# HYDROGEN PEROXIDE IN EUTROPHIC LAKE TAIHU, CHINA: ADDITION EFFECTS ON PHYTOPLANKTON AND DIEL VARIABILITY IN NATURAL CONCENTRATIONS

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ABSTRACT

Amy L. Pflaumer: Hydrogen peroxide in eutrophic Lake Taihu, China: Addition effects on phytoplankton and diel variability in natural concentrations
(Under the direction of Hans Paerl)

Hydrogen peroxide (HOOH) has demonstrated potential for algicidal use due to selective suppression of cyanobacteria relative to other phytoplankton. Bioassays were used to assess the effects of HOOH on Lake Taihu phytoplankton community and toxin concentrations. The relative contribution of cyanobacteria to phytoplankton biomass was reduced by >50% at 40-60 μM HOOH. Toxin concentrations did not increase above untreated controls at doses >20 μM. Although algicidal use of HOOH is not feasible in large lakes (e.g. Taihu), these results provide further evidence for selective suppression, possibly in smaller lakes. In addition to selective effects, HOOH is an ideal algicide because it is produced abiotically and biotically in lakes and decays into water and oxygen. During a diel study in Taihu, surface HOOH concentrations ranged from 17 ± 6 nM to 168 ± 8 nM and demonstrated diel cycling. Concentrations may be relatively low due to heightened biological decay during blooms.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| allo | alloxanthin |
| ANOVA | Analysis of Variance |
| anth | antheraxanthin |
| *β* Car | *β*-carotene |
| C | carbon |
| ºC | degrees Celsius |
| CDOM | chromophoric dissolved organic matter |
| chl *a* | chlorophyll *a* |
| chl *b* | chlorophyll *b* |
| cm | centimeter |
| $C_{SA}$ | hydrogen peroxide concentration of the standard addition |
| $C_{Sam}$ | hydrogen peroxide concentration of the sample |
| $C_{Std}$ | hydrogen peroxide concentration of the standard |
| Cu(I) | copper(I) |
| Cu(II) | copper(II) |
| cyanoHAB | cyanobacterial harmful algal bloom |
| diad | diadinoxanthin |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DO | dissolved oxygen |
| DOC | dissolved organic carbon |
| DOM | dissolved organic matter |

| | |
|---|---|
| ε | molar extinction coefficient |
| $EC_{50}$ | concentration at which 50 percent inhibition of photosynthetic yield occurs |
| ech | echinenone |
| ELISA | enzyme linked immunosorbent assay |
| Fe(II) | $Fe^{2+}$, iron(II) |
| Fe(III) | $Fe^{3+}$, iron(III) |
| fuco | fucoxanthin |
| Fur | ferric uptake regulator |
| $F_V/F_M$ | photosynthetic yield |
| g | gram |
| GF/A | glass fiber filter grade A |
| GF/F | glass fiber filter grade F |
| h | hour |
| *h* | Planck's Constant |
| H | hydrogen |
| $HO_2$ | hydroperoxyl |
| HOOH | hydrogen peroxide |
| HPLC | high-performance liquid chromatography |
| km | kilometer |
| L | liter |
| lut | lutein |
| m | meter |
| M | molar |

| | |
|---|---|
| mg | milligram |
| mL | milliliter |
| mm | millimiter |
| mM | millimolar |
| myx | myxoxanthophyll |
| n | group size |
| N | nitrogen |
| NaOH | sodium hydroxide |
| neo | 9'cis neoxanthin |
| $NH_4^+$ | ammonium |
| $NH_4^+$-N | nitrogen constituent of ammonium |
| nm | nanometer |
| nM | nanomolar |
| $NO_2^-$ | nitrite |
| $NO_2^-$-N | nitrogen constituent of nitrite |
| $NO_3^-$ | nitrate |
| $NO_3^-$-N | nitrogen constituent of nitrate |
| NtcA | global nitrogen regulator |
| $O_2$ | oxygen |
| $O_2^-$ | superoxide |
| $O_2$ % | oxygen saturation |
| P | phosphorus |
| PAR | photosynthetically active radiation |

| | |
|---|---|
| $PO_4^{3-}$ | phosphate |
| $PO_4^{3-}$-P | phosphorus constituent of phosphate |
| POC | particulate organic carbon |
| PON | particulate organic nitrogen |
| ROS | reactive oxygen species |
| rpm | revolutions per minute |
| s | second |
| SOD | superoxide dismutase |
| TLLER | Taihu Laboratory for Lake Ecosystem Research |
| µg | microgram |
| µL | microliter |
| µm | micrometer |
| µM | micromolar |
| µmol | micromole |
| UV | ultraviolet |
| UVR | ultraviolet radiation |
| ν | wavelength of the photon |
| viol | violaxanthin |
| $V_{Minicell}$ | volume in the minicell |
| $V_{Sam}$ | volume in the sample |
| $V_{Std}$ | volume of the hydrogen peroxide standard added |
| WSP | wastewater stabilization pond |
| w/w | weight per weight |

| | |
|---|---|
| $X_{Int}$ | x-intercept |
| zea | zeaxanthin |
| % | percent |

# CHAPTER 1: EFFECTS OF HYDROGEN PEROXIDE ON PHYTOPLANKTON COMMUNITY COMPOSITION AND PRODUCTION IN EUTROPHIC LAKE TAIHU, CHINA

## 1. Introduction

### 1.1 Cyanobacterial harmful algal bloom formation

Cyanobacteria form a phylum of photosynthetic bacteria widely studied due to their ability to rapidly accumulate into cyanobacterial harmful algal blooms, or cyanoHABs. In recent years, cyanoHABs have increased in intensity and frequency worldwide as a result of accelerating eutrophication and rising water temperatures due to global warming (Paerl and Huisman 2008, 2009). Ecosystems impacted by cyanoHABs exhibit loss of water clarity, resulting in inhibited growth of macrophytes and reduced fish and invertebrate habitats, oxygen ($O_2$) depletion (hypoxia and anoxia), reduction in aesthetic/recreational value, and toxicity to humans and animals (Chorus and Bartram, 1999; Landsberg, 2002; Malbrouck and Kestemont, 2006). In the aquatic ecosystem, bottom-up and top-down controls interact to regulate the rate of phytoplankton biomass production, or primary productivity. Net production is primarily controlled by light availability and nutrient supply (bottom-up) and herbivorous grazing (top-down). Under ideal growth conditions, biomass production may surpass consumption, resulting in blooms (Paerl *et al*., 2001).

#### *1.1.1 Light*

Photosynthetic organisms require light in the form of photosynthetically active radiation (PAR) for growth. Cyanobacteria use pigments including chlorophyll and phycobiliproteins to harvest light. Phycobiliproteins provide a competitive advantage by harvesting light in a portion

of the spectrum largely unused by other major phytoplankton groups (Mur *et al*., 1999). Although cyanobacterial growth is inhibited by long periods of excessive light (Van Liere and Mur, 1979), surface bloom-formers produce carotenoids to tolerate and quench higher light intensities (Paerl *et al*., 1983). Cyanobacteria also thrive under low light intensities. Since they use minimal energy for basic cellular functions relative to other phytoplankton, more energy can be directed to growth (Van Liere and Mur, 1979). Light intensity is lowered during a bloom because of shading caused by the bloom itself (Mur *et al*., 1999).

*1.1.2 Macronutrients: Nitrogen and phosphorus*

Nitrogen (N) and phosphorus (P) are required for phytoplankton growth. In most aquatic ecosystems, phytoplankton productivity may be limited by nutrient availability, particularly of N and P (Redfield, 1958). However, in recent years, anthropogenic nutrient over enrichment has caused widespread eutrophication. P in particular has been thought to play a key role in cyanobacterial bloom formation, especially under conditions of long residence times, high water temperatures (above 20 ºC), and strong stratification (Paerl 1988, 2008). The relative importance of N and P loading is complicated by the fact that some cyanobacterial genera are capable of obtaining N through N fixation (the process by which atmospheric dinitrogen gas is converted into bioavailable ammonia) (Gallon, 1992). Cyanobacteria tend to dominate at low molar N:P ratios (below 15). At high N:P ratios (above 20), eukaryotic algae dominate (Smith, 1983). However, molar N:P ratios are less important in highly eutrophic systems having excessive N and P inputs (Paerl *et al*., 2001). When both N and P are not limiting, other physical-chemical-biotic factors, such as light, flushing rates of water bodies, micronutrients, temperature, and zooplankton grazing, may control phytoplankton community production and composition (Paerl, 2008).

*1.1.3 Flushing rates of water bodies*

Flushing rates of water bodies influence cyanoHAB dynamics by affecting water column stability and nutrient delivery (Paerl, 2008). Low flushing rates are associated with high water column stability, which favors cyanobacterial growth and allows buoyant cyanobacteria to accumulate in surface scums (Paerl, 1988; Köhler, 1992). In Lake Albufera, a large, shallow lake on the coast of Spain, a 45% increase in water residence time during dry years coincided with an increase in *Microcystis* populations by 1-2 orders of magnitude (Romo *et al*., 2013). Rapid flushing rates remove cyanobacterial biomass from a water body and limit bloom development (Sherman *et al*., 1998; Mitrovic *et al*., 2003; Maier *et al.*, 2004), since cyanobacteria grow at a relatively slow rate compared to eukaryotic phytoplankton (Paerl *et al*., 2011; Paerl, 2014). However, enhanced freshwater discharge increases nutrient input, which promotes cyanobacterial growth (Paerl and Paul, 2012).

