1} after 24 hours. A $KMnO_4$ residual was still present after 7 hours with treatments where 3 and 5 mg L^{-1} were added initially, no residual was present in the 1 mg L^{-1} $KMnO_4$ treatment after 2 hours (Figure 5.1).

The percentage of intact cells remained constant at 98% with $KMnO_4$ concentrations of 1 and 3 mg L^{-1} (Figure 5.2a). Approximately 26% of cells exposed to 5 mg L^{-1} $KMnO_4$ lost integrity within 6 hours. The highest concentration of $KMnO_4$ (10 mg L^{-1}) resulted in consistent cell lysis with no intact cells remaining after 6 hours. While the cell integrity was compromised at 5 mg L^{-1} $KMnO_4$, the cells were not fully destroyed and cell densities remained fairly constant after 6 hours of treatment, similar to the results for 1 and 3 mg L^{-1} $KMnO_4$ (Figure 5.2b). A decline in cell density was evident in the 10 mg L^{-1} $KMnO_4$ treatment.

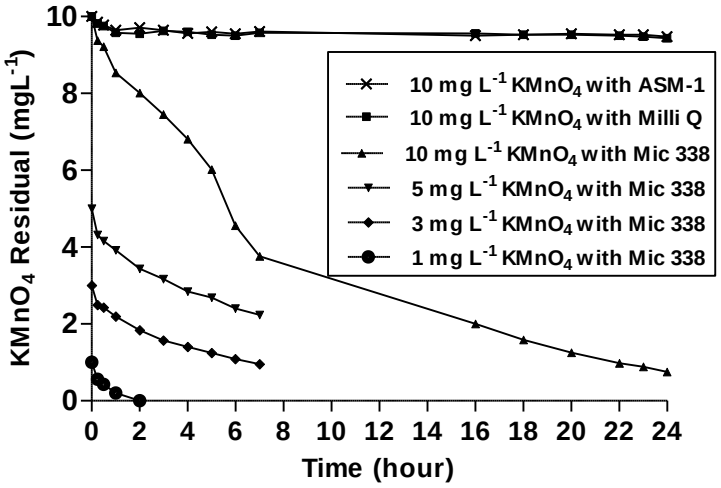

Figure 5.1 $KMnO_4$ residual of Microcystis aeruginosa cultures treated with $KMnO_4$ at 1, 3, 5 and 10 mg L^{-1}. $KMnO_4$ added to ASM-1 Medium and Milli Q water are included as control

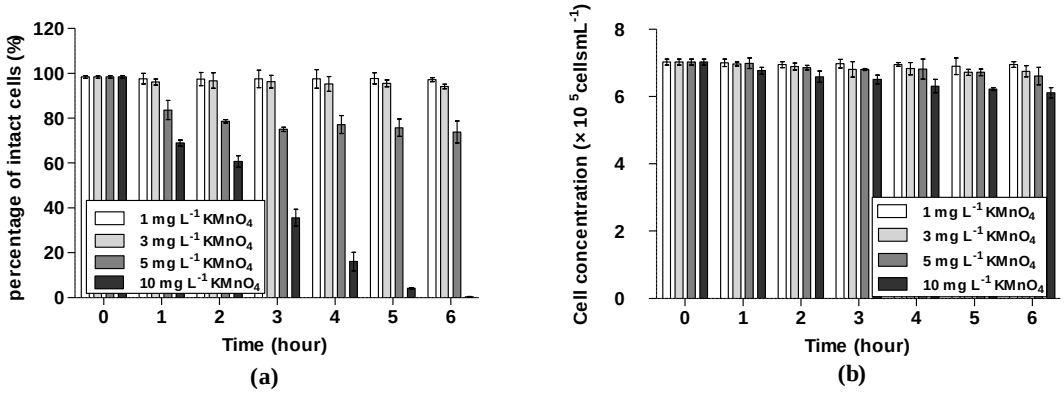

Figure 5.2 Microcystis aeruginosa cells treated with $KMnO_4$ at concentrations of 1, 3, 5 and 10 mg L^{-1} measured hourly for six hours. (a) Percentage of intact cells; (b) Cell densities. The initial cell numbers was approximately 7×10^5 cells mL-1. Error bars equal ± Standard Deviation.

