CHLORINE DIOXIDE IMPACTS ON *MICROCYSTIS AERUGINOSA* CELL STRESS, GROWTH AND MICROCYSTIN PRODUCTION

by

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Abstract

Cyanobacteria and their toxins severely contaminate water sources and pose a danger to the health of humans and animals. A way to disinfect water is needed to create safe drinking water without creating additional hazards**.** The use of disinfectants was hypothesised to create cellular stress. Cellular stress was hypothesized to trigger increased production of cyanotoxins.

Here, chlorine dioxide (ClO_2) was tested to inhibit a toxin-producing strain of *Microcystis aeruginosa*. Through a series of experiments, ClO_2 was exposed to *M. aeruginosa*, and the production of microcystin was monitored. The first experiment, batches of *M. aeruginosa* were exposed to ClO_2 (0-10 mg/L) for 30 minites. Chlorophyll *a* and microcystin concentrations were lowered at higher levels of disinfection, suggesting that ClO_2 can treat both *M. aeruginosa* and its toxins.

The second investigation focused on the effective treatment of ClO_2 to viability of cells, stress and microcystin production. *M. aeruginosa*, during four stages of population growth, were exposed to 0-5 mg/L ClO_2 for various contact times (3 hours to 3 days) Population growth was measured by chlorophyll *a* and optical density**.** Viability was assayed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and electrolyte leakage (conductivity). Cellular stress was measured by malondialdehyde (MDA) lipid peroxidation, catalase (CAT), superoxide dismutase (SOD), and carotenoids (CARs) levels. Microcystin was quantified by enzyme-linked immunosorbent assay (ELISA). ClO_2 affected the viability of cells**.** In addition, increased electrolyte leakage illustrated the membrane damage that directly related to the deterioration of cells in every growth phase. Early populations (or early blooms) were more responsive to ClO_2 inhibition than late-growth populations. In term of stress, ClO_2 treatments at 1.5 mg/L induced oxidative stress. SOD activity increased in all three quartiles of population growth**.** Carotenoids and chlorophyll *a* became oxidized**.** The residual carotenoids may react to quench stress induced by ClO_2. However, catalase activity increased to scavenge reactive oxygen species. The experiment suggests that crucial parameters for evaluation should include the viability of cells and metabolic activity. The study suggests relationships between ClO_2

treatment and stress as well as microcystins production exist. However, no relationship was observed between stress and microcystins production. Further, low population densities exhibited a greater response to the disinfection, and had the greatest ClO_2 related cyanotoxin release. These findings have implications that the timing and dosage of disinfection are very important in treating water with cyanobacteria to avoid the additional release of cyanotoxins and additional risk to human and animal health.

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List of Abbreviations and Symbols

ATP	Adenosine triphosphate
AWWA	American Water Works Association
CARs	Carotenoids
$CAR^{\bullet+}$	Radical cations
$CAR^{\bullet-}$	Radical anions
CAT	Catalase
Chl *a*	Chlorophyll *a*
Cl^-	Chloride ion
ClO_2	Chlorine dioxide
ClO_2^-	Chlorite
ClO_3^-	Chlorate
Ca^{2+}	Calcium II ion
CO_2	Carbon dioxide
Cu	Cupper
DBCM	tribromomethane
DBPs	disinfection by-products
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Fe	Iron
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
M. aeruginosa	*Microcystis aeruginosa*
MDA	malondialdehyde
MW	Molecular weight

Mn	Manganese
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NADP+	Nicotinamide adenine dinucleotide phosphate,
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
NOM	natural organic matter
NCCs	Nonfluorescent Chl-catabolites
O_2^-	Superoxide radicals
$^1O_2$	Singlet oxygen
OD	Optical density
ppb	Parts per billion
ppm	Parts per million
PSI	Photosynthesis system I
PSII	Photosynthesis system II
p-value	Probability value
r	Correlation coefficient
Ref	Reference
ROS	Reactive oxygen species
$RO^{\bullet}$	Free radical
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THMs	Trihalomethanes
TISTR	Thailand Institue Scientific and Technological Research

UV	Ultra violet
U.S. EPA	United State Environmental Protection Agency
WHO	World Health Organization
w/v	Weight by volume
Zn	Zinc

Chapter 1. INTRODUCTION

1.1 Background

Water resource contamination by cyanobacteria, or blue green algae as they are commonly known, is a widespread problem around the world (Walter et at., 2018). Countries with tropical climates are particularly affected because their warm, wet climates allow cyanobacteria to proliferate. In Thailand, key reservoirs used to supply drinking water are contaminated by cyanobacteria (Somdee et al., 2013) making it an urgent problem that requires attention. In conventional water treatment, the typical steps for treating cyanobacteria are two treatments with disinfecting chemicals. At intake, a high dose is applied, followed by a lower dose as disinfection after all of the other treatment steps. Conventional disinfection with chlorine can produce harmful by-products such as trihalomethanes (THMs). Chlorine dioxide (ClO_2) was introduced as an alternative disinfectant because it does not react with organic matter to form THMs associated with chlorine disinfection. ClO_2 has become widely adopted around the world for drinking water disinfection because of these benefits, but it is not yet commonly used in Thailand. The effectiveness of ClO_2 to inhibit cyanobacteria and, in particular, its sub-lethal effects have not been widely studied. This thesis addresses that knowledge gap.

1.2 Cyanobacteria Characteristics and Their Effects on Water Supply

Cyanobacteria are primitive organisms that have existed for almost 3.5 million years (Schopf, 1993). They are photoautotrophic organisms containing important pigments such as chlorophyll, especially chlorophyll *a*, for use in oxygenic photosynthesis. Blooms of cyanobacteria in eutrophic water can cause serious deterioration in the quality of water, such as the undesired visual appearance of scum forming; depletion of oxygen in the water; poor taste and odour; and toxins production (Lopez et al., 2008). Mowe et al (2007) reported that cyanobacteria proliferate in the tropical regions of Australia, Africa and Asia. *Microcystis aeruginosa* (*M. aeruginosa*) is the dominant species, comprising of both non-toxic and toxic strains. These harmful photoautotrophs have been found ubiquitously at or above 25 ^{o} C, which is their suitable temperature for growth (Paerl and Huisman, 2008). Furthermore, their presence and proliferation

become serious matters for public health when toxic strains grow in the reservoirs used as feedstocks for municipal water supply.

At present, climate change is a critical problem affecting the hydrological system (Metz et al, 2007). Eutrophic conditions are anticipated to become more common, favouring cyanobacteria proliferation (Elliott, 2012; Paerl and Paul, 2012). Elevation of water temperatures encourages further cyanobacteria bloom (Roberts and Zohary, 1987; Butterwick et al., 2005). Researchers had broadly studied effects of tropical and subtropical water sources on cyanobacterial proliferation in eutrophic water (Velzeboer et al., 2000; Komárková and Tavera, 2003; Havens et al.2003; Abrantes et at., 2006). Nevertheless, the blooming of harmful cyanobacteria is the biological complexity including many factors to provide growth (Heisler et al, 2008).

1.3 Cyanobacteria Proliferation in Thailand's Drinking Water Sources

As a result of its latitude and coastal position in Southeast Asia, Thailand has a tropical climate with hot weather and high humidity. Like the rest of the region, it has three main seasons: a rainy monsoon season (mid-May to mid-October); a mild, dry winter (mid-October to mid-February); and a hot, dry summer (mid-February to mid-May), consistent with the rest of Southeast Asia (Yokoi and Satoma 2005). These conditions promote cyanobacterial blooms. Anthropogenic nitrogen and phosphorus releases into water sources further promote cyanobacteria proliferation. Table 1.1 presents observations of cyanobacteria and cyanotoxins in Thai water sources. Many of these studies were conducted in the Northern regions of Thailand, but observations were noted elsewhere, too. The most frequent blooming strains are *M. aeruginosa*, *M. aeruginosa (*Kützing), and *Cynkindrospemopsis raciborskii* (Wolosz), all of which can produce cyanotoxins.

Table1.1: The observation of cyanobacteria and cyanotoxins in water resource of Thailand.

The location of cyanobacterial survey	Strains	Type of toxins	Amount of toxins	Impacts	References
1. Kang Krachan dam, Petchaburi (Western Thailand) Mae Kwang dam, Chiang Mai (Northern Thailand) and a duck husbandry pond in Bangkok	- *M. aeruginosa* -Pleurocapsalean filamentous cyanobactrium	Microcystins	0.76-0.81 mg/g dry cell	- Deteriorate quality of water source - Induce primary liver cancer (Ueno et al., 1996)	(Mahakhant et al., 1998)
2. Mae Kuang Udomtara dam, Chiang Mai (Northern Thailand)	-*M. aeruginosa (*Kützing) -*Cylindrospermopsis raciborskii* (Wolosz)	Microcystins	0.77-0.81mg/L dry cell	-Decline quality of water supply - Cause of hepatotoxin in human, livestock and animals (Carmichael, 1995)	(Peerapornpisal et al., 2000)
3. Bang Phra Reservoir, Chon Buri (Eastern Thailand)	- *M. aeruginosa*	Microcystin LR Microcystin YR Microcystin RR	0.046 µg/L 0.25 µg/L 1.29 µg/L	-Potential health risk to human, livestocks and aquatic animals	(Wang et al., 2002)
4. Two fresh water giant prawn farms in Teung District, Chiang Rai (Northern Thailand)	- *M. aeruginosa* - *M. Wesenbergii*	Microcystins	2.2- 9.4 µg/L	-Risk to bioaccumulation in prawn	(Prommana et al., 2006)
5. Four recreational reservoirs, Khon Kaen (Northeastern Thailand)	-*Microcystis* sp. - *Cylindrospermopsis* sp.	Microcystins Cylindrospermopsin	0.913 µg/L 0.463 µg/L	- Deteriorate water quality -Mortality of aquatic animals	(Somdee et al., 2013)
6. Phayao lake and Ing river (Northern Thailand	-*Microcystis* spp. -*Oscillatoria* spp. -*Cylindrospermopsis raciborskii* (Wolosz) -*Anabaena* spp.	Microcytins LR	Up to 7.56 µ/L	-Contaminant and deteriorate water quality - Bioaccumulation in fihesh	(Whangchai et al., 2013)
7. Freshwater ponds in ten provides, north, northeast and central Thailand	*Planktothricoides raciborskii* Suda and Watanabe	2-methylisoborneol (MIB)	Minimum	-Deteriorate quality of water resource -Affect aquatic animals and humans	(Tawong, 2017)

Figure 1.1 illustrates the proliferation of cyanobacteria in the reservoir of a water treatment plant in Bangkok, Thailand.

Figure 1.1: Algal blooms in the reservoir of Wang of Wang Noi Power Plant in Bangkok, Thailand (13 June 2011)

1.4 *Microcystis aeruginosa* and Cyanotoxins Production

M. aeruginosa is the most prominent species of cyanobacteria. Their morphologies are unicellular spherical configurations with Gram-negative cell walls (Hou, el al., 2008). Within the cells are gas vesicles that provide a source of buoyancy that supports survival of the cell by enabling photosynthesis and movement within the environment. Their buoyancy is valuable in seeking nutrient sources and avoiding harmful conditions, including vertical migration to find a suitable depth for intensive light. (Ibelings et at., 1991; Walsby et al, 1997 and Paerl and Paul, 2012). These reasons are why they are tolerant of extreme environments such as hot springs, freezing temperatures, and the deep-sea, and flourish in fresh water.

Proliferation of *M. aeruginosa* can produce considerable intracellular and extracellular toxins. Cyanotoxins released into a water source can cause harmful effects to aquatic life as well as livestock, domestic animals, or human beings unlucky enough to ingest or come into contact with the affected water. The World Health Organization set a provisional guideline for the safe limit of 1.0 µg/L for microcystin-LR, the most toxic of microcystins contained within drinking water (WHO, 1998). Bishop et al. (1959) were

the first group of the researchers to isolate microcystins from *M. aeruginosa*. They identified that these toxins are cyclic peptide hepatotoxins. Subsequently, these toxins were called by an abbreviated name, MCYST, by Konst et al. (1965) and Carmichael et al. (1992).

The general structure of MCYST, shown in Figure 1.2, is cyclic heptapeptides of cyclo (-D-Ala1-L-X2-D-MeAsp3-L-Z4-Adda5-D-Glu6-Mdha7). At the position of X and Z are variable L-amino acids. Adda is a C-20 β-amino acid unique to cyanobacteria (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-pheyldeca-4, 6-dienoic acid. D-MeAsp is D-erythro-β-methylaspartic acid. Mdha is N-methyldehydroalanine. These are non-proteinogenic amino acids produced by toxin-producing strains of *M. aeruginosa* (Mazur-Marzec et al. 2010). The toxic moiety of MCYST is produced by the Adda and Mdha configuration (Chorus and Bartram, 1999). The mechanism of this toxin was identified by MacKintosh et al (1990) to inhibit serine/threonine-specific protein phosphatases 1 and 2A (PP-1 and PP2A) within the liver.

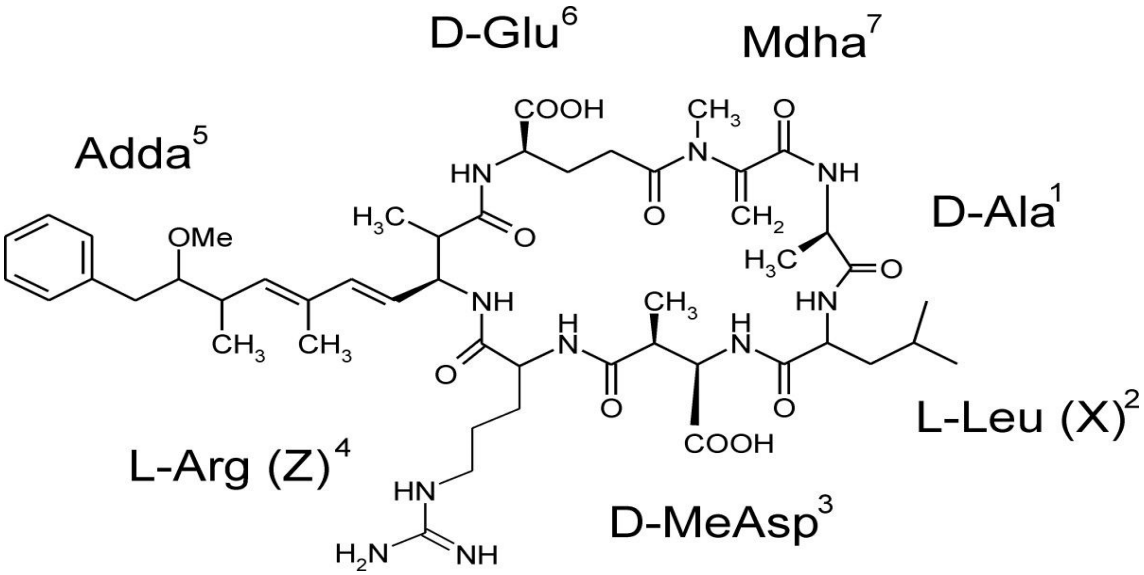


Figure 1.2: The general chemical structure of MCYST Fewer et al., 2007)

Cyanobacteria blooms consist of toxic and non-toxic strains that cannot always be classified. However, cyanotoxins production is facilitated by many conditions. Researchers indicate

that conditions such as intense light, high nutrient (nitrogen and phosphorous) loading, and elevated levels of potentially toxic elements stimulated toxin production (Sivonen and Jones, 1999). Cyanotoxins production has been hypothesised and investigated as a potential competitive advantage when cyanobacteria encounter attacks from invaders. Another hypothesis on cyanotoxin production investigated their potential roles as physiological aids for cellular benefits such as homeostasis, efficacy of photosynthesis, and encouragement of growth rate (Holland and Kinnear, 2013).

This research investigates the use of chlorine dioxide (ClO_2) disinfectant as cyanobacteria inhibiter and crucial factor that induces oxidative stress. Oxidative stress becomes apparent by increasing levels of reactive oxygen species (ROS) that consist of radicals and free radicals. ClO_2 treatment is believed to be an inducing agent to oxidative stress as an abiotic stress factor that is similar to a bactericide (Lu Z., el at., 2016). There are several intracellular mechanisms of cyanobacteria dealing with stress (Prosecká, 2010). For example, cells manipulate imbalances of redox reaction by enzymatic activity. In addition, antioxidant systems of cells that scavenge excess ROS in order to reduce oxidative stress. Furthermore, in response to stress conditions, cyanobacteria may be stimulated to produce cyanotoxins against injurious situations (Holland and Kinnear, 2013). More details about stress response will be illustrated in the Literature Review (Chapter 2).

1.5 Aims and Objectives

This research aims to investigate ClO_2 disinfection of cyanobacteria in water and the cyanotoxins that these cyanobacteria may release. Chlorine dioxide (ClO_2) was selected as the disinfectant due to its strong oxidizing properties, wide range of working pH (3-10), effectiveness to remove microorganisms compared to other chlorine-based compounds, and widespread use around the world. Although ClO_2 usage is widespread in water treatment processes, its effects on cyanobacteria and cyanotoxins formation are not well known. This research addresses this important knowledge gap.

The objectives are:

1. Identify a suitable strain of *M. aeruginosa* from Thailand that produces intracellular and extracellular cyanotoxins;

2. Develop assays to quantify *M. aeruginosa* health, stress, and intracellular and extracellular cyanotoxin formation;

3. Evaluate the effects of ClO_2 on:

    - Cyanobacteria stress and lethality (toxicology) and

    - Release of cyanotoxins; and

4. Develop recommendations for suitable drinking water pre-treatment & disinfection conditions, including timing and doses, to address cyanobacteria proliferation and cyanotoxin formation.

Key hypotheses that were evaluated were:

- ClO_2 will stress cyanobacteria by oxidizing amino acids within cyanobacterial cells.

- Stress of cyanobacteria after contact with ClO_2 will cause bacteria to release cyanotoxins as an adaptation for survival.

- Cyanotoxin release will be influenced by cellular growth phase (young populations through mature populations), producing differences in cyanatoxin concentration and interactions with the disinfectant.

These aim, objectives, and hypotheses were evaluated with a programme of laboratory work. Chapter 2 contains a literature review on the current body of knowledge on cyanobacteria, ClO_2 disinfection, and cyanotoxins formation. Chapter 3 presents common methodologies implemented across subsequent chapters. Chapter 4 examines the toxicity of *M. aeruginosa* strains and effectiveness of ClO_2 treatment of ClO_2. Chapter 5 investigates the effect of sublethal ClO_2 doses on the viability of cells and stress response in four different growth phases. Chapter 6 evaluates the relationships between ClO_2 treatment, cellular health, stress, and cyanotoxins formation in every growth phase. Finally, conclusions and recommendations are presented in Chapter 7.

Chapter 2. LITERATURE REVIEW

2.1 Cyanobacteria and Cyanotoxins

Cyanobacteria are prokaryotes, classified into the division *Cyanophyta* of Kingdom Monera. In 1962, blue-green algae were classified under the bacterial genera (Stanier and Van Niel, 1962). Subsequently, Buchanan and Gibbons (1974) substituted "blue-green algae" with "cyanobacteria" for the formal name. Figure 2.1 illustrates the structure of cyanobacteria and the detail of cyanobacteria structure is clearly explained in Table 2.1. Cyanobacteria do not contain any membrane-bound organelles. Additionally, these organisms exhibit haploid cells, containing a single set of unpaired chromosomes. Their reproduction occurs by a fission system, and their DNA (Deoxyribonucleic acid) is coherent with a group of proteins (histone) in the eukaryotic cell. Furthermore, the cyanobacterial cell wall lacks cellulose (Clark, 1998).

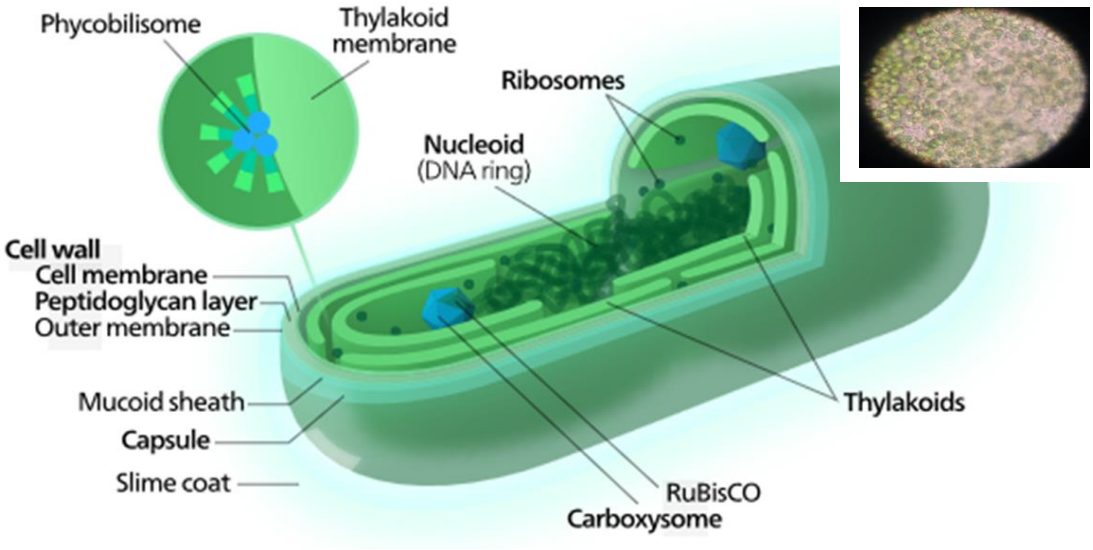


Figure 2.1: Intracellular physiology of a blue green algae cell (Kelvinsong, 2013)

Table 2.1: General structure of Cyanobacteria

Characteristics	Topographies
Morphology	1. Single cell; the unicellular cell that diving by binary fission
	2. Colonial to dive by multiple fission
	3. Filamentous with heterocysts for nitrogen fixation
	4. Filamentous with nonheteroccystous structure
	5. Branching filamentous structure
Size	0.5-1 µm, until 40 µm
Cell wall	Gram-negative bacteria with thick layer of peptidoglycan
Photosynthetic pigment	Chlorophyll a, Phycobilins and Carotenoids
Toxins	Cyanotoxins mainly Microcystins, Nodularins, Anatoxin and Cylindrospermopsins
Others useful structure	1. Gas vesicles for buoyancy to find the suitable environmental growth
	2. Heterocysts to fix nitrogen for the hetercystous cyanobacteria
	3. Cyanophycin; the nitrogen source when a cell encounters the lack of nitrogen from the environment
	4. Akinetes; the thick outer walls to protect cell from unsuitable growth condition such as drought, cold and longtime of darkness

Source: Biology of Microorganisms 13th ed. (Madgan et al., 2012)

Naturally, cyanobacteria utilize chlorophyll to absorb energy from sunlight and convert it into chemical energy via the electron transport chain. This results in producing carbohydrates from water and CO_2, liberating molecules of O_2 into the environment. For this reason, the disruption and removal of chlorophyll substantially inhibits the viability of *M. aeruginosa*. Fundamentally, the structure of all general chlorophylls constitutes a porphyrin head that forms a complex of four heterocyclic pyrrole rings, which chelate magnesium within their center. One pyrrole ringside is esterified with a

long chain hydrocarbon (called phytol chain). This long hydrophobic tail anchors the insoluble molecule proteins in the thylakoid membrane of the chloroplast, as shown in Figure 2.2.

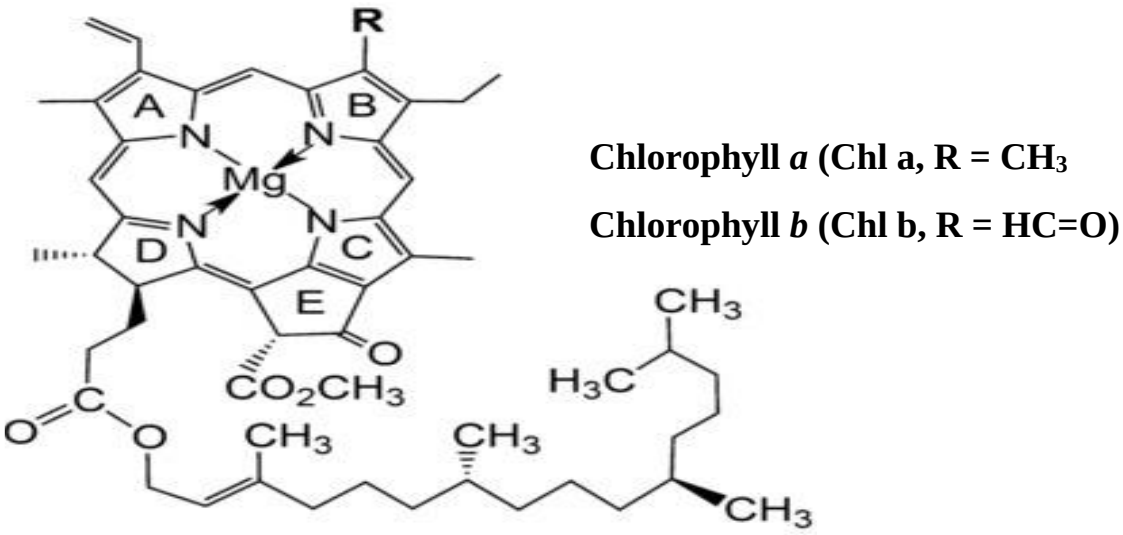


Figure 2.2: Chlorophyll structure. The different structural variation of chlorophylls (Kräutler, 2016)

Cyanobacteria are oxygenic, phototrophic bacteria, containing the photosynthetic pigments required to convert solar energy into chemical energy (Adenosine triphosphate; ATP) and release oxygen back to the environment. Chlorophyll *a* is a crucial pigment, which is green in color, because it absorbs red and blue light preferentially and reflects green light. This pigment can absorb a particularly high amount of 680 nm wavelength light. Generally, we see the green color when it blooms, hence why it was named "Blue-Green Algae". The general morphology of cyanobacteria is similar to other bacteria, with the addition of chlorophyll. Furthermore, the photosynthetic system of cyanobacteria contains other important pigments, such as carotenoids, phycocyanin, and allophycocyanin (Govindjee and Mohanty, 1972; Govindjee and Braun, 1974; Papageorgiou, 1996; Govindjee, 1999). Uniquely, photosynthesis and respiration are combined in the same compartment; the thylakoid membrane (Vermaas, 2001).

Consequently, cyanobacteria have evolved to survive in extreme environments, and are considered to resemble primitive creatures that first released oxygen into the earth atmosphere (Friend, 1999). Additionally, explorations have shown that cyanobacteria survive and proliferate in diverse conditions, such as hot springs, temporarily frozen ponds, deep marine environments, deserts, volcanoes, as well as in freshwater (Bold, 1985; Dvornyk and Nevo, 2003).

The report published in 1878 was described the occurrence of harmful thick green scum (blue-green algae) in the estuary of Murray River, Australia. Animals were reported to have died after drinking this heavily contaminated water (Francis, 1878). Since then, reports have shown that 25-75 % of cyanobacteria strains produce harmful cyanotoxins (Bláha and Maršálek, 2009) that cause ailments and fatalities for humans, livestock, domestic animals, and wildlife (Carmicheal, 2001; Chorus, 2001; Bláhová et al., 2007; 2008 and Codd et al., 2005). Cyanobacteria include various strains such as *Microcystis, Anabaena, Oscillatoria* and *Nodularia*, many of which are capable of producing and releasing cyanotoxins into the environment (Codd and Poon, 1988; Carmichael, 1992 and Rinehart et al., 1994).

Studies have shown that *Microcystis* species, including both toxic and non-toxic strains, are more proliferative in tropical lakes than in more temperate reservoirs (Te and Gin 2011). Mackintosh et al (1990) showed that most toxic strains of *Microcystis* can generate microcystins. These cyanotoxins are cyclic, water-soluble peptides, contained in the cyanobacterial cell. They can be released to contaminate the environment by an intact cell or by cell lysis (Harada, 1996). These harmful secondary compounds cause illness and fatalities in human beings and animals who consume water contaminated with cyanotoxins. *M. aeruginosa* is the species most frequently observed in eutrophication (Watanabe and Oishi, 1985). This toxic strain is known to produce cyanotoxins, especially microcystins.

Microcystins are cyclic heptapetides that are potently toxic, exposure to which is reported to increase the risk of cancer and death (Carmichael, 1994; Dawson, 1998). For instance, in Brazil, it was reported the numerous people died due to ingestion of water contaminated with cyanotoxins, which caused liver deterioration and eventually led to death (Jochimsen, et al., 1998). Accumulation of microcystins has been identified

in aquatic animals, such as the sea snail (Zhang et al., 2007), potentially offering another vector for human contact with these dangerous substances. Moreover, low concentrations of microcystins in drinking water have been associated with cancerous tumours in the liver and intestinal system in people who drink water contaminated with cyanotoxins (Martínez Hernandez et al., 2009). An example of a cyanobacteria bloom in Thailand is shown in Figure 1.1. The ill health effects associated with various cyanotoxins are listed in Table 2.2.

Table 2.2: Cyanobacteria and cyanotoxins, including the effects of toxicity with human, animals, and livestocks

Toxigenic genera	Cyanotoxins	Chemical structure	Mechanism of toxicity
Microcystis, Anabaena, Planktothrix (Os cillatoria), *Nostoc, Hapalosiphon, Anabae nopsis,*	Microcystins	Cyclic heptapetides	Hepatotoxin, Liver toxic to block protein phosphatases cause haemorrhaging of the liver, tumor promotor and cancer
Cylindrospermopsis raciborskii, Aphanizomenon, Umezakia	Cylindrospermopsin	Alkaloids	Hepatotoxins, Inhibition of protein synthesis substantial cumulative toxicity such as liver, kidneys, spleen, lung and intestine.
Nodularia spumigena	Nodularin	Cyclic heptapetides	Hepatotoxins, liver toxic, similarly with microcystins function to cumulate toxicity
Anabaena, Planktothrix (Oscillatoria), Aphanizomenon Cylindrospermum	Anatoxin-a (alkaloid)	Alkaloid (Tropane-related alkaloids)	Neurotoxins to depolarize acetylcholine receptors and to lead respiration arrest
Anabaena, Aphanizomenon	Anatoxin-a(s)(unique organophosphate)	Guanidine methyl phosphate	Neurotoxins to inhibit acetycholinesterase and breakdown acetylecholine
Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis	Saxitoxins	Carbonate alkaloids	Neurotoxins to block Sodium channel
Lyngbya, Schizothrix, Planktothrix (Oscillatoria)	Aplysiatoxins	Polyketides	Dermatotoxins (irritantants) and inflammation
All general cyanobacteria	Lyngbyatoxin-a and Aplysiatoxin	Alkaloids	Dermatotoxins (irritantants), inflammation and cytotoxins
All general cyanobacteria	Lipopolysaccharides	Lipopoly-saccharides	Endotoxins (irritants), inflammation and gastrointestinal irritants
All general cyanobacteria	B-Methylamino-L-alamine (BMAA)	Amino Acid	Neurotoxins to disorder neurodegenerative systems

Sources: Turner et al., 1990; Kuiper-Goodman et al., 1999; Sivonen and Jones, 1999; Carmichael, 2001; Bláha et al., 2009.

2.1.1 Environmental conditions influence cyanotoxins production in cyanobacteria

Several factors affect cyanotoxins production. Water sources may contain non-toxic and toxic strains; however, the microscope cannot differentiate the toxicity of cyanobacteria with the same species. Laboratory studies have evaluated differences and effects of environmental conditions effects on cyanotoxins production, including light intensities; nutrients and trace element effects (Kaebernick and Neilan, 2001); and water conditions such as temperatures (Kaebernick and Neilan, 2001; Neilan, et al., 2013).

Cyanotoxins concentrations have shown correlation with light intensities. Utkilen and Gjølme (1995) demonstrated that lower toxin concentration corresponded with low intensive light at 2-20 µmol photons m^{-2} s^{-1} and higher concentration corresponded with light intensity at 20-142 µmol photons m^{-2} s^{-1}. In addition, increasing intensive light together with increased iron uptake resulted in high cyanotoxins production. Oppositely, low iron concentration had a stronger association with low cell proliferation, although it was beneficial effect with high microcystins level (Lukač, and Aegerter, 1993). In addition, different light intensities seem to affect the isoforms of microcystins production (Tonk et at., 2005).

Effects of nutrients such as phosphorous, nitrogen and trace elements exhibit an important role with cyanobacterial growth and toxin production. Phosphorus has been identified as a crucial nutrient affecting proliferation and cyanotoxins production. Sivonen and Jones (1999) showed that low phosphorus level resulted in decreased cyanotoxins concentration whereas Oh et al (2000) showed a different observation with high microcystins concentration per dry weight under phosphorus limitation. Nitrogen has shown different effects with non-nitrogen and nitrogen fixating strains of cyanobacteria. In conditions with low nitrogen, nitrogen-fixing strains are capable of increasing cyanotoxins (Lehtimaki, et al., 1997). In contrast, non-fixating strains had the highest cyanotoxins concentrations in conditions containing high nitrogen levels (Sivonen and Jones, 1999). Nitrogen conditions can influence gene expression and microcystins production (Sevilla, et al., 2010). Although abundant nitrogen supports

cells growth, it does not correlate with microcystin biosynthesis gene transcription and toxin formation.

Temperature effects on cyanotoxins concentrations have been investigated in varying temperature ranges. Results have shown the suitable temperature to raise the high level of cyanotoxins that are between 18-25 ˚C (Sivonen and Jones 1999). In addition, there was correlation between low temperature and phosphorus limitation with increased toxin concentration with *Alexandrium* sp (Anderson, et al., 1990). Also, a relationship has been observed between changing temperatures and cyanotoxins formation (Rapala, et al., 1997). Microcystins-LR is synthesized by *Anabaena* spp. much more than microcystins-RR at temperatures below 25˚C comparing with higher temperature (Rapala, et al., 1997). In addition, *Microcystis* spp. also synthesized microcystin-LR more than microcystin-RR under conditions of phosphorus limitation and low intensive light (Oh, et al., 2000; Rapala, et al., 1997).

From the investigation, the highest rates of toxin biosynthesis of cyanotoxins correlated with optimum growth (Orr and Jones, 1998). Relationships were observed between cell division of *M. aeruginosa* and microcystins production under nitrogen and phosphorus-limited conditions (Orr and Jones, 1998; Oh, et al., 2000; Neilan, et al., 2013).

2.1.2 Epidemiological evidence of cyanotoxins

The phenomena of cyanobacteria blooms and cyanotoxins production have been reported around the world (Codd et al., 2005). In tropical and subtropical areas, *Microcystis* spp. was the most abundant genus that proliferates in eutrophic conditions particularly in Asia, Africa and Central America (Mowe, et al., 2015). Other strains such as *Cylindrospermopsis* spp. were prevalent in tropical zones of Australia and Brazil. Cyanobacteria blooms deteriorate drinking water quality, reduce aesthetics, and negatively affect recreation and tourism. Increasing cyanobacteria bloom strongly correlates with increased toxins that have serious impacts on human health, aquatic life, and animals including livestock. Cyanotoxins that are secondary metabolites of cyanobacterial production are classified by several criteria. Generally, their modes of action are focused on hepatotoxins, neurotoxins, dermatotoxins and cytotoxins (Carmichael,

1992; Codd et al., 2005). The well-known cyanotoxins are the cyclic heptapeptide hepatotoxins called microcystins. Their toxicity cause liver failure and tumour promotion in mammals (Svirčev, et al., 2017). Microcystins also have potent toxicity to the neurological system (Hu et al., 2016) and reproductive system (Chen et al., 2016). They also have genotoxicity which may cause carcinogenicity (Zegura, 2016).

World Health Organization has warned about toxicity from cyanobacteria for many years (WHO, 1998; Codd et al., 2005). However, the epidemiological aspect of cyanotoxins is greatly valuable comparing between toxicity and health effects. In Australia, animal deaths and human ailments have been associated with exposure to cyanobacteria since the 1870s (Codd et al., 1994; Falconer, 2001). In 1979, there was a significant incident in Palm Island off the Queensland coast where 138 children and 10 adults had a hepatoenteritis-like illness (EPA, 2015). In this outbreak, investigators postulated that patients consumed water treated by copper sulphate (algicide) to inhibit cyanobacteria and, therefore, the illness was associated with algicide effects and cyanotoxins release. Another study in Southeastern Australia (1992-1994) reported association of pregnancy, consumption of contaminated water, and birth ailments in 32,700 newborns from 156 communities (Pilotto, et al., 1999). Associations were found between cyanobacteria exposure in the first trimester and percentages of low and very low fetus development. In the last trimester, association was not clear.

In China, the association between microcystins and risk of cancer was first examined in the 1980s (Yu, 1989). During 1981 to 1984, a mortality rate of 100 per 100,000 from primary liver cancer was observed in people who consumed water collected from ponds and ditches that likely contained cyanobacteria and microcystins (Ueno et al., 1996). Students (248) who lived near Taihu Lake were exposed to microcystins for a long time via drinking water contaminanted by cyanobacteria. They experienced negative effects on liver function (Chen et al., 2002).