*1.1.4 Micronutrients*

In addition to macronutrients such as N and P, phytoplankton require several micronutrients for growth (Baptista and Vasconcelos, 2006). Iron is particularly important to phytoplankton growth. It is used in a variety of cellular processes including: photosynthesis, electron transport, N assimilation, N fixation, and energy transfer, and availability can serve as a limiting factor (Paerl *et al*., 2001; Morrissey and Bowler, 2012). However, cyanobacteria often dominate in areas in which phytoplankton biomass is limited by iron availability. Under low iron concentrations, cyanobacteria are able to produce siderophore chelators as part of a high-affinity iron acquisition system (Murphy *et al*., 1976). Other important trace metals include manganese, cobalt, copper, and zinc. However, these trace metals are not typically limiting in freshwater ecosystems (Paerl *et al*., 2001).

*1.1.5 Temperature*

Cyanobacteria thrive at relatively high temperatures, with maximum growth occurring at above 25 ºC, higher than in other major phytoplankton groups (Robarts and Zohary, 1987; Mur *et al*., 1999). Increased temperatures resulting from global warming favor cyanobacterial bloom formation (Paerl and Huisman, 2009). Global warming also enhances vertical stratification, which promotes bloom formation. Cyanobacteria are buoyant due to intracellular gas vesicles. Thus, under conditions of minimal vertical mixing, they can accumulate in surface blooms (Paerl and Huisman, 2008).

*1.1.6 Top-down controls*

Zooplankton grazers impose top-down control of bloom formation. However, the extent to which zooplankton can reduce cyanobacterial biomass is limited. Attempts to reduce cyanobacterial biomass by artificially enhancing zooplankton biomass have met with limited success (Scheffer and Rinaldi, 2000; Rondel *et al*., 2008). Cyanobacteria are poor quality food for zooplankton. Toxic secondary metabolites produced by cyanobacteria can be lethal to zooplankton upon ingestion (Leflaive and Ten-Hage, 2007). Nevertheless, *Daphnia*, a large generalist grazer, has been shown to control cyanobacterial biomass to some extent (Elser *et al*., 2000). Less studied but perhaps equally important in top-down control are cyanobacterial viruses (cyanophages). Up to 50% of cyanobacterial cell death may be due to cyanophages (Tucker and Pollard, 2005).

## 1.2 *Microcystis* and Microcystins

*1.2.1 Microcystis*

*Microcystis* is a genus of freshwater cyanobacteria, including 22 main European and tropical morphospecies such as *Microcystis aeruginosa, flos-aquae,* and *wesenbergii* (Komárek

and Komárková, 2002). *Microcystis* is a unicellular colony-forming genus. Because *Microcystis* cells contain gas vesicles, they are buoyant, which allows for the formation of surface scums. These high density surface scums are associated with a plethora of negative environmental consequences, including production of the hepatotoxin microcystin (Paerl *et al*., 2001).

Microcystins are harmful to organisms big and small, from microalgae to mammals. Humans are exposed to microcystins primarily through drinking contaminated water, but may also be exposed through recreational contact, food, or hemodialysis. Microcystin exposure causes both acute (WHO, 1998) and chronic (Zhou *et* al, 2002) effects in humans through inhibition of protein phosphatases 1 and 2. For example, long-term consumption of low levels of microcystins has been linked to increased risk of liver cancer (Ueno *et al.*, 1996) and colorectal cancer (Zhou *et al*., 2002).

*1.2.2 Microcystin biosynthesis*

Cyanobacteria produce a variety of bioactive secondary metabolites, including hepatotoxic microcystins. Microcystins are heptapeptides which are synthesized nonribosomally. Microcystins are the most structurally diverse group of cyanobacterial toxins, containing approximately 90 isomers, which differ in their level of toxicity as well as other factors including methylation and peptide sequence (Welker and von Döhren, 2006). The *mcy* gene cluster codes for microcystin production in *Microcystis*. The *mcy* gene cluster covers 55 kilobases and includes 10 genes, *mcyA-J*. The genes are divided into 2 operons, *mcyA-C* and *mcyD-J* (Tillett *et al*., 2000). The two operons are divided by a 750 base pair promoter region (Kaebernick and Neilan, 2001).

*1.2.3 Regulation of microcystin production*

Microcystin production has been correlated with a multitude of environmental parameters (nutrients, trace metals, light, pH, temperature, etc.), indicating that a wide variety of factors may play a role in *mcy* transcriptional regulation. However, many of these correlations are believed to reflect the effect of environmental parameters on *Microcystis* cell growth, as opposed to directly affecting microcystin transcriptional regulation, since microcystin production is believed to be proportional to cell growth rate (Kaplan *et al*., 2012; Orr and Jones, 1998). Nevertheless, a few key environmental stimuli are believed to have a direct effect on *mcy* gene transcription, chief among them light and iron-limiting conditions. The effects are manifested in small changes in *mcy* transcription as opposed to on-off regulation. For example, the abundance of *mcy* transcripts has been shown to be upregulated above a critical light threshold (Kaebernick *et al*., 2000) and under iron deplete conditions (Sevilla *et al*., 2008). Additionally, both *mcy* operons exhibit different start sites of transcription under altered light conditions (Kaebernick *et al*., 2002). It's also interesting to note that individual genes in the *mcy* cluster exhibit differing levels of upregulation under oxidatively stressful conditions, indicating that genes are individually regulated within the *mcy* gene cluster (Straub *et al*., 2011). The cause of this differential regulation amongst *mcy* genes is unknown.

Further evidence for the role of iron as well as N in the transcriptional regulation of microcystin biosynthesis is found in the presence of ferric uptake regulator (Fur) and three global N regulator (NtcA) transcription factor binding sites in the *mcy* promoter region (Kaebernick *et al*., 2002). Fur is involved in iron availability and redox status (which is affected by both iron and light conditions). NtcA, on the other hand, is involved in N availability and redox status. Since predicted changes in *mcy* transcript abundance under differing N conditions were not seen

experimentally, the importance of NtcA on microcystin regulation likely lies in its connection to redox status, as opposed to N availability (Sevilla *et al*., 2010).

*1.2.4 Non-toxic Microcystis*

Some *Microcystis* strains do not have the ability to produce microcystin. At the biochemical level, non-toxic strains are unable to produce microcystin because they lack some or all of the microcystin synthetase *mcy* genes (Meißner, *et al.*, 1996). Lack of even a single *mcy* gene results in the inability to produce microcystin (Dittmann *et al*., 1997). Phylogenetic evidence indicates that microcystin biosynthesis evolved early on in cyanobacterial evolutionary history. The last common ancestor of many species of cyanobacteria had the microcystin synthetase genes, which means that modern day non-toxic *Microcystis* have lost the ability over time. Despite their inability to synthesize microcystin, non-toxic strains do have the genes to produce other nonribosomal peptides, which may fulfill a similar role as the toxin (Rantala *et al*., 2004).

**1.3 Oxidative stress in cyanobacteria**

*1.3.1 Reactive oxygen species*

Prior to the evolution of cyanobacteria, the Earth had a reducing atmosphere (Dietrich *et al*., 2006). Around 3 billion years ago, cyanobacteria evolved and began oxygenating the atmosphere through photosynthesis (Brocks *et al*., 1999). As the first $O_2$-producers, cyanobacteria were also the first organisms to encounter the damaging effects of the reactive oxygen species (ROS) unavoidably produced as a byproduct of aerobic metabolism. ROS include the superoxide anion ($O_2^-$), the hydroxyl radical, and hydrogen peroxide (HOOH), among others (Latifi *et al*., 2009). As a stable diradical and a weak univalent electron acceptor, $O_2$ does not efficiently oxidize amino acids or nucleic acids. $O_2^-$, HOOH, and the hydroxyl

radical are stronger univalent oxidants and are highly reactive with most biomolecules. The hydroxyl radical in particular is extremely reactive, with reaction rates limited by diffusion rates (Imlay, 2003).

*1.3.2 Mechanisms of cellular damage*

Oxidative stress occurs when ROS accumulate within the cell. ROS are damaging to lipids, proteins, and DNA (Latifi *et al*., 2009). For example, the hydroxyl radical can deform the fatty acid side chains of membrane lipids by removing a hydrogen (H) from a carbon (C) in the fatty acid side chain, forming water. The C is left with an unpaired electron, becoming a radical. This radical then reacts with $O_2$ to form an alkyl peroxyl radical, which removes a H from a nearby side chain, creating another radical. In this manner, ROS result in chain reaction creation of additional ROS until the process is halted when two radicals encounter one another and form a covalent bond. Proteins are damaged through the oxidation of side chains and polypeptide backbones, resulting in the formation of carbonyl groups (Halliwell and Gutteridge, 2007; Latifi *et al*., 2009). ROS break DNA strands by attacking the backbone deoxyribose sugars or inflict damage through modifying and mutating the nucleotide bases (He and Häder, 2002).

**1.4 Hydrogen peroxide as an algicide**

*1.4.1 Support for the use of hydrogen peroxide as an algicide*

Nutrient reduction is widely regarded as the best strategy to combat cyanoHABs (Conley *et al*., 2009). However, improving water quality by nutrient reduction of a hypereutrophic lake is often a slow, difficult process, requiring several years before cyanoHAB occurrence is reduced. Thus, short-term strategies for eliminating cyanoHABs have been developed, including the application of HOOH. HOOH application is a method of rapid bloom suppression that has demonstrated success in small lakes. Upon application of 60 μM HOOH to a small, shallow lake

in the Netherlands experiencing a *Planktothrix agardhii* bloom, phytoplankton photosynthetic vitality was reduced to 70% within three hours (Matthijs *et al*., 2012). Furthermore, cyanobacterial abundance decreased from 600 x $10^3$ cells/mL prior to HOOH addition to 10 x $10^3$ cells/mL ten days post HOOH addition and remained low for seven weeks. The key to successful suppression of cyanoHABs by HOOH application is determining the lowest concentration of HOOH necessary to suppress the bloom, thereby limiting potential effects on other organisms. This concentration varies depending on a wide range of factors in each system and should be assessed through laboratory incubations prior to each HOOH application (Matthijs *et al*., 2012).