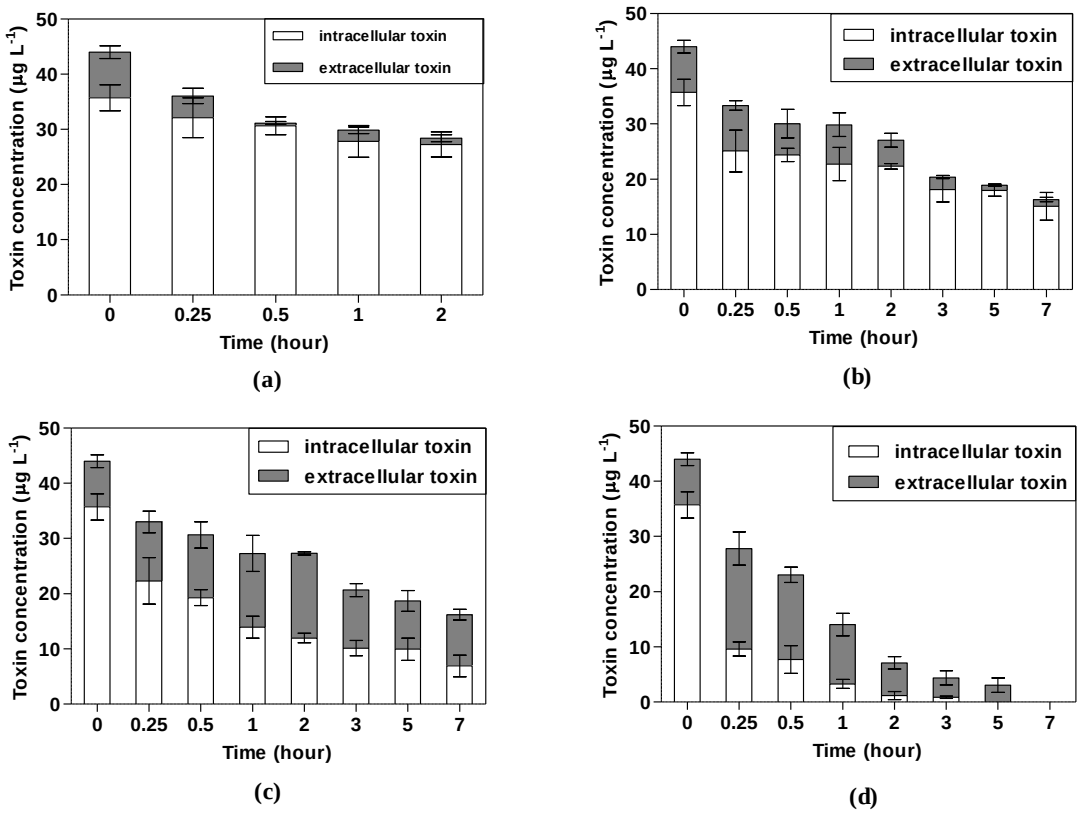

Figure 5.3 Intracellular and extracellular microcystin concentrations after treatment with $KMnO_4$ (up to 7 hours contact time) with $KMnO_4$ at concentrations of (a) 1 mg L^{-1}; (b) 3 mg L^{-1}; (c) 5 mg L^{-1}; and (d) 10 mg L^{-1}. Error bars equal ± Standard Deviation.

Toxin concentrations were measured for a reaction time of up to 7 hours except for the 1 mg L^{-1} $KMnO_4$ treatment as there was no $KMnO_4$ residual detected after 2 hours. The *M. aeruginosa* culture contained a total of 44 µg L^{-1} of microcystins with the majority proportion (81%) determined to be intracellular. The total toxin decreased with time for all cultures treated with $KMnO_4$ between 1 and 10 mg L^{-1}. A $KMnO_4$ concentration of 1 mg L^{-1} degraded 35% of the total toxin after 2 hours with a decrease of 24% and 87% of intracellular and extracellular toxin, respectively. There was no increase in intracellular toxin evident during the treatment (Figure 5.3a). A fairly consistent decrease of the intracellular and extracellular toxin was observed at 3 mg L^{-1} $KMnO_4$ with a final reduction of 58% and 85%, respectively (Figure 5.3b). The concentration of total toxin reduced to16 µg L^{-1} for both the 3 mg L^{-1} and 5 mg L^{-1} $KMnO_4$ treatments after 7 hours. Although the total toxin remaining after 7 hours of 5 mg L^{-1} $KMnO_4$ treatment was similar to 3 mg L^{-1}, the toxin release and oxidation process

was different. There was up to an 86% increase of extracellular toxin due to the leakage of intracellular toxin by 5 mg L^{-1} $KMnO_4$ oxidation after 2 hours. The extracellular toxin was degraded by following $KMnO_4$ oxidation to a final concentration of 9 μg L^{-1} after 7 hours (Figure 5.3c).