In 1988, eutrophication of water sources in Thailand were evaluated for cyanobacteria and cyanotoxins. Toxic strains contained, on average, microcystins levels of 0.7-0.8 mg/g dried weight (Mahakhant, et al., 1998). Monitoring indicated that cyanobacteria and cyanotoxins deteriorated water quality and posed hazards to humans, aquatic life, agriculture and animals including livestock. However, there are no records of associated

mortality. However, the epidemiological effects of cyanotoxins upon human health and animal deaths may be many. Increasing population results in increasing anthropogenic contributions of pollution to water sources that affect cyanobacteria proliferation. Cyanobacteria and cyanotoxins levels depend on nutrients (nitrogen, phosphorus and trace elements); water condition as temperature and circulation; watershed effects; and light intensities. The longer-term effects of climate change may include further eutrophication with increased cyanobacterial blooms.

2.2 Water Treatment and Disinfection

Water treatment is practiced to improve the safety and quality of water for people to use. By following water quality standards, such as U.S. EPA (United States Environmental Protection Agency), best practices for water treatment can be established. Treatment is carried out to limit and, where possible, eliminate contamination of water supplies by inorganic or organic matter, including microorganisms, before distributing to the population. Water treatment unit operations are outlined in the remainder of this section.

Coagulation is a chemical process used to remove particles (flocs) from sediment in reservoirs or other raw water storage facilities. A coagulant material, such as alum, iron salt, or other metal salts, is added to raw water and mixed turbulently for 20-30 minutes. Colloids and other negatively charged particles will destabilize and bind with the coagulant, forming heavy particles in a process known as flocculation.

In sedimentation, the next treatment step, no mixing is performed. Flocs are left to settle while water is passed slowly through a sedimentation tank. This physical process employs gravitational force to precipitate flocs. The typical time for this process is approximately one hour. Sedimentation is followed by filtration, where water is passed through filter media to remove any remaining suspended solid particles.

Finally, disinfection is performed. This process is an important and quite complex task of trying to eliminate microorganism that may cause ailments, such as diarrhoea, and other negative health effects for consumers.

Approved storage and distribution systems are then used to maintain the cleanliness standards of drinking water. Figure 2.3 shows the practical process of water treatment to

treat a cyanobacteria-contaminated water source and how both cyanobacteria and cyanotoxins may proceed through the water treatment unit operations if not fully removed upon intake. Typical treatments for cyanobacteria use high concentration of ClO_2 for initial treatment (>1.4 mg/L) and lower concentration ClO_2 in the final disinfection step (≤1.4 mg/L) (Gates, 1998, Chen and Regli, 2002). For intake water, many recommendations have not preferred to use high ClO_2 doses for treatment because of possible formation of chlorate and chlorite by-products (Westrick, et al., 2010; Kull et al., 2004, 2006). Balance must be found between effective inactivation to reduce cyanobacteria and cyanotoxins with generation of regulated contaminants (Westrick, et al., 2010).

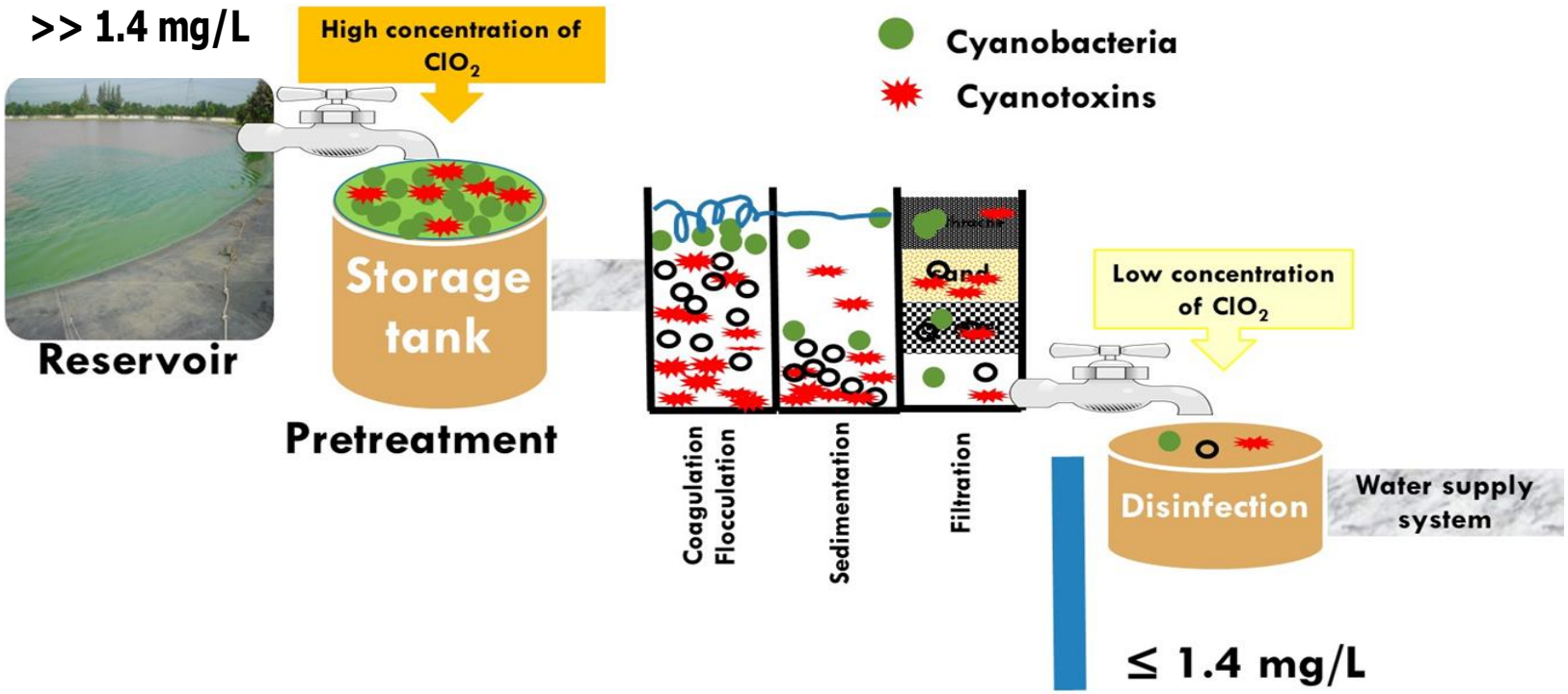


Figure 2.3: Schematic diagram of how a typical water treatment process is adapted to address a cyanobacteria bloom in a reservoir, including fate of cyanobacteria and cyanotoxins

2.3 Disinfection system

The objective of water disinfection is to inhibit growth and wherever possible eliminate waterborne microorganisms that may otherwise cause disease (Maris, 1995). Factors thought to impact the efficacy of disinfectants include pH, salinity, contamination with organic and inorganic matter, and the morphology of microorganisms themselves (Bellon-Fontaione and Cerf, 1988; Dauphin and Darbord, 1988; and Russell 1983). The mechanisms of disinfection are listed in Table 2.3.

Table 2.3: The main potential targets of disinfectants (Maris, 1995)

The bacterial target to attack	
The external membrane	To crack the chemical bond of phospholipids and lipopolysaccharides
The cell wall	To deteriorate rigidity structure especially the water solution disinfectants
The cytoplasmic membrane	To disturb the transport membrane system
The metabolic activity	To impact with the chemical energy within cell (eg. ATP)
The cytoplasm and nucleus	To cause abnormal chromosome function
The bacterial spores (the impermeable structure to consist with dipicolinic acid)	To destabilize with the strong oxidizing disinfectants such as chlorine, chlorine dioxide and hydrogen peroxide

Furthermore, Sorlini (2004) suggested that there are several other crucial factors that must be considered before designing an effective disinfection system:

- The quality of raw water: the quantity of microorganisms, chemicals (i.e. hardness), pH, turbidity, and heavy metals.
- Potable water quality requirements.
- The toxicity of the disinfectant how to manage its use.
- The types and amounts of disinfectant by-products.
- Budget, construction, operating, and maintenance costs.

Water treatment manual guidelines (U.S. EPA, 1999; Crittenden, et al., 2012) outline practical disinfection kinetics for the efficient disposal of microorganisms, and protection of human health. However, many formulations can be used, depending on the convenience of the maintainer. For example, Chick's law, described by Dr. Dame Harriette Chick, provides a basic first-principle method to eliminate microorganisms using chemicals for disinfection. This hypothesis was first referred to as first-order kinetics (Chick, 1908). Subsequently, Chick's Law was developed and applied by Dr. Herbert Watson, and then became known as the Chick-Watson Equation. This model is currently used some instances for water sanitation. However, the most popular kinetic method, due to its ease of use and maintenance, is known as C t- values, which is based on the concentration of disinfectant and time to inactivate or disinfect microorganisms. This kinetic was firstly practiced by the U.S. EPA to remove pathogens in water treatment plants. In this approach, the following criteria are controlled to optimise the efficacy of disinfection (U.S. EPA, 1999):

- Decomposition time of the disinfectant, especially oxidizing chemicals such as chlorine, chlorine dioxide, and hydrogen peroxide.
- Temperature influence on C t-values.
- Equal distribution of disinfectant in the water supply, which may require a process of additive mixing to disinfect evenly.

Methods of disinfection applied in the treatment of household water supplies should ensure to inactivate pathogenic microorganisms. There are many chemicals and types of disinfection systems that can inhibit growth of pathogenic organisms. However, the most appropriate method for a given situation depends on various conditions related to the quality of raw water and the environment where it is contained. Selection of the optimum process ought to considerate the final result of drinking water quality as well. The advantages and disadvantages of common disinfectants are listed in Table 2.4. At present, disinfection is primarily aimed at inactivating waterborne diseases. In addition, several other factors influence the choice of disinfection process including cost, maintenance, and complexity of the system. The control of disinfection by products is also a crucial factor for consideration when developing a water treatment system.

Table 2.4: Comparison of ordinary Disinfections applied in water treatment plants to improve drinking water quality

Disinfectants	Chlorine	Chloramine	Chlorine dioxide	Ozone	UV light
The used form on site	Solid, Liquid or Gas	Liquid	Liquid	8-7 kWh/kg O_3	5-140 mW•s/cm^2
Influence of pH (disinfection)	Less effective at high pH	Less effective at high pH	Wide rage pH 3-10	Wide range pH (suitable 6-9)	Wide range pH (suitable 6-9)
Disposal; soluble iron, manganese and sulfides	***	***	***	***	Not suitable
Disposal; color, test, and odor	*** but more dosage cause the test and odor	**	***	***	**
Inhibit pathogens and vegetable such as algae	***	**not work for Giardia and Cryptosporidium	***	***	*** not work for Giardia and Cryptosporidium
Removal biofilm	0	*	***	0	0
Create disinfection by-products	THMs, HAAs, and others	DBPs	Chlorite and Chlorate	Bromines, Aldehyde, and ketones	DBPs
Corrosive (with distribution system	**	**	0	0	0
Cost; equipment, chemicals, and maintenance	*	*	**	***	*** suitable for small scale
Complexity	*	*	**	***	**

*** = Very good, ** = Moderate, * = few, scant, and 0 = incapable. **Sources**: U.S. EPA, 1999; Lin et at., 1998; Radziminski et al., 2002; Zhang, 2009.

2.4 Chlorine Dioxide (ClO_2)

In the 1950's, the biocidal capability of chlorine dioxide (ClO_2) was discovered and it was found to be particularly potent at high pH. This was later introduced more generally as a disinfectant for drinking water because it produces fewer by-products than chlorine and other chlorine compounds. The general properties of ClO_2 are listed in Table 2.5.

Table 2.5: Physical and chemical properties of chlorine dioxide

Properties	Chlorine dioxide
CAS registry number	10049-04-4
Molecular formula	ClO_2
Molecular weight	67.46
Melting point, (°c)	-59
Boiling point, (°c)	11
Water solubility, g/L	3.0 at 25°
Specific gravity	1.642 at 0°

Source: Budavari et al., 1989.

ClO_2 is effective as a primary disinfectant at low concentrations. It is highly effective in controlling waterborne pathogens such as viruses (Junli et al., 1997) while minimizing halogenated disinfection by-products (Gray, 2014). One application of ClO_2 in drinking water treatment has been to control tastes and odours associated with algae and decaying vegetation. It is particularly effective at destroying taste and odour producing phenolic compounds. ClO_2 is a strong oxidant that can also be used to control levels of iron and manganese. ClO_2 reacts with the soluble forms of iron and manganese to form precipitates that can be removed through sedimentation and

filtration. Furthermore, it is highly soluble in water (particularly at low temperatures). Compared to chlorine, ClO_2 is a more effective disinfectant across a broader pH range (roughly between 3 and 10) (Gates, 1998). Although ClO_2 is an excellent oxidizing agent to disinfect water borne pathogens and particulates, it can generate potentially by-products of chlorite and chlorate that would be consumed in drinking water. To protect from these compounds, ClO_2 dosing for pre-oxidation of surface water plants is restricted to 1 to 1.4 mg/L, to decrease the formation of chlorite (ClO_2^-) and chlorate (ClO_3^-) (Gates, 1998, Chen and Regli, 2002). In addition, U.S. EPA (2010) set a residual disinfection concentration of ClO_2 at 0.8 mg/L to prevent over-production of chlorite by-product. The U.S. EPA's maximum concentration level of ClO_2^- is 1 mg/L (U.S. EPA, 2010). Chlorite may be associated with hemolysis and nervous system, particularly in infant and children. (WHO, 2005). In Europe, the guideline for ClO_2 concentration to disinfect water distribution systems is set at 0.05 to 0.1 mg/L (Gates, 1998). However, these only typical guidelines must be considered to control the quality of drinking water. In practice, the quantity of ClO_2 for pre or post disinfection and water distribution systems depends on the quality of raw water, concentrations of microorganism, efficacy of water treatment, and the quality of effluent water before distribution.

ClO_2 is a yellowish-green gas, which is highly soluble in aqueous solutions up to 20 g/L (Sharma and Sohn, 2012). ClO_2 effectively oxidizes across a broad range of pH values (3 to 10); it is also considered to exhibit stable and selective oxidization (Sharma and Sohn, 2012).In water treatment, ClO_2 has the advantage of being a strong disinfectant, but not forming THMs or oxidizing bromide to bromate. This is because chlorine dioxide exists in a + 4 oxidation state, compared with the +1 state of free chlorine. This means that chlorine dioxide has different pathways for disinfection and formation of by-products when used in drinking water treatment. Consequently, chlorine dioxide does not produce significant levels of halogenated organic by-products. The oxidizing properties of ClO_2 are shown in Equations 2.1, 2.2 and 2.3.

$$ClO_2 + e^- \rightleftharpoons ClO_2^- \qquad E^\circ = 0.950V \qquad (2.1)$$

$$ClO_2 + 4e^- + 4H+ \rightleftharpoons Cl^- + 2H_2O \qquad E^\circ = 0.936V \qquad (2.2)$$

$$ClO_2 + 5e^- + 4H+ \rightleftharpoons Cl^- + 2H_2O \qquad E^\circ = 1.510V \qquad (2.3)$$

Additionally, ClO_2 may decompose to chlorate (ClO_3^-) and chlorite (ClO_2^-) via three different possible pathways (Odeh et al, 2002). The three decomposition pathways of ClO_2 are shown in Figure 2.4, Figure 2.5 and Figure 2.6.

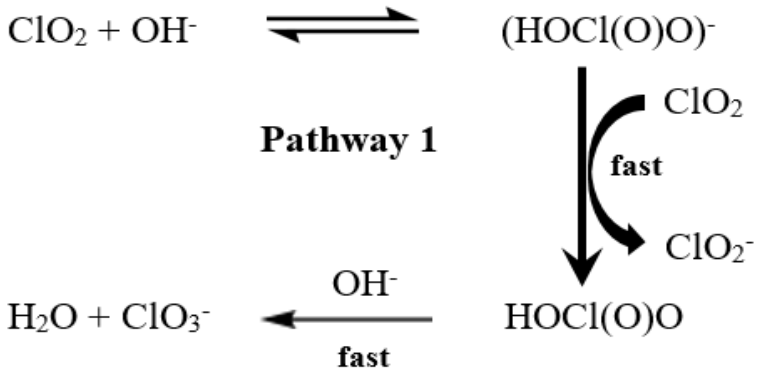


Figure 2.4: Pathway 1 is the first-order kinetic reaction of decay of ClO_2 in aqueous solution. The significant product is chlorate (ClO_3^-)

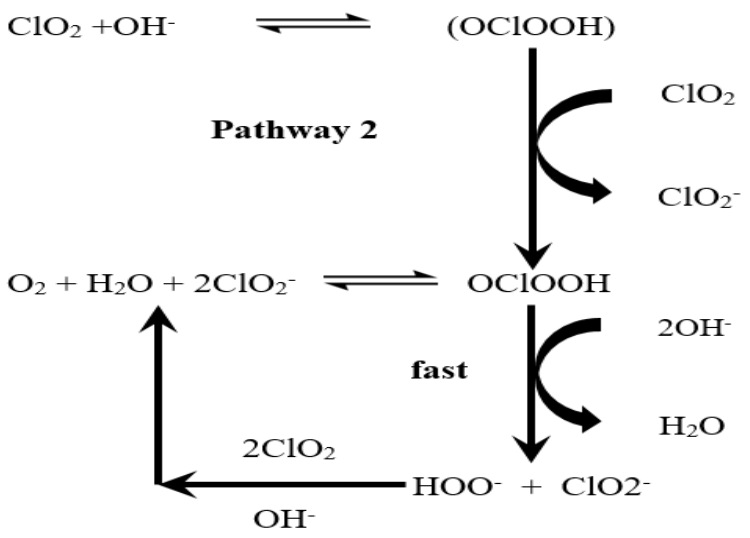


Figure 2.5: Pathway 2 is also a first-order decomposition of ClO_2, in which there are two steps of reaction to form chlorite (ClO_2^-)

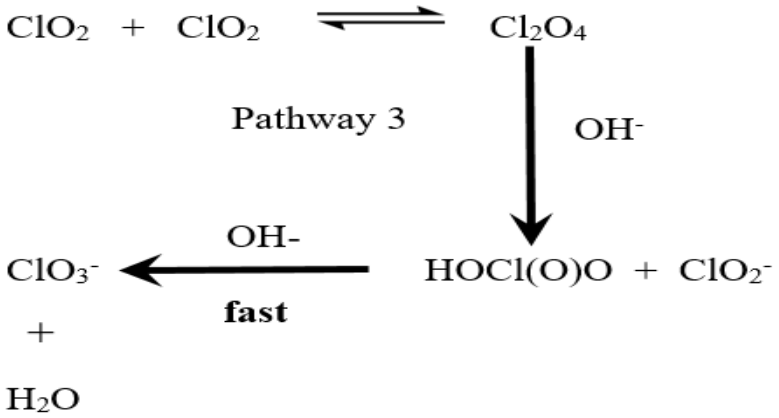


Figure 2.6: Pathway 3 is a second-order of kinetic decomposition of ClO_2 in aqueous solution. This pathway can generate two kinds of by-products: ClO_2^- and ClO_3^-

Furthermore, information from water treatment with low concentrations of ClO_2 suggests that it generates a greater proportion of ClO_2^- than ClO_3^- (Odeh, et al., 2002).

More specifically, it has been estimated that decomposition of ClO_2 produces approximately 50 to 70 % ClO_2^- and 30 to 50 % ClO_3^- and Cl^- (Werdehoff and Singer, 1987).

2.5 ClO_2 and Treatment of Cyanobacteria and Cyanotoxins

Evidence indicates that ClO_2 is more efficient than chlorine and chlorine compounds to inhibit water-born diseases (Metcalf and Eddy, 2003). Moreover, the main advantage of ClO_2 over chlorine is that it does not produce the same types or amounts of harmful by-products such as THMs while reacting with organic matter contained in raw water (McVeigh et al., 1985; Lykins and Griese, 1986; Gordon and Bubnis, 1995, and Long et al., 1997). Additionally, reports indicate that there are reductions of chloroform and other THMs when using ClO_2 to treat water compared with Cl_2 (Richardson et al., 1994; Li et al., 1996 and Richardson et al., 2003).

Natural raw water contains various humic substances, of which amino acids are an important component (Thurman, 2012; Chinn and Barrett 1999, 2000). These organic contaminants in surface water have been broadly characterised to include free amino acids, peptides, and proteins, found in levels ranging from ng/L to mg/L (Lytle and Perdue, 1981; Duguet et al., 1988; Chinn and Barrett, 1999, 2000). In general, Figure 2.7 exhibits amino acids that are foundational building-blocks of cells. They are bound by peptide bonds to create long chain peptides; polypeptides, and to make up the entire primary structure of simple proteins (Abdelmoez, et al., 2007). Amino acids exhibit common patterns, such as RCH (NH_3^+) COOH, where R groups can be aliphatic, aromatic, or sulfur moiety (Sharma and Soln, 2012). Amino acids containing sulfur or aromatic rings were determined to be the most reactive towards ClO_2. Cystine with an -SH group, the most nucleophilic group studied, was the most reactive amino acids with ClO_2 (Sharma and Soln, 2012). Additionally, previous research reported that an organosulfur compound (R-SH; thiol group) in biologicals was oxidised by ClO_2 to sulphoxide or sulphone group ($RSO_2Ŕ$), and this reaction caused a permanent biochemical change (Roller et al., 1979).

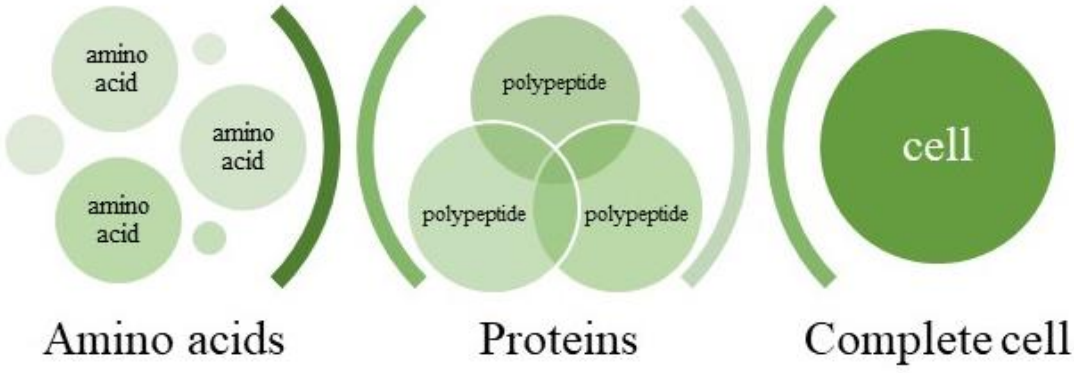


Figure 2.7: Cell formation by the grouping of amino acids such as glycine, proline, histidine, cysteine, tyrosine, glutathione, etc. They are gathered together by α-carbon L-isomers to form proteins and cells (Alberts et al., 1994)

Algal cells have been identified in various morphologies, containing many different organic compounds; proteins and polysaccharides (Fimmen et al., 2008). In consequence, they are vulnerable to oxidization by ClO_2. Accordingly, cyanobacteria are classified as a bacterial species, containing photosynthetic pigments, redox reactions can be established by ClO_2 within the cells, causing damage that can lead to cell death. The toxic strains of cyanobacteria can spontaneously produce secondary metabolites, including cyclic peptides, such as microcystins (Stewart et al., 2008). These secondary metabolites can also be oxidized by ClO_2. In addition, examination of cyanobacteria treatment by oxidizing agents, such as pyrogallol, to inhibit micocystins, suggest that effective treatment concerns inactivate protein function (Spencer et al., 1988). This also includes disturbance of electron transport chains in the photosynthesis system (Leu et al., 2002; Dziga et al., 2007), and causing oxidation damage to polyphenol, thereby inhibiting algal growth (Nakai et al., 2001). Similarly, the functions of herbicides directly involve increased formation of reactive oxygen species (ROS) and/or restraint of biosynthetic pathways (Hess, 2000; Garcia-Plazaola et al., 2002; Kim and Lee, 2005).

To summarize, prokaryotic and eukaryotic cells are composed of several primary structures; the cell wall/membrane and organelles, formed by amino acids and proteins. These may be susceptible to attack by ClO_2, including various enzymes and

toxic peptides in cyanobacteria. This is partly why ClO_2 is used widely as an adjunct treatment for the disinfection of water supplies (Sussman and Ward, 1979; White, 1992).

2.6 Oxidative Stress in Cyanobacteria

2.6.1 Cause of Oxidative Stress; Reactive Oxygen Specie (ROS)

Reactive oxygen species comprise normal and free radicals that are formed from several *in vivo* sources, such as autoxidation, photochemical, and enzymatic reactions (Kehrer, 2000). However, excessive ROS can be stimulated *in vitro* by biotic and abiotic causes of oxidative stress. In phytoplankton, ROS are generated from oxygen in water by the photogeneration sites of chloroplasts and thylakoids. Other mechanisms for producing activated oxygen (i.e. ROS) in plant life are believed to reside in various sites of chloroplast (Elstner, 1991), such as:

1. Photosystem I (PSI) by the Mehler reaction to reduce O_2 to O_2^-. This process is stimulated reductions of NADP to NADPH in the Calvin cycle cannot be oxidized, electrons will be provided to the electron transport system by PSI.

2. Photosynthetic reaction centres: chlorophyll captures light energy, entering into an excited state (triplet state), and transfers energy to oxygen (ground state or triplet state), generating excited oxygen (singlet oxygen; $^1O_2$). Singlet oxygen is also stimulated during potentially deleterious conditions; for example, nutrient deficiency, drought environments, and exposure to harmful chemicals.

3. The oxidizing site of Photosystem II (PSII): by transferring electrons from water to produce O_2 and O_2^- simultaneously.

Photorespiration: this system is identified as an important source of the oxygenation pathway. Although this is not the most straightforward way to generate activated oxygen, metabolism of glycolysis in peroxisomes can generate ROS.

In many cyanobacterial species, cellular photosynthesis and respiration systems are contained in the same compartment within the thylakoid membrane (Paumann et al., 2005). Thus, this dual-function organ is a crucial source for generating intracellular

ROS. This activated molecular oxygen may be classified into two main types; free radicals and non-radicals. Molecules that have unpaired electrons and are therefore ready to interact freely with other molecules are called free radicals; these include O_2^- and Hydroxyl radical (OH•). On the other hand, non-radicals do not exhibit unpaired electrons, and are sometimes formed by sharing electrons with free radicals, such as singlet oxygen ($^1O_2$) and Hydrogen peroxide (H_2O_2). They are intermediate molecules that are highly reactivity; therefore, they have been identified as a cause of extensive cell damage and fatality (Apel and Hirt, 2004; Foyer and Noctor, 2005). In addition, Asada (2006) reported that surplus ROS molecules cause damage to pigments, proteins, and lipids of cyanobacteria.

Conventionally, the precursor of the triplet state of chlorophyll (Chl*) is the Chl (ground state) that it is stimulated by UV radical following Equation 2.4. Then, photosensitization is an important source of $^1O_2$ production in photosynthetic organisms. After that, $^1O_2$, being a non-radical and consisting an unpaired electron, is produced by the physical excitation of Chl*, which is gained from the Equation 2.4. Finally, an excited state of oxygen singlet oxygen can be obtained following Equation 2.5.

$$\text{Chl (ground state)} \longrightarrow \text{*Chl (triplet; excited state)} \qquad (2.4)$$

$$\text{*Chl} + {}^3O_2 \text{ (ground state)} \longrightarrow \text{Chl} + {}^1O_2 \text{ (excited state; singlet state)} \qquad (2.5)$$

Additionally, the generation of $^1O_2$ can be stimulated by various severe environments such as drought, high salinity (causing stomatal closure leading to limited CO_2 concentration; (Das and Roychoudhury, 2014), and xenobiotics (Devasagayam and Kamat, 2002).

Superoxide anions (O_2^-) are classified as free radicals because they contain an unpaired electron. This ROS is the primary reduction product of the Mehler reaction (1951). Ground state oxygen is reduced by losing an electron via the electron transport chain (Allen and Hall, 1973) in photosynthesis and respiration. Superoxide anions form a negative charge, hence cannot permeate the cell membrane, and can only cross through

the anion channel of the membrane. Although it is localized and considered moderately harmful, O_2^- continuously establishes chain reactions to generate more dangerous ROS; H_2O_2 and OH•.

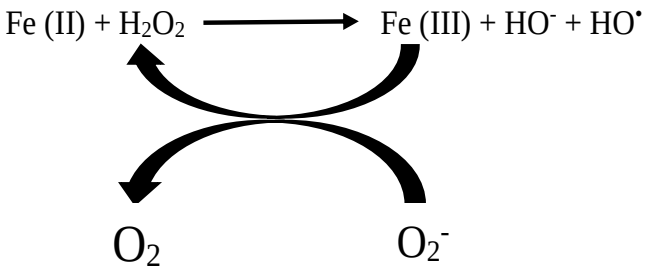
H_2O_2 is a non-radical, but it can participate in oxidation and reduction within cells. There are assumed to be two possible pathways to generate H_2O_2: dismutation of O_2^- by SOD, and by oxidation by oxidases, such as amino and oxalate oxidase (Asada, 2006; Hu et al., 2003).

Hydroxyl radical (OH•) is considered the most damaging ROS, because no specific enzymes are known to scavenge it. Furthermore, it can easily diffuse from the generation site to other components in a diffusion-controlled rate (108-109 $M^{-1}s^{-1}$) (Asada, 1999). Hydroxyl radicals (OH•) are generated principally by oxidation of H_2O_2 and O_2^- with metal ions; Fe (II) or Cu (I); called Fenton and Haber-Weiss reactions following Equation 2.6 and 2.7 in Figure 2.8 (Fenton, 1894: Branchaud, 1999).

Fenton Reaction

$$Fe\ (II) + H_2O_2 \longrightarrow Fe\ (III) + HO^- + OH\bullet \qquad (2.6)$$

Haber-Weiss reaction (using of Superoxide to drive Fenton Reaction)



And the net Haber-Weiss Reaction

$$O_2^- + H_2O_2 \longrightarrow O_2 + HO^- + OH\bullet \qquad (2.7)$$

Figure 2.8: The possible reactions to produce hydroxyl radicals by hydrogen peroxide and superoxide substrates via Fenton Haber-Weiss reactions (Barbusiński, 2009)

2.6.2 Damage from Oxidative Stress

In aerobic organisms, oxygen behaves as an electron acceptor in cellular metabolism, providing non-radical and free radical by-products. Cells naturally adjust and develop an *in vivo* biochemical system to regulate the balance of ROS, specifically via enzymatic and non-enzymatic antioxidants. This equilibrium becomes disturbed incertain environments, such as high-intensity light, nutrient deficiency, drought, freezing conditions, and harmful chemicals (e.g. herbicide). These environmental stimuli disrupt the balance of intracellular redox reactions, causing an overproduction of ROS (Mitter, 2002; Karuppanapandian and Manoharan, 2008; Mafakheri et al., 2010). This biochemical phenomenon, characterised by an imbalance between generations and scavenging of ROS by antioxidants, is known as oxidative stress (Davies, 1995). Researchers normally focus on four major ROS types, which are presented in Table 2.6.

Table 2.6: The major ROS produced via oxidative stress and their molecular targets, including systematic scavenging mechanisms

ROS Types	Producing site	Mode of reaction	Scavenging system
$^1O_2$	Chloroplast; photosynthesis and respiration	Directly oxidizes protein, PUFAs and DNA	Carotenoids and α-tocopherols
O_2^-	Chloroplast; photosynthesis and respiration	Reacts double bond site of compounds	SOD
H_2O_2	Chloroplast; photosynthesis and respiration, Peroxisomes	Creates the oxidation with protein and to be the substrate to form HO•	CAT and Peroxidases
OH•	Chloroplast; photosynthesis and respiration	Extremely harmful ROS to react with all biomolecules in cells	Nonspecific enzyme

Source: Karuppanapandian et al., 2011; Das and Roychoudhury, 2014.

For example, xenobiotics, such as herbicides, have been demonstrated to disturb metabolic functions of algae cells, causing oxidative stress (Romero et al., 2011). In addition, the photosynthetic system PSII was attacked by herbicide blocking the electron transport chain, inevitably resulting in oxidative stress (Rutherford and Krieger, 2001). A previous study examined environmental impacts on ROS production in cyanobacteria, highlighting serval effects on cells that are illustrated in Figure 2.9 (Hsieh and Pedersen, 2000).

Cyanobacteria produce ROS via intracellular mechanisms that support redox reactions in the electron transport chain of the photosynthetic respiratory system. However, extraneous stimuli such as high intensity light, drought conditions, algaecide, herbicide, and starvation trigger increased oxidative stress in cyanobacteria. To maintain homeostasis, cyanobacteria employ survival strategies to manage excessive ROS and reduce their harmful effects within the cell. Detoxification is implemented by both non-enzymatic and enzymatic systems, which scavenge surplus ROS that would otherwise cause imbalances in noxious oxygen species. For example, non-enzymatic antioxidants: carotenoids, have the capacity to function as light harvesters in the cyanobacterial cell (Schafer et al., 2005). In terms of scavenging tactics, carotenoids dissipate the energy of excited oxygen (singlet oxygen) converted to ground stage ($^3O_2$); therefore, they control surplus $^1O_2$. In consequence, studies have reported that carotenoids absorb excessive energy to protect from photo-inhibition damage when cyanobacteria are confronted with high intensity sunlight, releasing the excess light energy as heat (Powles, 1984; Aro et al., 2005).

As mentioned in the previous chapter, O_2^- is a free radical produced by several pathways leading to the redox reaction, and causes damage to intracellular molecules. It directly attacks polyunsaturated fatty acids of the cell membrane, producing lipid peroxidation. Additionally, the protein structure of iron-sulphur has a particularly susceptible moiety to interact with damaging superoxide free radicals. (Li, et al., 2002; Regelsberger et al., 2004). One of the by-products released from O_2^- scavenging by SOD is hydrogen peroxide, which is relatively stable, although it can also be oxidized to release harmful peroxide free radicals (HO^-) (Fridovich, 1997). Particularly the PSI site, that produces O_2^-, causes H_2O_2 generation by the Mehler reaction (Asada, 1994).

The severe effects of H_2O_2 have been reported numerous times. For instance, it oxidizes cysteine to form sulphenic acid, leading to generation of di-sulfide that can crosslink with other cysteines (Imlay, 2003). However, stable hydrogen peroxide radicals can be transformed into more harmful HO^- free radicals by the Fenton reaction via reduction of Fe^{2+} into Fe^{3+} (Halliwell and Gutteridge, 1986). Spontaneously, the formed Fe^{3+} is then oxidized by O_2^-, producing Fe^{2+} (Haber-Weiss reaction), continuously supporting the Fenton reaction. Thus, controlling and eliminating H_2O_2, O_2^-, and Fe^{2+} is a beneficial strategy to prevent dangerous continuation of the Fenton reaction, which would result in cell harm. In correlation with this, scavenging of O_2^- and H_2O_2 by enzymatic antioxidants SOD and CAT are essential to protecting the cell from damage.

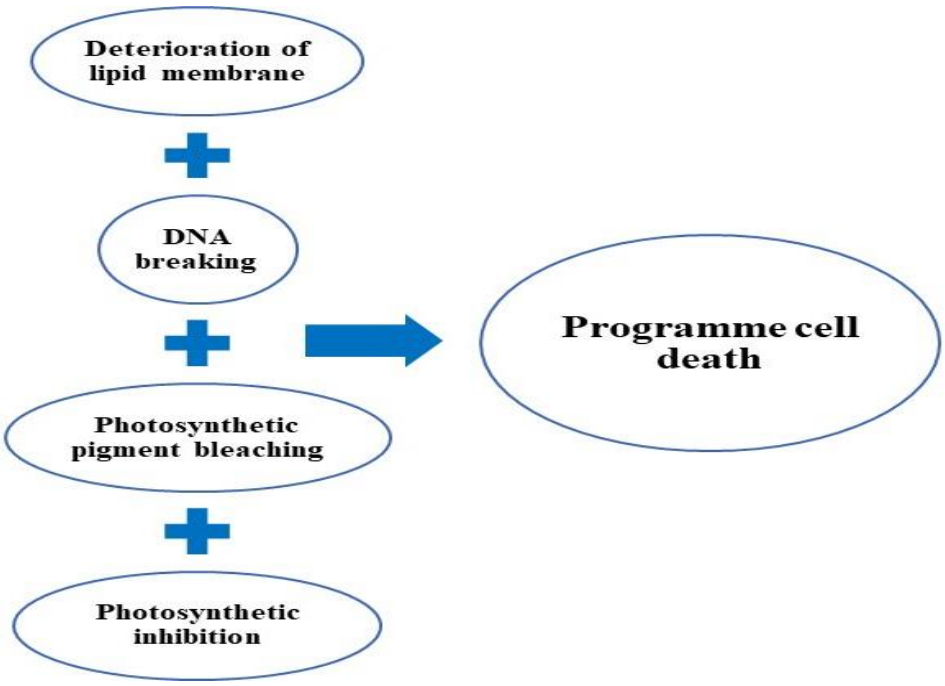


Figure 2.9: The effects of cyanobacteria undergoing oxidative stress

2.6.3 Scavenging Systems; Enzymatic and Non-enzymatic Antioxidants

2.6.3.1 Enzymatic Antioxidant; Superoxide Dismutase (SOD)

The photosynthesis system in cyanobacterial cells, especially PSI, has been identified as an important source of O_2^- generation (Mehler, 1951). Singlet oxygen (O_2^-) is a free radical that exhibits negative charge; hence, it is unable to transfer across the plasma membrane, which consists of a negatively charged phospholipid bilayer. In consequence, it reacts within its production source (Ke et al., 2014). Enzymatic antioxidants (e.g. SODs) in cyanobacteria perform the crucial function of defending against O_2^-, mitigating the amount of photo-oxidation damage incurred. Generally, SODs are categorized into four types; Fe-SOD, Cu/Zinc-SOD, Mn-SOD, and Ni-SOD (Fridovich, 1997). Particularly, filamentous and coccoid cyanobacteria have been shown to contain more Fe-SOD and Mn-SOD (Canini et al., 1991; Campbell and Laudenbach, 1995; Grilli Caiola et al., 1996). In contrast, spherical marine cyanobacteria contain both Fe-SOD and Cu/Zinc-SOD, and exhibit an absence of Mn-SOD (Chadd, et al., 1996). Although, the unicellular marine strain of cyanobacteria contains only Ni-SOD. Researches have described how a general strain of cyanobacteria undergoes increased expression of Fe-SOD activity while encountering stress induced by high dose radiation (Canini et al., 1991; Grilli Caiola and Canini, 1993). The mechanism of scavenging employed by SODs is dismutation of O_2^- to cope with the imbalance of the excess free radicals induced by oxidative stress. The quenching reaction of SOD activity is defined as Equation 2.6, 2.7 and 2.8.

$$\text{Oxidized dismutase} \xrightarrow{O_2^{\bullet}} \text{Reduced dismutase} + O_2 \qquad (2.6)$$

$$\text{Reduced dismutase} \xrightarrow{2H^+ + O_2} \text{Oxidized dismutase} + 2H_2O_2 \qquad (2.7)$$

$$2O_2^{\bullet} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad (2.8)$$

Numerous studies have implicated increased SOD activity in prokaryotic and eukaryotic cells with increased levels of oxidative stress (Scandalios, 1990; Bowler et al., 1992; Gralla and Kosman, 1992). Furthermore, xenobiotic chemicals such as herbicides present as electron acceptors at the PSI, disturbing the electron transport chain and generating oxidative stress that activates SODs to manage cell damage (Scandalios, 1993). Previously, Monk and colleagues (1989) reported that unsuitable environmental conditions for human existence, such as drought, freezing temperatures, absence of oxygen, etc, were paired in a direct relationship with increased SOD activity. Additionally, there was a report of cyanobacterial Fe-SOD activity increasing in response to greater light intensity, specifically in species *M. aeruginosa*. These observations implicate enzymatic dismutation, or scavenging, of ROS by SOD in the damage-control response to photo-oxidative stress (Canini et al., 2001).