HOOH application for bloom removal has also been tested in waste stabilization ponds in Australia. Upon addition of 3.8 µmol HOOH/µg chl *a*, cyanobacterial chl *a* decreased from 59.0 µg/L to 22.4 µg/L after 24 hours. In a second test, 5.9 µmol HOOH/µg chl *a* decreased total chl *a* to 40% after one week. Cyanobacteria were selectively suppressed relative to other phytoplankton, including diatoms and cryptophytes, for two weeks (Barrington *et al*., 2013). Most laboratory studies indicate that cyanobacteria may be more sensitive to HOOH than other phytoplankton, allowing for a reduction in cyanoHAB biomass with limited damage to the eukaryotic component of the phytoplankton community (Stratford *et al*., 1984; Barroin and Feuillade, 1986; Schrader *et al*., 1998, Drábková *et al*., 2007a,b; Weenik *et al*., 2015).

A study by Drábková and colleagues compared the HOOH-induced reduction in photosynthetic yield in five cyanobacterial species, three green algal species, and a diatom (2007b). Photosynthetic yield ($F_v/F_M$) represents the maximal yield of photosystem II and indicates the general fitness level of an organism. Photosynthetic yield was inhibited by 50% in all five cyanobacterial species upon addition of 15 to 74 µM HOOH while two algal species and the diatom were unaffected. The HOOH concentration that caused a 50% inhibition of

photosynthetic yield ($EC_{50}$) varied from 8 to 51µM in cyanobacteria as opposed to 183 to 619 µM in green algae and 294 µM in the diatom (Drábková *et al*., 2007b).

Although chemical methods of cyanoHAB suppression are not ideal, HOOH is easily preferable to more traditional chemical algicides such as aluminum and copper sulfate. HOOH is naturally produced in freshwater systems in low concentrations, by photolysis of dissolved organic matter (Cooper *et al*., 1988; Draper and Crosby, 1983; Cooper and Zika, 1983) and also biologically by phytoplankton (Asada, 2006). HOOH decays into $O_2$ and water (Cooper and Zepp, 1990), such that it does not remain in the system for long or result in the accumulation of harmful by-products such as heavy metals (Matthijs *et al*., 2012). The half-life of HOOH in highly productive freshwaters is typically less than 3 h (Richard *et al*., 2007; Mostofa *et al*., 2013).

*1.4.2 Limitations to the use of hydrogen peroxide as an algicide*

Nevertheless, HOOH application poses some difficulties. To date, whole-lake HOOH application has only been tested in relatively small lakes due to the expense and logistical difficulty of applying HOOH homogenously to high volume lakes. Special devices, such as a "water harrow" must be developed for successful application (Matthijs *et al*., 2012). Additionally, the massive cyanobacterial cell lysis resulting from HOOH application can cause the release of intracellular toxins, such as microcystin, from toxic blooms. However, because HOOH enhances the degradation of microcystin (Cornish *et al*., 2000), studies show a decrease in extracellular microcystin concentrations post application (Matthijs *et al*., 2012). In some cases, the decrease is not attributed to degradation by HOOH, however. Microcystin concentrations may increase immediately after HOOH addition and fall below detection levels after several days. Since HOOH decays within hours (Cooper *et al*., 1994), it would not remain to degrade

microcystins after several days. Thus, other environmental effects must be responsible for microcystin degradation (Barrington *et al*., 2013).

Further research is required on ecosystem effects of HOOH addition. Zooplankton are sensitive to HOOH. One study found that upon addition of 1470 μM HOOH to a toxic *Alexandrium* bloom, zooplankton abundance drastically decreased from 40,000 organisms/L to 15 organisms/L (Burson *et al*., 2014). Similarly, addition of small amounts of HOOH has been shown to decrease prokaryotic heterotrophic production, altering competition dynamics and C flow (Xenopoulos and Bird, 1997).

## 1.5 Mechanisms employed by cyanobacteria to protect against oxidative stress

### *1.5.1 Preventative mechanisms and nonenzymatic antioxidants*

Like all aerobic organisms, cyanobacteria have evolved mechanisms for preventing damage by unavoidably present ROS. The cells' first line of defense includes preventative mechanisms including energy dissipation and ultraviolet (UV) sunscreens. Dissipation of excess energy and limitation of UV light input reduces formation of ROS. Excess energy may be dissipated through blue light-induced non-photochemical quenching involving carotenoids (El Bissati *et al*., 2000; Rakhimberdieva *et al*., 2004) and through regulation of tetrapyrrole biosynthesis by high light-inducible proteins (Xu *et al*., 2004; Havaux *et al*., 2003). Cyanobacteria also contain nonenxymatic antioxidants, primarily carotenoids such as β-carotene, myxoxanthophyll, zeaxanthin, and echinenone. Carotenoids dissipate energy from singlet oxygen or photosensitized chlorophyll. When the ability to produce zeaxanthin was removed in a mutant *Synechocystis* PCC 6803 strain, increased sensitivity to high light intensity and oxidative stress resulted (Schäfer *et al*., 2005). α-Tocopherol, more commonly known as vitamin E, is another important nonenzymatic antioxidant produced by some cyanobacteria. Specifically, α-

Tocopherol protects cyanobacterial cells from lipid peroxidation. Tocopherol-deficient mutants were more sensitive to oxidative stress induced by high light and linoleic or linolenic acid addition than wild type (Maeda *et al*., 2005).

*1.5.2 Enzymatic antioxidants*

Antioxidant enzymes are the most widely studied mechanism for protection against oxidative stress, particularly superoxide dismutases (SODs). SODs are metalloenzymes, enzymes which contain metal cofactors (Chelikani *et al*., 2004). Cyanobacterial SODs contain either iron, copper and zinc, nickel, or manganese. SODs scavenge the primary agent of photooxidation, $O_2^-$, and HOOH in a very efficient reaction. HOOH is dismutated by catalases. Peroxidases dismutate a variety of peroxides (Chelikani *et al*., 2004). Peroxiredoxins are catalysts for the reduction of HOOH, peroxynitrite, and alkyl hydroperoxides (Wood *et al*., 2003).

*1.5.3 Microcystin production*

Microcystin production has been proposed as a potential mechanism for protection against oxidative stress. Studies indicate HOOH-induced oxidative stress reduces chl *a* content and growth of toxic cyanobacterial cells to a greater extent than non-toxic cells (Dziallas and Grossart, 2011). Gene transcripts of the gene cluster coding for toxin production, *mcy*, are elevated under high light intensity (Kaebernick *et al*., 2000). Interestingly, under conditions of oxidative stress, individual genes in the *mcy* gene cluster are upregulated to varying degrees, with *mcyB* upregulated 20% compared to upregulation of *mcyD* by 370% (Straub *et al*., 2011). Transcriptomic analysis indicates that microcystin biosynthesis occurs during the light period of the light/dark cycle (Nishizawa *et al*., 1999; Straub *et al*., 2011).

Zilliges and colleagues (2011) proposed a mechanism by which microcystin protects the cell against oxidative stress. Under oxidatively-stressful conditions, microcystin molecules bind

to various proteins (including redox-sensitive cysteines) within the cell, protecting the proteins from damage by ROS. In a microcystin-deficient mutant, differential accumulation of redox-sensitive proteins has been demonstrated. Furthermore, microcystin-deficient mutants are more sensitive to conditions of high light and oxidative stress induced by HOOH addition (Zilliges *et al*., 2011).

The hypothesized role of microcystin in protection against oxidative stress is reasonable from an evolutionary perspective as well. Today, cyanoHABs are dominated by both toxic and non-toxic *Microcystis* strains. However, phylogenetic analyses indicate that all ancestral cyanobacteria were capable of toxin production (Rantala *et al*., 2004). Microcystin genes are believed to have coevolved with housekeeping genes and to have been lost in modern non-toxic lineages. Loss of microcystin synthesis may have occurred as cyanobacteria evolved other mechanisms of protection against oxidative stress that were less energetically expensive to maintain than microcystin production. In modern day cyanobacterial lineages, toxin production may only be evolutionarily advantageous for some strains, such as cyanobacteria growing under surface bloom conditions. Surface bloom-formers experience heightened oxidative stress due to high light levels and $O_2$ supersaturation (Zilliges *et al*., 2011).

**1.6 Study goals**

Recent whole lake and waste pond studies indicate a promising role for HOOH in controlling cyanoHABs in small bodies of water (Matthijs *et al*., 2012; Barrington *et al*., 2013). Although laboratory studies demonstrate selective suppression of cyanobacteria by HOOH (Stratford *et al*., 1984; Barroin and Feuillade, 1986; Schrader *et al*., 1998, Drábková *et al*., 2007a,b; Weenik *et al*., 2015), microcosm and mesocosm level studies are relatively limited (Barrington and Ghadouani, 2008; Barrington *et al*., 2011, 2013; Matthijs *et al*., 2012). In this

study, two HOOH addition bioassays were performed to determine the effect of HOOH addition on phytoplankton community composition and production, as well as microcystin concentrations, in cyanobacteria bloom-dominated Lake Taihu, China.

## 2. Materials and Methods

### 2.1 Study site and bioassay design

Lake Taihu ("great lake" in Mandarin) is the third largest freshwater lake in China, spanning 2338 $km^2$, with a volume of 4.4 billion $m^3$ (Pu and Yan, 1998; Qin *et al*, 2007, 2010). Taihu is a shallow, polymictic lake, with an average depth of 1.9 m. It is located in China's coastal plain, about 150 km west of Shanghai (centered at 30º55'40"-31º32'58"N; 119º52'32"-120º36'10"E; Figure 1.1) in the Yangtze River delta. The Lake Taihu watershed is home to 40 million people. The Taihu Basin is a high population density and high gross domestic product per capita region (Xie *et al*., 2007). The lake has been plagued by seasonal cyanoHABs on a yearly basis since the mid-1980's. The blooms occur as a result of excessive nutrient inputs from industrial sources, including sewage, livestock drainage, and agricultural runoff (Lai and Yu 2006). Major nutrient sources are located northwest from the lake, resulting in most intense blooms being located in the highly-eutrophied northern portion of the lake, particularly Meiliang and Zhushan Bays. Lake Taihu's phytoplankton community, once diatom-dominated, is now cyanobacteria-dominated (Chen *et* al., 2003a,b). In 2007, over 1 million people in the city of Wuxi experienced a drinking water shortage as the result of a Lake Taihu bloom (Qin *et al*., 2010). In addition to drinking water shortages, blooms threaten fishing and tourism.