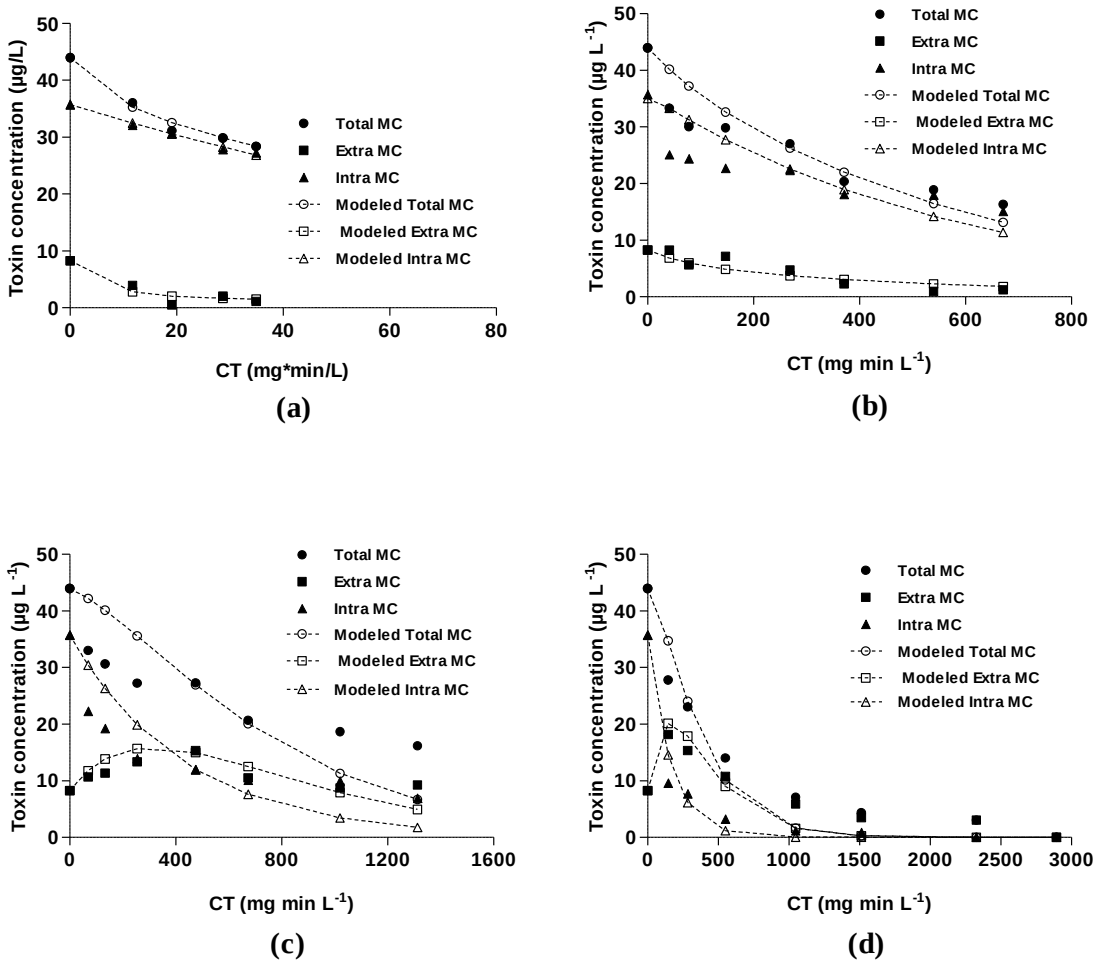

Figure 5.4 Comparison of experimental and model predicted results of Microcystin concentrations after treatment with $KMnO_4$ at initial concentrations of (a) 1 mg L^{-1}; (b) 3 mg L^{-1}; (c) 5 mg L^{-1}; and (d) 10 mg L^{-1}.

More rapid toxin oxidation was observed with the high concentration of $KMnO_4$ (10 mg L^{-1}). There was only 7 μg L^{-1} of total toxin remaining (a decrease of 84%) after 2 hours oxidation (Figure 5.3d). The release of intracellular toxin was observed after 0.25 hour resulting in a 120% increase in the extracellular toxin concentration. This corresponded to a reduction of 73% of the intracellular toxin. Simultaneously, a decline of 37% of the total toxin occurred

which indicated that the intracellular toxin was not only being released but was being degraded rapidly. Both of the intracellular and extracellular toxins were degraded and there was degradation of total toxin to below analytical detection after 7 hours $KMnO_4$ treatment.

The kinetics of cell lysis depends on the initial $KMnO_4$ concentration (Appendix C). For the lower $KMnO_4$ concentrations (1 and 3 mg L^{-1}), the percentage of intact cells were relatively stable even when the CT values increased. The cell lysis occurred between 0 and 674.7 mg min L^{-1} at an initial $KMnO_4$ concentration of 5 mg L^{-1}. However, the kinetics of cell rupture stopped even though the CT values continued to increase after the residual $KMnO_4$ was below 3 mg L^{-1}. These findings suggest there may be a threshold concentration (3 mg L^{-1}) of $KMnO_4$ that is required to lyse cyanobacterial cells. For 10 mg L^{-1} $KMnO_4$, the cell lysis increased with the increasing CT values and all the cells lost cell integrity at a CT value of 2642.6 mg min L^{-1}. The degradation of toxin progressed with different initial $KMnO_4$ concentrations in accordance with increasing CT values, ranging from 0 to 2892.4 mg min L^{-1} (Appendix C).

The results of toxin release and degradation were modelled by fitting the change of intracellular (k_i) and extracellular toxin (k_e) with CT using a first order model (Figure 5.4). The fitted k_i for initial $KMnO_4$ concentration as 1, 3, 5 and 10 mg L^{-1} is 0.00817, 0.00171, 0.00230 and 0.00618 L mg^{-1} min^{-1}, respectively. The fitted k_e for initial KMnO4 concentration as 1, 3, 5 and 10 mg L^{-1} is 0.156, 0.0122, 0.00250 and 0.00391 L mg^{-1} min^{-1}, respectively (Table 5.1). The results from 1, 3 and10 mg L^{-1} $KMnO_4$ experiments were explained well by the model while for 5 mg L^{-1} $KMnO_4$ the model did not fit data very well as indicated by a low Nash–Sutcliffe model efficiency (Table 5.1).