2.6.3.2 Enzymatic Antioxidant; Catalase (CAT)

Catalase (CAT) is another type of enzymatic antioxidant used to scavenge free-radicals while cells are exposed to oxidative stress. The mechanism of CAT to combat damage from oxidative stress relies on dismutation of H_2O_2 into H_2O and O_2 (Zámocký et al., 2008a). In cyanobacteria, the regular enzymatic antioxidants to scavenge H_2O_2 are CAT and/or thioredoxin peroxidase, because they lack an ascorbate peroxidase (Regelsberger et al., 2002). Particularly, increased CAT activity is suggested to be an adaptive mechanism used by aerobic organisms to decrease toxicity induced by H_2O_2, which would otherwise result in greater cell damage (Karuppanapandian, et al., 2011). The two types of CAT are shown in Table 2.7.

Table 2.7: The family of CAT to decompose H_2O_2 to H_2O and O_2

Types of CAT	Family	Source	Reference
Haem containing enzyme	1. Monofunctional catalase	All domains of life	Zámocký et al., 2008a
	2. Haem peroxidase	Plants, fungi, protists and bacteria	Welinder, 1992 and Passardi et al., 2007
	3. Peroxidase-cyclooxygenase	All domains of life	Zámocký et al., 2008b
Non-haem containg enzyme	1. Manganese catalase		Zámocký et al., 2008a
	2. Vanadium peroxidases		Littlechild, 1999
	3. Ubiquitous thiol peroxidase (peroxidredoxins and glutathione peroxidases)		Rouhier and Jacquot, 2005

Haem-catalases consist of a cyanide-sensitive catalytic component and comprise different sequences, structures, and mechanisms (Passardi et al., 2007). Non-haem catalases are thought to be less diverse and less widespread; For example, manganese-catalase (non-haem) is found to have lower antioxidant activity than the haem-containing group (Latifi et al., 2009).

2.6.3.3 Non-enzymatic Antioxidant; Carotenoids

Structurally, carotenoids (CARs) constitute 40 carbon atoms and approximately 3 to 13 conjugated double bonds (Hsieh and Pedersen, 2000). In addition, Havaux (2013) classified carotenoids into two types; those without oxygen (e.g. β-carotene and lycopene) and those containing oxygen, called xanthophylls (e.g. lutein and zeaxanthin). CARs are the lipophilic compounds since they are capable of dissolving in lipids. This causes them to accumulate in the hydrophilic side or lipoproteins of the cell membrane. This property of lipophilicity essentially affects membrane absorption, transportation, and excretion of cells (Stahl et al., 2002). In cyanobacteria, the ubiquitous carotenoids are B-carotene, zeaxanthin, echinenone, and myxoxanthophyll (Takaichi and Mochimaru,

2007). In their two main functions, CARs serve as an accessory pigment in the photosynthesis light-harvesting system (Goedheer, 1959) and as a protective antioxidant pigment, degrading injurious photodynamic reactions stimulated by high-intensity light (Krinsky, 1968). In terms of their antioxidant functions, the following four mechanisms are described in Figure 2.10.

- To react with intermediate compounds of lipid peroxidation to force the terminal stage of lipid damage (Burton and Ingold, 1984).
- To scavenge $^1O_2$ by absorbing its energy and transforming it into heat (Mathis and Kleo, 1973).
- To react with triplet or excited state chlorophyll to reduce the formation of $^1O_2$.
- To dissipate excess energy by releasing it through the xanthophyll cycle (McKersie, 1996).

Furthermore, under oxidative stress conditions, as various radicals are generated within cells, carotenoids play an important role as a scavenger by reacting with peroxyl radicals (Burton and Ingold, 1984); as illustrated in Figure 2.10.

$$RO\bullet + CAR \longrightarrow RO^- + CAR\bullet^+$$

$$RO\bullet + CAR \longrightarrow RO\bullet + CAR\bullet^-$$

$$RO\bullet + CAR \longrightarrow ROH\bullet + CAR(-H)\bullet$$

$$RO\bullet + CAR \longrightarrow (RO\text{-}CAR)\bullet\text{; inactivated product}$$

Figure 2.10: The scavenging pattern of CAR to react with peroxyl radicals, producing harmless products within the cell (Edge and Truscott, 2009)

Sie and Stahl (1995) found that the lipophilicity and antioxidant function of CARs to react with peroxyl radicals are implicated in protecting the cellular membrane and lipoproteins against oxidative damage. In cyanobacteria, CARs carotene and xanthophyll are contained

in both the cytoplasm and thylakoid membrane; in addition, ß-carotene has been detected in PSII. In summary, CARs exhibit a protective role by scavenging $^1O_2$ and mitigating membrane damage (Packer et al., 1981).

2.7 Summary of Key Findings

Contamination by cyanobacteria and cyanotoxins causes deteriorated water quality, undesirable taste and odours, and, most importantly, exposure to potent toxins. Water treatment processes have to address this problem if water intake supplies are contaminated with cyanobacteria. Cyanobacteria are prokaryotic organisms that obtain chemical energy through photosynthesis and release oxygen back to their environment. The pigment, chlorophyll *a*, absorbs a particularly high amount of light at 680 nm wavelength. Cyanobacteria contain other important photosynthetic pigments such as carotenoids, phycocyanin, and allophycocyanin.

ClO_2 is a strong, stable, selective oxidant that is highly soluble in water and can be applied over a wide range of pH (3-10). Although ClO_2 has good disinfectant quality and avoids reacting with organic matter, it can generate by-products of chlorite, chlorate, and chloride. In consequence, U.S. EPA recommends limiting concentration of ClO_2 for water treatment disinfection at 0.8 mg/L. In Europe, the provisional guideline is set at 0.05 to 0.1 mg/L. The recommendation of ClO_2 for pre-oxidation of surface water is suggested at 1 to 1.4 mg/L. These recommendations do not take into account water resource contamination with cyanobacteria.

Water treatment processes are adapted to address reservoir intake water contaminated with blooming cyanobacteria by applying high dose disinfectant at intake followed by low dose disinfectant after all of the other treatment steps. As a disinfectant, ClO_2 oxidises susceptible moiety sites (i.e. porphyrin rings). Photosynthetic pigments are bleached by ClO_2 oxidation, reflecting disturbances of electron transport chains in Photosystems I and II. In consequence, redox reactions become imbalanced with significant effects on viability of cell. In another inhibition mechanism, ClO_2 oxidizes thiol groups of amino acids to sulphoixide or sulphone compounds, which is a permanent transformation resulting in abnormality and fatality. ClO_2 is an abiotic factor that induces oxidative stress. Viability of cyanobacteria cells can be characterized by membrane

leakage, chlorophyll bleaching, and metabolic activity. Stress response can be determined by quantifying secondary metabolites of lipid peroxidation and non-enzymatic and enzymatic antioxidants.

Although not all cyanobacteria produce toxins, this research focuses on a species that produces toxins. In this research, ClO_2 induced oxidative stress is hypothesised to cause cyanobacteria to produce cyanotoxin (microcystins) as part of its protective system. The effects of lethal and sub-lethal concentrations of ClO_2 are investigated for inhibition, cell viability, stress response and cyanotoxin production. Details of these experiments are presented in subsequence

Chapter 3. MATERIALS AND METHODS

This research is interested in the efficacy of ClO_2 to treat water affected by cyanobacterial blooms focusing on *M. aeruginosa.* Two key experiments were conducted. The first experiment was designed to assess the effects of ClO_2 exposure on *M. aeruginosa*, including cell lethality and cyanotoxins production, at different concentrations and exposure times. Building on this experience, the second experiment evaluated the effects of sublethal ClO_2 exposure on *M. aeruginosa* at different growth phases from low to mature population densities. Key parameters included cell health, stress response, and cyanotoxins. The two experiments are detailed in Chapters 4 and 5. This chapter outlines common methodologies that were developed and used in these experiments**.**

3.1 Synthesis of the oxidizing agent chlorine dioxide (ClO_2)

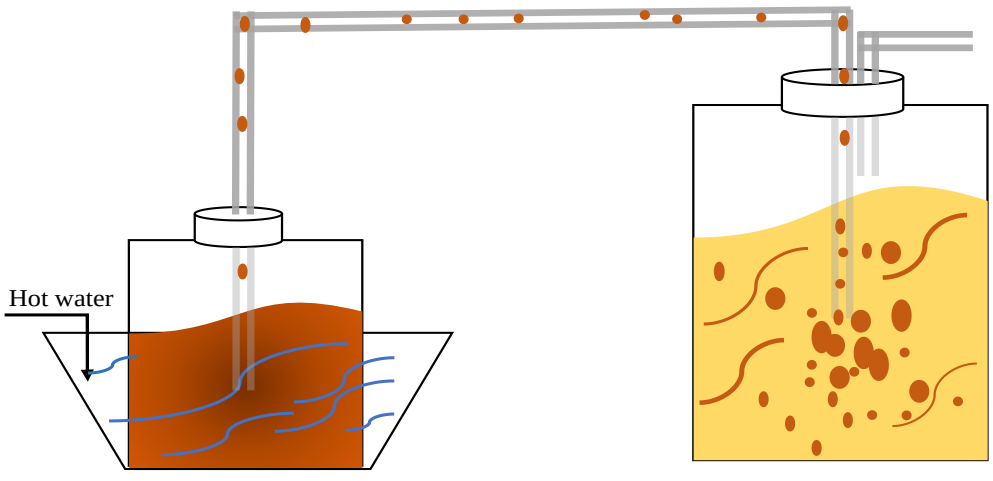
Chlorine dioxide is a strong oxidising agent, synthesised by the acidic reaction between sodium chlorite ($NaClO_2$; Laboratory grade) and citric acid ($C_6H_8O_7$; ACS grade), causing the acidification of chlorite Thangaraj et al., 2001). This oxidant was prepared with 20 mL of $NaClO_2$ (50% w/v) mixed together with 20 mL of citric acid (50% w/v) (Sigma-Aldrich, Gillingham, UK) to produce chlorine dioxide (ClO_2) gas inside a Nalgene HDPE bottle with a cover to prevent the escape of chlorine dioxide gas. Equipment was cleaned with and rinsed with deionised water before every synthesis. The chlorine dioxide gas was dissolved in cold saline solution (0.9%, w/v) until the reaction reached an equilibrium state whereby there was simultaneously a decrease in bubbling gaseous ClO_2 and a deep yellow colour in the cold saline solution. The ingredients and amounts are highlighted in Table 3.1. The chemical reactions are shown as Equations 3.1 and 3.2 and the process is illustrated in Figure 3.1.

Table 3.1: Chemicals for acid reaction of chlorine dioxide synthesis

Name	IUPAC name and formula	Concentration
Sodium chlorite	Sodium chlorate ($NaClO_2$) MW = 90. 438g/mol	50% (w/v)
Citric acid	Citric acid ($C_6H_8O_7$) MW = 192.012 g/mol	50% (w/v)
Saline solution	Sodium chloride (NaCl) MW = 58.44 g/mol	0.9%(w/v)

$$H^+ + NaClO_2 \rightleftharpoons HClO_2 + Na^+ \quad (3.1)$$

$$5ClO_2^- + 5H^+ \rightleftharpoons 4ClO_2 + HCl + 2H_2O \quad (3.2)$$



50% $NaClO_2$ (20 ml) + 50% Citric acid (20 ml)

ClO_2 solution in cool saline solution

Figure 3.1: Chlorine dioxide synthesis reactions from sodium chlorite ($NaClO_2$) and citric acid ($C_6H_8O_7$)

The concentration of synthesized chlorine dioxide was determined by the classical method of Iodometric titration, 4500-ClO_2 B (APHA et at., 1998) with 0.1N sodium thiosulfate ($Na_2S_2O_3$; Analytical grade ; Sigma-Aldrich, Gillingham, UK) as the titrant. Synthesised ClO_2 was processed in acidic conditions by adding 2 ml of 0.1 N HCl (ACS grade). Then, 0.5 g of KI (Analytical grade) was added into the solution to liberate iodine from iodide. The solution was allowed to rest for 5 minutes to allow this reaction before titration. Subsequently, 1ml of 1% starch indicator solution was added. The titration endpoint was at the beginning of emergence of the permanently dark blue color solution. Titration followed with gradual additions of $Na_2S_2O_3$. The volume of titrate in both sample and blank were used to determine ClO_2 concentration, which was calculated by Equation 3.3. Titration was done in in the fume cupboard under dark conditions to protect against iodine degradation.

$$\text{mg } ClO_2 \text{ /L} = \frac{[(A \pm B) \times N \times 13490}{mL\ of\ sample} \quad (3.3)$$

Where:

A = mL titrant solution for sample

B = mL titrant solution for blank (the deionized water)

N = normality of $Na_2S_2O_3$

In addition, the ClO_2 concentrations was double-checked using a colorimeter (series DR/850; HACH Company, USA) to detect the formed compound (pink colour) produced by the reaction between ClO_2 solution and a testing chemical (Commercially coded, from HACH Company).The concentration of chlorine dioxide was displayed on the portable device. Afterwards, the ClO_2 solution was stored in an amber glass bottle to protect it from deteriorating under light exposure and kept below 20°C to reduce its oxidation rate. High concentrations of synthesized ClO_2 were determined to be stable for 3 months. Concentration of stock chlorine dioxide solution was always rechecked before starting an experiment.

3.2 Growth Procedure for *M. aeruginosa*

M. aeruginosa (TISTR 8305) was used in all experiments. This strain was a confirmed cyanotoxin producer (Mahakhant et al., 1999) supplied by Thailand Institute of Scientific and Technological Research (TISTR). The ingredients for *M. aeruginosa* media (MA media; Table 3.2) were dissolved in distilled water, with its pH adjusted to 8.6 using NaOH solution or HCl, to make up a volume of 1 L. This media solution was then sterilized in an autoclave at 121 ˚C at 15 Ibf/sq in (psi) for 30 min before use (Ichimura, 1979; Gupta, et al., 2002). All analytical grade of chemicals were purchased from Sigma-Aldrich (Gillingham, UK).

Table 3.2: *M. aeruginosa* media (MA media) ingredients and amounts used in 1L water

Name	IUPAC name and formula	Weight
Calcium nitrate	$Ca(NO3)_2$; MW = 164.088 g/mol (anhydrous) $Ca(NO_3)_2.4H_2O$; MW = 236.15 g/mol (tetrahydrate)	0.05 g
Potassium nitrate	KNO_3; MW = 101.1032 g/mol	0.01 g
Sodium nitrate	$NaNO_3$; MW = 84.9947 g/mol	0.05 g
Sodium sulfate	Na_2SO_4; MW = 142.04 g/mol (anhydrous) = 322.20 g/mol (decahydrate)	0.04 g
Magnesium Chloride Hexahydrate	$MgCl_2.6H_2O$; MW = 203.31 g/mol (hexahydrate) = 95.211 g/mol (anhydrous)	0.05 g
Disodium beta-glycerophosphate pentahydrate	β-Na_2 glyerophosphate.$5H_2O$ MW = 216.04 g/mol	0.1 g
Disodium edetate dihydrate	Na_2EDTA; MW = 372.24 g/mol	0.005 g
Ferric chloride hexahydrate	$FeCl_3.6H_2O$; MW = 270.295 g/mol (hexahydrate) = 162.204 g/mol (anhydrous)	0.0005 g
Manganese (II) chloride tetrahydrate	$MnCl_2.4H_2O$; MW = 197.91 g/mol (tetrahydrate) = 125.844 g/mol (anhydrous)	0.005 g
Zinc chloride	$ZnCl_2$; MW = 136.315 g/mol	0.0005 g
Cobalt chloride hexahydrate	$CoCl_2.6H_2O$ MW = 237.93 g/mol (hexahydrate) = 129.839 g/mol (anhydrous)	0.005 g
Sodium molybdate dihydrate	$Na_2MoO_4.2H_2O$; MW = 241.95 g/mol (dihydrate) = 205.92 g/mol (anhydrous)	0.0008 g
Boric acid	H_2BO_3; MW = 61.83 g/mol	0.02 g
Bicine	Bicine ($C_6H_{13}NO_4$); MW = 163.17 g/mol	0.5 g

Cells were acclimated at room temperature on a shaker at the slow speed for 3 days. *Microcystis aeruginosa* were cultured in MA media while applying artificial light (GRO-LUX, F 36 W/GRO-T8, Feilo Sylvania, Germany) on a cycle of 12 hours in light followed by 12 hours in dark to imitate the cycle of photosynthesis and respiration that occurs during natural growth. This was done to observe cell growth before treating them with chlorine dioxide (Figure 3.2). Some portion of cells were then preserved by modified mixing solution of dimethyl sulfoxide (DMSO) and methanol (MeOH) at final ratio of 3 and 5% (v/v) in a freezer at -80°C using a cryopreservation procedure (Rastoll, et al., 2013) following steps illustrated in Figure 3.3 for stock in further experiments.

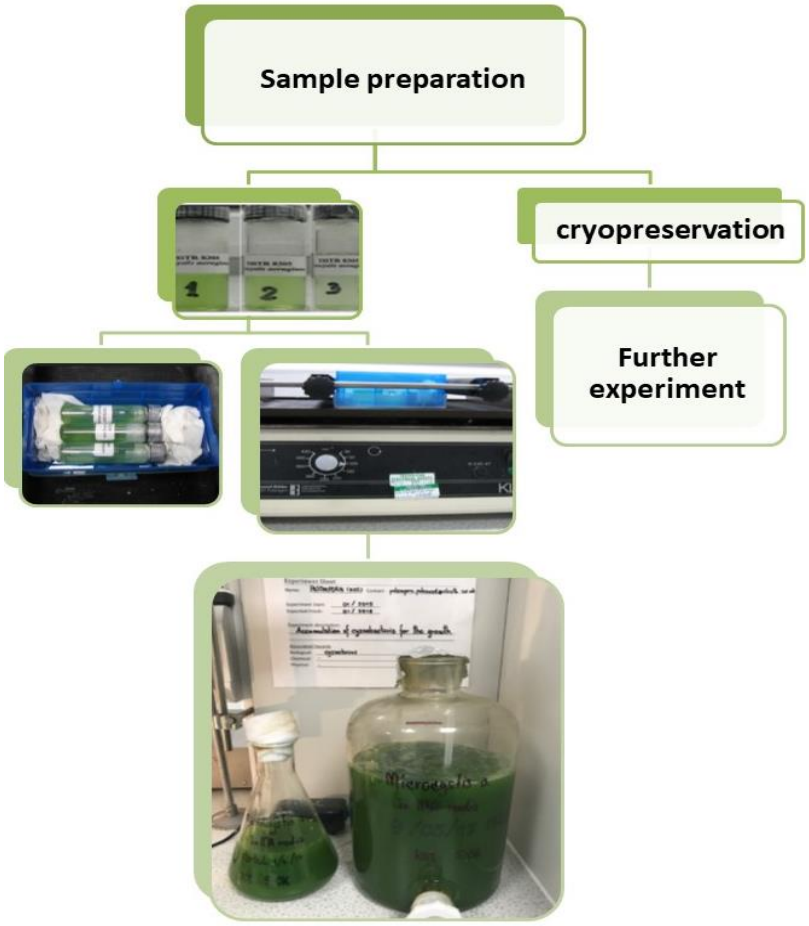


Figure 3.2: Steps to maintain healthy cyanobacteria before studying oxidative stress induced by chlorine dioxide

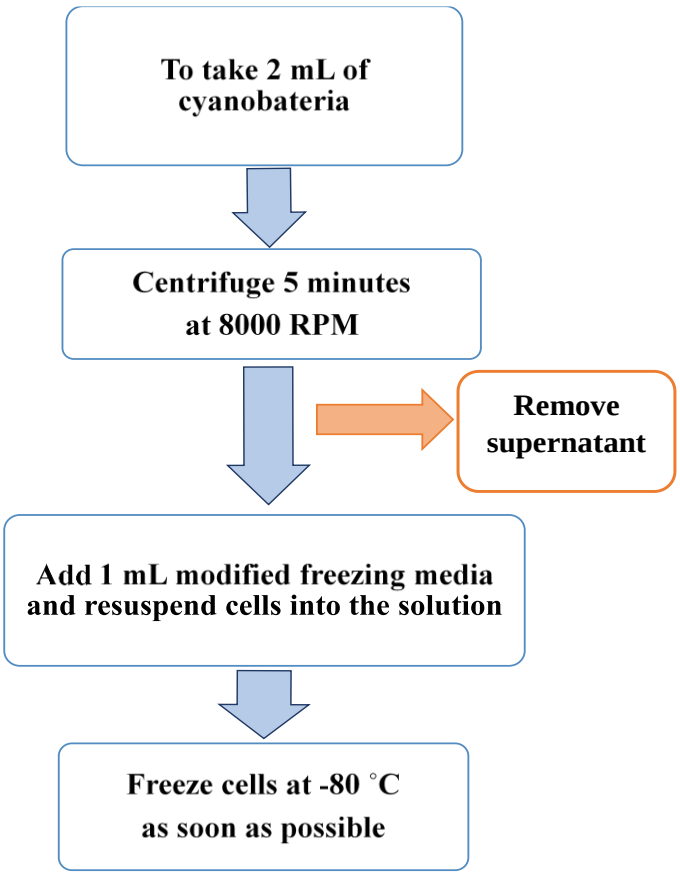


Figure 0.3: Cryopreservation of cyanobacteria for preserving cultures

3.3 Assessment of *M. aeruginosa* proliferation

3.3.1 Optical density (OD 680) correlates with cyanobacterial pigment absorption

The growth rate of cyanobacteria was measured using a spectrophotometer (Helios Zeta UV/VIS, Thermo Fisher Scientific, Leicester, UK) at 680 nm. This wavelength correlates with absorbance by important pigments (e.g. chlorophyll *a*) of the photosynthesis system (Li et al., 2014), which provides a good viability marker for cyanobacteria cultures. The cyanobacteria were agitated into a homogeneous mixture before taking a sample of 1.5 mL. Each sample was then placed inside a cuvette, to prevent cell precipitation, and inserted into the spectrophotometer to measure light absorbance at 680 nm.

3.3.2 Chlorophyll a represents the cell viability

Chlorophyll *a* is the most important pigment in the photosynthesis system of cyanobacteria and is also representative of the overall biomass of cells in a cyanobacterial culture. In this experiment, was slightly adapted from analysis of chlorophyll *a* in cyanobacteria (Zavřel et al, 2015). Taking a homogeneous cyanobacteria sample of 1.5 mL, this was precipitated by centrifugation at 9000 RPM for 10 minutes to separate cells and media. The supernatant was then discarded to harvest live cyanobacteria cells. In the next step, bacterial chlorophylls (chlorophyll *a* and carotenoids) were extracted by adding 1.5 mL of methanol (CH_3OH; HPLC grade, Sigma –Aldrich, Gillingham, UK) in the same vial then mixing with cells using a vortex machine to break down the cell wall of cyanobacteria. Bacterial chlorophyll was thereby extracted and dissolved in methanol, ready for the next stage in the process. The extracted solution was then pre-cooled in a fridge at 4 ˚C for 20 minutes. Next, the cooled sample was centrifuged again at 9000 RPM for 10 minutes, completing the extraction process in preparation for analysis. The supernatant (green colour) was then analysed for light absorbance at 470, 665 and 720 nm using the spectrophotometer (Zavřel et al, 2015). The concentration of chlorophyll *a* contained within cyanobacterial cells was calculated according to Equations 3.4 and 3.5 (Ritchie, 2006; Zavřel, 2015). The chemicals involved in this assay and their concentrations are listed in Table 3.3 and the procedure steps are outlined in Figure 3.4

Table 3.3: Extract solution for chlorophyll *a* extraction

Name	IUPAC name and formula	Concentration
Methanol	methyl alcohol (CH_3OH or CH_4O) MW = 32.04 g/mol	≥ 99.99% (GC)

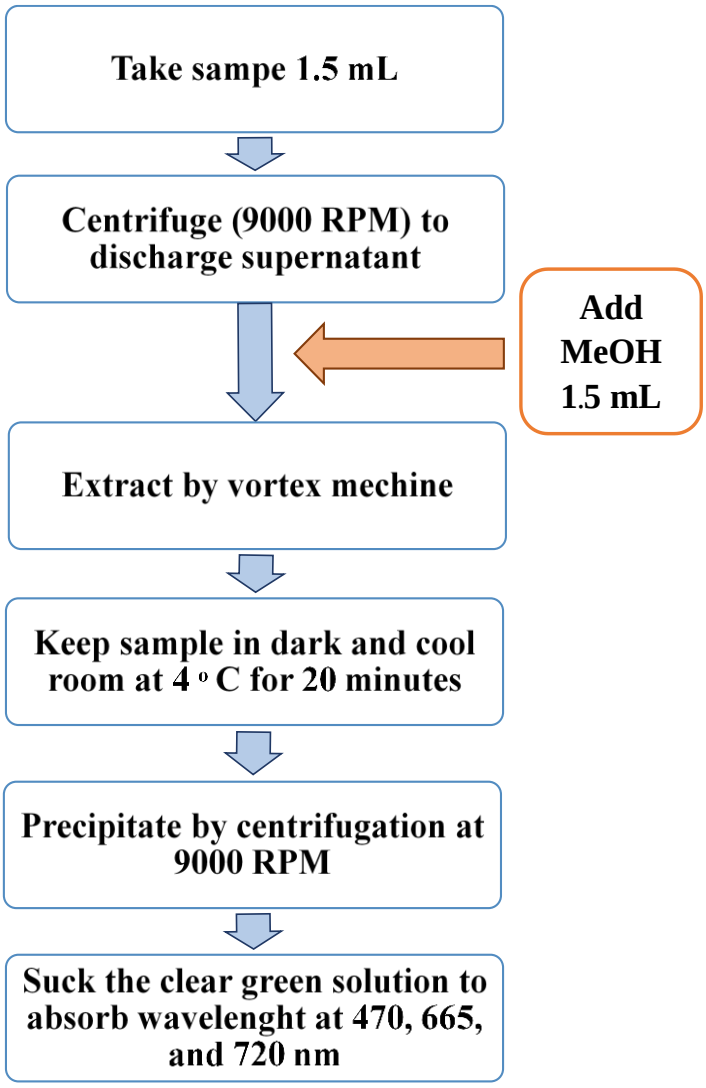


Figure 3.4: The procedure to analyse chlorophyll *a* from *M. aeruginosa*

$$\text{Chlorophyll } a \text{ (µg)/mL)} = 12.9447\times(A665 - A720) \quad (3.4)$$

Where:

A665 = absorbance at 665 nm

A720 = absorbance at 720 nm

Total chlorophyll (chlorophyll *a*) concentration in sample:

$$\text{Total chlorophyll } a\ (\mu\text{g/mL}) \times \frac{\text{Volume of methanol (mL)}}{\text{Volume of Sample (mL)}} \tag{3.5}$$

This procedure can also enable an analysis of the concentration of carotenoids that is used in a later part of this investigation to examine oxidative stress. Furthermore, the monitoring of the specific growth rate (μ) was calculated by Equations 3.6 and 3.7 (Imai, et al., 2009):

$$\mu = \frac{\left[\text{In (OD 680 (F))} - \text{In}\left(\text{OD 680(I)}\right)\right]}{(\text{t(F)} - \text{t(I)})} \tag{3.6}$$

Where OD 680 (I) and OD 680 (F) are the initial and final optical densities at 680 nm, respectively, while t(I) and t(F) are the initial and final time of the experiment.

$$\mu = \frac{\left[\text{In (Chl a (F))} - \text{In}\left(\text{Chl a (I)}\right)\right]}{\left(\text{t(F)} - \text{t(I)}\right)} \tag{3.7}$$

Where Chl a (I) and Chl a (F) are the initial and final concentrations of chlorophyll *a*, and t(I) and t(F) are the initial and final times of the experiment. Either of these Equations (3.6 and 3.7) be used to calculate exponential growth rate (log phase). In this research, OD 680 (Equation 3.6) was used.

3.4 Evaluation of membrane malfunction by electrolyte leakage

In this research, a conductivity was identified the electrolyte leakage from treated cells that were inhibited by ClO_2 treatment. The demonstration was the direct evaluation, which no steps preparing cells or washing. This leakage was measured by Benchtop

pH meter (Seven Muti, probe type In Lap 731, METTLER TOLEDO, Beaumont Leys, UK). The experiment was set up the blank control for each concentration of ClO_2 including media, $BC_{0.0,}$ $BC_{1.5}$, $BC_{3.0}$ and $BC_{5.0}$, respectively. These electrolyte values indicated the innately electrical ions causing overestimated conductivity. Therefore, the untreated and treated samples had to subtract with blank control. Figure 3.5 revealed the step of experiment.

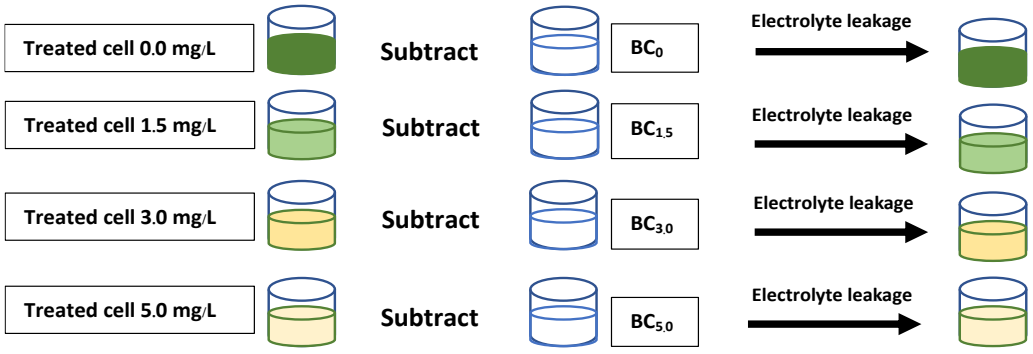


Figure 3.5: Steps to measure electrolyte leakage after *M. aeruginosa* inhibited with different concentration of ClO_2

3.5 Assessment of viable cell by MTT (assay)

The MTT (3-(4, 5-dimethylthiazolyl-2-yl)-2, 5-diphenyltetrazolium bromide; Analytical grade) assay was used to detect viable cells using a colorimetric method. MTT (Thermo Fisher SCIENTIFIC, UK) is part of the tetrazolium salt group. It is reduced by oxidoreductase within the cell to form the purple colour of formazan (Berridge et al, 2005). The developing colour solution was prepared in phosphate buffered saline (all chemicals are the analytical grade from Sigma – Aldrich, Gillingham UK (MTT 0.4143 g/PBS 100 mL at pH 6.75) that was filtered through a 0.2 µm filter. This can then be stored for up to 2 weeks at 4 ºC. Cyanobacterial cell culture samples of 200 µL were harvested and placed inside 96 well plates. Then 50 µL of MTT solution was added to each well before covering the plate with aluminium foil to protect the cells from UV-

light. The 96 well plate was then incubated in the incubator at 37 ºC for four hrs. Subsequently, the plate was carefully taken out of the incubator and the supernatant was removed before the colour forming process. In the next step, 200 µL of dimethyl sulfoxide (DMSO; GC grade from Sigma – Aldrich, Gillingham, UK) was added to each well to dissolve formazan crystals into a clear purple solution. Light absorbance at 556 nm was measured (Li and Song, 2007) with a spectrophotometer. The results of treated cells were compared with an untreated control sample to evaluate the change in cellular metabolic activity induced by treatment with ClO_2. The chemicals used in the assay are listed in Table 3.4. The steps involved in the assay are illustrated in Figure 3.6.

Table 3.4: Chemicals used to detect cellular metabolic activity

Name	IUPAC name and formula	Concentration
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); $C_{18}H_{16}BrN_5S$; MW = 414.32	10 mM
DMSO	Dimethyl sulfoxide; $(CH_3)_2SO$ MW = 78.129 g/mol	
PBS	Phosphate Buffered Saline	

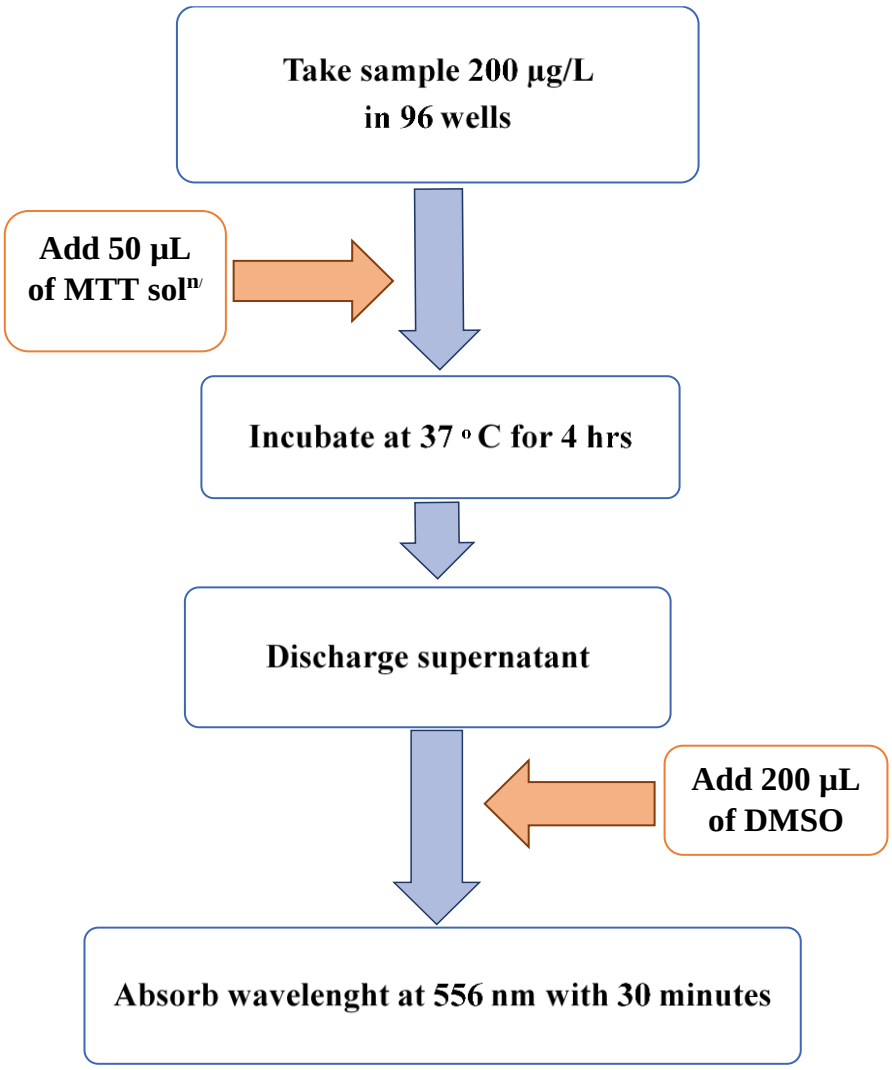


Figure 3.6: Flow chart of steps taken to quantify the metabolic activity of viable cells

3.6 Assessment of membrane damage by lipid peroxidation

One of the by-products of lipid oxidation is malondialdehyde (MDA). This can be used to quantify the degree of lipid cell membrane damage by ROS induced by treatment with an oxidizing agent (Gutteridge, 1995). MDA formation may be used to infer the amount of oxidative stress suffered by the cell. To detect the quantity of MDA, we mainly follow the method employed by Heath and Packer (1968). The chemicals used in this assay obtained from Sigma – Aldrich, Gillingham, UK. The extraction solution is prepared by mixing 0.5% thiobarbituric acid (TBA; Pharmaceutical grade) in 20%

trichloroacetic acid (TCA; ACS grade). This is then made into a dilution with 80% ethanol in water to induce cell lysis. Firstly, a homogeneous cyanobacterial cell culture sample of 20 mL was taken and centrifuged at 7500 RPM for 5 minutes to precipitate cells and discard the supernatant. Next, the precipitated sample was added to 1.5 mL of the prepared ethanol (ACS spectrophotometric grade) dilution (80%) and shaken for 30 minutes. This shaken solution was then further added to 1.5 mL of diluted ethanol, producing a total sample volume of 3.0 mL. Afterwards, the sample solution was incubated in a heating bath at 95ºC for 30 min, before being cooled down at the room temperature. The sample was then centrifuged again at 7500 RPM for 5 minutes to separate the precipitation and keep the supernatant (pink colour solution) for analysis. Finally, light absorbance of the sample solution at 532 and 600 nm was measured using a spectrophotometer to identify the degree of MDA generated by lipid membrane peroxidation. The level of MDA generated by thiobarbituric acid reactive substances (TBARS) was calculated using Equation 3.8, as followed by Heath and Packer (1968) including Hodges and colleagues (1999). The chemicals and concentrations used in this assay are listed in Table 3.5. In addition, the procedure are illustrated in Figure 3.7.