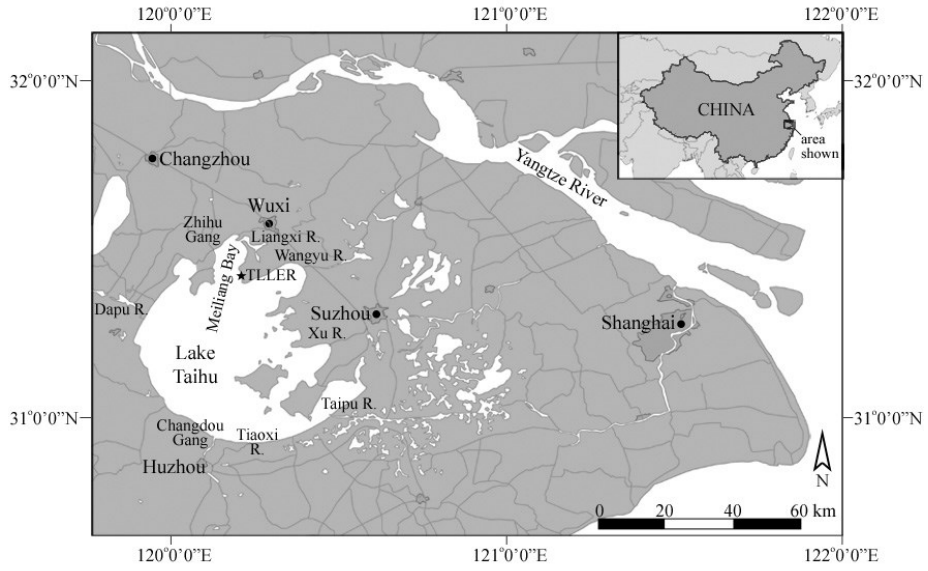


Figure 1.1 Map of Lake Taihu showing Meiliang Bay and the Taihu Laboratory for Lake Ecosystem Research (TLLER), Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences (figure from Paerl *et al*., 2014).

Water was collected from the northern part of the lake in the highly eutrophic Meiliang Bay on June 12$^{th}$, 2014 for the concentrated *Microcystis* bioassay and on June 22$^{nd}$, 2014 for the natural assemblage bioassay. In the concentrated *Microcystis* bioassay, water was collected in two 100 L buckets and allowed to settle, while covered, overnight. This allowed the buoyant *Microcystis* colonies to rise to the surface for collection. The lake water was then filtered through 20 µm mesh followed by 5 µm mesh, and finally 47 mm GF/A glass fiber filters (1.6 µm nominal pore size) to remove the phytoplankton. 900 mL filtrate was combined with 100 mL *Microcystis* surface scum in 1 L semitransparent polyethylene Cubitainers. Cubitainers are chemically inert and allow 80% of PAR (400-700 nm). In the natural assemblage bioassay, the water was not pre-filtered. Instead, 3.5 L were directly added to 4 L Cubitainers.

Bioassays were performed at the Taihu Laboratory for Lake Ecosystem Research (TLLER), Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, in an outdoor concrete enclosure adjacent to the water collection site into which lake water was

pumped. Sealed Cubitainers were placed in the enclosure under one layer of neutral density screening (30% reduction in incident irradiance) to prevent photoinhibition. The concentrated *Microcystis* bioassay was carried out for three days and the natural assemblage bioassay ran for four days. The difference in duration of the two bioassays was due to logistical considerations unrelated to bioassay design. Nutrients were added on day 0 (initiation of experiment) and day 2 to prevent nutrient depletion: nitrate as $KNO_3$ (21.6 µM $NO_3^{2-}$-N), ammonium as $NH_4Cl$ (14.4 µM $NH_4^+$-N), phosphate as $KH_2PO_4$ (1.6 µM $PO_4^{3-}$-P), and iron as $C_{10}H_{12}FeN_2O_8^{2-}$ (0.2 µM $Fe^{2+}$). Bicarbonate was added as $NaHCO_3$ (304.7 µM $HCO_3^-$-C) to prevent inorganic C limitation. Four HOOH treatments were tested in each bioassay, 20 µM, 40 µM, 60 µM, and 80 µM HOOH, as well as an untreated control. Three replicate Cubitainers were used in each treatment and the untreated control. Because HOOH degrades rapidly, HOOH was added daily to maintain conditions of oxidative stress throughout the bioassays. Prior to addition, HOOH was standardized spectrophotometrically (molar extinction coefficient Ɛ=38.1 $M^{-1}$ $cm^{-1}$). Parameters, including pH, dissolved oxygen (DO), oxygen saturation ($O_2$ %), and temperature, were measured daily. On the initial and final days of each bioassay, subsamples were collected for various parameters including: nutrients, particulate organic carbon (POC) and particulate organic nitrogen (PON), total microcystin concentrations, phytoplankton biomass and phytoplankton community composition.

**2.2 Bioassay parameters**

*2.2.1 pH, dissolved oxygen, oxygen saturation, and temperature*

Daily measurements of pH, DO, $O_2$ %, and temperature were taken from replicate Cubitainers using a Hach HQ30d portable pH, conductivity, optical DO, ORP, and ISE multi-parameter meter.

*2.2.2 Nutrients*

Subsamples were collected for nutrient analysis on the initial and final days of each bioassay. In both bioassays, a single sample was collected per treatment by combining equal volumes from each replicate Cubitainer of the treatment. Phosphate ($PO_4^{3-}$), ammonium ($NH_4^+$), nitrate ($NO_3^-$), and nitrite ($NO_2^-$) were measured. Water was syringe filtered through 0.45 µM Millipore HA filters and frozen until analysis. Nutrients were analyzed according to the Standard Methods for the Examination of Water and Wastewater (Eaton *et al*., 1995). $PO_4^{3-}$ was measured spectrophotometrically by the molybdenum blue method. $NH_4^+$ was determined by the indophenol blue method. $NO_2^-$ and $NO_3^-$ were determined by the cadmium reduction method using a flow injection analyzer (Skalar SAN++, Breda, Netherlands).

*2.2.3 Particulate organic carbon and nitrogen*

Subsamples were collected for POC and PON analysis on the initial and final days of each bioassay. In the natural assemblage bioassay, individual subsamples were collected from replicate Cubitainers. In the concentrated *Microcystis* bioassay, a single sample was collected per treatment by combining equal volumes from each replicate Cubitainer of the treatment. Water was filtered through pre-combusted (500 ºC) 25 mm GF/F glass fiber filters (nominal pore size 0.7 µm) and filters were patted dry, folded, and frozen in foil until analysis. Analysis was performed on a Costech CHN analyzer (Model ECS 4010). Prior to analysis, filters were dried for 24 hours at 60 ºC, fumed for 8 hours with 12 M hydrochloric acid to remove inorganic C, and redried at 60 ºC.

*2.2.4 Total microcystin concentrations*

Individual subsamples were collected from replicate Cubitainers for total microcystins (intracellular + extracellular, all isoforms) on initial and final days of the bioassays. Water

samples were frozen in 2 mL centrifuge vials until analysis. Prior to analysis, samples underwent three freeze/thaw cycles to lyse cells. Analysis was performed with an Abraxis enzyme linked immunosorbent assay (ELISA) on a Thermo MultiSkan Spectrum plate reader. The assay is an indirect competitive test in which microcystins present in the sample compete with a microcystins-protein analogue for antibody binding sites. A label is added and the intensity of the color signal generated is inversely proportional to the concentration of microcystins in the sample. The assay's limit of detection is 0.10 µg/L.

*2.2.5 Phytoplankton biomass*

Chlorophyll *a* (chl *a*) is representative of total phytoplankton biomass. Individual subsamples were collected from replicate Cubitainers and filtered by vacuum filtration onto 25 mm GF/F filters (nominal pore size 0.7 µm), patted dry, folded, and frozen for fluorometric analysis. Extracted chl *a* was measured according to a modified version of the fluorometric method in EPA Method 445.0 (Arar and Collins, 1997). Briefly, filters were ground into a slurry in 10 mL 90% acetone, shaken, and frozen at -20 ºC overnight. Subsequently, samples were shaken and centrifuged for 10 minutes at 5800 rpm. The supernatant was poured into a syringe filter and filtered through a GF/F filter before measurement on a Turner Designs TD-700 Trilogy fluorometer.

*2.2.6 Phytoplankton community composition*

Pigment analysis allowed identification of major algal taxonomic groups and was performed by high-performance liquid chromatography (HPLC) on a Shimadzu HPLC (Model LC-20AB). In the natural assemblage bioassay, individual subsamples were collected from replicate Cubitainers. In the concentrated *Microcystis* bioassay, a single sample was collected per treatment by combining equal volumes from each replicate Cubitainer of the treatment. Water

was filtered through 47 mm GF/F filters (nominal pore size 0.7 μm), patted dry, folded and frozen until analysis. In preparation for analysis, filters were extracted in 100% acetone using a Sonics Vibra-Cell sonicator (Model VCX130) and frozen for 24 hours. Pigments were identified and quantified according to their absorption spectra compared to purified pigment standards (Danish Hydraulic Institute, Denmark). Contributions of the 4 dominant freshwater algal classes (chlorophytes, cryptophytes, cyanobacteria, and diatoms) to total chl *a* were determined using ChemTax (Mackey *et al*., 1996). A ChemTax pigment input matrix (Table 1.1) previously developed for a Lake Taihu study was used (Paerl *et al*., 2014). This matrix was adapted from a study of freshwater phytoplankton species (Schlüter *et al*., 2007).