5.4 Discussion

For $KMnO_4$ to be applicable as a pre-treatment oxidant in the presence of cyanobacteria, it is necessary to understand how $KMnO_4$ affects cell integrity and toxin degradation. It was necessary to use *Microcystis aeruginosa* at a high density (7.0×10^5 cells mL^{-1}) to ensure sufficient toxin was present for analysis by HPLC in order to characterise toxin release and oxidation. *M. aeruginosa* cells exposed to low concentration of $KMnO_4$ (1 and 3 mg L^{-1})

maintained cell integrity which is in agreement with studies on other species of cyanobacteria (Chen and Yeh, 2005; Ho et al., 2009). It has been demonstrated that $KMnO_4$ can oxidise pure cyanobacterial toxin (Chen et al., 2005; Rodriguez et al., 2007a); however, this study has suggested that at low concentrations $KMnO_4$ was not only oxidising extracellular toxin (Figure 5.3a; 5.3b) but was also entering cells and oxidising intracellular toxin with cell integrity remaining otherwise intact (Sytox negative) (Figure 5.3a; 5.3b). Alternatively there could have been leakage of toxin which was oxidised extracellularly.

Table 5.1 The rate constants and evaluation of model performance.

$[KMnO_4]_0$ (mg L^{-1})	Nash–Sutcliffe Coefficient			ki ($L\ mg^{-1}\ min^{-1}$)	ke ($L\ mg^{-1}\ min^{-1}$)
	Total toxin	Extra toxin	Intra toxin		
1	0.98	0.90	0.99	0.00817	0.156
3	0.78	0.83	0.40	0.00171	0.0122
5	0.31	0.09	0.63	0.00230	0.00250
10	0.93	0.81	0.97	0.00618	0.00391

Significant cell lysis and release of intracellular toxin were evident as extracellular toxin concentration increased within 0.25 hour following $KMnO_4$ addition (10 mg L^{-1}). Futhermore total toxin was degraded after 7 hours. This finding contrasts that of Rositano (1996) who documented 42% microcystin remained after 8 hours treatment with 10 mg L^{-1} $KMnO_4$. Consequently, the result of Rositano (1996) suggests that $KMnO_4$ was unable to lyse the cells effectively and therefore could not decrease the intracellular toxin and total toxin efficiently. Although Lam et al. (1995) reported that 10 mg L^{-1} $KMnO_4$ released microcystin-LR from a bloom sample of *M. aeruginosa* and *Aphanizomenon* sp. which was subsequently oxidised, the observations were measured over 2 days, a much longer contact time than this study. This study has demonstrated that a high concentration of $KMnO_4$ can degrade toxin, but may present difficulties in a typical treatment plant with short contact time.

Many authors have found CT values useful to define the toxin degradation ability of oxidants. For instance, Daly et al (2007) documented microcystin degradation with chlorine CT values from 0 to 30 mg min L^{-1} using pure toxin in reservoir water. In this study, $KMnO_4$ was added to cyanobacterial cultures (as opposed to pure toxin) and required larger CT values to degrade toxins (Appendix C). Our results demonstrated that $KMnO_4$ can degrade microcystins at a large range of CT values in *Microcystis* cultures. However, the degradation rates observed in this study (Table 5.1), where cell lysis occurred (Sytox positive), were slower than published values for MC-LR in Milli-Q water (0.136 L mg^{-1} min^{-1}, Rodriguez et al., 2007a). This variation may be attributed to the competing reactions with other molecules from the lysed cyanobacterial cells. Daly et al. (2007) reported similar observations that the toxin degradation rates in *Microcystis* cultures were appreciably slower than in the absence of the cyanobacterial cells during chlorination.

The ability of $KMnO_4$ to oxidise toxin without significantly disrupting cyanobacterial cell integrity at low concentrations (Figure 5.2), suggests that it may be possible to use $KMnO_4$ as a pre-treatment agent without adversely impacting water quality. At a $KMnO_4$ concentration of 5 mg L^{-1} the majority (74%) of cells remained intact after 6 hours treatment; however, the concentration of extracellular toxin increased. To overcome this deficiency, the contact time would need to be increased to oxidise the released toxin. This would be dependent upon the concentration of residual $KMnO_4$. To avoid cell lysis and associated toxin release, the concentration of $KMnO_4$ should not exceed 3 mg L^{-1} which would be sufficient to remove low concentrations of Fe and Mn particularly when relatively short contact times are used, approximately 1 hour. However, a high $KMnO_4$ concentration (> 3 mg L^{-1}) may be necessary when large amounts of Mn and Fe are present. However, the contact time should be increased to oxidise the total toxins. Although most studies and treatment plants use a short contact time (~1 hour) for pre-oxidation using $KMnO_4$ (Carlson and Knocke, 1999; Roccaro et al., 2007; Chen and Yeh, 2005) there are examples where a longer contact time, up to 20 hours, has been applied (Liu et al., 2011).