Table 3.5: Chemicals used for analysis of MDA produced by lipid peroxidation

Name	IUPAC name and formula	Concentration
Thiobarbituric acid (TBA)	2-Sulfanylidene-1,3-diazinane-4,6-dione ($C_4H_4N_2O_2S$); MW = 144.15 g/mol	0.5%(w/v)
TCA or TCAA	Trichloroacetic acid ($C_2HCl_3O_2$) MW = 163.38 g/mol	20% (v/v)
Ethanol	C_2H_5OH; MW = 46.07 g/mol	80% (v/v)

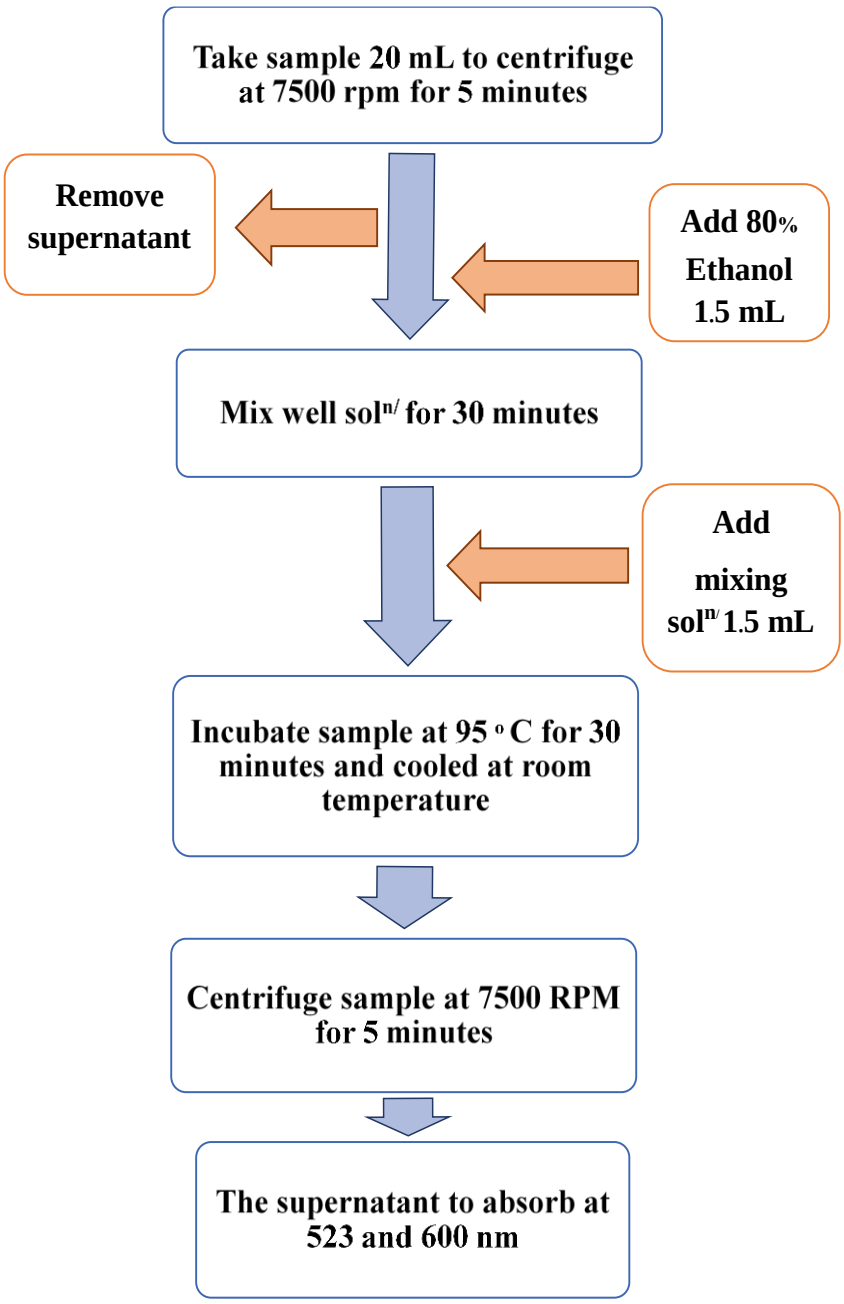


Figure 3.7: Flow chart of steps take to measure MDA produced by lipid peroxidation induced by chlorine dioxide

$$\text{MDA equivalents (nmol. mL}^{-1}) = \left[\frac{(A532 - A600)}{155000}\right] \times 10^6 \qquad (3.8)$$

Where:

A532 = absorbance at 532 nm that is the maximum of TBA-MDA complex

A600 = absorbance at 600 nm that is estimated the nonspecific turbidity

155000 = the represent value of the molar extinction coefficient for MDA

3.7 Estimation of antioxidation activity by non-enzymatic antioxidants (**Carotenoids**)

Carotenoids are produced within intact cells of cyanobacteria in the thylakoid membrane of the photosynthesis system (Loll et al., 2005; Umena et al., 2011). In a survival response against harmful conditions, these pigment compounds play an important role in scavenging ROS produced when cells encounter biotic or abiotic stress. Pigment was extracted using a methanol solution using the same process of extraction as chlorophyll *a* (Section 3.3.2). The extract solution was analysed using a spectrophotometer to measure absorbance at 470 and 720 nm wavelengths. Carotenoid levels were then calculated according to Equations 3.9 and 3.10 (Wellburn, 1994):

$$\text{Carotenoids } (\mu g/mL) = \frac{[1{,}000(A470 - A\ 720) - 2.86(\text{Chlo } a(\mu g/mL))]}{221} \qquad (3.9)$$

Where:

A470 = absorbance at 470 nm

A720 = absorbance at 720 nm

Total carotenoids in cyanobacteria

$$\text{Carotenoids}(\mu g/mL) \times \frac{\text{Volume of methanol}(mL)}{\text{Volume of sample}(mL)} \qquad (3.10)$$

3.8 Estimation of antioxidation activity by enzymatic antioxidations

3.8.1 Detection of Superoxide dismutase (SOD) activity to respond oxidative stress

To determine superoxide dismutase (SOD), a cyanobacterial sample of 20 mL was harvested. These cells were lysed with a cold solution of 0.1 M Tris/HCl (Analytical grade). This extract solution was produced by 6.057 g of Tris-base, dissolved in 350 ml of deionized water. Its pH was then adjusted to 7.4 with 12 M of HCl. Triton X-100 (Laboratory grade) of 2.5 mL was then added to the final solution. In the last preparative step, 0.20 g of β-ME (2-Mercaptoethanol; Analytical grade) and 50 mg of PMSF (phenylmethanesulfonyl fluoride; analytical grade) were added to the solution, making up a total volume of 500 mL. In total, this 0.1 M Tris/HCl solution contained 0.5% Triton X-100, 5 mM β-ME, and 0.1 mg/mL PMSF. All chemicals for extraction solution were ordered from Sigma-Aldrich, Gillingham, United Kingdom.

The harvested cyanobacteria were precipitated to separate the media solution by centrifugation at 10,500 RPM for 5 minutes. The supernatant was discarded before starting of the cracking step. The cold solution of 0.1 M Tris/HCl (0.2 mL) was added to the harvested cells and mixed well. This solution was then centrifuged again at 10,500 RPM for 5 minutes to extract super dioxide dismutase (SOD). The supernatant was then transferred to a new tube. Next, 20 µL of the collected solution was taken to analyse the antioxidant enzyme using an SOD colorimetric assay (Abcam #65354; Cambridge, UK). Alternatively, if the assay was not carried out the same day as harvesting and preparation, the extracted solution was collected and transferred immediately into a freezer for storage at -80°C. When ready to analyse, the sample was thawed. It should be noticed that the results from pre-frozen samples may be artificially lower that the real value, because of altered sample stability. Thus, the sample was stored in dark bottles for double protection. The chemicals used in this assay and their concentrations are shown in Table 3.6 and the steps involved in this assay are illustrated in Figure 3.8.

Table 3.6: Chemicals used for detecting SOD

Name	IUPAC name and formula	Concentration
Tris-base	2-Amino-2-(hydroxymethyl)-1,3-propanediol ($NH_2C(CH_2OH)_3$; MW = 121.14 g/mol	0.1M
Triton X-100	4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol, Polyethylene glycol tert-octylphenyl ether ($C_{14}H_{22}O(C_2H_4O)_n$) (n = 9-10); MW = 647 g/mol	0.5% (v/v)
β-ME	2-Mercaptoethanol (C_2H_6OS) MW = 78.13 g/mol	5 mM
PMSF	phenylmethane sulfonyl fluoride, phenylmethylsulfonyl fluoride ($C_7H_7FO_2S$) MW = 174.194 g/mol	0.1 mg/mL

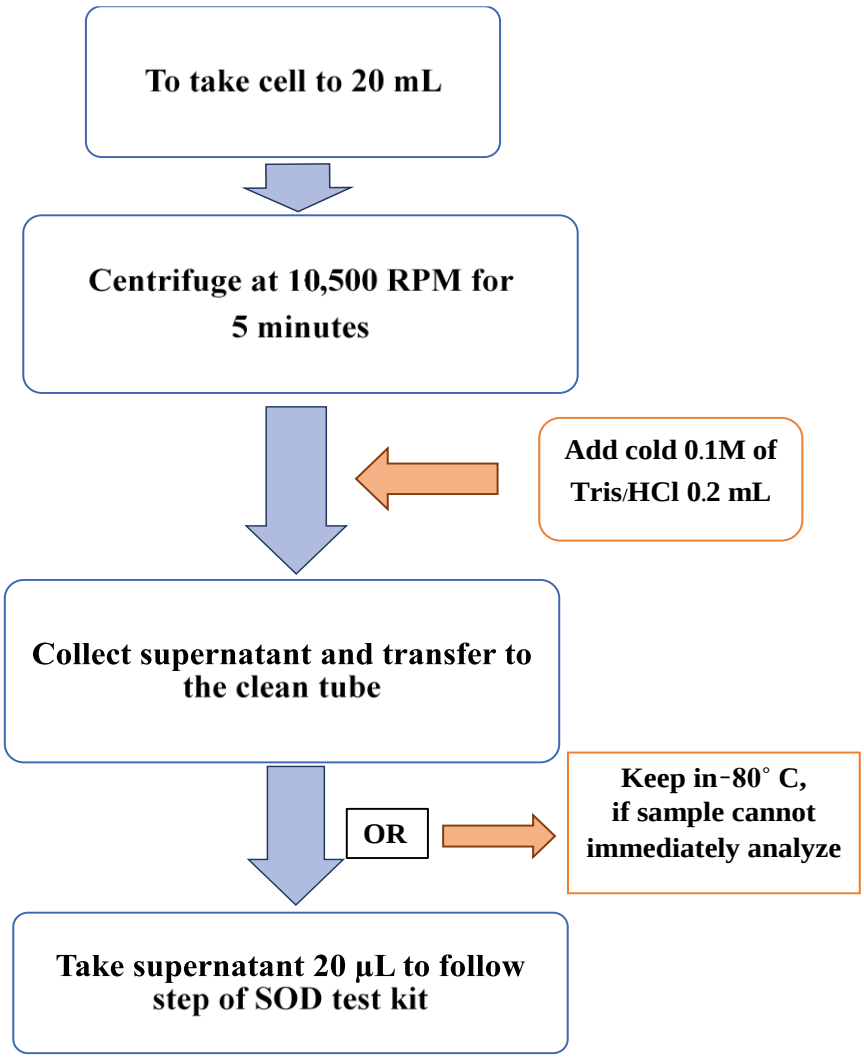


Figure 3.8: Flow chart of steps taken to analyse SOD using a test kit assay

3.8.2 Detection of Catalase (CAT) activity to quench oxidative stress

The catalase (CAT) assay is a general assay used to detect a detoxification enzyme of ROS that is generated to manage the imbalance of redox reactions occurring within the cell. CAT scavenging of ROS decreases H_2O_2, which is oxidized and transformed into water and oxygen gas (Hong et al., 2009). Cyanobacterial cells were lysed before beginning CAT detection with a test kit (Cayman Chemical, #707002; Ann Arbor, MI, USA). Following cell lysis step, 20 mL of cyanobacteria were harvested and precipitated by centrifugation at 9000 RPM for 10 minutes to separate the supernatant. Next, 1-2 mL of cool buffer solution (50mM potassium phosphate (ACS grade)containing 1mM EDTA (ACS grade) at pH 7) was added. To disrupt the physical structure of cells, they were sonicated in an ice bath for 10 minutes to reduce the decomposition of CAT enzymes due to vibration heating. The sample solution was then precipitated again by centrifugation at 9000 RPM for 15 minutes at 4 ºC to control enzyme degradation. The supernatant was then kept in an amber microcentrifuge tube with cap at -80°C in a freezer; if not analysed immediately. The supernatant was then added to the CAT assay, forming a compound solution. Light absorbance at 540 nm was measured by a spectrophotometer to quantify the amount of catalase in the sample. The degree of cellular CAT activity was then determined following Equations 3.11 and 3.12 (from instruction manual; Cayman Chemical, #707002; Ann Arbor, MI, USA). The calculation was performed by the formaldehyde detection with standardized distributions for each sample following the manual step. The chemicals involved in this assay and their concentrations are listed in Table 3.7 (all chemicals from Sigma-Aldrich, Gillingham, UK) and the procedure steps are outlined in Figure 3.9.

Table 3.7: Chemicals to prepare the buffer solution to analyse CAT activity

Name	IUPAC name and formula	Concentration
Potassium phosphate	Monopotassium phosphate (KH_2PO_4) MW = 136 g/mol	50 mM
EDTA	Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2O_8$); MW = 292.24 g/mol	1 mM

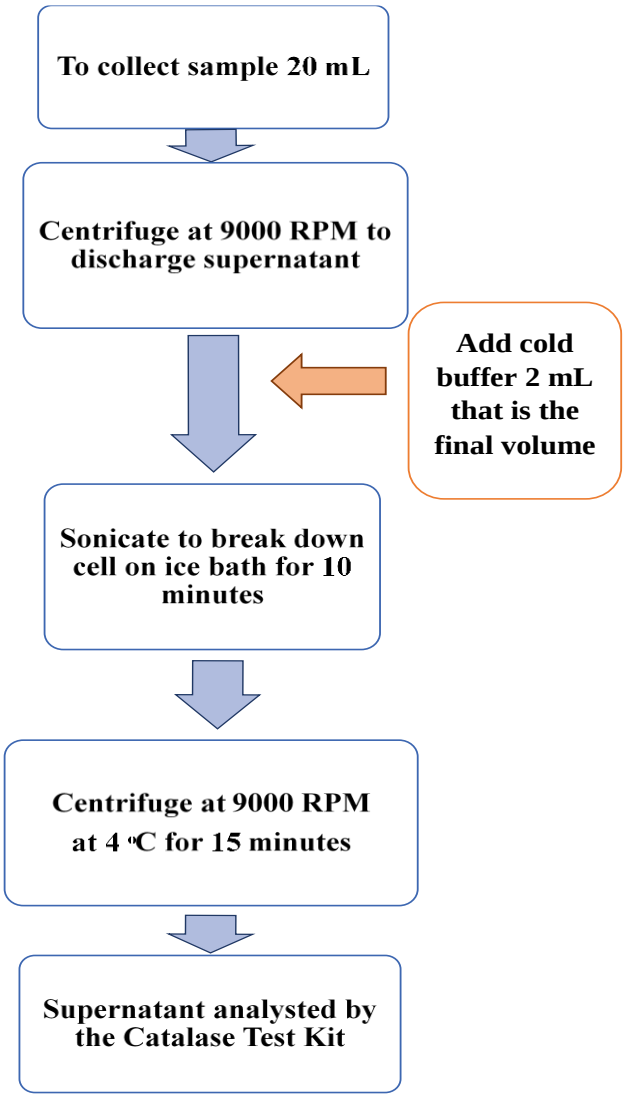


Figure 3.9: Sample preparing steps before CAT activity testing

$$\text{Formaldehyde } (\mu M) = \left[\frac{\text{Sample absorbance} - (\text{y-intercept})}{\text{Slope}}\right] \times \frac{0.17 \text{ mL}}{0.02 \text{ mL}} \quad (3.11)$$

To calculate CAT activity within cells, the amount of this enzyme required to cause formation of 1.0 nmol of formaldehyde per minute at room temperature is used:

$$\text{CAT Activity} = \frac{\mu M \text{ of Sample}}{20 \text{ min}} \times \text{Sample dilution } \left(\frac{\text{nmol}}{\text{min.mL}}\right) \quad (3.12)$$

3.9 Detection of cyanotoxins

The group of cyanotoxins includes various toxins, such as microcystins, saxitoxin, cylindrospermopsin, and others, that are produced by toxic strains of cyanobacteria. In the case of *Microcystis aeruginosa*, the dominant and the most harmful toxins are microcystins (Jacoby et al., 2000 and Li and Xiao, 2016.). Therefore, this research focusses on detecting intracellular microcystins produced before being released into the environment.

The extraction solution was prepared with 80% MeOH (for HPLC grade), mixing with water containing 0.1% of Trifluoroacetic acid (TFA; Analytical grade) and 0.1% of Tween 20 (Analytical grade), all chemicals obtained fromSigma-Aldrich, Gillingham, UK. In the pre-treatment step war followed the guideline in the sample preparation in ELISA test kit (Microcystins-ADDA ELISA, part no. ALX-850-319; Abraxis Company, USA). A homogeneous 50 mL of cyanobacteria culture was taken and filtered with 0.8 µm of nylon membrane filter paper. The filter paper was then cut into small pieces and stored in an amber bottle to prevent decomposition of cyanotoxins by UV-light. In the next step, 10 ml of the extraction solution was added into the sample bottle and placed in an ultrasonic bath for 10 min to stimulate the cell envelope into releasing cyanotoxins. The sample was stirred for 30 minutes in the absence of light to ensure the cells are thoroughly broken. After this, the sample was centrifuged at 6000 RPM

for 5 minutes to separate the precipitate. The supernatant was then used to verify the amount of cyanotoxins produced by *M. aeruginosa*. If the sample was not used to analyse cyanotoxins within 24 hours, was stored in an amber bottle at -80°C. In this research, we used an enzyme-linked immunosorbent assay (ELISA) designed specifically to verify microcystin concentration produced by cyanobacteria (Microcystins-ADDA ELISA; Abraxis Company #ALXX-850-319, Los Angeles, CA, USA). The chemicals used in this assay and their concentrations are listed in Table 3.8 and the procedure is illustrated in Figure 3.10.

Table 3.8: Chemicals used for extraction of cyanotoxins

Name	IUPAC name and formula	Concentration
Methanol	CH_3OH or CH_4O; MW = 32.04 g/mol	
TFA	Trifluoroacetic acid ($C_2HF_3O_2$) MW = 114.02 g/mol	0.1% (w/v)
Tween 20	Polyoxyethylene (20) sorbitan monolaurate ($C_{58}H_{114}O_{26}$); MW = 1227.54 g/mol	

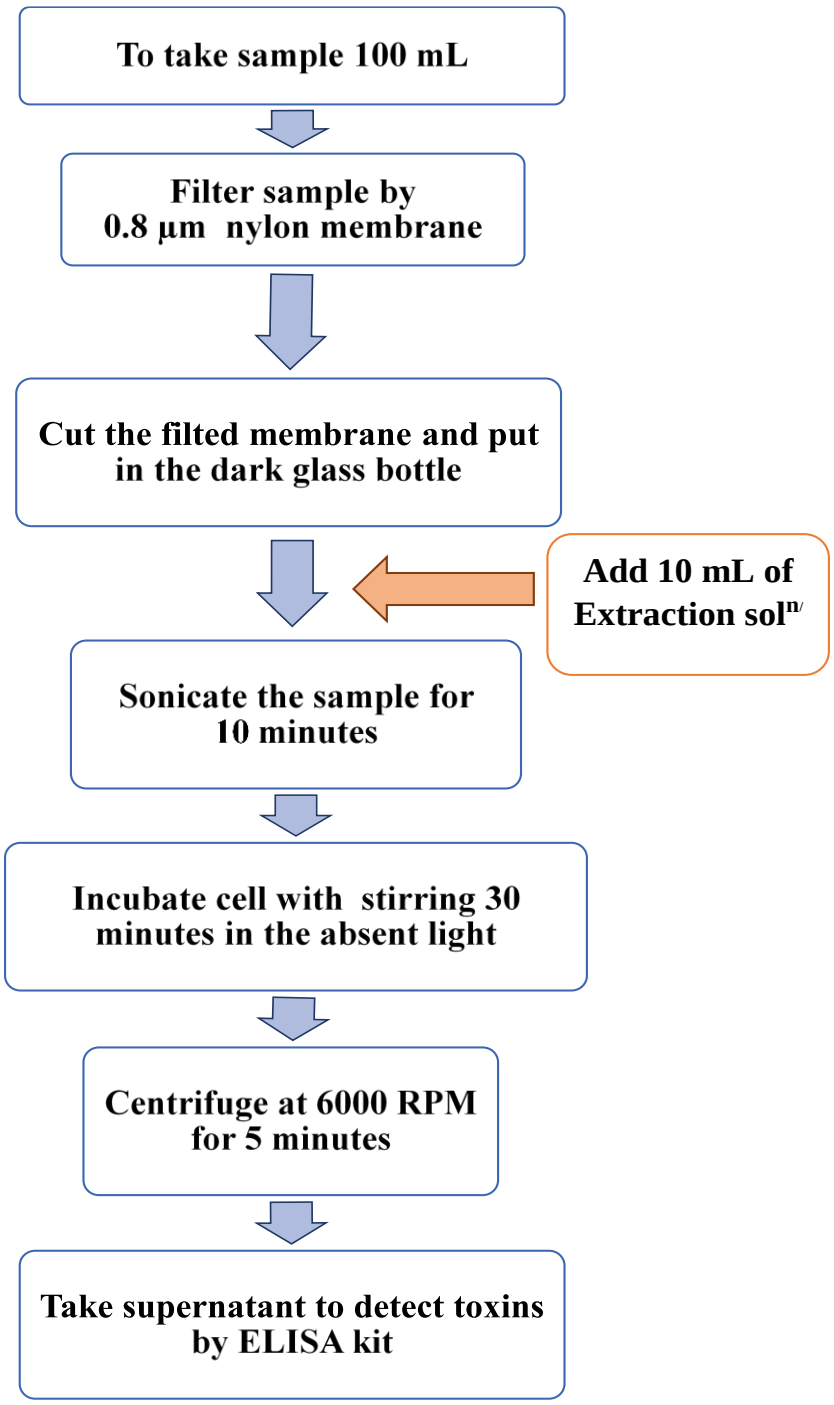


Figure 3.10: Flow chart of steps taken to extract cyanotoxins from cells before measurement by ELISA kit

3.10 Statistical analysis

Data were statistically analysed with SPSS (Version 24; IBM Corp, USA). Two-way analysis of variance (ANOVA) tests were performed to evaluate the effects of ClO_2 treatment on cyanobacteria, with two fixed factors: concentration and treatment contact time. Moreover, data was separated into four intervals of growth (Qurartiles1-4): the early stage (lag phase; Quartile1), exponential stage (log phase; Quartile 2), late exponential stage (Quartile 3), and stationary stage (Quartile 4). Tukey's honestly significant difference (HSD) post-hoc tests were employed to correct for multiple comparisons, with a significance threshold value of α (p-value) = 0.05. Cyanotoxins produced were interpreted by two-way ANOVA, also known as the Kruskall-Wallis test. Finally, relationships between experimental observations with treatment concentration and contact time were evaluated by Spearman's rho correlation analysis.

Chapter 4. PRELIMINARY TEST

4.1 Introduction

Chlorine dioxide (ClO_2) offers a promising choice for use as pre-and post-treatment disinfectant for water treatment plants instead of traditional chemicals such as chlorine and other chlorine compounds that react with humic acid and produce harmful by-products such as trihalomethanes (Aieta, and Berg, 1986; Volk et al., 2002). ClO_2 is more effective than other chlorine compounds for eliminating protozoa such as *Cryptosporidium* and *Giardia* (U.S. EPA, 1999). Although ClO_2 does not generate THMs, ClO_2 decomposes to chlorate, chlorite, and chloride as by-products. Other by-products may be possible (Richardson et al., 2000). Only chlorite has a guideline value set by WHO. Beneficially, Benarde et al., (1965) reported that effective disinfection with ClO_2 did not change with pH conditions. However, its effectiveness as a disinfectant can be reduced over long exposure time, light exposure, or presence of organic matter. In addition, higher concentrations of chlorine dioxide demonstrate increasing deterioration rates relative to those of lower concentrations of ClO_2 (Medir and Giralt , 1982).

Gordon et al (1972), who derived the following Equation 4.1, studied the stability of ClO_2 solution in aqueous buffer.

$$2ClO_2 + H_2O \longrightarrow ClO^-_3 + ClO^-_2 + 2H^+ \qquad (4.1)$$

Today, various sources of water in Thailand have been reported to exhibit cyanobacterial blooms and contamination by microcystins. For example, in June 2009, a large fish kill was reported at Kaen Nakhon Reservoir, Khon Kaen Province, coinciding with a large bloom of *Microcystis* sp. (Peerapornpisal et al., 2000). This experiment was designed to study the effectiveness of ClO_2 treatment on a toxic strain of *Microcystis* sp. that was found to proliferate in Thailand. Growth and toxin production of these cyanobacteria were examined. The efficacy of ClO_2 treatment to remove cyanobacteria and the effects on cyanotoxin production at various concentrations and contact times were also examined.

4.2 Analytical procedures

4.2.1 Preparation of ClO_2 stock solution and evaluation of its stability in water

ClO_2 is typically generated at the site of work in quantities depending upon the specific requirements at the time of use (U.S. EPA, 1999). The majority of chlorine dioxide syntheses are launched by a chlorine compound (gas or solution) reacted with an acid solution, which is convenient for small scale, laboratory applications. In this research, a batch reactor was set up for small scale ClO_2 production by citric acid anhydrous and sodium chlorite ($NaClO_2$) (Sigma-Aldrich, Dorset, UK). These chemical precursors are used in their aqueous forms, which are highly corrosive. Chlorine dioxide was synthesized by the acidification of sodium chlorite by adding citric acid. To convert chlorine dioxide gas into an aqueous solution, the gas was passed through a saline solution (<0.9% w/v) chilled to 10-15 °C. The high concentration stock solution of chlorine dioxide was kept in a sealed amber glass bottle and refrigerated at 4 °C. The concentration of the ClO_2 stock solution was determined by Iodometric titration (Section 3.1) before starting the experiment.

The experiment to observe ClO_2 degradation was set up at room temperature and ambient fluorescent-light conditions of the laboratory. ClO_2 decomposition was studied at 0.0, 1.0, 5.0, 10.0, and 20.0 mg/L starting concentrations in 500 mL solutions. Extracts of 20 mL were taken on a daily basis for titration with 0.1 N sodium thiosulfate to determine the decomposition rate.

4.2.2 Cyanobacterial cultivation and growth

The proliferation of *M. aeruginosa* was observed in a 2 L solution in an Erlenmeyer flask. That was covered with cotton to prevent contamination by other microorganisms but allow oxygen (air) to support bacterial growth. Measurements were taken every other day starting with the lag phase and continuing until the decline stage (Figure 4.1). They were provided nutrients (MA media; Section 3.2), 12/12 hrs light and dark cycle of intensive light (GRO-Lux, F 36 W/GRO-T8, Feilo Sylvania Europe Limited, Newhaven, UK), and constant room temperature at 20 ± 1 °C. At each sampling time, a sample of 10 mL was collected and two different, complementary methods were used

to evaluate viability (Figure 4.1). Firstly, chlorophyll *a* was extracted with methanol (HPLC grade; Sigma-Aldrich, Gillingham, UK) and detected by UV-Vis spectrometry (665, and 720 nm; Chapter 3). This photosynthesis pigment is representative of intact cyanobacteria. Simultaneously, whole-cell spectrometry (OD 680) represented a simple procedure of measuring cell density by light absorbance by cyanobacterial chlorophyll, which absorbs wavelengths of 680 nm. OD680 is an improper method for detecting viable cells because dead cells can increase turbidity and obscure the absorption measurement by the spectrophotometer.

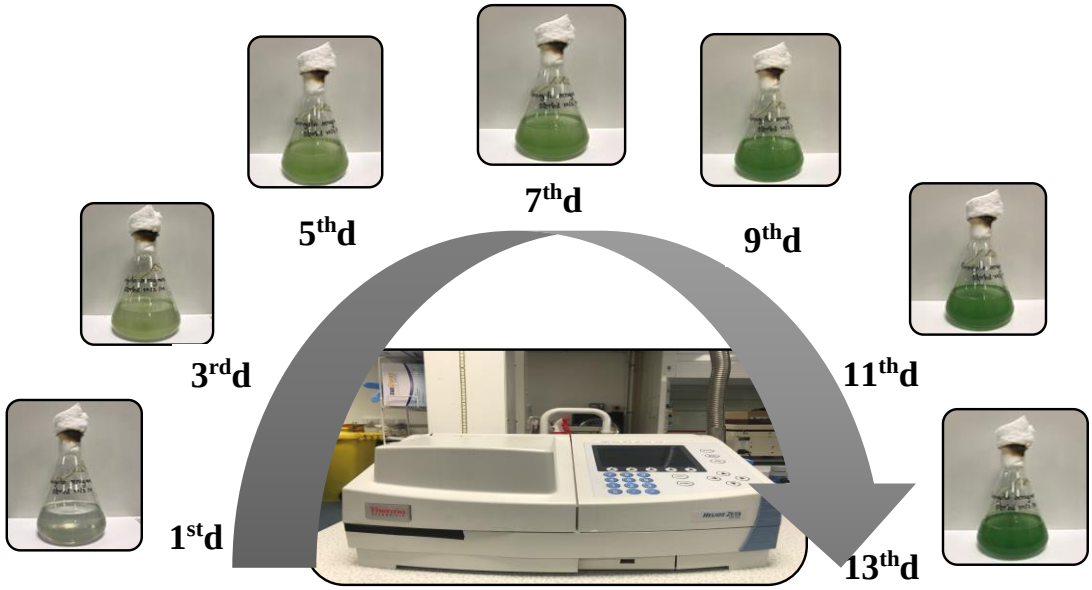


Figure 4.1: The growth of *M. aeruginosa* was evaluated by measuring OD 680 on the spectrophotometer and chlorophyll *a* extraction

4.2.3 Measurement of cyanotoxins production

To confirm the toxicity of this strain, enzyme-linked immunosorbent assay (ELISA) was used to evaluate cyanotoxin production. For our experiment, the Microcystin-ADDA ELISA Kit (microliter plate; Abraxis Company) was used for measurement of cyanotoxins. The sensitivity of this ELISA kit is in the parts-per-billion range (Abraxis,

2007). The colour developed exhibits an inverse relationship to the concentration of cyanotoxins. Hence, resulting dark colors reflect low microcystins concentration, whereas lighter colors identify high concentrations of microcystins, relative to reference standards. Microcystins analysis consisted of a multistep process to filter, extract and detect intracellular microcystins following Figure 4.2. Filtration separated the extracellular toxins from the cells for quantification. Extraction involved breaking the cell membranes to release intracellular toxin (Section 3.9). Detection of intra and extracellular microcystins was conducted by ELISA kit.

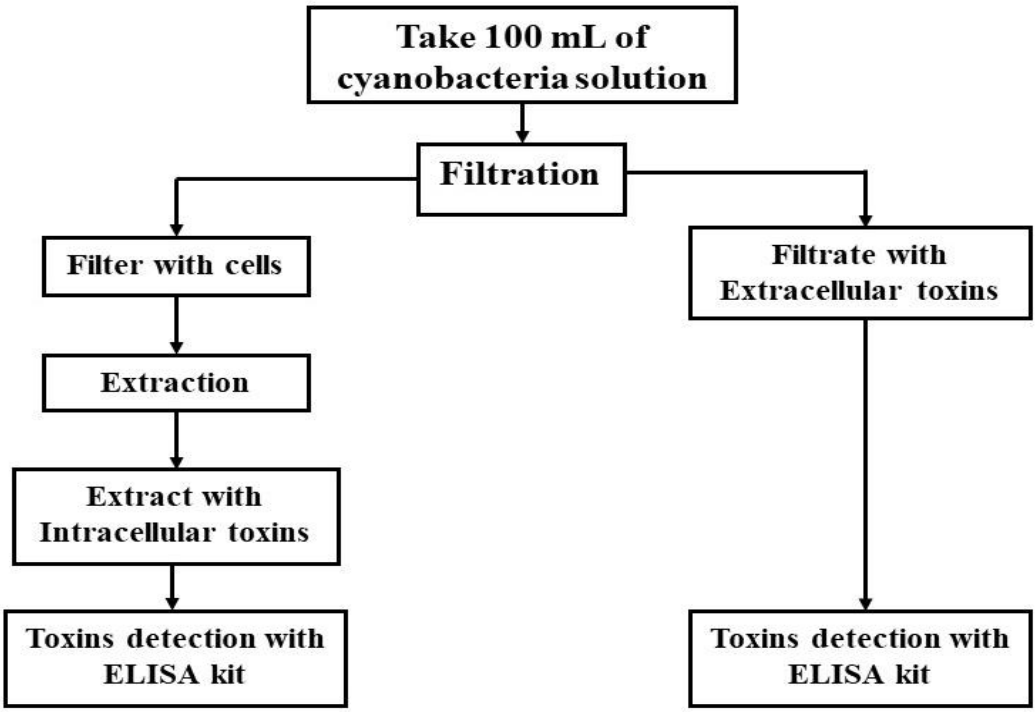


Figure 4.2: The analytical steps to detect cyanotoxins using an immunoassay

4.2.4 Effects of ClO_2 exposure on cyanobacteria and cyanotoxins formation

4.2.4.1 Sample preparation steps to evaluate treatment by ClO_2

This experiment was designed to evaluate the disinfection efficacy of ClO_2 to inhibit cyanobacteria and decrease toxin production. The investigation focuses on cyanobacteria during an interval of rapid growth (the exponential phase) because during this period most of the cells are intact. This investigation was performed with batches of ClO_2 prepared at 0.0 (control sample), 0.5, 1.0, 1.5, 3.0, 5.0, and 10.0 mg/L ClO_2. These concentrations (C_2) were prepared from ClO_2 solution at 200 mg/L (C_1) that was itself prepared by dilution from the ClO_2 stock solution. Each resulting solution was made up to 100 mL (V_2) following the calculation of Equation 4.1.

$$C_1V_1 = C_2V_2 \quad (4.1)$$

Where:

C_1 = The initiate ClO_2 concentration (stock solution)

C_2 = The final concentration of ClO_2

V_1 = The initiate volume of ClO_2

V_2 = The final volume of ClO_2

Cyanobacteria cell samples (X mL) were collected at X d of growth, which corresponded to the exponential growth stage and placed into [container type and size] for treatment with ClO_2. After cell samples were exposed to ClO_2 at the different concentrations for 30 minutes, the ClO_2 reaction was immediately quenched with 1 mL of 0.1% (w/v) sodium thiosulfate (Sigma-Aldrich, Gillingham, UK). Solutions exposed to ClO_2 were subsequently evaluated for the level of chlorophyll *a* and OD 680, comparing with the control sample (untreated), as described above. Simultaneously, a portion of the treated samples and untreated control sample were collected to estimate cyanotoxins formation, including both intracellular and extracellular cyanotoxins. Samples were filtered, and the filter paper (containing cyanobacterial cells) was kept in an amber glass bottle for

extraction with solution of 80% methanol + 0.1% of Trifluoroacetic acid (TFA) + 0.1% of Tween 20. Finally, intracellular toxins from broken cells were separated by centrifugation (6000 rpm for 5 minutes) and evaluated using the ELISA kit. Filtrate stored in amber glass bottles was used to directly measure the extracellular cyanotoxins, also using the ELISA kit. Figure 4.3 illustrates these steps.