Table 1.1 ChemTax input matrix. Crypto= cryptophytes, chloro= chlorophytes, cyano= cyanobacteria, fuco= fucoxanthin, neo= 9'cis neoxanthin, viol= violaxanthin, diad= diadinoxanthin, anth= antheraxanthin, myx= myxoxanthophyll, allo= alloxanthin, lut= lutein, zea= zeaxanthin, chl *b*= chlorophyll *b*, *β* Car= *β*-carotene, ech= echinenone (table adapted from Paerl *et al*., 2014).

| | **Fuco** | **Neo** | **Viol** | **Diad** | **Anth** | **Myx** | **Allo** | **Lut** | **Zea** | **Chl *b*** | ***β* Car** | **Ech** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Diatoms | 0.51 | 0 | 0 | 0.074 | 0 | 0 | 0 | 0 | 0 | 0 | 0.003 | 0 |
| Crypto | 0 | 0 | 0 | 0 | 0 | 0 | 0.37 | 0 | 0 | 0 | 0.004 | 0 |
| Chloro | 0 | 0.038 | 0.026 | 0 | 0.016 | 0 | 0 | 0.15 | 0 | 0.36 | 0.003 | 0 |
| Cyano | 0 | 0 | 0 | 0 | 0 | 0.14 | 0 | 0 | 0.28 | 0 | 0.097 | 0.76 |

*2.2.7 Statistical analyses*

One-way Analysis of Variance (ANOVA) tests were used to analyze the differences in growth responses between the untreated control and the various HOOH treatments. Statistically significant differences in mean values were analyzed by post-hoc comparison using Tukey's least significant difference procedure. The level of significance used was $p<0.05$. Statistical analysis was performed using the Matlab R2013a program.

## 3. Results and Discussion

### 3.1 Bioassay pH, dissolved oxygen, oxygen saturation, and temperature

pH, DO, $O_2$ %, and temperature were monitored daily as a broad indicator of phytoplankton growth in the bioassay. In the natural assemblage bioassay, pH varied from 9.26 to 10.71 (Table 1.2). In freshwater lakes, pH typically ranges from 6 to 9 in most lakes (Wetzel, 1983). Under conditions of high phytoplankton photosynthetic activity experienced in hypereutrophic lakes, pH is raised as dissolved carbon dioxide is depleted. For example, cyanobacteria-dominated Santa Olalla lake in Spain is a stable system with a biologically-produced alkaline pH. Santa Olalla's average pH is 9.52, and can reach as high as 10.5 (López-Archilla *et al*., 2004). This is similar to pH ranges seen in the natural assemblage bioassay used here. As a result of increasing phytoplankton biomass, pH increased throughout the bioassay, with all treatments showing a higher pH on the final day than the initial day of the bioassay. On the final day, pH was significantly lower in all HOOH treatments than in the untreated control (Figure 1.2).

Table 1.2 Natural assemblage bioassay daily record of pH, dissolved oxygen (DO), oxygen saturation ($O_2$ %), and temperature. Day 0 represents initial conditions in Cubitainers prior to first HOOH addition.

| Day / Time | HOOH Treatment (µM) | Cubitainer | pH | DO (mg/L) | $O_2$ % | Temperature |
|---|---|---|---|---|---|---|
| 0 13:30 | 0 | 1 | 9.36 | 15.35 | 190.8 | 26.3 |
| Initial | | 2 | 9.39 | 15.01 | 185.2 | 26.4 |
| | | 3 | 9.26 | 15.86 | 198.5 | 26.4 |
| 1 12:45 | 0 | 1 | 9.67 | 14.72 | 180.0 | 25.2 |
| | | 2 | 9.67 | 14.50 | 176.6 | 25.5 |
| | | 3 | 9.70 | 15.81 | 195.1 | 25.4 |
| | 20 | 4 | 9.53 | 13.58 | 165.8 | 25.3 |
| | | 5 | 9.65 | 15.02 | 185.6 | 25.5 |
| | | 6 | 9.59 | 13.79 | 169.1 | 25.5 |
| | 40 | 7 | 9.60 | 14.48 | 179.8 | 25.7 |
| | | 8 | 9.46 | 13.46 | 166.8 | 25.8 |
| | | 9 | 9.63 | 14.64 | 181.1 | 25.8 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 60 | 10 | 9.51 | 13.86 | 170.9 | 25.8 |
| | | | 11 | 9.57 | 13.99 | 172.4 | 25.5 |
| | | | 12 | 9.57 | 13.51 | 166.5 | 25.6 |
| | | 80 | 13 | 9.60 | 14.12 | 174.1 | 25.6 |
| | | | 14 | 9.61 | 14.01 | 173.4 | 25.7 |
| | | | 15 | 9.53 | 13.75 | 169.0 | 25.5 |
| 2 | 13:15 | 0 | 1 | 10.39 | 13.23 | 173.4 | 29.0 |
| | | | 2 | 10.40 | 13.06 | 171.0 | 29.2 |
| | | | 3 | 10.40 | 14.22 | 187.9 | 29.4 |
| | | 20 | 4 | 10.20 | 13.55 | 178.2 | 29.3 |
| | | | 5 | 10.24 | 13.78 | 182.4 | 29.0 |
| | | | 6 | 10.18 | 13.68 | 180.7 | 29.3 |
| | | 40 | 7 | 10.04 | 13.68 | 179.9 | 29.2 |
| | | | 8 | 10.01 | 13.76 | 181.4 | 29.3 |
| | | | 9 | 10.10 | 13.48 | 179.0 | 29.0 |
| | | 60 | 10 | 10.00 | 13.77 | 182.7 | 29.6 |
| | | | 11 | 10.04 | 14.21 | 188.4 | 29.5 |
| | | | 12 | 10.04 | 14.18 | 187.5 | 29.6 |
| | | 80 | 13 | 10.02 | 14.31 | 188.6 | 29.3 |
| | | | 14 | 10.04 | 13.96 | 184.6 | 29.3 |
| | | | 15 | 10.04 | 14.65 | 192.5 | 29.1 |
| 3 | 13:30 | 0 | 1 | 10.61 | 12.19 | 149.8 | 25.4 |
| | | | 2 | 10.61 | 11.67 | 143.3 | 25.5 |
| | | | 3 | 10.65 | 12.02 | 147.8 | 25.6 |
| | | 20 | 4 | 10.49 | 12.07 | 148.4 | 25.4 |
| | | | 5 | 10.55 | 12.61 | 155.0 | 25.6 |
| | | | 6 | 10.46 | 12.18 | 149.5 | 25.5 |
| | | 40 | 7 | 10.35 | 12.88 | 158.3 | 25.5 |
| | | | 8 | 10.36 | 12.30 | 151.2 | 25.5 |
| | | | 9 | 10.35 | 12.88 | 158.2 | 25.5 |
| | | 60 | 10 | 10.39 | 13.70 | 168.2 | 25.4 |
| | | | 11 | 10.37 | 13.75 | 168.7 | 25.5 |
| | | | 12 | 10.38 | 13.26 | 162.9 | 25.4 |
| | | 80 | 13 | 10.45 | 14.15 | 173.3 | 25.5 |
| | | | 14 | 10.46 | 13.70 | 168.1 | 25.4 |
| | | | 15 | 10.49 | 14.26 | 174.7 | 25.5 |
| 4 | 14:45 | 0 | 1 | 10.69 | 8.89 | 105.5 | 23.6 |
| | | | 2 | 10.68 | 8.67 | 102.5 | 23.7 |
| | | | 3 | 10.71 | 8.76 | 104.1 | 23.7 |
| | | 20 | 4 | 10.55 | 9.04 | 107.2 | 23.7 |
| | | | 5 | 10.62 | 9.21 | 109.8 | 23.8 |

| | | | | | |
|---|---|---|---|---|---|
| | 6 | 10.47 | 8.77 | 104.4 | 23.8 |
| 40 | 7 | 10.43 | 9.24 | 110.0 | 23.7 |
| | 8 | 10.45 | 9.51 | 112.9 | 23.7 |
| | 9 | 10.36 | 9.26 | 110.2 | 23.8 |
| 60 | 10 | 10.43 | 9.05 | 107.8 | 23.7 |
| | 11 | 10.38 | 9.06 | 108.1 | 23.8 |
| | 12 | 10.29 | 8.88 | 105.8 | 23.8 |
| 80 | 13 | 10.53 | 9.33 | 111.3 | 23.7 |
| | 14 | 10.51 | 9.26 | 110.4 | 23.9 |
| | 15 | 10.48 | 9.32 | 111.2 | 23.9 |

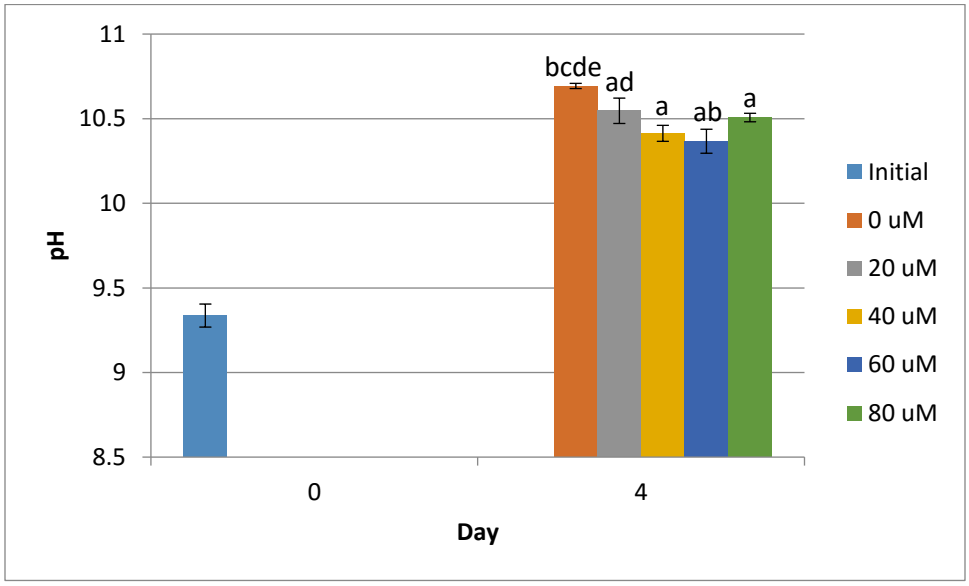


Figure 1.2 Natural assemblage bioassay pH in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