This study has shown that a high concentration of $KMnO_4$, 10 mg L^{-1}, can completely oxidize microcystins in 7 hours which corresponded to a detectable residual after 24 hours. However, precise oxidant application would be required since a residual $KMnO_4$ of 0.05 mg L^{-1} or greater will result in a pink taint to the drinking water (Welch, 1963). For complete toxin

degradation, use of an alternative oxidant such as chlorine where a residual is not problematic may be a more suitable management option.

As an example the model has been applied to a scenario where $KMnO_4$ could be used as a pretreatment to remove soluble Mn and Fe from reservoir water. Historically the highest Mn and Fe concentrations in Myponga Reservoir (South Australia) between 1/10/2005 to 27/06/2011 were 0.077 and 0.361 mg L^{-1}, respectively. From theoretical equivalents the $KMnO_4$ concentration required for oxidation is 1.92 mg mg^{-1} Mn and 0.94 mg mg^{-1} Fe, so 0.49 mg L^{-1} is required to consume all Mn and Fe. At a pH of 7.5 the reaction time is estimated to be from 5-10 minutes (Kawamura, 2000). For the purposes of estimating the CT exposure, we assumed that the $KMnO_4$ residual would decay following a first order process over a 10 min reaction time (reaction complete after 10 half lives, k = 0.693 min^{-1}). The CT estimated from this curve was then used in the model to give the resultant soluble toxin concentration in the water following treatment. A range of k values were determined by fitting the experimental data for this study. Using the k values estimated in the 1 and 3 mg L^{-1} $KMnO_4$ experiments, there was no increase in soluble toxin concentration. For the k values in the 5 and 10 mg L^{-1} $KMnO_4$ experiments, extracellular toxin increased to 0.04 and 0.14 µg L^{-1}, respectively. These final increasing toxin concentrations are below 1.3 µg L^{-1} (Australian Drinking Water Guidelines, 2011). In this scenario a very high density of cells (5.67×10^6 cell mL^{-1}) would be needed in solution to increase of extracellular toxin to exceed the guideline value (1.3 µg L^{-1}).

This scenario demonstrates that at $KMnO_4$ concentrations on the scale used for Mn and Fe removal, $KMnO_4$ CT values are small compared to those required for undesirable levels of extracellular toxin to be released. Higher levels of Mn and Fe would require more $KMnO_4$ and therefore higher CT values. This model provides a useful tool for estimating cell lysis, toxin oxidation rates and ultimately the risk to water quality when $KMnO_4$ is used as a pretreatment oxidant to remove Mn and Fe in the reservoir when associated with cyanobacterial blooms.

5.5 Conclusions

$KMnO_4$ was shown to be an efficient oxidant to degrade microcystins, however, it can lyse cyanobacterial cells at concentrations > 3 mg L^{-1}. This study has suggests that the lower $KMnO_4$ concentrations (1 and 3 mg L^{-1}) may decrease both intracellular and extracellular toxin concentrations without lysing cells completely. Therefore, it should be feasible to use $KMnO_4$ pre-treatment at the lower concentrations to remove Fe and Mn when the cyanobacteria are also present. Higher $KMnO_4$ concentrations may be necessary when high concentrations of Mn and Fe prevail but care must be taken when cyanobacteria are present as intracellular metabolites are likely to be released. In this scenario it may be possible to extend contact times to degrade the released toxins or use activated carbon for toxin or taste and odour removal.

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Chapter 6. Conclusions and Recommendations

6.1 Summary

The presence of cyanobacteria and their harmful secondary metabolites in water bodies compromise water quality and pose significant risks to consumers. Chemical-based technologies for control of bloom-forming cyanobacteria and cyanotoxin removal have been considered as effective strategies to guarantee immediate access to drinking water and irrigation. However, chemical treatments may compromise membrane integrity of cyanobacteria cells resulting in a leakage of intracellular metabolites into surrounding waters. The preferred option is to remove toxins contained in whole cells when using conventional water treatment processes as they are ineffective in removing extracellular metabolites.

Various chemical compounds have been trialled for use as algicides or pre-treatment oxidants such as copper sulphate ($CuSO_4$) and chlorine. However, a comparative evaluation of these technologies and their impact on cyanobacterial cell rupture is limited. Chapter 2 of this thesis addressed this knowledge gap by assessing and comparing a wide range of algicides/oxidants for effective cyanobacteria control with an emphasis on how membrane integrity of cyanobacterial cells was disrupted during treatment. The combination of flow cytometry with SYTOX Green stain provided a rapid analysis to determine changes in membrane integrity of *M.aeruginosa* cells during $CuSO_4$, chlorine, potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2) and ozone treatments. The experiments detailed in Chapter 2 demonstrated that all the chemicals could impair cell integrity of *Microcystis aeruginosa* to varying degrees. Chlorine was the most effective chemical as it inactivated the majority of *M. aeruginosa* cells with a faster cell lysis rate than the other chemicals. $CuSO_4$ and H_2O_2 showed both algistat potential in preventing the growth of *M.aeruginosa* over the 7 day treatment as well as an algicidal activity.