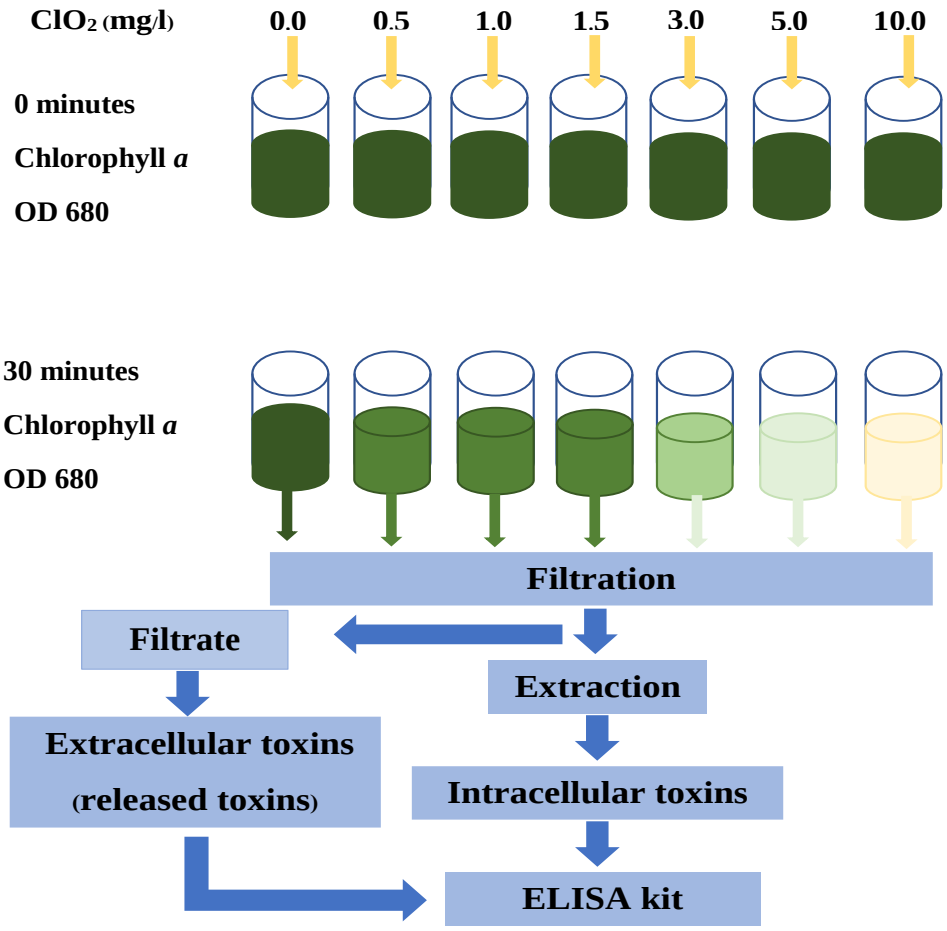


Figure 4.3: The experimental steps to monitor the effective treatment of ClO_2 to impact cyanobacteria viability and cyanotoxins formation

4.2.4.2 ELISA step to evaluate microcystins produced after M. aeruginosa treated by ClO_2 at various concentration

The ELISA immunoassay consists of eight steps. Firstly, 50 µL of standard and sample solutions were added to the microcystin plate. Secondly, 50 mL of antibody solution was added to individual wells by a multi-channel pipette. Wells were then covered with parafilm or tape. The well plate was rapidly swirled by hand for 30 s to mix well. The plate was left to incubate for 90 minutes in the laboratory. Thirdly, the washing step was launched after incubation by removing the cover and shaking the solution out. The microplate wells were washed three times with 250 µL of 1x buffer solution for each well and then dried by vigorous shaking on the paper towels. Fourthly, 100 µL enzyme conjugate solution was added to individual wells by multi- channel pipette. This microplate was covered again with parafilm, mixed by hand, and incubated for 30 minutes at laboratory conditions. Fifthly, the washing procedure in the third step was repeated. Sixthly, 100 µL of substrate solution was added individual wells by multi-channel pipette and incubated for 30 minutes in laboratory conditions, in the dark. Seventhly, after incubation, 50 µL of stop solution was added to individual wells successively using multi-channel pipette to stop the reactions. Finally, from the microplate, absorbance was recorded at 450 nm by microplate spectrophotometer (Epoch, BioTek, Agilent, UK).

4.3 Result and Discussion

4.3.1 ClO_2 synthesis and stability in water

The synthesised ClO_2 stock solution had a concentration of 10,200 ± 100 mg/L, as determined by titration. Degradation of synthesized chlorine dioxide was studied at 1.0, 5.0, 10.0 and 20.0 mg/L to represent conditions normally used in water treatment. Results revealed degradation of 34, 28, 30 and 43% over 8 days from these initial concentrations, respectively. Declining amounts of ClO_2 were observed for every starting concentration that have shown in Figure 4.4. The highest starting concentration experienced the most decline. However, in all but the lowest concentration, the decreased ClO_2 concentrations remained suitable for use as a disinfectant (>1 mg/L).

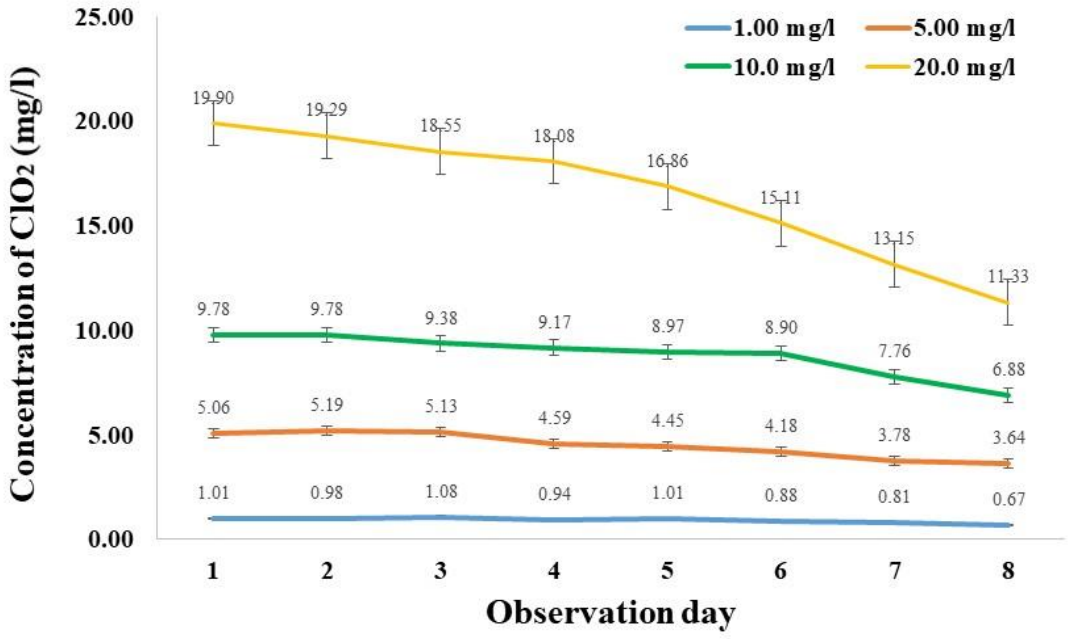


Figure 4.4: Decomposition of chlorine dioxide from starting concentrations of 1.0, 5.0, 10.0 and 20.0 mg/L

Based on these observations, further experiments were designed with a maximum contact time of 3 days because minimal degradation was observed in all but the highest ClO_2 concentration (Figure 4.5).

4.3.2 M. aeruginosa growth and cyanotoxins production

Growth rate and cyanotoxin production (intracellular and extracellular) of *M. aeruginosa* were monitored for fourteen days (Figure 4.5). Results show that exponential growth started at observation day 5 and was detected by initial OD 680 nm (I) on 0.13 nm. In the last sampling interval (day 13), rapid growth continued and was measured as final OD 680 nm (F) at 3.08 nm. Following Equation 3.6, the speed of *M. aeruginosa* strain growth was quantified as 1.05 d^{-1}.

Intracellular toxin concentration was observed to steeply increase, accompanying the proliferation of cyanobacterial cells. Extracellular cyanotoxins (i.e. released) were detected at relatively lower concentrations, although also increasing with cell proliferation. At 13 d, intracellular toxin concentration was 152 µg/L for the highest level and

extracellular toxin concentration was 7.9 µg/L as well. Extracellular toxins released into the environment are less than intracellular toxins because released toxin is associated with cell lysis but not healthy, intact cells (Leda et al, 2016). The WHO has a provisional guideline of microcystin-LR that is less than 1.0 µg/L (WHO, 2011) for health risks to humans and animals. After 8 d of observation, extracellular toxins exceeded this amount, suggesting *M. aeruginosa* toxin levels became problematic to human and environmental health during the exponential growth stage. Although intracellular toxins exceeded this level after only 3 d of observation, these toxins are not problematic until released from the cells.

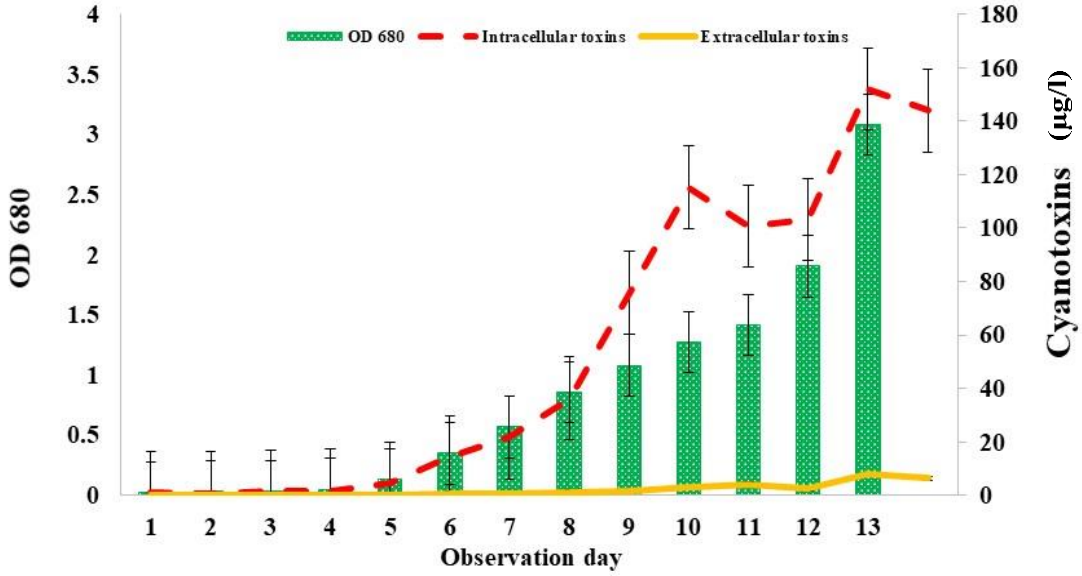


Figure 4.5: Monitoring the growth of *M. aeruginosa* and microcystins production, from lag phase to 14 days of growth

4.3.3 M. aeruginosa inhibition by ClO_2 and effects on cyanotoxins production

Evaluation of the efficacy of ClO_2 on *M. aeruginosa* was initiated at 0.0 (control), 0.5, 1.0, 1.5, 3.0, 5.0 and 10.0 mg/L ClO_2, which were each applied with an exposure time of 30 minutes. As the concentration of ClO_2 increased, chlorophyll and intracellular cyanotoxin concentrations decrease in Figure 4.6. The effects on OD 680 and extracellular

cyanotoxin release were less clear. Lower concentrations of ClO_2 (0.5, 1.0 and 1.5 mg/L) do not differ substantially in their effect to inhibit *M. Aeruginosa*, given the comparable levels of chlorophyll and OD 680 observed. On the other hand, higher concentrations of ClO_2 (3.0, 5.0 and 10.0 mg/L) were more effective than lower concentrations at disinfecting *Microcystis sp*. OD 680 decreased slightly compared with the control sample; however, OD 680 must be combined with other measurements to evaluate cell viability. Chlorophyll *a* decreased sharply after cells were exposed to higher concentrations of ClO_2. The reaction of cyanobacteria and ClO_2 is hypothesized to start with disruption of the permeability of the outer membrane proteins and lipids, which form a considerable component of the cyanobacterial cell wall (Aieta and Berg, 1986). Subsequently, ClO_2 oxidizes the olefin structure (unsaturated hydrocarbons, C=C) at the long hydrophobic tail of chlorophyll (Matile, et al., 1996). ClO_2 also reacts with the pyrrole chain ring of chlorophyll causing deterioration and bleaching of the pigment color. Sharp decreases in the chlorophyll *a* concentration observed in Figure 4.6 likely resulted from these reactions.

Extracellular toxin levels released by *M. aeruginosa* were very low and accurate measurements were difficult to obtain, which is consistent with previous observations showing low extracellular toxins during the exponential growth stage (Figure 4.5). As a result, the effects on extracellular cyanotoxins were similar between cells treated with ClO_2 and the control sample.

Intracellular toxins production by *M. aeruginosa* also decreased sharply after exposure to high concentrations of ClO_2 (3, 5 and 10 mg/L). Conversely, low concentrations of ClO_2 (<3 mg/L) were less capable of reducing the production of microcystins. This finding is important because the recommended treatment level with ClO_2 is 1.5 mg/L to avoid generation of inorganic by-products chlorite and chlorate (Gray, 2014). That concentration is ineffective to inhibit *M. aeruginosa* and prevent microcystins production in the early, exponential growth stage. These results also suggest a relationship between chlorophyll content and intracellular microcystin concentration. Further exploration of growth phase (cell density) and ClO_2 dose is needed to understand how low concentrations of ClO_2 relevant to water treatment affect cyanobacteria.

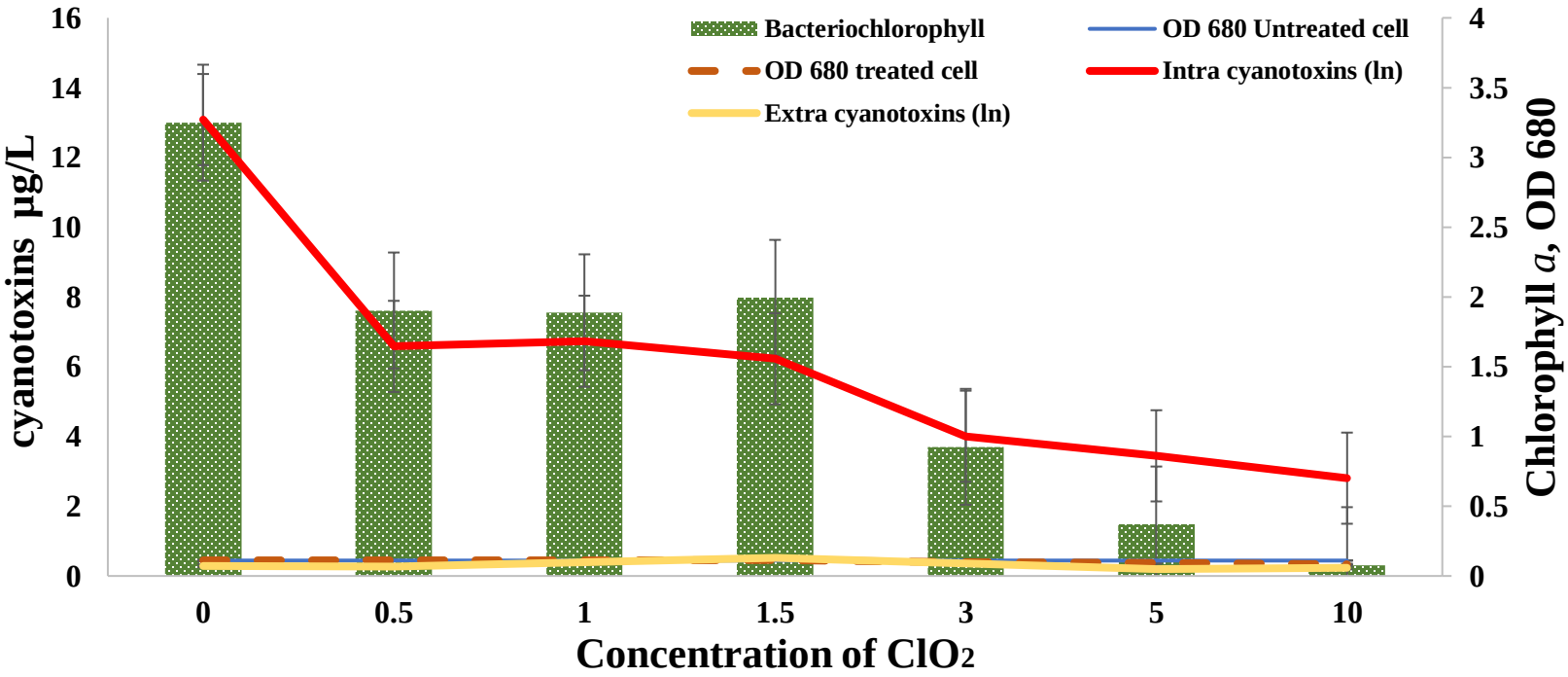


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Figure 4.6: Examination of *M.aeruginosa* and microcystins (concentration) following 30 minutes exposure to different concentrations of ClO_2.

4.4 Summary of Key Findings

This work provides evidence to support the use of ClO_2 as a qualifying treatment for inhibiting *M. aeruginosa* and avoiding microcystin release. Progressively higher concentrations of ClO_2 have demonstrated the ability to reduce chlorophyll *a* and production of microcystins.

Preliminary tests established that ClO_2 concentration was stable for 3 days and then declined, reaching a loss of 50% in 8 days. Experiments were designed for contact time ≤ 3 days to avoid this ClO_2 decomposition.

Growth of *M. aeruginosa* showed that it proliferated in MA media. ELISA kit confirmed that this strain was toxic and produced microcystins. Intracellular toxins above the method detection limit were first measured at 3 days and extracellular toxins were first measured at 8 days

Finally, high-dose treatment (≥ 3 mg/L) with ClO_2 inhibited *M. aeruginosa* and minimised intracellular cyanotoxin production, whereas the results from lower dose treatment, in line with what is used in water treatment processes, showed ineffective inhibition and less influence on cyanotoxin production. Further exploration of growth phase (cell density) and ClO_2 dose is needed to understand how low concentrations of ClO_2 that are relevant to water treatment affect cyanobacteria.

Chapter 5. STRESS FACTORS

5.1 Introduction

By-products of cyanobacterial biosystems include reactive oxygen species (ROS), namely $^1O_2$, O_2^-, H_2O_2, OH^-, and others. Thus, cyanobacteria cells inevitably encounter radicals generated from these species. ROS was identified to harm with various cellular components, such as reaction with side-chains of amino acid groups, leading to deterioration of protein structures (Davies, 2004). Particularly, the polyunsaturated fatty acids (PUFA) site of the lipids membrane is vulnerable to losing electrons by ROS, resulting in malfunction and unstable membrane structure (Triantaphylidès et al., 2008). The most harmful ROS is OH^- due to its non-selective destruction within the cell and its ability to fluently diffuse into various biomolecules (Czapski, 1984). On the other hand, O_2^- is less reactive species because of its negative charge can pass through certain anion channels. Although there is a limitation to the reaction, this initiates H_2O_2 production by superoxide dismutase (SOD), scavenging and releasing O_2 and Fe^{2+} from iron-sulphur bonds in proteins ($[4Fe\text{-}4S]^{2+}$). These substrates promote OH^- free radicals via the Fenton reaction (Latifi et al., 2009).

Our research observed stress induced by ClO_2 treatment, the production of ROS in *Microcystis* sp. may be accelerated. Theoretically, ClO_2 acts as an electron accepter. Then, it can take electrons from intracellular cyanobacteria, resulting in redox imbalance. This disturbed equilibrium accelerates ROS production within cells that is supported to induce oxidative stress and cell damage (Gill and Tuteja, 2010).

Cyanobacterial cells are believed to have one or more mechanisms that allow them to manage excess levels of ROS and protect them from damage. This experiment focuses on *M. aeruginosa* treated with ClO_2, which causes ROS production and ensuing oxidative stress. This will be observed by measuring the viability of cells, including a biomarker of cell damage: lipid peroxidation. In addition, the extent of anti-oxidation activities required to detoxify ROS is examined.

5.2 Experimental Procedures

5.2.1 Experimental design and operation

Growth phases were considered to be important points for evaluating the impact of oxidizing agents on the health and stress response of cells, as the population will be experiencing different levels of resource availabilities including light and nutrients. The efficacy of ClO_2 to inhibit *M. aeruginosa* was observed by measuring oxidative stress response compared with untreated cyanobacterial cells.

A 35 L aerobic reactor was used to grow *M. aeruginosa* in MA media (Section 3.2). An initial seed of 1 mL from high population density was added to MA media to create the initial low population density. The reactor was run at 20 ± 2 °C in a cycle of 12 hours in darkness and 12 hours in light (GRO-Lux, F 36 W/GRO-T8, Feilo Sylvania Europe Limited, Newhaven, UK). For each factor of treatment, cyanobacterial samples were collected (95 mL) from the aerobic reactor and placed into a 150 mL plastic bottle, which was cleaned with Neutracon (Decon Laboratories, Hove, UK) and subsequently rinsed at least three times by deionized water before use. After 7 days of incubation, samples were collected every 5 days for ClO_2 treatments as the *M. aeruginosa* culture progressed from low population density to high population density (Quartiles 1-4; Figure 5.1). In each quartile, ClO_2 treatment was applied at 0.0, 1.5, 3.0 and 5.0 mg/L for 3 hr, 1 day, 2 days and 3 days of contact time. These contact times were selected based on the decline of ClO_2 concentration with time. After 3 days, the last contact time, ClO_2 concentration degradation was observed (Chapter 4, Section 4.3.1). Treatment dosage was calculated as specified in Equation 4.1 (Section 4.2.4). A total of 16 treatments combinations (concentration-time) were applied in each quartile. All treatments were done in triplicate. Controls (0.0 mg/L ClO_2) were also done in triplicate. The sequence of the experiment is displayed in Figure 5.1.

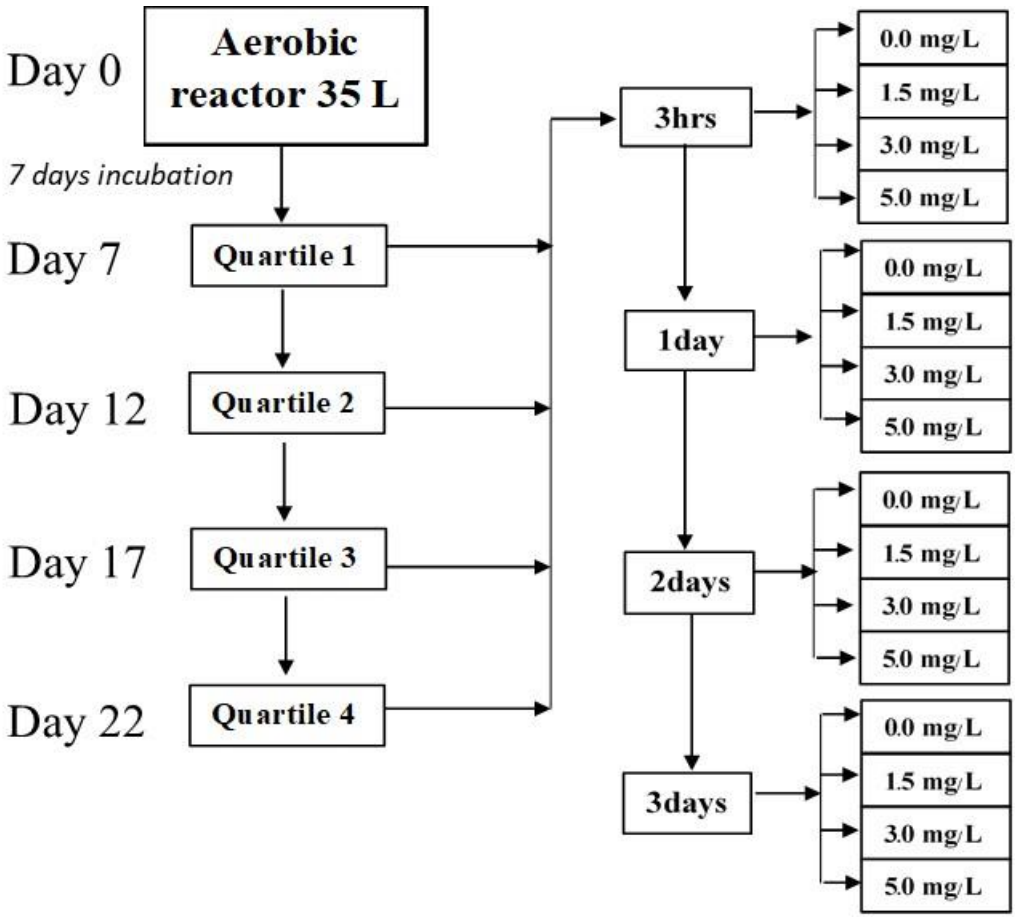


Figure 5.1: Experimental step to observe the efficacy of ClO_2 treatment to inhibit *M. aeruginosa* overall of four quartiles at various contact times and ClO_2 concentrations.

After each contact time had elapsed, the reactions of ClO_2 treatment were stopped by 1ml of 1% sodium thiosulfate ($Na_2S_2O_3$) (Section 4.2.4). Treated cell and control solutions were evaluated for cell health and stress response as shown in Figure 5.2.

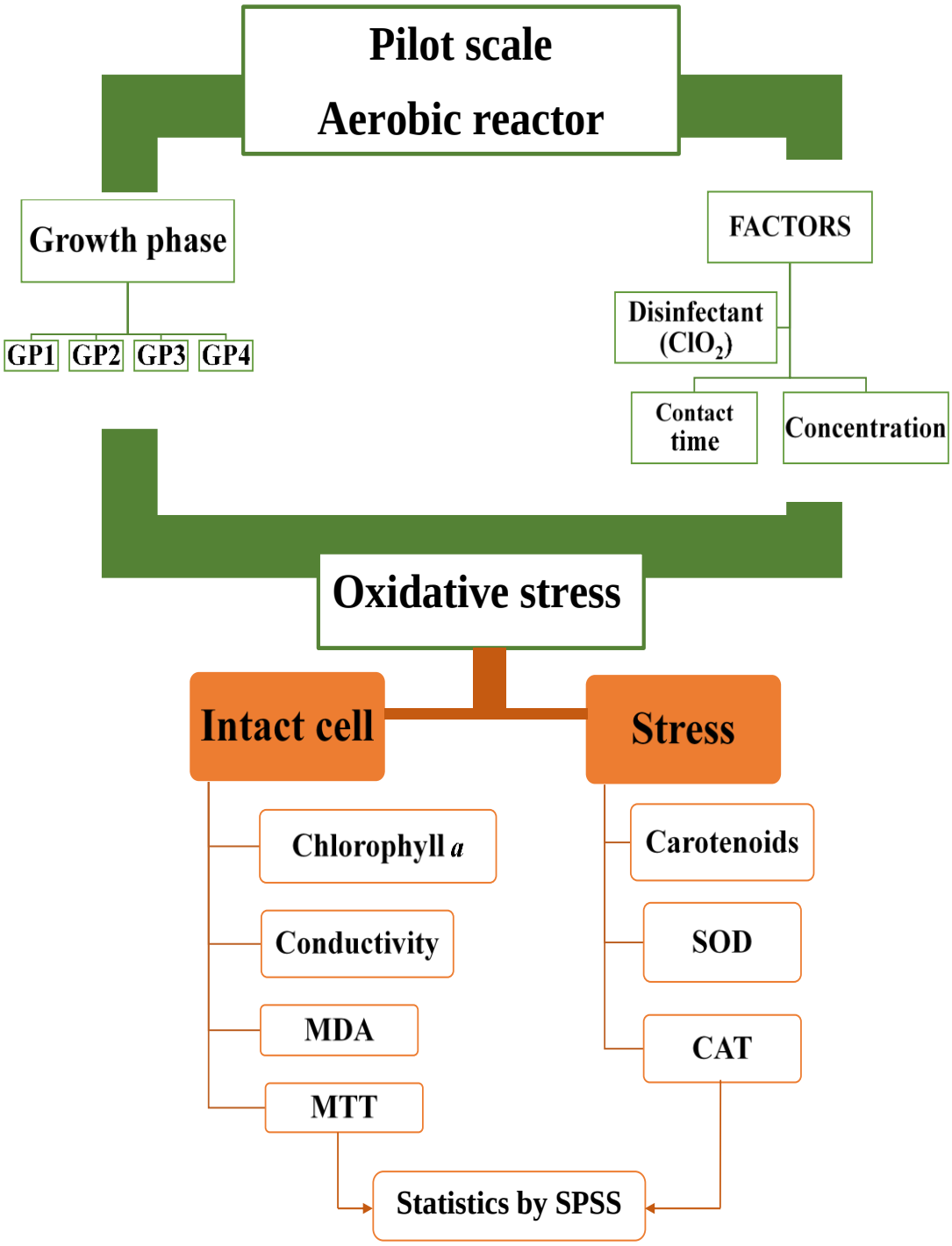


Figure 5.2: Experimental plan to investigate *M. aeruginosa*, cell healthy and stress induced by the disinfectant: ClO_2 exposure in each growth phase

5.2.2 Analytical measurements

All samples were analyzed for chlorophyll *a,* membrane malfunction by leakage (conductivity), (MDA) and metabolic activity (MTT). Stress response was examined by the level of antioxidants produced to reduce oxidative damage and involved measurements of CARss, SOD and CAT.

All samples were analyzed for chlorophyll *a* by centrifuging 1.5 mL of cyanobacteria solution; extraction of photosynthesis pigments with 99.9% methanol to dissolve the cell membrane, releasing chlorophyll *a* and other pigment compounds; and centrifugation again (Section 3.3.2). The resulting solution was analysed by spectrophotometer and concentration of chlorophyll *a* was calculated using Equations 3.4 and 3.5.

Electrical leakage, correlated with cell membrane permeability, was determined by direct measurement of electrical conductivity of each solution with a conductivity probe (Section 3.4).

In the MTT assay, 200 µL of each solution was pipetted into the microplate and incubated at 37˚C for 4 hours. The resulting formazan crystals were dissolved by DMSO (Dimethyl sulfoxide), producing a clear solution, which was then assessed with the spectrophotometer (Section 3.5).

To quantify the secondary product of lipid peroxidation, malondialdehyde (MDA), 20 mL samples were centrifuged at 7500 RPM for 5 min. The precipitate was reacted with thiobarbituric acid (TBA) at 95˚C for 30 minutes and analysed by spectrophotometer at 532 and 600 nm (Section 3.6).

Carotenoids extraction are followed an identical procedure to chlorophyll *a* extraction (Section 3.7). The same extracted solution was analysed for absorbance at 470 and 720 nm by spectrophotometer and a calculation step followed using Equation 3.9.

Enzymatic antioxidants SOD and CAT were measured. For SOD, a 20 mL sample was harvested and centrifuged at 10,500 RPM for 5 minutes. The supernatant was discarded and the precipitate was extracted with 0.2 mL of Tris/HCl (Section 3.8.1). The extract (20 µL) was analysed by SOD test kit (Section 3.8.1). For catalase analysis, a 20 mL sample was collected and centrifuged (9000 RPM for 15 minutes). The

supernatant was discharged. Buffer solution (2 mL) was added and cell membranes were broken by sonication for 10 minutes. The solution was stored at 4 °C for 10 minutes and then centrifuged at 9000 RPM for 15 minutes. The supernatant solution was recovered and evaluated by the catalase activity test kit (Section 3.8.2).

5.2.3 Statistical analysis

Mean values were determined for chlorophyll a, conductivity, MTT, MDA, CARs, SOD, and CAT for each treatment condition and graphed to evaluate differences. Data for MTT, MDA, CARs, SOD, and CAT were normalised by chlorophyll *a* concentration of the individual sample at those treatment conditions. Error bars were calculated as 95% confidence intervals from the mean values. Two-way analysis of variance (ANOVA) was used to evaluate all results using SPSS (version 24; IBM Corp, USA). Results were grouped by quartile. The General Linear Model was the core function and the fixed factors were contact time and concentration of ClO_2. Tukey's honestly and significant difference (HSD) post-hoc tests were used to employ for multiple comparisons. Significance was established by a confidence value of 95 % ($P < 0.05$). The F-statistic was the ratio of overall variation of means of multiple groups to between-subjects variation. We use the F-test to determine if the standard deviations from two sets of data are statistically different.

5.3 Cellular Health

5.3.1 Presence and degradation of chlorophyll a

Cells were inhibited by ClO_2, as measured by a reduction in chlorophyll *a* concentration (Figure 5.3). Control samples (untreated cells) show that levels of chlorophyll *a* continually increase with time. For the first and second quartiles (Quartiles 1 and 2), the growth of *M. aeruginosa* is gradual, indicative of lag phase, as apparent by a slight increase of chlorophyll *a.* During Quartile 3 to Quartile 4, the concentration of chlorophyll *a* dramatically increased, indicating the increasing of cyanobacterial proliferation consistent with the exponential phase.

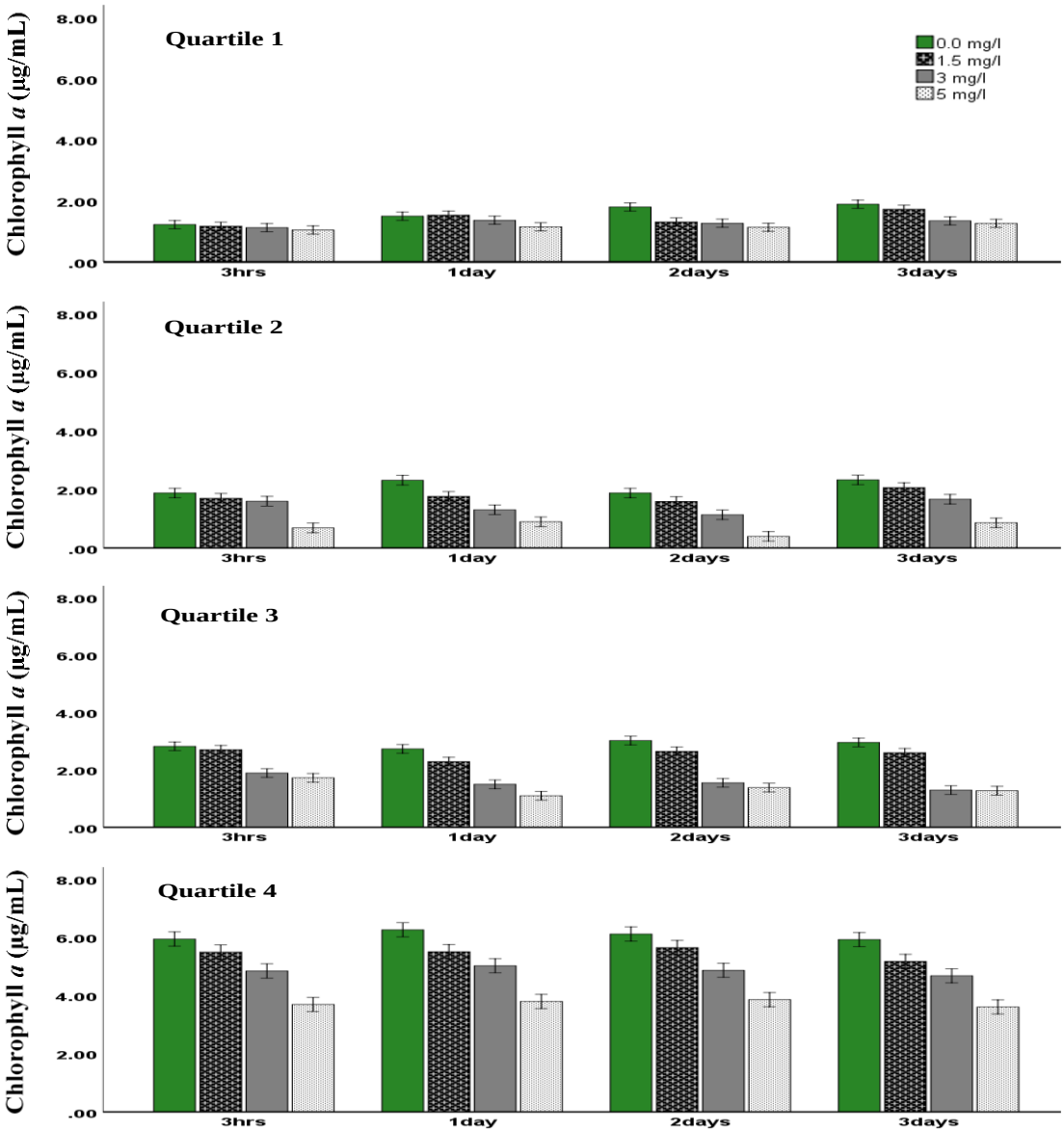


Figure 5.3: Change in chlorophyll *a* concentration through the lifespan after ClO_2 treatment at 0.0, 1.5, 3.0, and 5.0 mg/L and observation at 3hr, 1d, 2d and 3d of exposure time

After *M. aeruginosa* was exposed to ClO_2, the levels of chlorophyll *a* began to decrease compared with untreated cells, due to the oxidizing agent. In Quartile 1, the combined factor (contact time and concentration of ClO_2) significantly affected the change of chlorophyll *a* concentration following exposure ($F [9, 15] = 4.30$, $p = 0.001$).