DO and $O_2$ % levels were very high throughout the bioassay (8.67-15.86 mg/L, corresponding to 102.5-198.5 $O_2$ %, respectively), indicating $O_2$ supersaturation as a result of photosynthetic $O_2$ production by the high levels of phytoplankton biomass in the natural assemblage bioassay. High $O_2$ levels are typical of surface blooms (Ibelings and Mur, 1992) and may contribute to the production of free radicals and oxidative stress when $O_2$ passively diffuses

into cells. In *Escherichia coli*, for example, $O_2$ diffuses into the cell and is reduced to $O_2^-$ and HOOH (Imlay, 2003; Giorgio *et al*., 2007). Bioassay temperatures ranged from 23.6 ºC to 29.6 ºC, within the range of expected temperatures during summer blooms in Meiliang Bay (Chen *et al*., 2003a,b). However, water temperature in the relatively low volume, high-surface-area-to-volume concrete enclosure in which the bioassays were conducted likely demonstrated higher temperatures and greater fluctuation than temperatures in Lake Taihu during the same time period. High temperatures promote bloom growth. Cyanobacteria have a competitive advantage over other phytoplankton groups at temperatures above 25 ºC because their growth rates are maximized at these high temperatures (Jöhnk *et al*., 2008).

The concentrated *Microcystis* bioassay demonstrated a much narrower pH range than that seen in natural assemblage bioassay, from 8.27 to 8.90 (Table 1.3). Although the pH in all treatments was higher on the final day of the bioassay than the initial day, there were no significant differences in pH amongst treatments (Figure 1.3). The lower pH range in this bioassay is likely due to the limited ability of the much lower biomass in this bioassay to raise pH. Similarly, the lower DO and $O_2$ % values in this bioassay (7.31-9.57 mg/L and 90.3-119.3%, respectively) indicate lower levels of $O_2$ production due to much lower phytoplankton biomass in this bioassay compared to the natural assemblage bioassay. The temperature varied from 25.2 ºC to 26.6 ºC. The limited range of temperatures measured in the concentrated *Microcystis* bioassay likely reflects lesser variation in air temperature and incident solar radiation (likely due to cloud cover) during this bioassay than during the natural assemblage bioassay.

Table 1.3 Concentrated *Microcystis* bioassay daily record of pH, dissolved oxygen (DO), oxygen saturation ($O_2$ %), and temperature. Day 0 represents initial conditions in Cubitainers prior to first HOOH addition.

| Day / Time | HOOH Treatment (µM) | Cubitainer | pH | DO (mg/L) | O2 % | Temperature |
|---|---|---|---|---|---|---|
| 0 9:00 | 0 | 1 | 8.50 | 8.42 | 104.7 | 26.6 |
| | | 2 | 8.39 | 8.65 | 108.0 | 26.3 |
| | | 3 | 8.50 | 8.64 | 107.9 | 26.5 |
| 1 9:00 | 0 | 1 | 8.39 | 8.47 | 105.2 | 26.3 |
| | | 2 | 8.44 | 8.27 | 102.8 | 26.3 |
| | | 3 | 8.40 | 8.22 | 102.2 | 26.6 |
| | 20 | 4 | 8.38 | 8.42 | 104.8 | 26.4 |
| | | 5 | 8.47 | 8.53 | 106.2 | 26.4 |
| | | 6 | 8.51 | 8.54 | 106.3 | 26.5 |
| | 40 | 7 | 8.47 | 8.64 | 107.6 | 26.5 |
| | | 8 | 8.50 | 8.62 | 107.5 | 26.5 |
| | | 9 | 8.53 | 8.65 | 107.6 | 26.5 |
| | 60 | 10 | 8.51 | 8.45 | 105.3 | 26.4 |
| | | 11 | 8.58 | 8.72 | 108.4 | 26.5 |
| | | 12 | 8.57 | 8.71 | 108.4 | 26.4 |
| | 80 | 13 | 8.52 | 8.70 | 108.4 | 26.4 |
| | | 14 | 8.55 | 8.84 | 110.2 | 26.4 |
| | | 15 | 8.50 | 8.78 | 109.4 | 26.5 |
| 2 9:00 | 0 | 1 | 8.40 | 8.27 | 101.4 | 25.4 |
| | | 2 | 8.41 | 8.09 | 99.2 | 25.4 |
| | | 3 | 8.27 | 7.97 | 97.4 | 25.3 |
| | 20 | 4 | 8.33 | 8.27 | 101.3 | 25.2 |
| | | 5 | 8.42 | 8.30 | 101.4 | 25.3 |
| | | 6 | 8.45 | 8.18 | 100.1 | 25.2 |
| | 40 | 7 | 8.45 | 8.48 | 103.7 | 25.3 |
| | | 8 | 8.44 | 8.38 | 102.5 | 25.2 |
| | | 9 | 8.44 | 8.33 | 101.8 | 25.3 |
| | 60 | 10 | 8.38 | 8.32 | 101.9 | 25.2 |
| | | 11 | 8.48 | 8.52 | 104.2 | 25.4 |
| | | 12 | 8.43 | 8.48 | 103.9 | 25.3 |
| | 80 | 13 | 8.37 | 8.54 | 104.4 | 25.3 |
| | | 14 | 8.39 | 8.45 | 103.3 | 25.2 |
| | | 15 | 8.42 | 8.62 | 105.4 | 25.2 |
| 3 12:45 | 0 | 1 | 8.74 | 8.67 | 106.3 | 25.2 |
| | | 2 | 8.63 | 8.30 | 101.6 | 25.3 |
| | | 3 | 8.33 | 7.31 | 90.3 | 25.5 |
| | 20 | 4 | 8.66 | 8.78 | 108.0 | 25.6 |

| | | | | | |
|---|---|---|---|---|---|
| | 5 | 8.86 | 9.41 | 116.7 | 25.7 |
| | 6 | 8.89 | 9.30 | 115.2 | 25.9 |
| 40 | 7 | 8.90 | 9.57 | 119.3 | 25.9 |
| | 8 | 8.87 | 9.10 | 113.2 | 26.2 |
| | 9 | 8.82 | 9.02 | 112.5 | 26.0 |
| 60 | 10 | 8.49 | 8.36 | 103.9 | 26.1 |
| | 11 | 8.67 | 8.76 | 109.3 | 26.1 |
| | 12 | 8.67 | 8.59 | 107.1 | 26.2 |
| 80 | 13 | 8.55 | 8.45 | 105.5 | 26.1 |
| | 14 | 8.53 | 8.33 | 103.7 | 26.2 |
| | 15 | 8.67 | 8.70 | 108.8 | 26.3 |

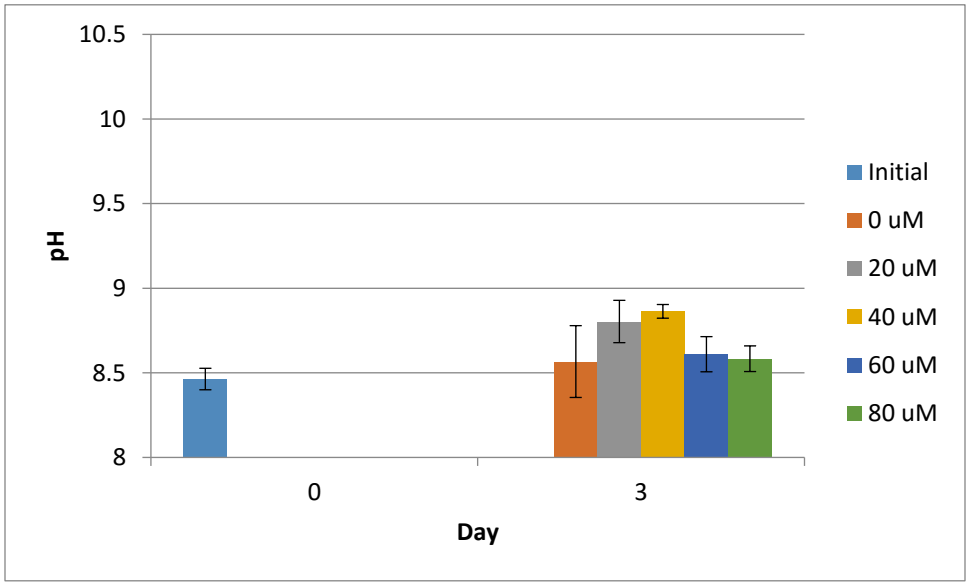


Figure 1.3 Concentrated *Microcystis* bioassay pH in HOOH treatments on initial and final days of the bioassay. One-way ANOVA ($p<0.05$) indicated no statistically significant differences between mean values. Error bars represent one standard deviation (n=3).