All the chemicals including $CuSO_4$, H_2O_2, chlorine, ozone and $KMnO_4$ may be considered as algicides as they all showed activity against cyanobacterial cell integrity (Chapter 2). However, these technologies may also cause an increase in extracellular toxins due to cell lysis. Experiments presented in Chapter 3 assessed toxin degradation/oxidation in addition to

cell lysis and toxin release. These experiments explore MC release and degradation kinetics associated with cell lysis of *M. aeruginosa* exposed to various chemicals. Interestingly, H_2O_2 (10.2-102 mg L^{-1}), chlorine (3-5 mg L^{-1}) and $KMnO_4$ ($\leq$ 3 mg L^{-1}) resulted in a decrease in extracellular MCs during treatments. This toxin oxidation which occurred as toxins leaked from cells is beneficial as it reduced the total extracellular toxin concentration. In contrast, copper and ozone induced a noticeable increase in extracellular MCs which persisted in the culture medium. To better understand the interaction of these chemicals with cyanobacteria, a new model was developed and presented in Chapter 3. This model integrates the rates of cell lysis, toxin release and toxin degradation and provides a useful tool for reservoir or treatment plant operators to predict toxin concentration following different oxidant exposures.

With the demands on water resources increasing there is pressure to find alternative water sources to supplement traditional surface water and groundwater resources. Consequently wastewater is increasingly viewed as a resource that could be exploited for industrial or agricultural reuse. Alternative water sources present additional risks and challenges which need to be appropriately managed to ensure water quality and protection of human health. Wastewater often undergoes a stabilisation step in wastewater treatment lagoons to inactivate pathogens and reduce nitrogen concentrations. Cyanobacteria are one challenge that can present in waste-stabilisation lagoons. Chapter 4 of this thesis evaluated the effectiveness of the chemical technologies in treating cyanobacterial cells in wastewater. $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone were applied to *M. aeruginosa* cells in wastewater to determine cell inactivation and concomitant MC release and degradation. All the treatments were shown to inactivate cyanobacterial cells to varying degrees but there was typically a higher oxidant demand in waste water relative to treatments in ASM-1 culture medium (Chapter 2). The effectiveness of H_2O_2, chlorine, $KMnO_4$ and ozone on MC degradation was reduced due to insufficient oxidant residual. The rates of MC degradation in wastewater were considerably lower than ASM-1 culture medium for both chlorine and $KMnO_4$ treatments (Chapter 3). An increase of extracellular MCs was observed during chlorine treatment and a higher dose and longer contact time was needed to oxidise the resultant extracellular MCs. These findings suggest that oxidant doses and contact times need to be carefully assessed for the water of interest to account for variations in dissolved organic carbon and chemical oxidant demand.

$KMnO_4$ is recognised as a reliable pre-treatment oxidant and is primarily added to oxidise dissolved manganese (Mn) and iron (Fe) ions. Mn and Fe problems are commonly associated with thermal stratification in reservoirs and they frequently coincide with the presence of cyanobacteria. Results presented here have shown that $KMnO_4$ doses above 3 mg L^{-1} could impair cyanobacterial cell integrity resulting in release of intracellular toxins (Chapter 3 and 4). However, sometimes it is necessary to apply high doses of $KMnO_4$ when large amounts of Mn and Fe and cyanobacteria are simultaneously present. Chapter 5 provided a more detailed investigation of $KMnO_4$ to help water authorities better understand its use in such a situation in relation to cell integrity and the concomitant toxin release and toxin degradation. High $KMnO_4$ doses (10 mg L^{-1}) could oxidise the total MCs below analytical detection with adequate contact time, thus $KMnO_4$ could be a feasible pre-treatment for Mn and Fe removal when cyanobacteria are also present. More specifically, Chapter 5 tested a toxin kinetics model which was developed in Chapter 3 and Chapter 4 using different realistic scenarios involving cyanobacteria in the presence of Mn and Fe. Highest concentrations of Mn and Fe were selected from historical data collected from Myponga Reservoir (South Australia) to estimate $KMnO_4$ demands. The scenario showed that at $KMnO_4$ concentrations in the range used for Mn and Fe removal would not cause concomitant release of cyanobacterial toxins to undesirable levels. This strengthened confidence in the use of $KMnO_4$ as an effective pre-treatment oxidant to remove Mn and Fe in the reservoir in the presence of cyanobacterial blooms. The scenario also provided a good example of this model applied as a useful tool for assessing the risk to water quality when $KMnO_4$ is used as a water treatment chemical.

6.2 Recommendations

In conclusion, the work presented in this thesis has provided important scientific knowledge on the impacts of various chemicals on *M. aeruginosa* cells and their associated MCs. With these findings, water authorities can better tailor the use of chemical treatment to improve the management of water quality issues caused by cyanobacterail blooms. The application of $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone must be considered in combination with the different physical-chemical properties and cyanobacterial densities in particular water bodies. The benefits and negative impacts of each treatment (Table 1) also need be taken into account

for water authorities to find a balance when dealing with cyanobacteria issues. Recommendations for the use of each chemical are as follows:

- The application of $CuSO_4$ as algicide should be applied at the early stages of cyanobacterial blooms when cell densities are low to avoid high levels of extracellular toxins. It is still an option for impulsive cyanobacterial bloom control when immediate action is required, however, water use for irrigation and drinking water should be stopped for an extended period of time to ensure all extracellular toxin is removed. However, $CuSO_4$ acts as a broad spectrum biocide and has been shown to have adverse impacts on aquatic ecosystems and copper, as a heavy metal, will accumulate and thus it is not a sustainable option for cyanobacteria control.
- H_2O_2 shows potential as an algicide for use in either reservoirs or wastewater stabilization ponds due to its effectiveness of cyanobacteria control without adverse environmental impacts. Furthermore, H_2O_2 has the added benefit of also degrading both intracellular and extracellular toxins. However, care must be taken when using this chemical due to its strong oxidising ability.
- Chlorine is a feasible option as a pre-treatment oxidant in drinking water systems, even in the presence of high cyanobacteria numbers, providing sufficient chlorine residual and contact time are used. Proper dosing of chlorine is a safe barrier as a post-oxidation step in wastewater treatment processes to inactivate the breakthrough cyanobacterial cells and breakdown the cyanotoxins. However, water authorities need to balance its use against the potential for harmful by-products like trihalomethanes to form in the presence of dissolved organic compounds (DOC).
- $KMnO_4$ at lower doses is an appropriate pre-treatment option to remove Fe and Mn even when the cyanobacteria are present. Higher $KMnO_4$ doses may be necessary when high concentrations of Mn and Fe prevail but care must be taken when cyanobacteria are present as intracellular toxins are likely to be released. However, higher $KMnO_4$ doses can be used as a post-oxidation treatment to inactivate cyanobacterial cells but sufficient contact time is needed to ensure any released toxins are degraded.
- Ozone is unsuitable to apply as a pre-treatment process at high cyanobacterial cell densities due to significant release of toxins and the residual ozone, which is consumed by competing DOC resulting in incapability of the released toxin

degradation. As a post-disinfection process in wastewater treatment systems, ozone is effective in inactivating cyanobacterial cells however higher doses are recommended to degrade the resultant increase of extracellular toxins. Ozone is a relatively expensive chemical and has inherent complexity in operation both in water and wastewater treatment systems.

Table 1 Advantages and disadvantages of each chemical on cyanobacteria treatment

treatment	advantages	disadvantages
$CuSO_4$	- economical - effective - easy to apply	- heavy metal accumulation - toxic to other organisms - increase of extracellular toxins
H_2O_2	- innocuous by-products - selectively toxic to cyanobacteria - oxidise toxins - no increase in extracellular toxins	- risky application in its pure form
chlorine	- economical - effective - oxidise toxins - no increase in extracellular toxins when residual available	- by-products like trihalomethanes
$KMnO_4$	- lower doses do not lyse cells - oxidise toxins - effective	- over dosing inducing pink colour
ozone	- effective - oxidise toxins	- high cost, - difficult to apply, - bromate formation, - increase of extracellular toxins

6.3 Future Direction

Technologies assessed in this study including $CuSO_4$, H_2O_2, chlorine, $KMnO_4$ and ozone were all shown to inactivate high densities of *M.aeruginos* cells. However, whether these chemical compounds have similar activities towards other cyanobacteria species are uncertain.

Therefore, further work is required to systematically evaluate various treatments on other common species such as *Anabaena*, *Cylindrospermopsis* and *Planktothrix* (*Oscillatoria*) and their respective toxins.

Although H_2O_2, chlorine, $KMnO_4$ degraded dissolved toxins during treatments, however, whether there is an increase of other dissolved metabolites (e.g. taste and odour) is unknown. Hence, further investigations are needed to determine the activity and kinetics of these chemicals against other cyanobacterial metabolites.

Each of the chemicals tested have advantages and disadvantages which need to be assessed for a particular situation. Detailed investigations including both laboratory based and small scale trials are required before full-scale applications of these chemicals are attempted. The laboratory based experiments conducted in this study have provided important information on the applicability and efficacy of various chemical treatments on cyanobacterial cells and the toxins they produce. This information will be of significant benefit to water authorities as they develop their own treatment regimes to control both cyanobacteria and the toxins they produce.

Appendix A

The cell integrity data for for algicides/oxidants in Microcystis aeruginosa cultures

Chlorine			$KMnO_4$			H_2O_2			$CuSO_4$			Ozone		
$[Chlorine]_0$ (mg L^{-1})	time (min)	intact cells (%)	$[KMnO_4]_0$ (mg L^{-1})	time (min)	intact cells (%)	$[H_2O_2]_0$ (mg L^{-1})	time (day)	intact cells (%)	$[CuSO_4]_0$ (mg L^{-1})	time (day)	intact cells (%)	$[Ozone]_0$ (mg L^{-1})	time (min)	intact cells (%)
5	0	98.8	1	0	98.4	0	0	98.2	0	0	99.5	0	5	99.5
	1	11.6		60	97.7		1	96.0		1	99.0	2	5	49.2
	2	2.1		120	97.4		2	96.3		2	98.1	4	5	28.8
	5	0.2	3	0	98.4		3	94.0		3	98.2	6	5	8.6
	10	0.2		60	96.1		7	90.7		7	93.7			
	20	0.2		120	96.8	10.2	0	98.2	0.5	0	99.5			
	30	0.3		180	96.4		1	86.1		1	94.6			
	60	1.3		240	95.3		2	15.6		2	80.1			
4	0	98.8		300	95.6		3	22.3		3	66.9			
	1	4.8		360	94.2		7	49.9		7	63.6			
	2	18.2	5	0	98.4	51	0	98.2	1	0	99.5			
	5	1.8		60	83.7		1	45.7		1	32.8			
	10	0.1		120	78.6		2	30.7		2	8.0			
	20	0.1		180	75.1		3	35.1		3	8.5			
	30	0.1		240	77.2		7	82.3		7	51.5			
	60	0.1		300	75.8	102	0	98.2	1.5	0	99.5			
3	0	98.8		360	73.8		1	50.9		1	2.2			
	1	2.9	10	0	98.4		2	7.1		2	0.1			
	2	7.2		60	69.0		3	19.2		3	0.0			
	5	3.2		120	60.8		7	72.6		7	0.3			
	10	0.9		180	35.6									
	20	0.3		240	16.1									
	30	0.3		300	4.2									
	60	0.1		360	0.5									