In addition, both of these factors exhibit a significant effect on the capacity of ClO_2 to decrease the amount of chlorophyll *a* produced by cyanobacteria. Individual treatment factors of contact time and concentration significantly affect increased chlorophyll *a* F [3, 15] = 27.1, $p < 0.001$; F [3, 15] = 36.0, $p < 0.001$)

In Quartile 2, comparable observations were noted. The combined factor (contact time and concentration of ClO_2) significantly decreased the amount of chlorophyll *a* (F [9, 15] = 3.05, $P = 0.009$). Individual treatment factors of contact time and concentration significantly affect decreased chlorophyll *a* concentration (F [3, 15] = 24.93, $p < 0.001$; F [3, 15] = 220, $p < 0.001$).

Similarly, in Quartile 3, the combined factor (contact time and concentration of ClO_2) significantly decreased the amount of chlorophyll *a* (F [9, 15] = 4.57, $p = 0.001$) and individually, contact time and concentration both significantly decreased the levels of chlorophyll *a* measured from the cyanobacteria sampled (F [3, 15] = 18.9, $p < 0.00$; F [3, 15] = 394, $p < 0.001$).

In Quartile 4, the combined factor of contact time and concentration was not a cofactor to significantly reduce the level of chlorophyll *a* (F [9, 15] = 0.491, $p = 0.869$). However, individual treatment factors of contact time and concentration significantly affect decreased chlorophyll *a* concentration (F [3, 15] = 5.30, $p < 0.001$; F [3, 15] = 272, $p = 0.004$).

In the early stage of growth (Quartile 1), cyanobacteria are developing, intracellular organelles to be intact thus; they are vulnerable to chlorophyll bleaching. In Quartiles 2 and 3, chlorophyll *a* degradation increased with increasing concentration of ClO_2 and contact time. In the last growth phase (Quartile 4), ClO_2 treatment exhibited efficacy to gradually break down chlorophyll *a* of *M. aeruginosa*. Based on Figure 5.4, concentration of ClO_2 treatment seems to influence chlorophyll *a* degreening more than contact time. The cells in this quartile were likely towards the end of their proliferation and entering into their steady phase having an accumulation of both viable and dead cells. ClO_2 oxidizes both viable and dead cells, reducing overall oxidization efficacy.

Oxidative damage not only deteriorates the chemical structure of chlorophyll (Djapic, 2013), but also causes loss of function such as the ability to harvest electrons from water; absorb energy from sunlight; or donate electrons to the electron transport system during photosynthesis (Foyer and Shigeoka, 2011). The malfunction of chlorophyll ultimately results in sustained photo-inhibition. Eventually, this decline in photosynthetic reactions induced by oxidative stress ends in cell death (Hörtensteiner, 1999).

5.3.2 Electrical conductivity and membrane dysfunction

Comparing cellular conductivity with that of the culture media enables an assessment of changes in functional integrity of the cell membrane, taken to infer the degree of leaked ions following oxidization by ClO_2. This experiment observed the electrolyte leakage induced by ClO_2 via increased conductivity. The efflux of ions (and corresponding increase in conductivity) is shown in Figure 5.4 and Figure 5.5. The line graphs in Figures 5.4 and 5.5 illustrate the percentage of leakage in treated cells compared with untreated cells as calculated by electrolyte leakage of treated cells subtracted by the electrolyte leakage of untreated cells, and divided by electrolyte leakage of untreated cells then multiplied by 100 %.

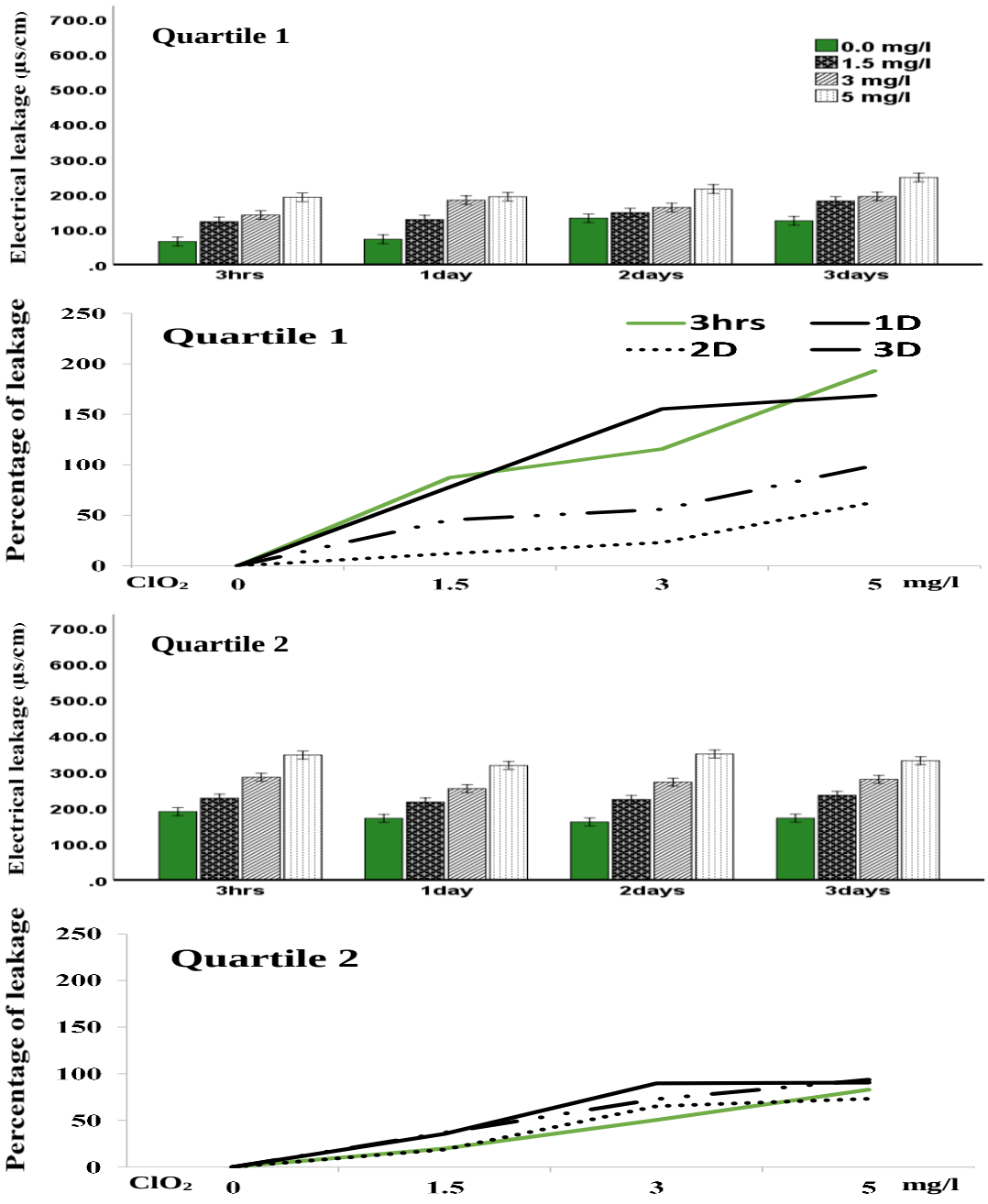


Figure 5.4: Electrical leakage assessment before and after cyanobacteria were treated by ClO_2 in the first and second growth intervals. This was performed to investigate membrane dysfunction

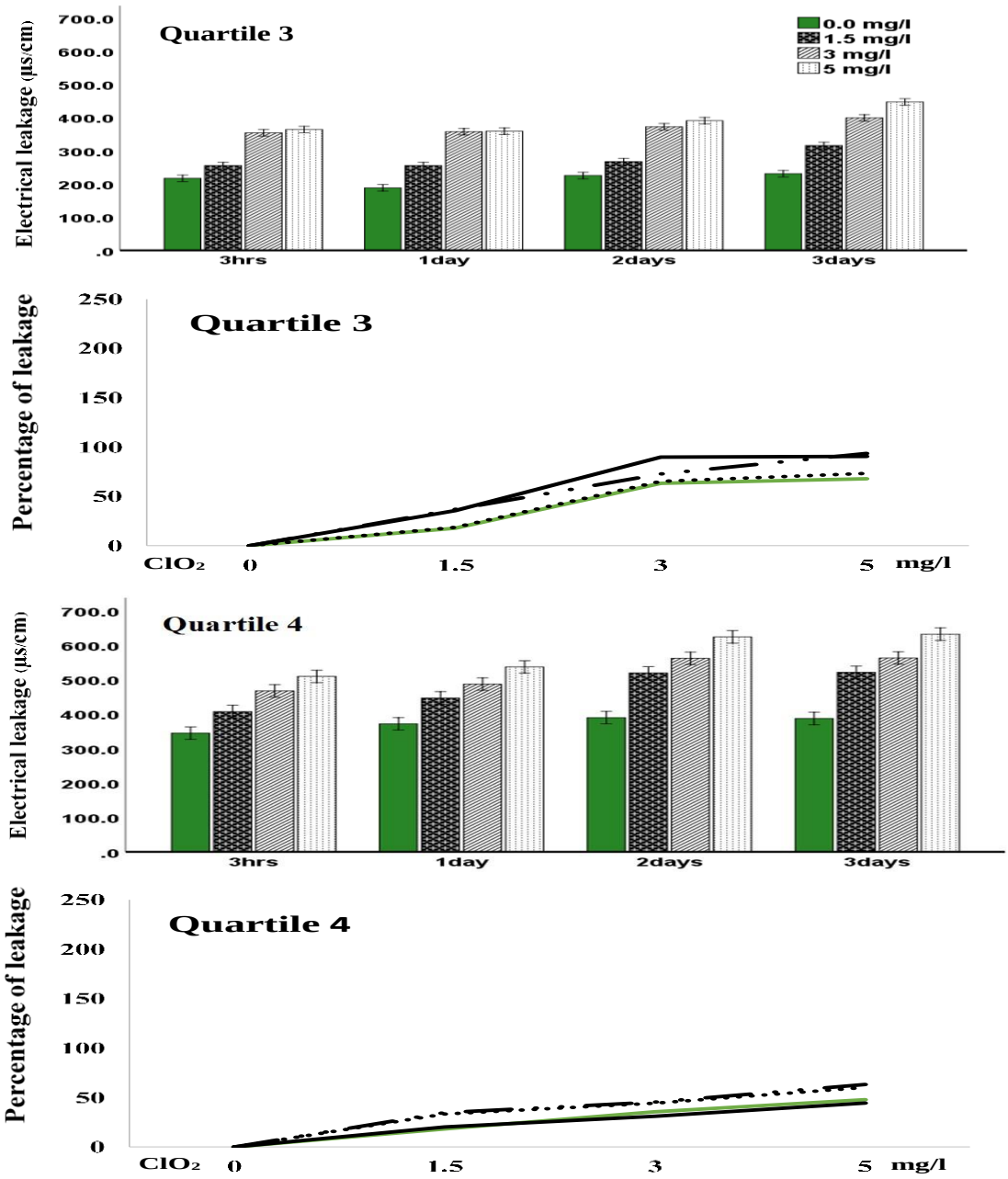


Figure 5.5: Electrical leakage assessment before and after cyanobacteria were treated by ClO_2 in the third and fourth growth intervals. This was performed to investigate membrane dysfunction

Measurements at each growth phase show similar trends with increases in ions leakage correlated with time, higher concentrations of ClO_2, and longer ClO_2 contact

times. In Quartile 1, statistical test of between-subject effects, the combined factor (contact time and concentration of ClO_2) indicates that increase of effective ClO_2 treatment significantly affects increased ions leakage ($F [9, 15] = 6.34$, $p < 0.001$). Individual treatment factors of contact time and concentration significantly affect increased ions leakage ($F /3, 15] = 66.5$, $p < 0.001$; $F /3, 1] = 245$, $p < 0.001$).

In Quartile 2, the combined factor exhibits a significant effect on increased ions leakage ($F [9, 15] = 2.97$, $p = 0.011$. Individual treatment factors of contact time and concentration significantly affect increased ions leakage (; $F [3, 15] = 11.7$, $p < 0.001$; $F [3, 15] = 651$, $p < 0.001$). In Quartile 3, the combined factor exhibits a significant effect on leakage of ions ($F [9, 15] = 8.12$, $p < 0.001$). Individual treatment factors of contact time and concentration significantly affect ions leakage ($F [3, 15] = 114$, $p < 0.001$; $F [3, 15] = 1170$, $p < 0.001$). In Quartile 4, the combined factor exhibits a significant effect on ions leakage ($F [9, 15] = 4.78$, $p < 0.001$). For the individual treatment factors of contact time and concentration significantly affect ions leakage ($F [3, 15] = 110$, $p < 0.001$; $F [3, 15] = 369$, $p < 0.001$).

The gradual growth of the first stage, low population density implies that cells in this phase are sensitive and susceptible to oxidative stress induced by ClO_2. This observation leads us to postulate that the cell wall of this stage of cyanobacterial cells may be quicker to become compromised and release intracellular ions into the culture media solution. Low population density may contribute to these effects. The next growth phases (Quartiles 2 and 3) had increased numbers of cells. The further advanced state of cellular development may consist of more fully functional cells with intact morphologies, which provide protection during potentially harmful environmental stressors. However, effective ClO_2 treatment still caused ions leakage. Finally, the last growth phase (Quartile 4) has even greater density of cells, including both viable and dead cells. This high density may reduce the efficiency of ClO_2 to inhibit *M. aeruginosa*. Nevertheless, there was still ions leakage induced by ClO_2 treatment.

Cellular leakage can be caused by oxidation of ClO_2 through several mechanisms. Amino acid groups of peptidoglycans and tetrapeptide cross-linkages in the cell wall can be oxidized by ClO_2 (Sharma and Sohn, 2012), which alters its integrity and

rigidity (Zhou, et al, 2014). Cell walls become susceptible to further damage from continued exposure. Loss of transmembrane protein structure is observed in porin channels (Benz and Bauer, 1988). Also, disruption of the electron transport chain can prevent the cell from generating enough life-sustaining energy (Zhou, et al, 2014).

5.3.3 Metabolic activity of intact cells (MTT assay)

The efficacy of ClO_2 to inhibit metabolic activity of *M. aeruginosa* was measured by the conversion of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) to the formazan salt, which forms a vibrant purple colour. The results of the MTT assay are shown in Figure 5.6.

In Quartile 1, the combined factor (contact time and concentration) exhibits significant effect on MTT level ($F [9, 15] = 12.8$, $p < 0.001$). Individual treatment factors of contact time and concentration significantly affect MTT level ($F [3, 15] = 251$, $p < 0.001$; $F [3, 15] = 33.7$, $p < 0.001$), which acted to reduce the MTT level. However, anomalously, cells that were exposed to 1.5 mg/L of ClO_2 for 3 days showed increasing metabolic activity.

In Quartile 2, the combined factor exhibits the significant effect on MTT level ($F [9, 15] = 35.7$, $p < 0.001$). With the individual factors of contact time and concentration significantly affect MTT level ($F [3, 15] = 167$, $p < 0.001$; $F [3, 15] = 287$, $p < 0.001$). The highest concentration of ClO_2 (5.0 mg/L) showed more effective treatment than other doses to decrease the MTT level.

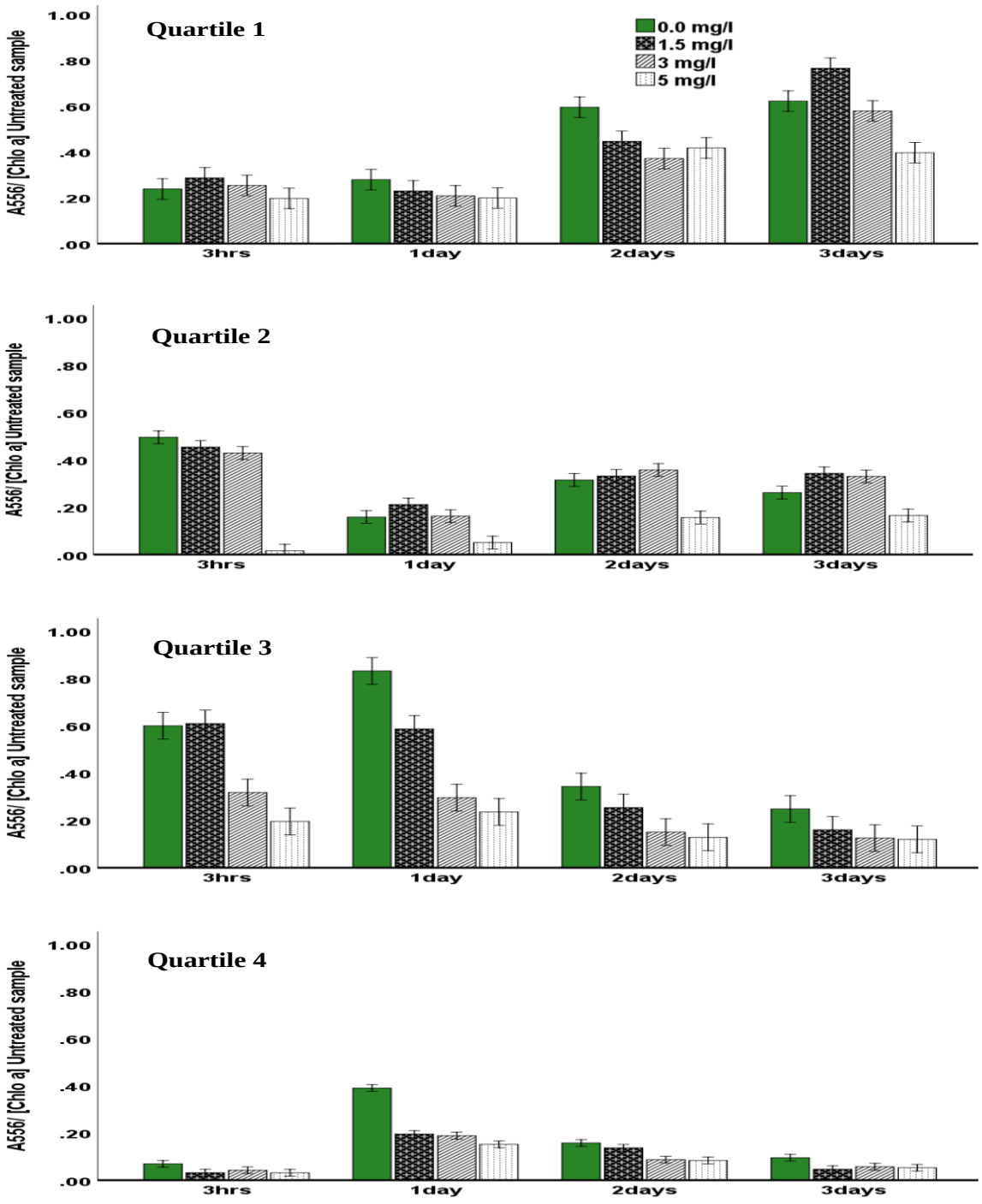


Figure 5.6: Metabolic activity of cyanobacteria evaluated via MTT assay for cell viability, observed by the degree of change in light absorbance at 556 nm (A 556)

In Quartile 3, the combined factor exhibits a significant effect on MTT reduction ($F [9, 15] = 15.0$, $p < 0.001$). Individual treatment factors of contact time and concentration significantly affect MTT reduction ($F [3, 15] = 130$, $p < 0.001$; $F [3, 15] = 127$, $p < 0.001$). In Quartile 4, the combined factor exhibits significant effect on MTT reduction ($F [9, 15] = 40.4$, $p < 0.001$). Individual treatment factors of contact time and concentration significantly affect MTT reduction ($F [3, 15] = 578$, $p < 0.001$; $F [3, 15] = 157$, $p < 0.001$).

Lower cell density in first stage of growth may has made it easier for ClO_2 to cause redox reactions and induce oxidative stress upon the cells. As population density increased (Quartiles 2-4), cells are more response to ClO_2 treatment due to full function of organelles having more oxidoreductase to convert MTT to formazan salt. In the most cells are thought to be intact with full biochemical functions. Therefore, intracellular redox reactions responded more clearly to oxidative stress induced by ClO_2. Furthermore, Quartile 4 seems to be in the late exponential phase with both viable and dead cells. while cells encountered oxidative stress induced by ClO_2, the response was slightly diminished. Cells exhibit less oxidoreductases activity and MTT was not reduced generally. Cells were probably also exhibiting stress from the higher population density. Nevertheless, this response showed a similar pattern with the preceding findings. The results showed that reduction of a tetrazolium salt (MTT) to formazan was lower in stressed cells than healthy cells, presumably caused by metabolic inhibition induced by ClO_2. Oxidative stress induced by ClO_2 markedly affected this decrease in MTT conversion, indicative of compromised cellular viability.

5.3.4 Lipid peroxidation (MDA)

The effectiveness of ClO_2 to inhibit *M. aeruginosa* was also implied by lipid peroxidation, another biomarker of membrane damage that has been described in Figure 5.7.

In Quartile 1, the combined factor of contact time and concentration) exhibits a significant effect on MDA production ($F [9, 15] = 3.28$, $p = 0.006$). Individual treatment factors only contact time significantly affects MDA production ($F [3, 15] = 9.56$, $p < 0.001$). Increased ClO_2 concentration does not significantly affect MDA production ($F [3, 15] = 1.74$, $p = 0.179$). The concentration of 5.0 mg/L and contact time of 1 day induced the greatest amount MDA production relative to the untreated sample.

In Quartile 2, the combined factor exhibits a significant effect on MDA production ($F [9, 15] = 3.28$, $p = 0.006$). Individual treatment factors of contact time and concentration significantly affect MDA production ($F [3, 15] = 52. 8$, $p < 0.001$; $F [3, 15] = 8.99$, $p < 0.001$). In Quartile 3, the combined factor exhibits a significant effect on MDA production ($F [9, 15] = 34.1$, $p < 0.001$). Individual treatment factors of contact time and concentration significantly affect MDA production ($F [3, 15] = 93.4$, $p < 0.001$; $F [3, 15] = 44.2$, $p < 0.001$). In Quartile 4, the combined factor exhibits a significant effect on MDA production ($F [9, 15] = 3.08$, $p = 0.009$). Individual treatment factors of contact time and concentration significantly affect MDA production ($F [3, 15] = 9.68$, $p < 0.001$; $F [3, 15] = 9.03$, $p < 0.001$).

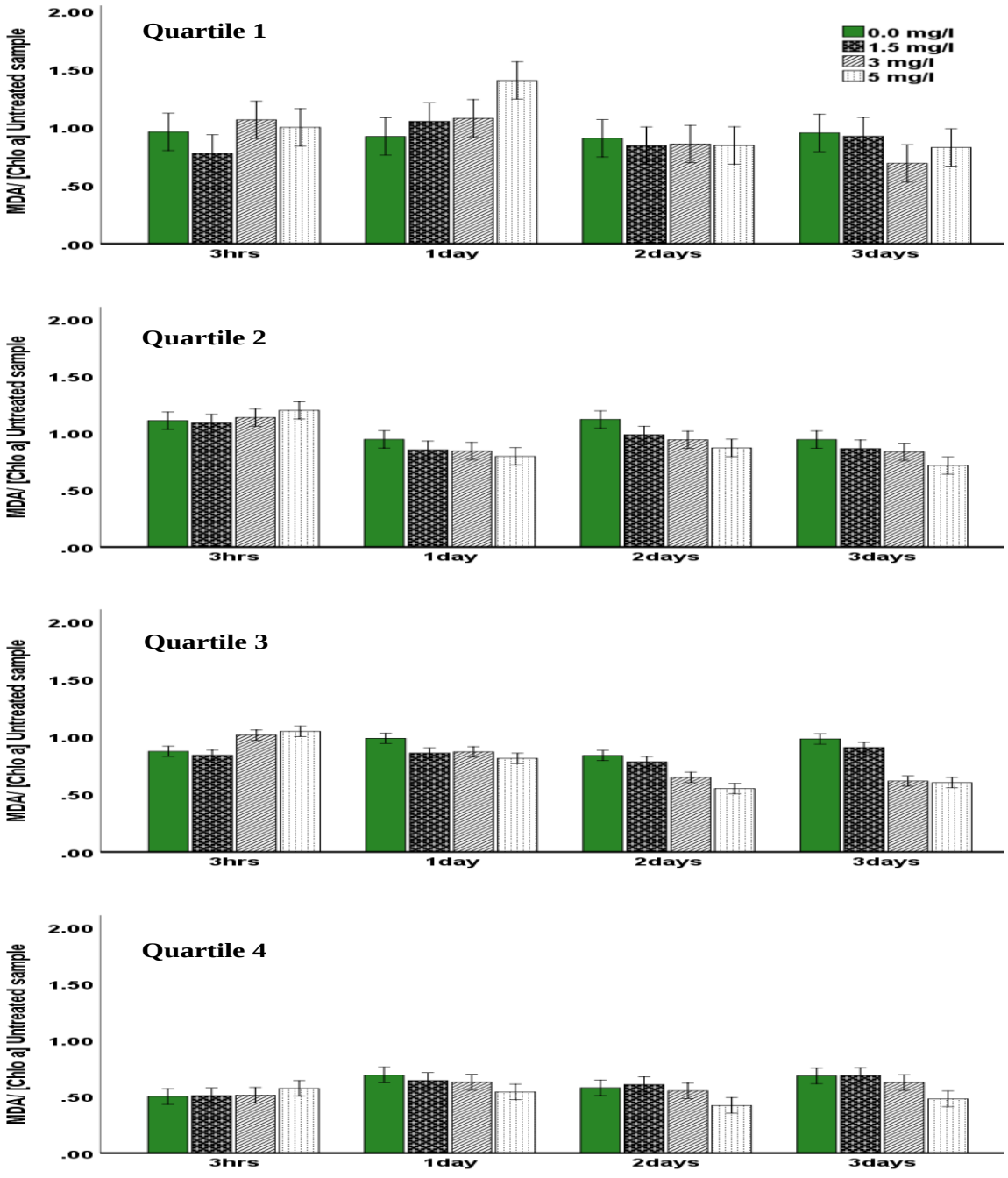


Figure 5.7: The level of MDA produced by lipid peroxidation of cyanobacterial cells induced by ClO_2 treatment

The degree of MDA change is presumably caused by peroxidation of the lipid membrane, causing membrane degradation. The unsaturated lipid membrane bond is vulnerable to attack by free radicals released by ClO_2. In the first stage of growth with its low cell density, cell organs and incomplete structures were susceptible to degradation induced by ClO_2. Contact time had more effect than ClO_2 concentration on the degree of membrane degradation, lipid peroxidation, and thus, creation of MDA compounds (Yin et al., 2011, Ayala, et al., 2014). In Quartiles 2 and 3, contact time and ClO_2 concentration both affected MDA production. Although the same observation was made in Quartile 4, MDA production was lower than in previous growth stages. MDA level was decreased for the extended time because it had low stability in biological specimens. It can cross-react with proteins, amino acids, etc (Khoubnasabjafari et al., 2015). Lipid peroxidation impairs membrane integrity, flexibility, and the transport of signaling molecules to stimulate proliferation and cell survival (Catala, 2006). Ultimately, lipid peroxidation can induce cell death.

5.4 Substances associated with elimination of reactive oxygen species

5.4.1 Carotenoids (CARs) activity

Each growth phase of *M. aeruginosa* showed a change in intracellular activity in response to ClO_2 treatment. Figure 5.8 represents the results of carotenoids (CARs) activity, reflecting antioxidant response of cells to stress. In Quartile 1, the combined factor (contact time and concentration) does not exhibit a significant effect on CARs activity ($F [9, 15] = 1.66$, $P = 0.140$). However, individual treatment factors of contact time and concentration significantly affect decline of CARs activity ($F [3, 15] = 9.75$, $p < 0.001$; $F [3, 15] = 15.5$, $p < 0.001$). In Quartile 2, the combined factor does not exhibit a significant effect on CARs activity ($F [9, 15] = 1.66$, $p = 0.140$). The individual treatment factor of increased contact time does not significantly affect CARs activity ($F [3, 15] = 0.382$, $p = 0.767$). On the other hand, increased concentration significantly affects decline of CARs activity ($F [3, 15] = 46.7$, $p < 0.001$). In Quartile 3, the combined factor does not exhibit a significant effect on CARs activity ($F [9, 15] = 1.27$, $p = 0.291$). However, individual factors of contact time and concentration significantly affect decline of CARs activity; ($F [3, 15] = 4.07$, $p = 0.015$;

F [3, 15] = 539, $p < 0.001$). In Quartile 4, the combined factor does not exhibit a significant effect on CARs activity (F [9, 15] = 0.66, $p = 0.738$). The individual treatment factor of contact time does not significantly affect CARs activity (F [3, 15] = 0.901, $p = 0.451$. However, increased ClO_2 concentration significantly affects decline of CARs activity (F [3, 15] = 98.6, $p < 0.001$).

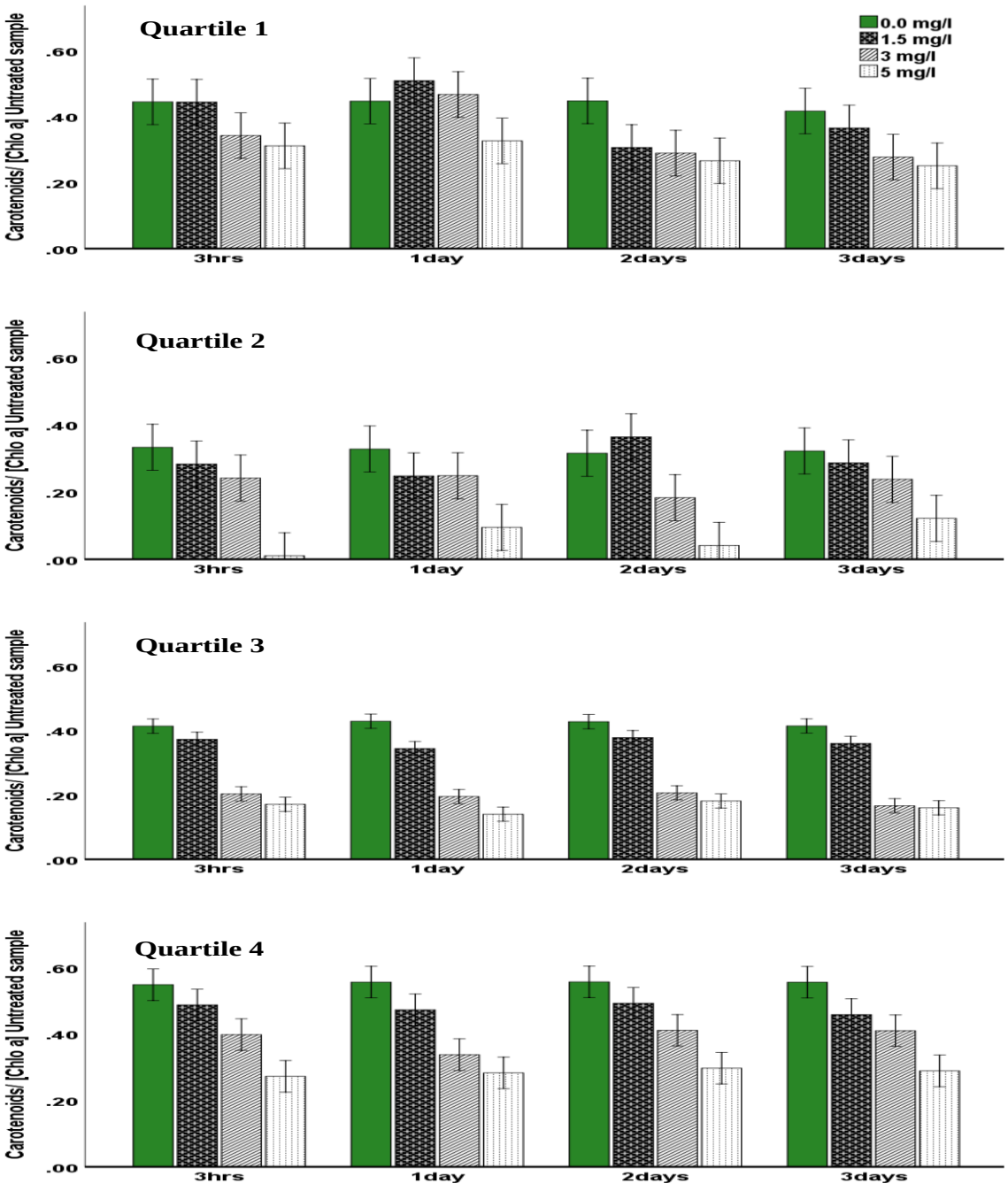


Figure 5.8: CARs level change in cyanobacteria confronted with oxidative stress induced by ClO_2 treatment

CARs functions have two key roles, a pigment of the light-dependent photosynthesis system (Goedheer, 1959) and an antioxidant that can absorb the oxidizing power of radicals (Boucher et al., 1977). Differential responses were observed across different intervals of *M. aeruginosa* growth. Decreasing CARs levels with ClO_2 treatment are direct measurements of the loss of pigment and therefore loss of photosynthetic function. Indirectly, these measurements indicate the ability of CARs antioxidants to quench oxidative stress. CARs levels increase with increases in population density and are decreased by ClO_2 treatment. Although, there were two instances that showed the increased CARs level with increasing ClO_2 treatment, they are not sufficient results supporting the antioxidative role of CARs. Mostly, in Quartiles 1 and 2, CARs level decreased with increasing ClO_2 treatment. Similar responses are observed in the exponential and late growth phases (Quartiles 3 and 4). Reducing CARs levels are most affected by increased ClO_2 concentration, but, by Quartile 4, CARs levels remain higher overall. With a higher density and integrity of cells, the protective systems in these phases may not rely only on CARs activity to resist the toxicity of ROS. Also, greater numbers of cells, both active and inactive, provide more reactive sites for ClO_2 treatment, reducing its effectiveness.

5.4.2 Superoxide dismutase (SOD)

Changes in SOD activity were apparent following ClO_2 treatment at the different concentrations and exposure times. Figure 5.9 demonstrates the results of the SOD assay. Samples from the first quartile of growth (Quartile 1) were lost because the freezing at -80˚C of storage room cracked a number of glass sample vials. In Quartile 2, the combined factor (contact time and concentration) exhibits a significant effect on the SOD activity to scavenge excess superoxide radicals ($F [9, 15] = 2.35$, $P = 0.036$). Individual treatment factors of contact time and concentration significantly affect increase in SOD activity ($F [3, 15] = 10.3$, $p < 0.001$; $F [3, 15] = 26.8$, $p < 0.001$). In Quartile 3, the combined factor also exhibits a significant effect on increased SOD activity ($F [9, 15] = 2.25$, $p = 0.044$). Individual treatment factors of contact time and concentration significantly affect SOD activity ($F [3, 15] = 26.9$, $p < 0.001$; $F [3, 15] = 26.5$, $p < 0.001$). In Quartile 4, the combined factor does not exhibit a significant effect on increased SOD activity ($F [9, 15] = 0.666$, $p = 0.733$). However, individual

treatment factors of contact time and concentration significantly affect increased SOD activity (F [3, 15] = 21.9, $p < 0.001$; F [3, 15] = 10.5, $p < 0.001$).