### 3.2 Nutrients

$PO_4^{3-}$-P, $NH_4^+$-N, $NO_3^-$-N, and $NO_2^-$-N measurements were taken on the initial and final days of the natural assemblage bioassay (Table 1.4) and the concentrated *Microcystis* bioassay (Table 1.5). In the natural assemblage bioassay, initial $PO_4^{3-}$-P, $NH_4^+$-N, $NO_3^-$-N, and $NO_2^-$-N concentrations (prior to first nutrient addition) were 0.06, 61.08, 32.60, and 15.01 µM, respectively. On the final day of the bioassay, $PO_4^{3-}$-P concentrations ranged from 0.09 to 0.11

μM, $NH_4^+$-N from 1.07 to 2.14 μM, $NO_3^-$-N from 26.52 to 68.32 μM, and $NO_2^-$-N from 8.10 to 14.01 μM.

Table 1.4 Natural assemblage bioassay nutrient measurements from initial and final days of the bioassay. Nutrients measured include phosphate ($PO_4^{3-}$-P), ammonium ($NH_4^+$-N), nitrate ($NO_3^-$N), and nitrite ($NO_2^-$-N). For nutrient analysis, a single sample was collected per treatment by combining equal volumes from each replicate Cubitainer of the treatment.

| Day | Time | HOOH Treatment (μM) | Cubitainer | $PO_4^{3-}$-P (μM) | $NH_4^+$-N (μM) | $NO_3^-$-N (μM) | $NO_2^-$-N (μM) |
|---|---|---|---|---|---|---|---|
| 0 | 16:30 | 0 | 1+2+3 | 0.06 | 61.08 | 32.60 | 15.01 |
| 4 | 15:50 | 0 | 1+2+3 | 0.10 | 1.36 | 26.52 | 8.10 |
| | | 20 | 4+5+6 | 0.09 | 1.07 | 41.75 | 10.38 |
| | | 40 | 7+8+9 | 0.11 | 1.71 | 54.73 | 12.67 |
| | | 60 | 10+11+12 | 0.11 | 2.14 | 60.57 | 13.22 |
| | | 80 | 13+14+15 | 0.09 | 1.14 | 68.32 | 14.01 |

Table 1.5 Concentrated *Microcystis* bioassay nutrient measurements from initial and final days of the bioassay. Nutrients measured include phosphate ($PO_4^{3-}$-P), ammonium ($NH_4^+$-N), nitrate ($NO_3^-$-N), and nitrite ($NO_2^-$-N). For nutrient analysis, a single sample was collected per treatment by combining equal volumes from each replicate Cubitainer of the treatment.

| Day | Time | HOOH Treatment (μM) | Cubitainer | $PO_4^{3-}$-P (μM) | $NH_4^+$-N (μM) | $NO_3^-$-N (μM) | $NO_2^-$-N (μM) |
|---|---|---|---|---|---|---|---|
| 0 | 10:30 | 0 | 1+2+3 | 0.06 | 2.89 | 75.54 | 2.70 |
| 3 | 1:00 | 0 | 1+2+3 | 0.10 | 6.14 | 61.63 | 2.78 |
| | | 20 | 4+5+6 | 0.07 | 1.64 | 57.68 | 2.60 |
| | | 40 | 7+8+9 | 0.10 | 1.43 | 43.72 | 3.36 |
| | | 60 | 10+11+12 | 0.12 | 1.64 | 40.74 | 2.85 |
| | | 80 | 13+14+15 | 0.12 | 1.93 | 41.90 | 2.29 |

In the concentrated *Microcystis* bioassay, initial $PO_4^{3-}$-P, $NH_4^+$-N, $NO_3^-$-N, and $NO_2^-$-N concentrations (prior to first nutrient addition) were 0.06, 2.89, 75.54, and 2.70 μM, respectively. On the final day of the bioassay, $PO_4^{3-}$-P concentrations ranged from 0.07 to 0.12 μM, $NH_4^+$-N from 1.43 to 6.14 μM, $NO_3^-$-N from 40.74 to 61.63 μM, and $NO_2^-$-N from 2.29 to 3.36 μM. Although $PO_4^{3-}$ was added to each bioassay (in the form of $KH_2PO_4$) to prevent nutrient

limitation, both bioassays were P-limited by the final day. The P concentration required for saturated growth yield in *Microcystis aeruginosa* is 7.10 μM in culture (Baldia *et al*., 2007). In Taihu, growth of *Microcystis* spp. is P-limited at concentrations < 6.46 μM (Xu *et al*., 2010). P concentrations on the final days of these bioassays were most similar to Lake Taihu P concentrations during the spring, when P-limitation may occur (Xu *et al*., 2010). P limitation provides those phytoplankton that most effectively compete for available P with a competitive advantage. In general, cyanobacteria, diatoms, and cryptophytes are good competitors when P is limiting, unlike green algae (Holm and Armstrong, 1981; Grover, 1989; Baldia *et al*., 2007). Green algae have a high demand for nutrients due to their high growth and loss rates (Reynolds, 1988). Thus, P limitation during these bioassays may have reduced the growth of green algae relative to the other major phytoplankton groups in all HOOH treatments as well as the untreated control.

### 3.3 Particulate organic carbon and nitrogen

POC was analyzed in the natural assemblage bioassay. POC includes C from phytoplankton, zooplankton, bacteria, and detritus. POC increased from an initial value of 16.0 mgC/L to final values ranging from 19.5 to 25.9 mgC/L (Figure 1.4). On the final day, there was a significant increase in POC concentrations at higher HOOH concentrations, indicative of the increasing phytoplankton biomass at high HOOH concentrations. Similar trends in POC and chl *a* data suggest that POC was primarily composed of phytoplankton C. PON values were not obtained for the natural assemblage bioassay due to instrument error during analysis.

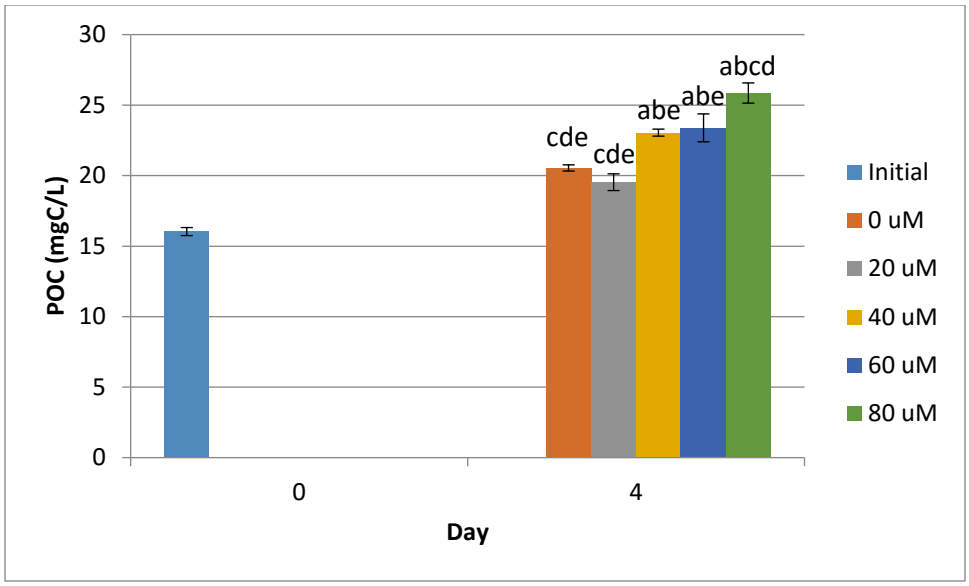


Figure 1.4 Natural assemblage bioassay POC concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

In the concentrated *Microcystis* bioassay, initial POC was 1.62 mgC/L and final POC values varied from 1.69 to 2.43 mgC/L, lower than those in the natural assemblage bioassay, again due to the much lower phytoplankton biomass (Figure 1.5). POC increased in all treatments throughout the bioassay, with highest values in the 20 and 40 µM HOOH treatments, but the data could not be tested for statistical significance, since a single sample was taken per treatment due to volume constraints.

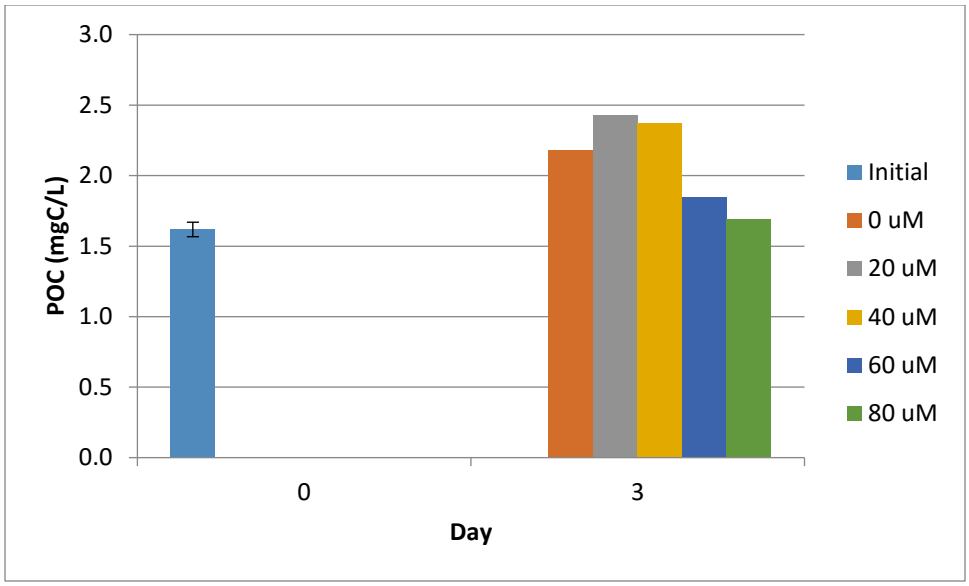


Figure 1.5 Concentrated *Microcystis* bioassay POC concentrations in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

Unlike POC, PON concentrations decreased from initial values during the concentrated *Microcystis* bioassay (Figure 1.6). Initial PON was 0.39 mgN/L, and final values were 0.27 to 0.38 mgN/L. Final PON was highest in the untreated control and decreased with increasing HOOH concentration. The data could not be tested for statistical significance, since a single sample was collected per treatment due to volume constraints. The resulting POC:PON molar ratio was 4.85 initially and varied from 6.74 to 8.96 on the final day of the bioassay (Figure 1.7). Initial values were below the Redfield molar POC:PON ratio of 6.7 (Redfield, 1958) while final values were above the Redfield ratio. This minor deviation might be caused by physical and chemical factors that affect the elemental composition of phytoplankton, such as light and temperature (Geider and La Roche, 2002). Changes in the POC:PON ratio may also result from changes in the relative abundance of the major phytoplankton groups present (cyanobacteria, diatoms, cryptophytes, chlorophytes). POC:PON ratios vary amongst phytoplankton groups

whose different growth strategies result in different ratios of C to N drawdown (Arrigo *et al*., 1999; Klausmeier *et al*., 2004; Martiny *et al*., 2013; Singh *et al*., 2015).