Appendix B

The concentration and objective in water treatment processes of $KMnO_4$.

[$KMnO_4$]	Application	Reference
0.85 mg L^{-1}	removal of turbidity, chemical oxygen demand (CODMn), and color	Liu et al., 2011
1.0 -1.5 mg L^{-1}	Microcystins	Rodriguez et al., 2007a
1-1.25mg L^{-1}	Microcystins	Rodriguez et al., 2007b
0.5 mg L^{-1}	Anabaena circinalis	Ho et al., 2009
1-3 mg L^{-1}	green algal, Chodatella sp.	Chen and Yeh, 2005
	Fe and Mn	Ellis et al., 2000
1.0 mg L^{-1}	nitrogenous disinfection by-products (N-DBPs)	Chu et al., 2011
2-18 mg L^{-1}	Antibiotics	Hu et al., 2010
0.15-0.7 mg L^{-1}	Mn	Carlson and Knocke, 1999
6.7 mg L^{-1}	Mn	Lee et al., 2003
1.74 and 3.47 mg L^{-1}	Mn	Roccaro et al., 2007
5 -10 mg L^{-1}	Microcystins	Chen et al., 2005
10.1 mg L^{-1}	Odors and tastes	Cherry, 1962

Appendix C

Summary of $KMnO_4$ exposure (CT) and *Microcystis aeruginosa* cell lysis and total microcystin concentrations during $KMnO_4$ treatment.

$[KMnO4]_0$ (mg L^{-1})	$[KMnO4]_t$ (mg L^{-1})	time (min)	CT (mg min L^{-1})	intact cells (%)	[toxin] (µg L^{-1})
1	1.0	0	0	98.44 ±0.55	44.0 ±1.1
1	0.6	15	11.7		36.1 ± 1.4
1	0.4	30	19.1		31.2 ± 0.3
1	0.2	60	28.7	97.65 ±2.29	29.8 ± 0.6
1	0.0	120	34.9	97.42 ±2.89	28.4 ± 0.6
3	3.0	0	0	98.44 ±0.55	44 ± 1.1
3	2.5	15	41.2		33.3 ± 0.9
3	2.4	30	78.2		30.1 ± 2.6
3	2.2	60	147.5	96.14 ±1.29	29.9 ± 2.2
3	1.8	120	268.5	96.79 ±3.45	27.1 ± 1.3
3	1.6	180	370.7	96.38 ±2.78	20.4 ± 0.3
3	1.4	240	460.0	95.31 ±3.28	
3	1.2	300	539.7	95.59 ±1.52	18.9 ± 0.2
3	1.1	360	609.8	94.17 ±1.02	
3	1.0	420	671.0		16.3 ± 0.4
5	5.0	0	0	98.44 ±0.55	44 ± 1.1
5	4.3	15	69.9		33 ± 2.0
5	4.2	30	133.5		30.6 ± 2.4
5	3.9	60	254.7	83.69 ±4.28	27.3 ± 3.3
5	3.4	120	475.5	78.61 ±0.81	27.3 ± 0.3
5	3.2	180	673.7	75.05 ±1.02	20.7 ± 1.2
5	2.8	240	854.2	77.19 ±4.01	
5	2.7	300	1020.3	75.79 ±3.88	18.7 ±

					1.9
5	2.4	360	1172.9	73.83 ± 4.89	
5	2.2	420	1312.1		16.2 ± 1.0
10	10.0	0	0	98.44 ± 0.55	44 ± 1.1
10	9.4	15	145.3		27.8 ± 3.0
10	9.2	30	284.8		23.1 ± 1.4
10	8.5	60	551.1	68.95 ± 1.29	14 ± 2.0
10	8.0	120	1047.7	60.75 ± 2.45	7.1 ± 1.1
10	7.5	180	1511.9	35.58 ± 3.78	4.4 ± 1.3
10	6.8	240	1940.0	16.13 ± 4.13	
10	6.0	300	2325.2	4.16 ± 0.27	3.1 ± 1.3
10	4.6	360	2642.6	0.47 ± 0.06	
10	3.8	420	2892.4		0.0