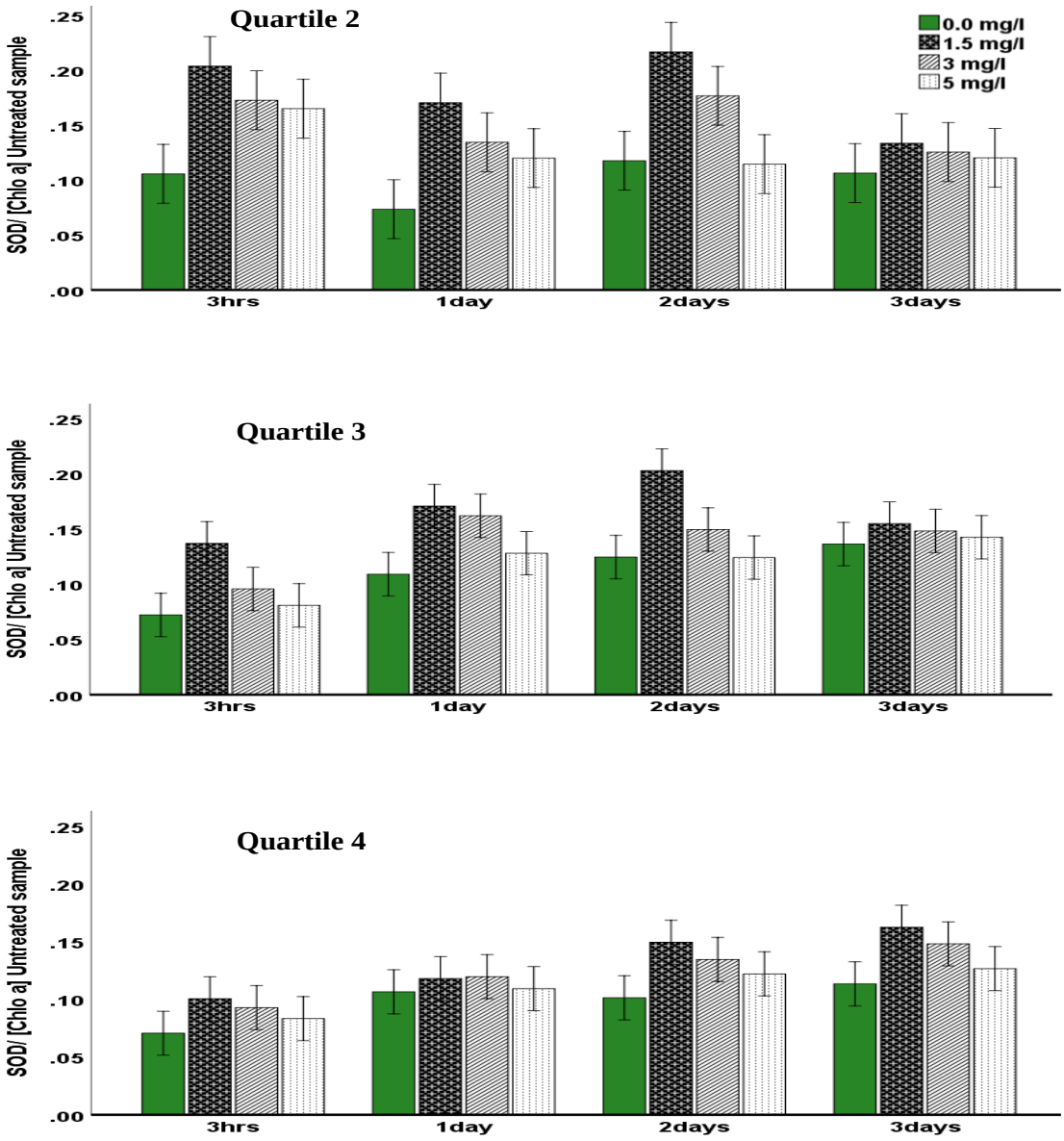


Figure 5.9: The SOD activity scavenges superoxide radicals generated by cyanobacteria that accumulate during oxidative stress induced by ClO_2

Chlorine dioxide treatment of *M. aeruginosa* resulted in higher rates of SOD activity compared with untreated samples. This observation is taken to reflect increased scavenging activity of SOD to cope with surplus ROS such as superoxide free radicals. Many studies have concluded that this antioxidative defense system is established to protect cells from oxidative damage by excess ROS generation (Hess, 2000; Garcia-Plazaola et al., 2002 and Kim and Lee, 2005). In this research, the protective role of SOD defends cells against oxidative damage directly related to increased dosage of ClO_2. Particularly during Quartile 2 where *M. aeruginosa* responded to abiotic stress induced by ClO_2 treatment, increasing levels of SOD activity illustrated the coping mechanism of cells trying to regulate ROS production. The ClO_2 concentration of 1.5 mg/L affects increased SOD activity whereas higher doses of ClO_2 treatment decreased SOD activity. SOD levels at higher concentrations are greater than or similar to SOD levels of untreated cells. High doses of ClO_2 may have caused cell death in addition to cell stress, which is supported by responses observed in chlorophyll *a*, electrolyte leakage, MTT level, MDA production and CARs level after ClO_2 treatment. Decreased chlorophyll *a* and CARs link with declines in cell numbers, photosynthesis capacity reduction, and antioxidant response. Increasing electrolyte leakage (conductivity) reflects malfunctions of membranes and cell integrity. Decline in metabolic activity as indicated by MTT reflects decreasing numbers of viable cells. Finally, MDA reduction reflects increases in membrane degradation and, therefore, decreasing viability of cells. The interpretation of decreased SOD activity after high doses of ClO_2 associated with cell death rather than cell stress is supported by these other lines of evidence. High concentrations may kill more cells but not all cells. Remaining cells seem to experience stress as evidenced by SOD level greater than or similar to untreated cells.

5.4.3 Catalase (CAT)

In this assay, the level of CAT generated to scavenge hydrogen peroxide in response to *M. aeruginosa* exposure to ClO_2 was investigated. As with SOD activity, samples from Quartile 1 were lost. The graph in Figure 5.10 presents the results of the CAT activity. In Quartile 2, the combined factor (contact time and concentration) does not exhibit a significant effect on influenced CAT level ($F [9, 15] = 1.59$, $p = 0.161$).

Conversely, individual treatment factors of contact time and concentration significantly affect changeability of CAT activity (F [3, 15] = 5.08, $p = 0.005$; F [3, 15] = 5.333, $p = 0.004$). In Quartile 3, the combined factor does not exhibit a significant effect on CAT activity (F [9, 15] = 1.63, $p = 0.150$). However, individual treatment factors of contact time and concentration significantly affect CAT activity (F [3, 15] = 39.4, $p < 0.001$; F [3, 15] = 6.34, $p = 0.002$). In Quartile 4, the combined factor does not exhibit a significant effect on CAT activity (F [9, 15] = 1.01, $p = 0.455$). The individual treatment factor of contact time significantly affects CAT activity (F [3, 15] = 15.0, $p < 0.001$). However, increased concentration does not significantly affect CAT activity (F [3, 15] = 1.97, $p = 0.139$).

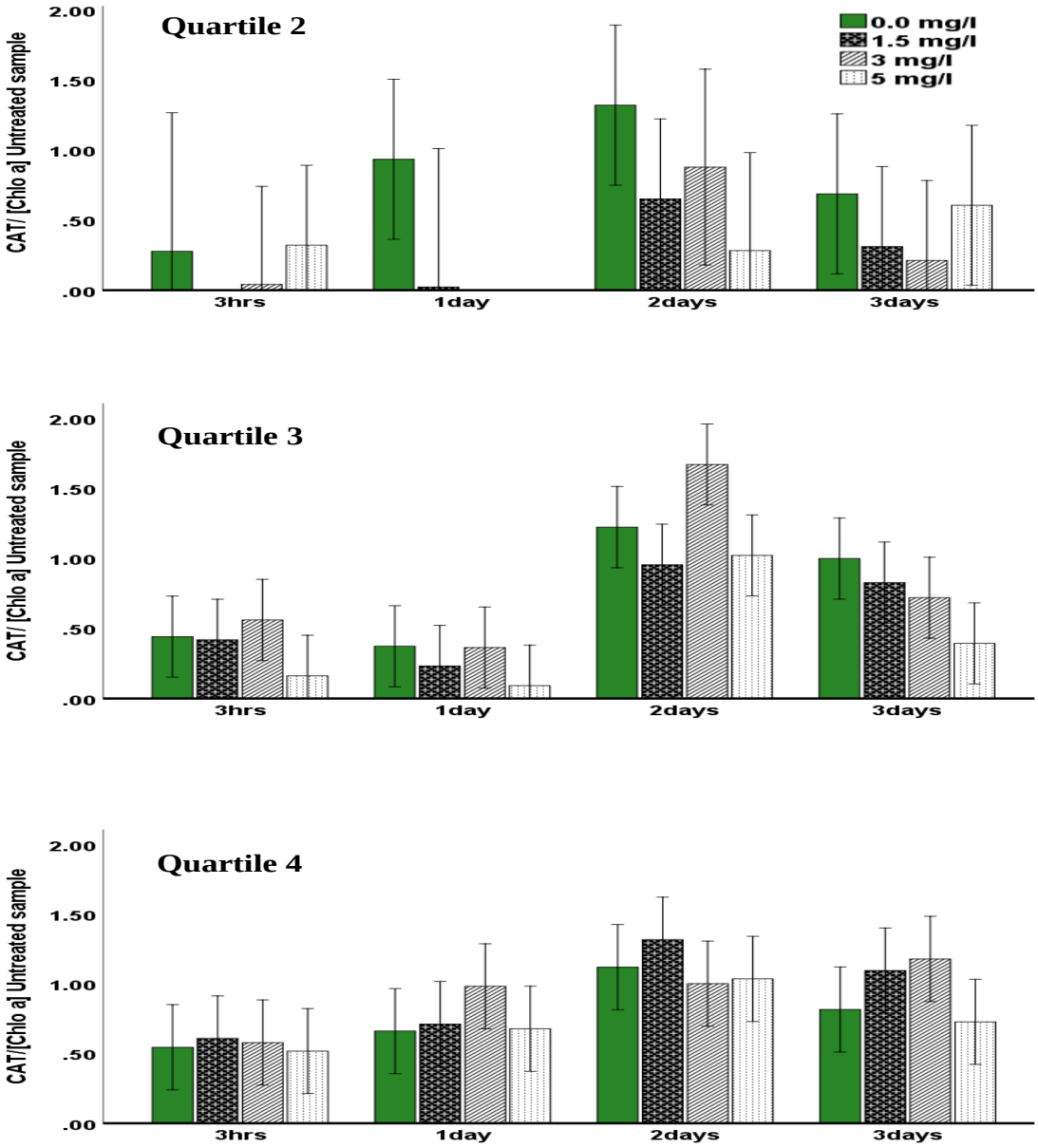


Figure 5.10: The activity of catalase to eliminate hydroxyl radicals created by cyanobacteria treated with ClO_2

The CAT levels of untreated cells increased from Quartiles 2-4. The measurements have a lot of variability, illustrated by the large 95 % CI error bars in Figure 5.11, in a number of cases. Statistically, CAT levels have differences with concentration but the pattern is not always clear. Decreasing CAT levels with treatment, as observed in Quartile 2, may be scavenging activity that converted H_2O_2 to O_2 and H_2O. However,

ClO_2 treatment does not always result in decreased CAT level (Quartiles 3 and 4); increasing ClO_2 concentration sometimes seems associated with increasing CAT level, which may reflect stress response but does not exhibit the expected role to quench H_2O_2 to a harmless form. Moreover, our research was always launched in the opened area exposed to the ambient light. This was the great cause to decrease CAT level and inactivation due to its sensitivity to light (Foyer et al., 1994; He and Häder, 2002). Accordingly, previous studies reported that low-density cell cultures show limited protective ability of CAT in response to high-intensity light and induced ROS such as superoxide radicals and H_2O_2 (He and Häder, 2002). These factors may have affected the high variability and inconsistent effects of ClO_2 treatment observed in this experiment.

5.5 Discussion

The relationship between ClO_2 treatment and factors of cell viability and stress factors is complex but clear trends arise among multiple lines of evidence.

ClO_2 directly influences the viability of cyanobacterial cells as evidenced by decreased chlorophyll *a* (Figure 5.3) after cells were exposed to chlorine dioxide in every growth phase. In addition, slopes of trendlines indicate declines of chlorophyll *a* that did not recover when compared with the untreated samples, particularly those in early population growth (Quartile 1). ClO_2 affects the viability of cells and effectively inhibits cyanobacteria.

ClO_2 treatment can cause membrane damage of cyanobacteria. As illustrated in Figure 5.4 and Figure 5.5, electrolyte leakage of treated cells exceeded that of untreated samples. Populations during early blooms seemed particularly susceptible to ClO_2 inhibition compared to later growth phases. This difference is apparent in the line graph of Figure 5.4 and 5.5 that shows the rates of electrolyte leakage compared to untreated samples.

Cyanobacteria during early growth phases responded greater to the ClO_2 treatment than the those approaching the stationary phase. Low population densities exhibited higher metabolic activity to survive the harmful experience than cells in later stages

(Figure 5.6). In addition, the lowest concentration (1.5 mg/L) stimulated metabolic activity of the cells in the low population densities despite the stressful conditions. On the other hand, later growth stages displayed low metabolic activity, thus the response to ClO_2 treatment was reduced.

Low concentrations of ClO_2 causes the cyanobacterial stress as evidenced by CARs activity to quench ROS (Figure 5.7) and SOD (Figure 5.8). The lost data of the first growth phase was expected to show an increase SOD activity similar to the other growth phases. The low concentration of ClO_2 caused cell stress, resulting in increased SOD activity. Subsequently, SOD decreased with increasing ClO_2 concentration. High concentrations may kill more cells but not all cells. The remaining cells may experience stress as evidenced by SOD level greater than or similar to that of untreated cells. CAT activity from Figure 5.9 does not exhibit a strong antioxidant role to scavenge ROS induced by ClO_2 to protect oxidative stress. Summary effects of ClO_2 treatment to inhibit *M. aeruginosa* is shown in a conceptual graph in Figure 5.11.

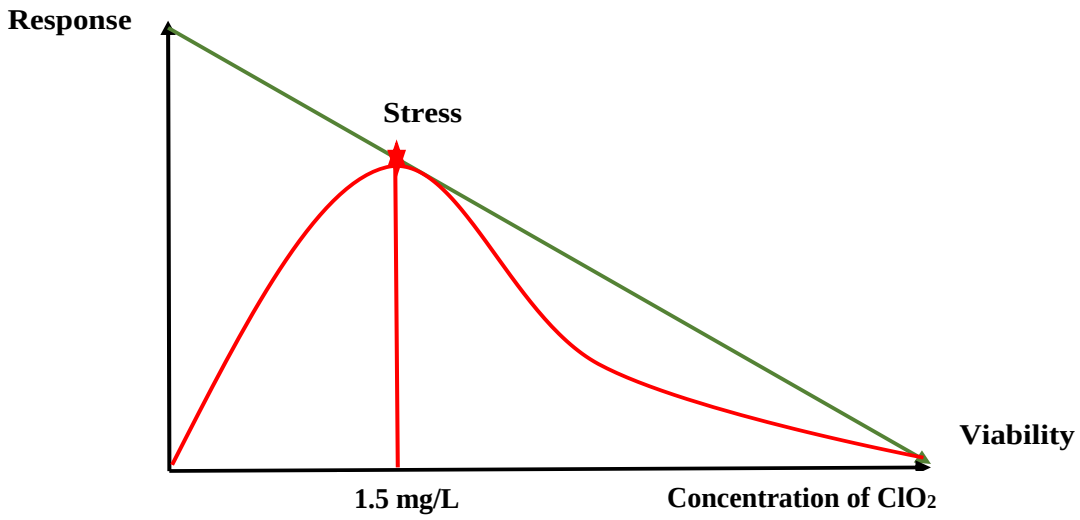


Figure 5.11: The conceptual relationship of *M. aeruginosa* response to ClO_2 treatment affecting cell viability (green line) and stress (red curve)

In drinking water treatment, 50-70 % of ClO_2 decomposes to chlorite, chlorate, and chloride ion (Chapter 2 Section 2.4; Odeh et al., 2002) that can cause blood poisoning (hemolytic anemia) and other health effects (Werdehoff and Singer, 1987). In consequence, WHO (2011) set a guideline of maximum contaminant level (MCL) for byproducts of ClO_2 treatment at 1.0 mg/L. The American Water Works Association suggested that the range of concentration of ClO_2 for pre-oxidation of surface water should be limited to 1.0-1.4 mg/L to control excess ClO_2 byproducts (Gates, 1998). However, this research suggests that concentration of ClO_2 treatment should depend on the quality of raw water and the degree of contamination with cyanobacteria. In the presence of cyanobacteria, the recommended levels may be too low. At these low concentrations (1.5 mg/L), cyanobacteria exhibit stress. Particularly during early blooms, which may only appear as thin green films on surface water, oxidative stress may stimulate metabolic activity in response to harmful conditions leading to further adaption and survival. These guideline values may need to be revisited to account for treatment of cyanobacteria blooms in intake water. Alternatively, ClO_2 may need to be combined with other treatments to remove cyanobacteria from intake water.

5.6 Summary of Key Findings

The effectiveness of treatment with ClO_2 to inhibit *M. aeruginosa* was studied since an early phase until a late potential phase of cyanobacterial growth. Cell proliferation and viability were quantified by measuring chlorophyll *a*. The findings suggest that initial blooms *M. aeruginosa* (approximately 1.2-1.9 µg/L of chlorophyll *a*) were the most sensitive to ClO_2 treatment, while greater concentrations of ClO_2 dramatically increased the leakage of intracellular ions. Cells during early stages of population growth are thought to be susceptible to attack at the PUFA moiety site. Furthermore, lipid peroxidation was analyzed by the presence of secondary metabolites resulting of membrane damage. This damage directly correlates with malfunction of the cell membrane, when it cannot perform its regular functions. Eventually, this leads to cellular dehydration, starvation, or over-osmosis, causing cells to break and die. Low dosing of ClO_2 impacted the metabolic activity of cells, including stimulation of oxidative stress. This stress response is linked with the protective system, which increases the level of antioxidants to regulate the imbalance of ROS induced by ClO_2

treatment. The low population density reacted to oxidative stress more than the higher population densities via increases in antioxidants, notably SOD, and decreases in carotenoids to protect the cells from oxidative damage. This research identified that low concentration of ClO_2 treatment induces cells stress, whereas high concentrations induce cell inhibition and to a lesser extent, further cell stress.

Table 5.1: Key findings from experiments of *M. aeruginosa* inhibition by ClO_2

Appraisement of ClO_2 treatment	**Optimum condition**		
	Interval of growth Chlorophyll *a* (µg/m/L)	**Contact time (hour and day)**	**Concentration of ClO_2 (mg/L)**
Conductivity	Q1; chl *a* = 1.2-1.9	3 hrs and 1 day	Increasing dose correlating increased rate of leakage
Metabolic activity	Q1; chl *a* = 1.2-1.9	3 days	1.5 mg/L increased MTT level; more metabolic activity
MDA	Q1; chl *a* = 1.2-1.9	1 day	Increasing dose; increasing lipid peroxidation
CARs	Q1; chl *a* = 1.2-1.9	1 day	1.5 mg/L increased CARs scavenging
SOD	Q2; chl *a* = 1.9-2.3	3 hrs, 1 day and 2 days	1.5 mg/L stimulated SOD activity
CAT	Q3; chl *a* = 2.9-3.0	2 days	3 mg/L; increased CAT activity

Chapter 6. CYANOTOXINS AND CORRELATION ANAYLSIS

6.1 Introduction

Currently, inhibition of cyanobacteria from water supplies is a worldwide problem. Chlorine dioxide (ClO_2) is a strong oxidizing agent used in disinfection and investigated here for its effects on cell stress, disruption of cellular function, and cyanotoxins production. Here, concentration and contact time of ClO_2 treatment for cyanobacteria are examined for the relationships between their effects on intact cells and oxidative stress to the formation of microcystins. Cyanobacteria shunt metabolism with increasing intracellular secondary metabolites (toxins) while cells are confronted with environmental stressors. Aspects of this behaviour including toxins production dynamics, mechanisms, production rate and sites of cyanotoxins production are topics of ongoing research. The formation and release of secondary metabolites (toxins) is directly related to cyanobacterial blooming and proliferation (Paerl and Millie, 1996).

The purpose of this research is to understand oxidative stress in *M. aerugi*nosa and potential for microcystins formation induced by ClO_2 treatment. This information is useful for understanding the ecology and biology of cyanobacterial cells during different growth phases, and for gaining insights concerning best management of drinking water supplies in terms of cyanotoxin disinfection.

6.2 Methodology and Experimental Procedure

6.2.1 Cyanotoxin quantification

The formation of microcystins was investigated through comparisons between healthy cells and those treated with ClO_2. The efficacy of ClO_2 treatment to affect intact cells of *M. aeruginosa* was measured via biochemical correlations to the parameters identified and detailed in Chapter 5 (chlorophyll *a*, membrane leakage, MTT, MDA, CARs, SOD and CAT). Observations covered four growth intervals of cyanobacteria proliferation from initial lag toward late exponential growth stages (Quartiles 1 – 4) at several ClO_2 concentrations (0.0, 1.5, 3.0, 5.0mg/L) and 4 contact times (3hrs, 1 day, 2 days and 3 days). The methodology for this experiment is reported in Chapter 5 and the analytical testing procedures are described in Chapters 3 and 5. This chapter focuses on microcystins production by *M. aeruginosa*. Intracellular microcystins were extracted from cells and quantified by ELISA kit (Chapter 3, Section 3.9). Methodologies for both

of these steps are outlined in Chapter 3. Statistical analyses were conducted on correlation results for 3hrs, 1 day, 2 days and 3days using SPSS (IBM Corporation, Armonk, New York, USA). All analytical values for cyanotoxins are reported in Table a7 of Appendix. Figure 6.1 shows the correlation analysis of ClO_2 treatment that affected with cyanotoxins production, healthy cells and stress.

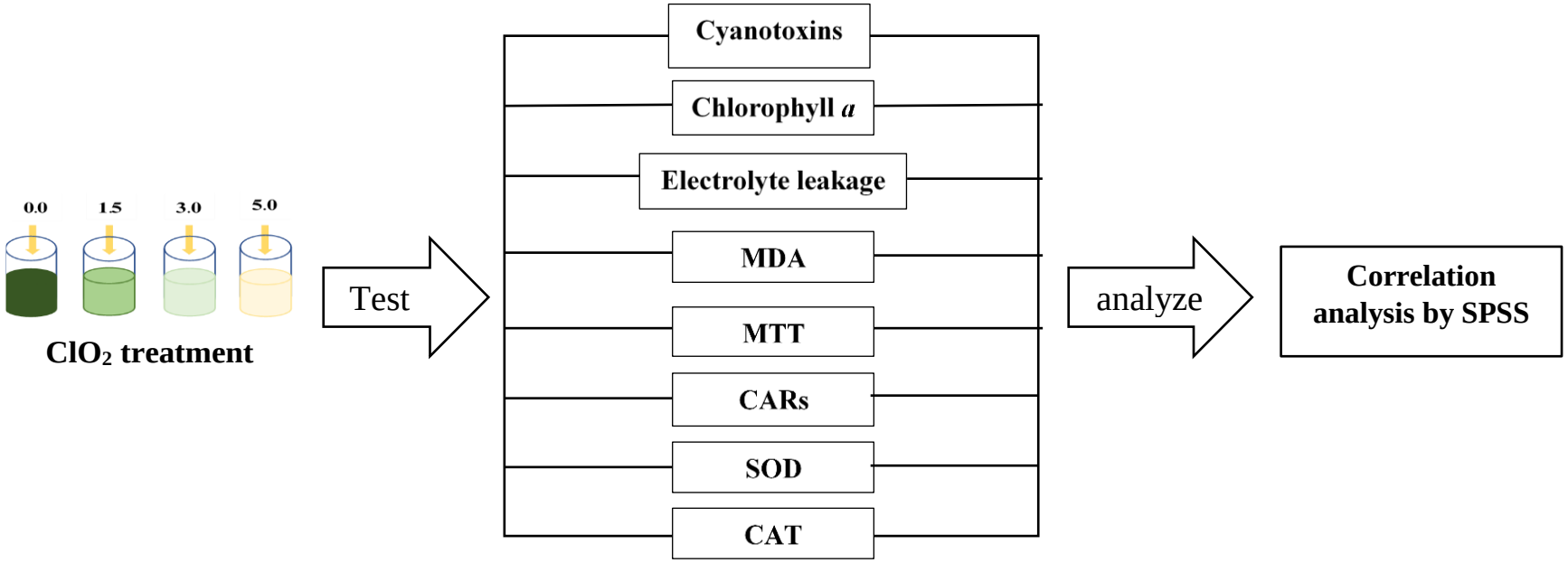


Figure 6.1: The statistic evaluation of correlation between ClO_2 treatment, cyanotoxins production, healthy cells (chlorophyll *a*, electrolyte leakage, MDA level, MTT) and stress response (CARs, SOD and CAT activity) of *M. aeruginosa*

6.2.2 Correlation analysis for cyanotoxin production

Measurements included chlorophyll *a*, membrane leakage, MTT, MDA, CARs, SOD, CAT, and microcystins production. Microcystins concentrations were divided by chlorophyll *a* measurements to normalise microcystins production to a cellular basis. To evaluate the relationships of ClO_2 treatment and microcystins with the observed changes in *M. aeruginosa*, statistical analyses were conducted with nonparametric, bivariate correlation functions. Analyses were conducted via splitting of growth phases and concentrations of ClO_2.

Cyanotoxins production on a cellular basis was evaluated against ClO_2 treatment with the Kruskal-Wallis Test, which is a one-way analysis of variance (ANOVA) on ranks. This test was selected because it does not require a normal distribution and allows comparisons of groups (i.e. ClO_2 concentration).

Spearman's rho correlation coefficient was chosen to determine the nonparametric relationship of ClO_2 treatment and microcystins production with changes in chlorophyll *a*, membrane leakage, MTT, MDA, CARs, SOD, and CAT.

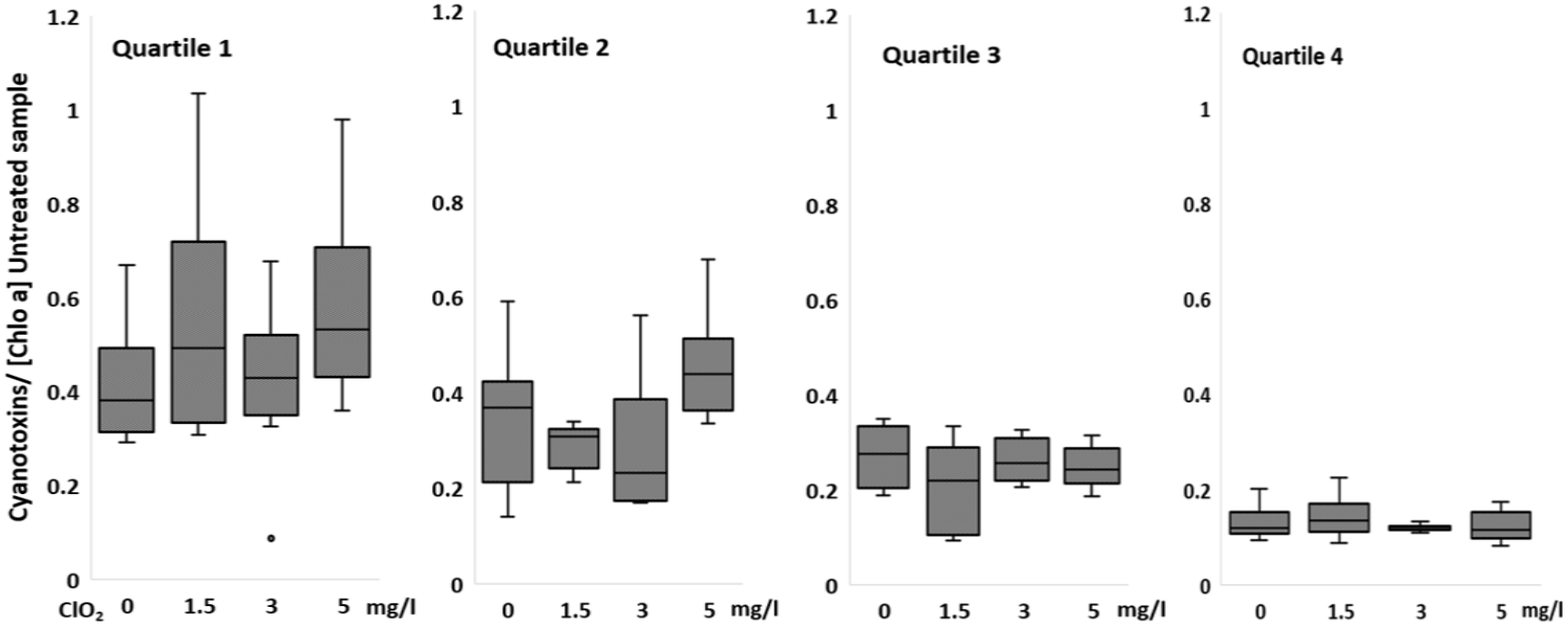
6.3 Result and Discussion

6.3.1 Cyanotoxin formation

In Figure 6.2, the relationship between cyanotoxin production and ClO_2 concentration after 3 d contact time is shown. Quartile 1 had the highest concentrations of microcystins regardless of ClO_2 treatment concentration. In Quartile 1, median microcystins production on a cellular basis shows variability and positively correlates with ClO_2 concentration (Table 6.1; $r = 0.347$, $p = 0.024$) and cyanotoxins production significantly; however, differences between treatments identified by Kruskal-Wallis (non-parametric method) are not statistically significant ($H = 6.191$, $p = 0.103$). ClO_2 treatment seemed to influence to increased cyanotoxins production. The results had significant variability, which affects the strength of the relationship between ClO_2 treatment and cyanotoxins production. Further research is necessary to evaluate this relationship. During the next growth stage (Quartile 2), there was no variability between cyanotoxins production and increased ClO_2 concentration (Table 6.2; r =

0.201, p = 0.262); however, differences between treatments were statistically significant (H = 8.832, p = 0.032) indicated by the Kruskal- Wallis test, which was, like Quartile 1 results, also likely affected by variability. The highest ClO_2 concentration (5.0 mg/L) was associated with the highest cyanotoxins production on a cellular basis.

In Quartile 3, there was no correlation between cyanotoxins production and increased ClO_2 concentration (Table 6.3; r = -0.013, p = 0.948); however, differences between treatments are not statistically significant (H = 2.358, p = 0.501) indicated by the Kruskal- Wallis test. Cells displayed a decline in the production of cyanotoxins and no differences among doses of treatment. Similarly, in Quartile 4, there was no correlation between cyanotoxins production and increased ClO_2 concentration (Table 6.4; r = -0.120, p = 0.415) and differences between treatments were not statistically significant (H = 2.975, p = 0.395). Cyanotoxins production declined further. These observations are in agreement with Orr and Jones (1998) who observed that although total microcystins concentration increased with cell density, the rate of concentration increase declined.



Test Statistic	6.191	Test Statistic	8.832	Test Statistic	2.358	Test Statistic	2.975
df	3	df	3	df	3	df	3
(p value)	0.103	(p value)	0.032	(p value)	0.501	(p value)	0.395

Figure 6.2: ln (Cyanotoxins concentration) per [Chlo a] of *M. aeruginosa* in the four growth stages exposed to ClO_2 at different concentrations (0, 1.5, 3, 5 mg/L) for all contact times (3hrs, 1d, 2d, 3d). Test statistics, degrees of freedom, and p values from the Kruskal-Wallis Test are also shown

Cyanotoxins production was hypothesised to protect the cell from oxidative stress by assisting acclimation to hazardous environments. A previous study identified that a mutant strain of cyanobacteria lacking a gene for toxin production was more susceptible to harmful conditions than non-mutated strains with the gene (Zilliges et al. 2011). Within 3 d of cells contact time at different ClO_2 concentration, our findings indicated that the change in microcystins level is not greatly affected by ClO_2 treatment. Cyanotoxins concentration correlates with ClO_2 concentration only in Quartile 1; however, the variability leaves questions about the strength of this relationship. More significant differences are associated with population density, particularly in Quartiles1-2. During early blooms, cyanobacteria are developing and consisting mainly of a low density of cells. Increasing ClO_2 concentration increases the oxidative stress in cells (Chapter 5), but this increase in stress does not seem to cause much increase in toxins production. For the next growth phases (Quartiles 3-4), population density has increased, and cells are more likely to efficiently respond to environmental changes. Cells have available resources and energy for protective mechanisms such as the cell envelope system and biochemical mechanisms to scavenge free radicals generated by harmful stimuli such as ClO_2. Also, ClO_2 reacts with the increasing numbers of inactive cells, which further protects active cells. These data are important for treatment of water contaminated with cyanobacteria and potential cyanotoxins. Early growth stages contain fewer cells but they are sensitive to harmful conditions causing increase toxins production. Cyanotoxins production may increase slightly with stress response. On the other hand, the increasing cell numbers (Quartiles 3-4) will burden effective ClO_2 treatment. Cells seemed to produce less cyanotoxins on a cellular basis and production is not affected by ClO_2 treatment. In consequence, the optimum ClO_2 concentration to inhibit cyanobacterial cells and prevent cyanotoxins release that may exceed WHO (2011) guideline must balance all factors. Based on this research, early blooms (Quartiles 1-2; 1.0 - 2.33 mg/L chlorophyll *a*) are the most effective growth stages to intervene to control cyanobacteria. Early intervention at high concentration of ClO_2 easily inhibits cyanobacteria and prevents cyanotoxins production (Chapter 4). Intervention with lower ClO_2 concentration treatment in line with current guidance may be inadequate. Treatment at this level ($\leq$1.5 mg/L ClO_2) causes stress (Chapter 5), which affects cyanotoxins production on a per-cell basis only

in early blooms. Although stressed early blooms may produce more toxins, the increase in total concentration may not be high enough to be harmful. Our research suggests that using high ClO_2 concentration for pre-treatment is beneficial. Initial, high concentration pre-treatment can be followed by low concentration disinfection after water treatment, under provisional guideline of WHO (2005) to oxidize remaining cells and control by-product creation.

6.3.2 Correlation analysis for cyanotoxins production

The correlations between ClO_2 treatments, cyanotoxins productions, cell health and stress response are described in Table 6.1-Table 6.4 for Quartiles 1-4, respectively. In Quartile 1, the correlation between cyanotoxins production has a positive relationship ClO_2 treatment (Table 6.1). This association indicates that ClO_2 treatment significantly affects the cyanotoxins production ($r = 0.347$, $p = 0.024$). Cyanotoxins production exhibits a positive correlation with electrolyte leakage significantly ($r = 0.413$, $p = 0.006$). Although correlation analysis shows increasing cyanotoxins concentration with increasing ClO_2 concentration, the variability of cyanobacteria measured at each ClO_2 concentration leads to some doubt. Further investigation is needed. The correlation between cyanotoxins production and electrolyte leakage may reflect the addition of ClO_2 to the system and, possibly, cyanotoxin release. However, there is no correlation with metabolic activity or stress response. Further correlations between cyanotoxins production and viability of cells are also shown in Table 6.1. Correlations of cyanotoxins production and SOD and CAT activity are not possible for Quartile 1 because of lost samples. With ClO_2 treatment, chlorophyll *a* has negative correlation with electrolyte leakage and positive correlations with metabolic activity including CARs level. Furthermore, the negative correlation between electrolyte leakage and CARs level is also significant. Importantly, cyanotoxins production does not exhibit correlation with CARs. We cannot identify that cyanotoxins will not exhibit correlation with any antioxidants because the SOD and CAT samples were lost.

Table 6.1: Correlation between ClO_2 treatments cyanotoxin productions, viable cell and stress response in the quartile 1st of growth

Q1			ClO_2 Exposure	Chl *a*	Electrolyte leakage	MDA	MTT	CARs	SOD	CAT
	Cyanotoxins	r	0.347*	-0.096	0.413**	-0.083	0.203	-0.130	.	.
		Sig	.024	0.544	0.006	0.601	0.196	0.412	.	.
		N	42	42	42	42	42	42	0	0
	Chl *a*	r		1	-0.310*	-0.111	0.534**	0.492**	.	.
		Sig		.	0.032	0.451	0.000	0.000	.	.
		N		48	48	48	48	48	0	0
	Electrolyte leakage	r			1	-0.153	0.097	-0.738**	.	.
		Sig			.	0.300	0.510	.000	.	.
		N			48	48	48	48	0	0
	MDA	r				1	-0.552**	0.397**	.	.
		Sig				.	.000	0.005	.	.
		N				48	48	48	0	0
	MTT	r					1	-0.231	.	.
		Sig					.	0.114	.	.
		N					48	48	0	0
	CARs	r						1	.	.
		Sig						.	.	.
		N						48	0	0
	SOD	r							.	.
		Sig							.	.
		N							0	0
	CAT	r								.
		Sig								.
		N								0

In Quartile 2, cyanotoxins production has negative correlation with metabolic activity (MTT) that reaches significance level (r = -0.543, p = 0.001), suggesting a metabolic cost for cyanotoxins production (Table 6.2). No other correlations with cyanotoxins production exhibited significance Chlorophyll *a* exhibits a negative correlation with electrolyte leakage and positive correlations with metabolic activity and CARs level. Electrolyte leakage has negative correlation with MDA level, metabolic activity and CARs level during cyanotoxins production that reach significance level. MDA level shows a negative relationship with metabolic activity and positive relationship with CARs. In addition, stress response has negative correlation with MDA. Metabolic activity has positive correlation with CARs level. However, cyanotoxins production does not exhibit correlation with antioxidants activity.

Table 6.2: Correlation between ClO_2 treatments cyanotoxin productions, viable cell and stress response in the quartile 2nd of growth

Q2			ClO_2 Exposure	Chl *a*	Electrolyte leakage	MDA	MTT	CARs	SOD	CAT
	Cyanotoxins	r	0.201	-0.180	0.244	-0.082	-0.543**	-0.056	-0.276	0.042
		Sig	0.262	0.315	0.171	0.651	0.001	0.755	0.120	0.851
		N	33	33	33	33	33	33	33	23
	Chl *a*	r		1	-0.873**	0.217	0.432**	0.857**	-0.295*	0.171
		Sig		.	0.000	0.139	0.002	0.000	.042	0.349
		N		48	48	48	48	48	48	32
	Electrolyte leakage	r			1	-0.317*	-0.403**	-0.857**	0.226	-0.346
		Sig			.	0.028	0.004	.000	0.122	0.052
		N			48	48	48	48	48	32
	MDA	r				1	0.463**	0.329*	0.198	0.099
		Sig				.	0.001	0.022	0.177	0.591
		N				48	48	48	48	32
	MTT	r					1	0.505**	0.267	-0.179
		Sig					.	0.000	0.066	.326
		N					48	48	48	32
	CARs	r						1	-.173	0.314
		Sig						.	.239	0.080
		N						48	48	32
	SOD	r							1	-0.139
		Sig							.	0.447
		N							48	32
	CAT	r								1
		Sig								.
		N								32

In Quartile 3, Table 6.3 shows the correlation analyses for the third growth stage. As observed in Quartile 2, cyanotoxins production has a negative correlation with metabolic activity. Chlorophyll a has negative correlation with electrolyte leakage and positive correlations with MDA, metabolic activity and CARs level. Electrolyte leakage has negative correlations with MDA level, metabolic activity, and CARs level that reach significance level. MDA shows positive correlations with metabolic activity and CARs level and negative correlation with SOD activity. Metabolic activity has negative correlations with SOD and CAT activity. In addition, metabolic activity has positive correlation with CARs level and CAT activity. As observed in Quartile 2, cyanotoxins production does not exhibit correlation with antioxidants activity.

Table 6.3: Correlation between ClO_2 treatments cyanotoxin productions, viable cell and stress response in the quartile 3rd of growth

Q3			ClO_2 Exposure	Chl *a*	Electrolyte leakage	MDA	MTT	CARs	SOD	CAT
	Cyanotoxins	r	-0.013	-0.091	0.212	0.188	-0.414*	-0.149	-0.355	0.009
		Sig	0.948	0.650	0.289	0.347	0.032	0.457	0.069	0.966
		N	27	27	27	27	27	27	27	27
	Chl *a*	r		1	-0.864**	0.444**	0.609**	0.963**	-0.114	0.238
		Sig		.	0.000	0.002	0.000	0.000	0.441	0.103
		N		48	48	48	48	48	48	48
	Electrolyte leakage	r			1	-.542**	-0.847**	-0.863**	0.181	0.022
		Sig			.	.000	0.000	0.000	0.218	0.884
		N			48	48	48	48	48	48
	MDA	r				1	0.539**	0.322*	-0.400**	-0.245
		Sig				.	0.000	0.026	0.005	0.094
		N				48	48	48	48	48
	MTT	r					1	0.620**	-0.205	-0.313*
		Sig					.	0.000	0.163	0.030
		N					48	48	48	48
	CARs	r						1	-0.071	0.286*
		Sig						.	0.629	0.049
		N						48	48	48
	SOD	r							1	0.091
		Sig							.	0.540
		N							48	48
	CAT	r								1
		Sig								.
		N								48

In Quartile 4, Table 6.4 shows the correlation analyses for the final growth stage. As observed in Quartiles 2 and 3, cyanotoxins production exhibits negative correlation with metabolic activity. Chlorophyll *a* exhibits negative correlation with electrolyte leakage and positive correlations with MDA, metabolic activity, and CARs level. In addition, electrolyte leakage has negative correlations with MDA level, metabolic activity, and CARs level and positive correlations with antioxidants activity, SOD and CAT activity. MDA has positive correlation with metabolic activity, SOD, CAT activity, and CARs level. Metabolic activity has positive correlation to CARs level. As observed in Quartiles 2-3, cyanotoxins production does not exhibit correlation with antioxidants activity.

Table 6.4: Correlation between ClO_2 treatments cyanotoxin productions, viable cell and stress response in the quartile 4th of growth

Q4			ClO_2 Exposure	Chl *a*	Electrolyte leakage	MDA	MTT	CARs	SOD	CAT
	Cyanotoxins	r	-0.120	0.136	-0.064	-0.124	-0.308*	0.149	-0.095	-0.128
		Sig	0.415	0.358	0.668	0.399	0.033	0.313	0.520	0.385
		N	48	48	48	48	48	48	48	48
	ClO_2 Exposure	r	1.000	-0.959**	0.814**	-0.446**	-0.343*	-0.952**	0.146	-.044
		Sig	.	0.000	0.000	0.001	0.017	0.000	0.322	0.768
		N	48	48	48	48	48	48	48	48
	Chl *a*	r		1.000	-0.806**	0.385**	0.415**	0.969**	-0.168	0.030
		Sig		.	0.000	0.007	0.003	0.000	0.253	0.838
		N		48	48	48	48	48	48	48
	Electrolyte leakage	r			1.000	-0.302*	-0.277	-0.749**	0.553**	.292*
		Sig			.	0.037	0.057	0.000	0.000	.044
		N			48	48	48	48	48	48
	MDA	r				1.000	0.333*	0.349*	0.291*	0.178
		Sig				.	0.021	0.015	0.045	0.227
		N				48	48	48	48	48
	MTT	r					1.000	0.323*	0.128	0.244
		Sig					.	0.025	0.384	0.094
		N					48	48	48	48
	CARs	r						1.000	-0.164	0.022
		Sig						.	0.266	0.882
		N						48	48	48
	SOD	r							1.000	0.540**
		Sig							.	0.000
		N							48	48
	CAT	r								1.000
		Sig								.
		N								48

Statistically significant correlations between cyanotoxins, cell viability and stress response after cells exposed to ClO_2.are summarised in Table 6.5. Blank spaces represent insignificant levels of correlation or, in the case of SOD and CAT in Quarttile 1 only, no data. At low population density (Quartile 1), significant positive correlations are between cyanotoxins and ClO_2 concentration ($r = 0.347$, $p = 0.024$) and electrolyte leakage ($r = 0.413$, $p = 0.006$). Although correlation analysis shows increasing cyanotoxins release with increasing ClO_2 concentration, the variability of cyanotoxins measured at each ClO_2 concentration that has been shown in Figure 6.3 leads to some doubt about the relationship. Further investigation is needed. Increased electrolyte leakage likely represents cyanotoxins release. Electrolyte leakage can be explained by the loss of strength, rigidity and permeability of cellular membrane. These losses lead to changes in membrane function, resulting in membrane damage. Also, some released cyanotoxins may be due to cell lysis. No significant correlation was observed with cell health or stress response.

In contrast, the next quartiles of growth phases (Quartiles 2, 3, and 4), which represent increasing population densities, exhibit significant negative relationships between cyanotoxins and metabolic activity (Quartile 2; $r = -0.543$, $p = 0.001$, Quartile 3; $r = -0.414$, $p = 0.03$ and Quartile 4; $r = -0.308$, $p = 0.03$). Metabolic activity is used to evaluate the proliferation and survival of the cells (Mosmann, 1983). Cyanotoxins are hypothesized to shunt intracellular metabolites while cells are confronted with environmental stressors (Paerl and Millie, 1996), which may be why correlations imply metabolic cost of cyanotoxins production in Quartiles 2-4. Simultaneously, cyanotoxins play a protective role against hazardous conditions (Holland and Kinner 2013). This mechanism seems to be important for the cells in the low population density (with high metabolic activity) to protect themselves. Meanwhile, mature populations densities were reducing metabolic activity and producing less cyanotoxins. This mechanism may be less important. However, cells produce secondary metabolites as a result of stress conditions. At low metabolic activity, cyanotoxins were produced in lower concentrations. Cyanotoxins production does not exhibit significant correlation with chlorophyll *a*, electrolyte leakage, MDA level or antioxidants activity.

Table 6.5: Statistically significant correlation between cyanotoxins, cell viability, and stress response by ClO_2 treatment

		ClO_2 Exposure	Chl *a*	Electrolyte leakage	MDA	MTT	CARs	SOD	CAT
Q1									
	r	0.347*		0.413**					
Cyanotoxins	Sig	0.024		0.006					
	N	42		42					
Q2									
	r					-0.543**			
Cyanotoxins	Sig					.001			
	N					33			
Q3									
	r					-0.414*			
Cyanotoxins	Sig					.032			
	N					27			
Q4									
	r					-0.308*			
Cyanotoxins	Sig					.033			
	N					48			

6.3.3 Discussion

In the early growth stage (Quartile1), cyanotoxins production after exposure to ClO_2 shows correlations with ClO_2 concentration and electrolyte leakage, although high variability leads to some doubt about the correlation with ClO_2 concentration. Cyanotoxins production does not correlate with stress response of cells as indicated by CARs. In the following growth phases (Quartiles 2-4), cyanotoxins production correlated to metabolic activity of cells, indicating the metabolic cost of cyanotoxins production. Cells reduce metabolic activity to regulate intracellular mechanisms. Although cells reduce metabolic activity, including less cyanotoxins production, cyanotoxins may still have a protective role. Cyanotoxins production does not correlate with stress response of cells as indicated by all three factors (CARs, SOD and CAT).These results imply that cells are not producing cyanotoxins in response to oxidative stress from ClO_2 treatment, which has important implications for water treatment.

High concentration ClO_2 treatment (up to 10 mg/L) inhibits cyanobacteria effectively and reduces cyanotoxin release (Chapter 4). Intake water contaminated with cyanobacteria, high concentration of ClO_2 is most appropriate treatment. Low concentration treatment in line with U.S. EPA and other guidance for disinfection (1.4 mg/L) causes cell stress in low population densities but does not inhibit them. Although ClO_2 treatment causes oxidative stress, cells do not produce cyanotoxins in response to this oxidative stress. The oxidation capacity of ClO_2 is not reduced by hydrolysis or inactivation with organic matter in water. In consequence, it seems to directly cause cell degradation and death more than causing cells stress and increased toxins.

When using high dose of ClO_2 treatment, excess ClO_2 risks decomposition to by-products of chlorite and chlorate, which may require further treatment. To avoid by-product formation, optimisation of ClO_2 treatment to inhibit cyanobacteria and reduce cyanotoxins, particularly at high population densities, may require integration with other methods such as activated carbon, membranes, or reverse osmosis. Furthermore, potential overdose of ClO_2 treatment can be monitored by by-product generation and

removed by ferrous iron or sulfite ion oxidation that is recommended from U.S. EPA (2010) or other treatments such as carbon absorption.

An interesting future direction to take this research would be to combine ClO_2 treatment with a method of suppressing cyanotoxin release such as ultrasonic irradiation. A promising approach involves disrupting the membranes of nitrogen-producing heterocysts in cyanobacteria (Kotopoulis, et al., 2009). This technique resulted in an ecological effect that suppressed release of toxins into the water. However, care should be taken when implementing in an environment where aquatic or semi-aquatic animals are present (i.e. before intake to water treatment) because the acoustic pressures associated with ultrasonic irradiation may generate harmful levels of noise. In addition, combined treatments may be particularly beneficial if they improve effectiveness of lower concentration ClO_2 treatment.

6.4 Summary of Key Findings

Cyanotoxin formation decreased with increasing population density. The lowest population density (Quartile 1) had the highest concentrations of microcystins regardless of ClO_2 treatment concentration. As population density increased (Quartiles 2-4), microcystins production declined. In the lowest population density (Quartile 1), based on correlation analysis, median microcystins production on a cellular basis seems to increase with increasing concentration of ClO_2. However, variability in microcystins concentration and the absence of statistically significant differences at different ClO_2 doses as determined by ANOVA calls into question the strength of this effect. In the next growth phase (Quartile 2), there was no correlation between microcystins and ClO_2 concentration; however, at least one difference was statistically significant. Further declines were observed in microcystins production as population density increased further (Quartile 3 and Quartile 4) and no differences were observed among doses of ClO_2. Our findings indicate that changes in microcystins production are not obviously affected by ClO_2. More significant differences are associated with increasing population density (Quartiles 1-4).

Correlation between cyanotoxins production and electrolyte leakage may reflect cyanotoxin release. However, there is no correlation with metabolic activity or stress response. Electrolyte leakage comes from malfunction of cell envelope (cell membrane and cell wall). It is plausible that cyanotoxins release occurs because of decreasing strength, rigidity, permeability of cell envelope, but there was no relation to stress response. Oxidation by ClO_2 deteriorates amino acids of cyanobacteria, reflecting cell degradation and potential toxins release. Although membrane damage and cyanotoxins production were measured, cyanotoxins release was not measured directly. In future research, damage to intact cells can be analyzed by a method such as flow cytometry to differentiate cell damage that is caused by ClO_2 treatment from other sources of membrane damage. Release of cyanotoxins induced by ClO_2 treatment can be measured by changes to extracellular cyanotoxins. Future research should evaluate if the ELISA kit (0.01 µg/L) is sensitive enough to detect releases by low population densities and the effects of ClO_2 on the ELISA assay.

Importantly, across all population densities, cyanotoxins production does not exhibit correlation with antioxidants activity. As population density increases (Quartiles 2-4), correlation between MTT with cyanotoxins suggests a metabolic cost for cyanotoxins production. However, cyanotoxins production does not exhibit correlation with antioxidants activity. Although cyanobacteria experience stress with ClO_2 treatment, this stress is not associated with cyanotoxins production at any population density.

Based on this work, low concentration treatment in line with U.S. EPA (2010) and other guidance (Gates, 1998, Chen and Regli, 2002) for disinfection (1.4 mg/l) causes cell stress in cyanobacteria but does not inhibit them. Although ClO_2 treatment at this level is ineffective for inhibition and causes oxidative stress, cells do not produce cyanotoxins in response to this oxidative stress. Contaminated water with cyanobacteria, high ClO_2 concentration is the most effective to inhibit cyanobacteria and reduce cyanotoxins release (Chapter 4). Low concentration treatment in line with U.S. EPA and other guidance for disinfection (1.4 mg/l) causes cell stress in low population densities but does not inhibit them. Cells do not produce cyanotoxins in response to this oxidative stress, meaning that inadequate treatment is only likely to increase cyanotoxins release by failing to inhibit cyanobacteria. High concentration treatment risks generation of chlorite and chlorate by-products, but these can be monitored and controlled. For example, chlorite can be oxidized by ferrous and sulfite ion (U.S. EPA, 2010) and both chlorate and chlorite can be controlled by activated carbon (Gonce and Voudrias, 1994)

Chapter 7. CONCLUSION

7.1 Conclusions

High concentration of ClO_2 treatment (up to 10 mg/L) shows effective inhibition of *M. aeruginosa.* Progressively high concentrations of ClO_2 demonstrated the ability to reduce chlorophyll *a*, microcystins production, and microcystins release. Chlorophyll becomes decolourised and serves as the distinct indicator of photosynthesis depletion, relating to viability of cells. These ClO_2 concentrations were much higher than published recommendations for disinfection. Typical published recommendations (e.g. U.S. EPA (2010) for maximum concentration of ClO_2 during disinfection are 1-1.4 mg/L to avoid excess chlorite formation. In Europe, the recommended residual dose of ClO_2 in a water distribution system is even lower: 0.05 - 0.1 mg/L.

Exploration of population density and ClO_2 dose was conducted to study how sublethal concentration of ClO_2 and various contact times affect cyanobacteria under relevant water treatment conditions. Treatment effects were observed on cells viability, stress response and cyanotoxins production. Low concentration of ClO_2 (1.5 mg/L) affected cellular health and influenced the stress response of *M. aeruginosa*; however, changes in microcystins production are not obviously affected by ClO_2. In the lowest population density (Quartile 1), based on correlation analysis, median microcystins production on a cellular basis seems to increase with increasing concentration of ClO_2. However, variability in microcystins concentration and the absence of statistically significant differences at different ClO_2 doses as determined by ANOVA calls into question the strength of this effect. In subsequent growth phases (Quartile 2), there were no correlations between microcystins and ClO_2 concentration More significant differences are associated with increasing population density (Quartiles 1-4).

Stress response by *M. aeruginosa* was apparent by increased CARs and SOD activity. While pigments can be oxidised by ClO_2, cells also display antioxidant roles in order to scavenge ROS that are causing oxidative stress. SOD level exhibits the most active scavenging to reduce oxidative stress that is induced by low ClO_2 concentration treatment.

Early experiments demonstrated that *M. aeruginosa* grows well in laboratory conditions with growth lights and sufficient nutrient; this species is toxic and produces microcystins; and decomposition of ClO_2 in water is 50 % in 8 days. Low concentrations of ClO_2 affected the viability of cells, particularly at low population density (Quartile 1). Impaired health of *M. aeruginosa* was apparent by decreases in chlorophyll a and metabolic activity (MTT) as well as increases in electrolyte leakage. The secondary product of lipid peroxidation (MDA) was not observed at elevated levels for the extended exposure times (3hr -3d) due to compound instability. However, increased MDA levels were evident during the shorter period of ClO_2 exposure (3 hrs). This evidence supports the idea that ClO_2 oxidises vulnerable sites of membranes, PUFA, causing MDA production. Unfortunately, SOD and CAT samples from the earliest growth phase (Quartile 1) were lost. SOD activity was expected to increase level while cells treated by ClO_2 comparing with control sample. The highest SOD activity was observed at low ClO_2 concentration (1.5 mg/L). SOD activity decreased with higher ClO_2 concentration but SOD activity was always greater than what was observed in untreated samples, likely reflecting increased cell death as well as cell stress. Increased activity of CAT was expected in this growth phase because low population densities tend to show more stress response than mature populations. CAT activity shows unclear scavenging reactions. Increased catalase (CAT) was not observed. Decreased CAT level was possibly as a result of quenching. However, the experiment was conducted in conditions such that cells were always exposed to light. Decreased CAT level may also be possible due to illuminant stress to inactivate CAT activity.

Cyanotoxins concentration increases with increasing population density; however, decreasing cyanotoxins formation is associated with increased cyanobacteria population density. Total concentration increases because the number of cells increases. The lowest population density produces the highest concentration of microcystins at all ClO_2 concentrations tested. Cyanotoxins production had correlation with electrolyte leakage, which may reflect cyanotoxins release. However, correlations between stress response and metabolic activity were not found. Electrolyte leakage resulted from malfunction of cell envelope (cell membrane and cell wall). Decline of strength, rigidity, and permeability of cell envelope causes cyanotoxins release but this

was not induced by stress response. As population density increased (Quartiles 2-4), microcystins production decreased. Correlation between MTT with cyanotoxins indicates a metabolic cost for cyanotoxins production. There were no correlations between cyanotoxins production and stress response or antioxidants activity at any population density. These results suggest that the changes in microcystins production are more strongly influenced by population density (Quartiles 1-4) than doses of ClO_2.

Published treatment recommendations from U.S. EPA and other organisations for ClO_2 pre-oxidation of surface water are 1 to 1.4 mg/L; however, these recommendations do not take into account water resource contamination with cyanobacteria. In this research, using low concentration of ClO_2 treatment near these recommended levels (1.5 mg/L) resulted in oxidative stress but was not effective for inhibition. However, excess cyanotoxins were not produced by cells in response to the oxidative stress. The early growth phases of *M. aeruginosa* responded greater to stress induced by ClO_2 treatment than later exponential phases. The low number of cells of the early phase make it a good target for intervention. For intake water, treatment of contaminated water by *M. aeruginosa*, and probably other cyanobacteria, this research suggests that using high concentration of ClO_2 is effective for inhibition and can minimize cyanotoxins release. However, at these concentration levels, further treatment of potential chlorite and chlorate by-products may be required. Low ClO_2 concentration treatment in line with the U.S. EPA guidelines is not suitable for pre-treatment to inhibit *M. aeruginosa* at any growth stage. Although low concentration treatment reduced viability and increased intracellular oxidative stress, it was insufficient to inhibit *M. aeruginosa.* Importantly, although low concentration ClO_2 treatment was inadequate, there was no correlation between stress and cyanotoxins production. Continued use of low concentration ClO_2 treatment for cyanobacteria likely requires integration with other methodologies such as carbon absorption, membrane, and reverse osmosis.

7.2 Recommendations for Future Work

Low concentration of ClO_2 showed ineffective treatment for inhibiting cyanobacteria but, apart from a limited effect at low population density, there was no effect to

increase cyanotoxins production. There are a number of factors related to the effect of the treatment that are worth exploring, such as increasing pH level and organic matter content to reflect the typical environmental conditions in water reservoirs. These factors may impact effectiveness of ClO_2 treatment and should be evaluated. For example, the effects of varied pH levels, particularly the mildly acidic to alkaline range ($6 < pH < 9$), should be evaluated to examine the efficacy of ClO_2 treatment and determine if treatment conditions need to be varied based on water intake conditions.

In addition to future laboratory study, this work should be up-scaled and evaluated in conditions typical for a water treatment system. As both pre-treatment and post-treatment with ClO_2 are interesting, a continuous water treatment system should be designed for high-dose and low-dose treatment to inhibit *M. aeruginosa* at different blooming stages. This continuous process should have conventional water treatment processes including coagulation-flocculation, sedimentation and filtration in between the two ClO_2 treatments such that pre-treatment and post-treatment (disinfection) with ClO_2 can be explored systematically. Fate of *M. aeruginosa* via viability of cells, stress response and cyanotoxins production in all treatment steps should be observed.

Treating with UV and ozone is more effective than chemical disinfection but has less longevity. Chang (2015) showed that a combined process was more effective compared with individual UV or ozone processes for the removal and mineralization of Microcystin-LR (i.e. the cyanotoxin) in water. Further study should examine the effectiveness of treatments on cyanobacteria as well as cyanotoxins released. To benchmark effectiveness, the treatment time and re-growth of cyanobacteria and cyanotoxins release should be evaluated. The efficacy of *M. aeruginosa* inhibition using this physical method should be compared with chemical disinfection as well. This inspection should focus on the pre-treatment according to the previous study. A wide variety of testing parameters including type of UV, hours of treatment and light intensity as well as ozone treatment time and concentration. Combined treatments may boost effectiveness and therefore permit use of lower ClO_2 concentrations in water treatment.

Finally, mechanisms to suppress release of cyanotoxins would be interesting to examine alongside chemical treatment. For example, disrupting the membranes of

nitrogen-producing cells in some cyanobacteria by means of ultrasound showed promise in suppressing toxin release. Additional water quality parameters and potential impacts from the acoustic pressures should be investigated. These techniques may be beneficial alongside chemical treatment and should be investigated further.

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APPENDICES

Table a1: The observation of *Microcystis aeruginosa* which is toxic strain to produce cyanotoxins and comparing with growth rate

Date	DO 680	Intracellular toxins	Extracellular toxins
	(nm)	µg/L	µg/L
1	0.02	0.84 ± 0.04	0.14 ± 0.008
2	0.03	0.80 ± 0.13	0.14 ± 0.01
3	0.03	1.59 ± 0.26	0.13 ± 0.004
4	0.05	1.78 ± 0.83	0.13 ± 0.007
5	0.13	4.54 ± 0.34	0.16 ± 0.01
6	0.35	14.16 ± 9.05	0.69 ± 0.07
7	0.57	21.60 ± 2.32	0.67 ± 0.01
8	0.86	36.30 ± 33.58	1.22 ± 0.008
9	1.08	75.75 ± 0.41	1.46 ± 0.29
10	1.27	115.25 ± 21.59	2.89 ± 0.27
11	1.42	100.61 ± 8.70	4.15 ± 0.87
12	1.91	103.07 ± 15.35	2.47 ± 0.10
13	3.08	151.93 ± 33.61	7.91 ± 0.19
14	Overage	143.90 ± 11.92	6.39 ± 2.03

Table a2: The investigation of efficiency treatment of ClO_2

ClO_2	OD 680 Control sample	OD 680 Treated cell	Chlo *a*	Intracellular toxins	Extracellular toxins
(mg/L)	(nm)	(nm)	(mg/L)	µg/L	µg/L
0	0.44	0.44	3.25 ± 0.01	13.08	0.29
0.5	0.44	0.45	1.90 ± 0.03	6.58	0.27
1	0.44	0.44	1.89 ± 0.03	6.73	0.39
1.5	0.44	0.46	1.99 ± 0.01	6.23	0.52
3	0.44	0.40	0.93 ± 0.02	4.01	0.36
5	0.44	0.37	0.37 ± 0.02	3.44	0.20
10	0.44	0.33	0.08 ± 0.03	2.80	0.23

Table a3: The detection of the intact cell from oxidative damage (1)

		1st Interval of growth				2nd Interval of growth			
		Chlorophyll *a*	**Conductivity**	**MTT**	**MDA**	**Chlorophyll *a***	**Conductivity**	**MTT**	**MDA**
Contact time		mg/L	µs/cm	Abs 556	nmol.ml^{-1}	mg/L	µs/cm	Abs 556	nmol.ml^{-1}
3hrs									
Conc.ClO_2	0.0	1.23±0.05	611.0±9.24	0.29±0.01	1.18±0.08	1.89±0.02	735.0±2.89	0.93±0.04	2.09±0.09
	1.5	1.17±0.02	668.0±0.58	0.35±0.01	0.95±0.15	1.70±0.13	772.5±11.26	0.86±0.04	2.05±0.08
	3.0	1.13±0.01	686.5±2.60	0.31±0.02	1.30±0.05	1.61±0.03	830.5±3.18	0.81±0.05	2.14±0.06
	5.0	1.05±0.04	737.0±1.15	0.24±0.03	1.22±0.02	0.69±0.02	892.0±5.20	0.03±0.01	2.26±0.14
1Day									
Conc.ClO_2	0.0	1.50±0.12	617.5±0.87	0.42±0.02	1.38±0.12	2.32±0.14	716.5±4.33	0.37±0.00	2.19±0.02
	1.5	1.54±0.06	673.5±3.75	0.34±0.03	1.58±0.07	1.77±0.06	762.0±8.08	0.49±0.02	1.98±0.05
	3.0	1.37±0.10	729.0±14.43	0.31±0.04	1.62±0.02	1.31±0.03	799.0±4.04	0.38±0.01	1.95±0.03
	5.0	1.15±0.03	738.5±2.60	0.30±0.04	2.10±0.33	0.90±0.12	863.0±0.00	0.12±0.01	1.85±0.03
2Days									
Conc.ClO_2	0.0	1.80±0.06	677.5±13.57	1.07±0.06	1.63±0.02	1.89±0.06	706.5±7.22	0.59±0.04	2.11±0.14
	1.5	1.31±0.03	693.5±2.60	0.80±0.04	1.52±0.04	1.60±0.04	769.0±3.46	0.62±0.01	1.86±0.06
	3.0	1.27±0.05	708.0±2.86	0.67±0.02	1.54±0.03	1.14±0.05	817.0±8.66	0.67±0.01	1.77±0.03
	5.0	1.14±0.08	760.5±1.44	0.75±0.00	1.52±0.05	0.41±0.17	895.0±2.89	0.29±0.02	1.64±0.11
3Days									
Conc.ClO_2	0.0	1.89±0.07	670.0±6.35	1.18±0.07	1.80±0.04	2.33±0.00	717.0±1.73	0.61±0.02	2.20±0.04
	1.5	1.73±0.10	726.5±3.75	1.45±0.06	1.75±0.01	2.07±0.08	780.5±2.02	0.80±0.02	2.02±0.03
	3.0	1.35±0.06	739.5±2.60	1.09±0.04	1.31±0.27	1.67±0.03	824.5±0.29	0.77±0.03	1.95±0.05
	5.0	1.26±0.04	793.5±1.44	0.75±0.01	1.56±0.02	0.87±0.07	876.5±6.64	0.38±0.04	1.67±0.05

Table a4: The detection of the intact cell from oxidative damage (2)

		3rd Interval of growth				4th Interval of growth			
		Chlorophyll *a*	**Conductivity**	**MTT**	**MDA**	**Chlorophyll *a***	**Conductivity**	**MTT**	**MDA**
Contact time		mg/L	µs/cm	Abs 556	nmol.ml-1	mg/L	µs/cm	Abs 556	nmol.ml^{-1}
3hrs									
Conc.$ClO_2$0.0		2.83±0.07	762.5±6.64	1.69±0.03	2.47±0.07	5.95±0.15	890.0±0.58	0.41±0.03	2.98±0.29
	1.5	2.71±0.04	801.0±1.73	1.72±0.08	2.37±0.01	5.50±0.12	952.5±0.29	0.19±0.03	3.02±0.34
	3.0	1.90±0.02	899.5±0.87	0.90±0.03	2.87±0.04	4.85±0.06	1012.5±2.02	0.25±0.04	3.05±0.28
	5.0	1.73±0.04	909.5±0.87	0.55±0.05	2.96±0.02	3.70±0.09	1054.5±2.02	0.19±0.01	3.41±0.46
1Day									
Conc.$ClO_2$0.0		2.74±0.02	734.0±0.00	2.28±0.25	2.70±0.03	6.27±0.17	917.0±2.31	2.44±0.10	4.33±0.23
	1.5	2.30±0.02	801.0±8.08	1.60±0.12	2.35±0.16	5.51±0.01	992.0±0.00	1.22±0.08	4.03±0.19
	3.0	1.51±0.26	903.0±2.89	0.81±0.04	2.38±0.06	5.03±0.16	1032.0±9.24	1.18±0.02	3.93±0.14
	5.0	1.10±0.02	904.5±6.64	0.64±0.02	2.23±0.01	3.81±0.03	1082.0±25.40	0.95±0.06	3.39±0.06
2Days									
Conc.$ClO_2$0.0		3.03±0.03	771.0±2.89	1.04±0.05	2.54±0.05	6.12±0.05	935.0±1.73	0.96±0.04	3.54±0.09
	1.5	2.65±0.06	813.0±6.35	0.77±0.01	2.37±0.02	5.66±0.16	1064.5±1.44	0.84±0.04	3.71±0.10
	3.0	1.56±0.01	918.0±1.15	0.46±0.02	1.96±0.06	4.88±0.08	1107.0±5.77	0.53±0.02	3.37±0.09
	5.0	1.39±0.02	936.0±1.15	0.39±0.02	1.67±0.05	3.87±0.20	1169.0±12.70	0.51±0.03	2.58±0.06
3Days									
Conc.$ClO_2$0.0		2.96±0.06	776.5±1.44	0.73±0.03	2.91±0.06	5.93±0.12	932.5±9.24	0.56±0.00	4.06±0.13
	1.5	2.61±0.04	861.5±4.33	0.47±0.03	2.69±0.03	5.18±0.05	1066.5±0.58	0.27±0.01	4.06±0.11
	3.0	1.30±0.00	944.5±2.60	0.37±0.02	1.82±0.08	4.69±0.11	1108.0±2.60	0.34±0.00	3.70±0.10
	5.0	1.28±0.04	992.5±11.26	0.35±0.02	1.78±0.07	3.62±0.16	1177.0±1.15	0.31±0.04	2.85±0.06

Table a5: The oxidative stress of *Microcystis aeruginosa* illustrated by the changed antioxidants level (1)

		1st Interval of growth				2nd Interval of growth			
		Chlorophyll *a*	**SOD**	**Catalase**	**Carotenoid**	**Chlorophyll *a***	**SOD**	**Catalase**	**Carotenoid**
Contact time		mg/L	Abs at 450 nm	Activity nmol/min/ml	mg/L	mg/L	Abs at 450 nm	Activity nmol/min/ml	mg/L
3hrs									
Conc.$ClO_2$0.0		1.23±0.05	-	-	0.55±0.03	1.89±0.02	0.20±0.01	0.52±0.41	0.63±0.01
	1.5	1.17±0.02	-	-	0.54±0.07	1.70±0.13	0.38±0.02	0.00±0.19	0.53±0.05
	3.0	1.13±0.01	-	-	0.42±0.01	1.61±0.03	0.33±0.02	0.16±0.89	0.46±0.01
	5.0	1.05±0.04	-	-	0.38±0.03	0.69±0.02	0.31±0.05	0.60±0.14	0.02±0.02
1Day									
Conc.$ClO_2$0.0		1.50±0.12	-	-	0.67±0.05	2.32±0.14	0.17±0.00	2.17±1.07	0.76±0.05
	1.5	1.54±0.06	-	-	0.76±0.10	1.77±0.06	0.40±0.03	0.05±0.20	0.58±0.02
	3.0	1.37±0.10	-	-	0.70±0.12	1.31±0.03	0.31±0.01	0.00±0.86	0.58±0.17
	5.0	1.15±0.03	-	-	0.49±0.02	0.90±0.12	0.28±0.05	0.00±0.015	0.22±0.04
2Days									
Conc.$ClO_2$0.0		1.80±0.06	-	-	0.81±0.03	1.89±0.06	0.22±0.00	2.49±0.45	0.60±0.03
	1.5	1.31±0.03	-	-	0.55±0.02	1.60±0.04	0.41±0.03	1.23±0.68	0.69±0.18
	3.0	1.27±0.05	-	-	0.52±0.02	1.14±0.05	0.33±0.02	1.66±1.38	0.35±0.00
	5.0	1.14±0.08	-	-	0.48±0.02	0.41±0.17	0.22±0.04	0.53±0.33	0.08±0.06
3Days									
Conc.$ClO_2$0.0		1.89±0.07	-	-	0.79±0.03	2.33±0.00	0.25±0.01	1.60±0.59	0.75±0.00
	1.5	1.73±0.10	-	-	0.69±0.05	2.07±0.08	0.31±0.03	0.73±0.30	0.67±0.04
	3.0	1.35±0.06	-	-	0.53±0.05	1.67±0.03	0.29±0.02	1.10±0.99	0.56±0.01
	5.0	1.26±0.04	-	-	0.48±0.02	0.87±0.07	0.28±0.02	1.41±0.52	0.28±0.01

Table a6: The oxidative stress of *Microcystis aeruginosa* illustrated by the changed antioxidants level (2)

		3rd Interval of growth				4th Interval of growth			
		Chlorophyll *a*	**SOD**	**Catalase**	**Carotenoid**	**Chlorophyll *a***	**SOD**	**Catalase**	**Carotenoid**
Contact time		mg/L	Abs at 450 nm	Activity nmol/min/ml	mg/L	mg/L	Abs at 450 nm	Activity nmol/min/ml	mg/L
3hrs									
Conc.$ClO_2$0.0		2.83±0.07	0.20±0.00	1.25±0.16	1.17±0.03	5.95±0.015	0.42±0.01	3.24±0.16	3.27±0.09
	1.5	2.71±0.04	0.39±0.01	1.18±0.37	1.05±0.03	5.50±0.12	0.60±0.03	3.62±0.48	2.90±0.07
	3.0	1.90±0.02	0.27±0.02	1.58±0.06	0.57±0.02	4.85±0.06	0.55±0.00	3.44±0.30	2.37±0.02
	5.0	1.73±0.04	0.23±0.01	1.06±0.60	0.48±0.01	3.70±0.09	0.50±0.09	3.08±0.11	1.62±0.04
1Day									
Conc.ClO_2 0.0		2.74±0.02	0.30±0.00	1.02±0.12	1.18±0.01	6.27±0.17	0.67±0.00	4.14±0.03	3.49±0.12
	1.5	2.30±0.02	0.47±0.02	0.64±0.014	0.94±0.01	5.51±0.01	0.74±0.03	4.46±0.74	2.97±0.05
	3.0	1.51±0.26	0.44±0.06	1.00±0.34	0.53±0.10	5.03±0.16	0.75±0.08	6.15±1.70	2.12±0.52
	5.0	1.10±0.02	0.35±0.03	0.61±0.36	0.38±0.00	3.81±0.03	0.68±0.08	4.25±0.55	1.77±0.02
2Days									
Conc.ClO_2 0.0		3.03±0.03	0.38±0.00	3.70±0.73	1.30±0.01	6.12±0.05	0.62±0.01	6.85±0.05	3.41±0.03
	1.5	2.65±0.06	0.61±0.03	2.89±0.19	1.15±0.03	5.66±0.16	0.91±0.02	8.06±0.26	3.02±0.08
	3.0	1.56±0.01	0.45±0.05	5.05±0.56	0.63±0.01	4.88±0.08	0.82±0.05	6.13±1.02	2.52±0.05
	5.0	1.39±0.02	0.38±0.03	3.09±0.77	0.55±0.01	3.87±0.20	0.75±0.12	6.34±0.43	1.82±0.09
3Days									
Conc.ClO_2 0.0		2.96±0.06	0.40±0.00	2.96±0.49	1.23±0.03	5.93±0.12	0.67±0.01	4.84±1.99	3.30±0.09
	1.5	2.61±0.04	0.46±0.02	2.45±0.18	1.07±0.02	5.18±0.05	0.96±0.06	6.50±0.65	2.72±0.02
	3.0	1.30±0.00	0.44±0.05	2.13±0.36	0.49±0.01	4.69±0.11	0.88±0.09	6.99±1.78	2.43±0.06
	5.0	1.28±0.04	0.42±0.02	1.17±0.31	0.48±0.02	3.62±0.16	0.75±0.04	4.31±0.40	1.71±0.09

Table a7: The detection of cyanotoxins produced by *Microcystis aeruginosa* after cell treated by ClO_2

Contact time	Intracellular cyanotoxins (µg/L)			
	1st growth phase	**2nd growth phase**	**3rd growth phase**	**4th growth phase**
3hrs				
Conc.ClO_2 0.0	0.45 ± 0.03	0.90 ± 0.11	-	0.87 ± 0.03
1.5	1.01 ± 0.14	0.54 ± 0.07	-	0.97 ± 0.13
3.0	0.50 ± 0.05	-	-	0.96 ± 0.19
5.0	1.01 ± 0.18	1.00 ± 0.14	-	0.68 ± 0.02
1Day				
Conc.ClO_2 0.0	0.47 ± 0.02	0.92 ± 0.05	-	0.64 ± 0.03
1.5	0.51 ± 0.02	0.73 ± 0.01	0.60 ± 0.01	0.80 ± 0.20
3.0	0.91 ± 0.06	0.90 ± 0.21	-	0.71 ± 0.02
5.0	0.92 ± 0.08	-	-	0.81 ± 0.15
2Days				
Conc.ClO_2 0.0	0.99 ± 0.11	0.38±0.04	0.63 ± 0.03	0.88 ± 0.01
1.5	0.81 ± 0.13	-	0.30 ± 0.01	0.86 ± 0.12
3.0	0.49 ± 0.17	-	0.67 ± 0.03	0.75 ± 0.01
5.0	0.99 ± 0.14	-	0.68 ± 0.06	0.56 ± 0.03
3Days				
Conc.ClO_2 0.0	-	0.69 ± 0.10	0.99 ± 0.02	0.67 ± 0.04
1.5	-	0.63 ± 0.07	0.90 ± 0.04	0.78 ± 0.05
3.0	0.82 ± 0.04	0.42 ± 0.02	0.90 ± 0.04	0.68 ± 0.00
5.0	0.74 ± 0.04	0.88 ± 0.06	0.81 ± 0.08	0.91 ± 0.11