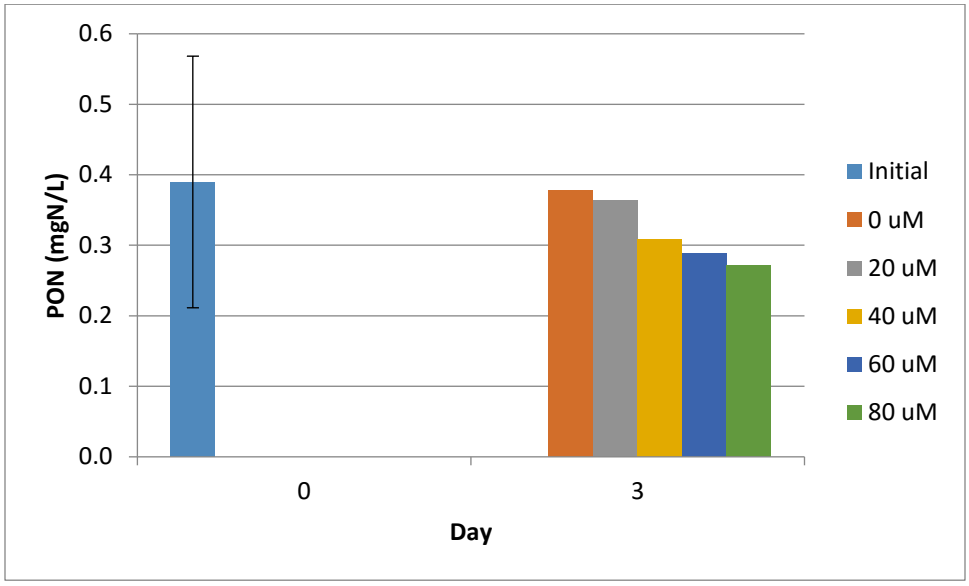


Figure 1.6 Concentrated *Microcystis* bioassay PON concentrations in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

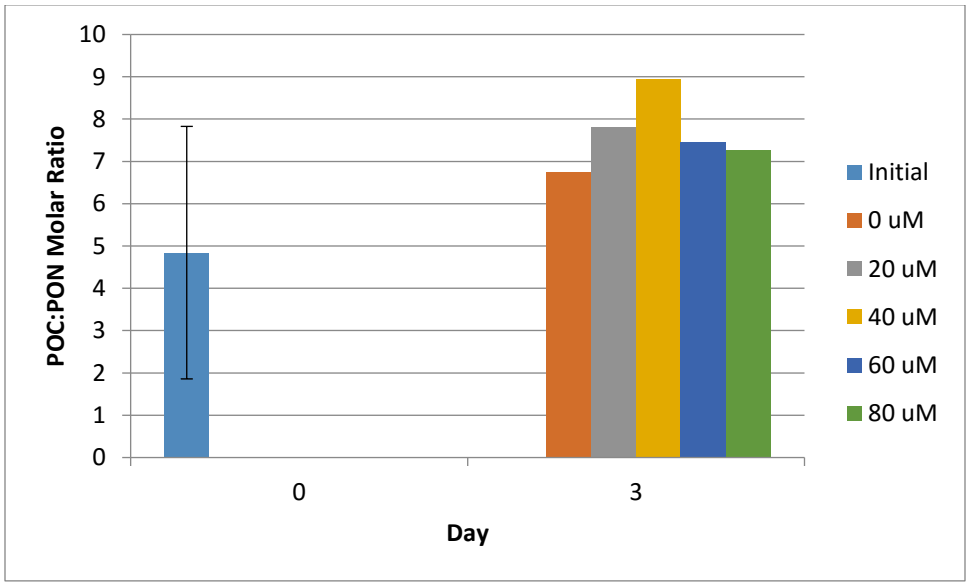


Figure 1.7 Concentrated *Microcystis* bioassay POC:PON molar ratio in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

### 3.4 Total microcystin concentrations

In the natural assemblage bioassay, the initial concentration of total microcystins (intracellular + extracellular) was 26.6 µg/L (Figure 1.8). This high concentration of microcystins is well above the WHO drinking water provisional guideline of 1 µg/L and recreational use guideline of 10 µg/L, but within the expected range found in Meiliang Bay. In the summers of 2009 and 2010, the average microcystin concentration in northwestern Lake Taihu was 28.7 µg/L (Otten *et al*., 2012). A high microcystin concentration is first and foremost a result of the high *Microcystis* biomass during a bloom. However, during a bloom, the extent of microcystin production is controlled by a wide variety of physical and chemical factors, including nutrient availability (N and P; trace metals), temperature, light, and pH (Neilan *et al*, 2013). Microcystin concentration increased from 26.6 µg/L to 41.5 µg/L by the final day, indicating microcystin production during the bioassay.

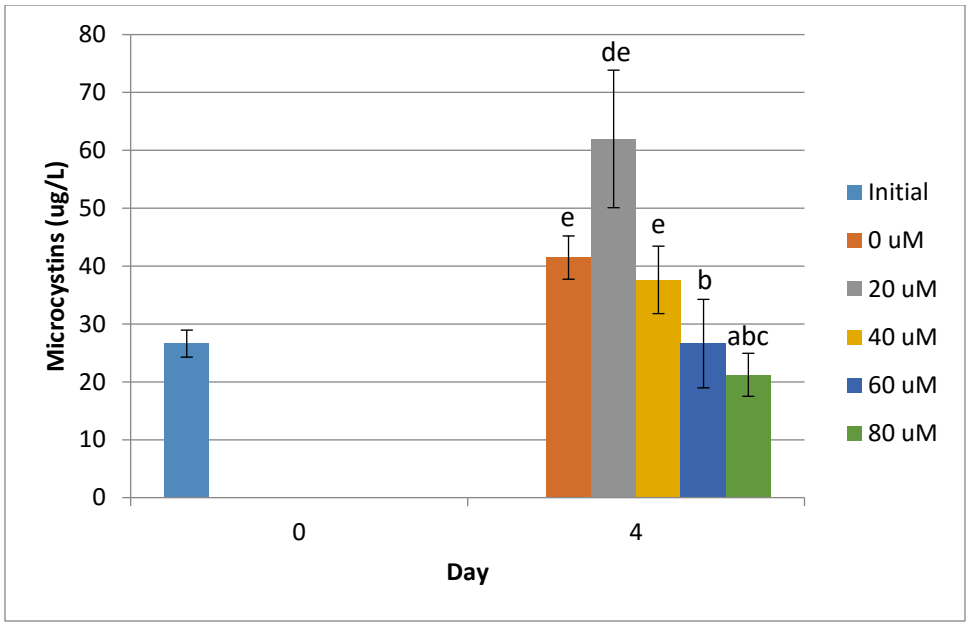


Figure 1.8 Natural assemblage bioassay total (intracellular + extracellular) microcystins concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

Microcystins were measured in the HOOH treatments as well. Microcystin concentrations were significantly lower in the 80 µM than in the untreated control. However, the measured microcystin concentrations are likely deceptive in terms of microcystin production. HOOH is a strong oxidant, which enhances photocatalytic degradation of microcystins (Cornish *et al*., 2000). Thus, the amount of microcystin produced in the HOOH treatments may be much higher than the quantity measured. Interestingly, the microcystin concentration was higher in the 20 µM HOOH treatment (62.0 µg/L) than in the untreated control (41.5 µg/L), despite the likelihood of enhanced degradation by HOOH. Since phytoplankton community composition analysis showed that cyanobacterial biomass decreased in the 20 µM treatment relative to the untreated control, these results indicate HOOH addition may stimulate increased microcystin production, supporting the hypothesis that microcystin plays a role in protection against

oxidative stress. This effect may only be detectable under relatively low HOOH concentrations at which HOOH-enhanced degradation does not fully mask enhanced microcystin production. However, no definitive conclusion regarding the effect of HOOH on microcystin production can be drawn from these results. Enhanced degradation of microcystins at high HOOH concentrations may have been minimal, with low microcystin concentrations instead resulting from lower cyanobacterial biomass and reduced rates of production.

The initial microcystin concentration in the concentrated *Microcystis* bioassay (2.2 µg/L; Figure 1.9) was much lower than in the natural assemblage bioassay. The low initial concentration highlights the fact that microcystins are primarily retained within the cell until cell lysis. The filtration procedures used to remove phytoplankton from the bioassay water before addition of *Microcystis* surface scum did not remove extracellular microcystins from the water. Thus, the low initial microcystin concentrations are a result of the lower biomass in the concentrated *Microcystis* bioassay. The final microcystin concentration in the 20 µM HOOH treatment (6.9 µg/L) is significantly higher than the untreated control (2.0 µg/L). Concentrations in all other HOOH treatments were not significantly different than control values. Together, these two bioassays indicate that HOOH addition may result in either significantly increased or decreased microcystin concentrations. Relatively low levels of HOOH (20 µM) may result in increased microcystin concentrations while relatively high levels (80 µM) may result in decreased microcystin concentrations.

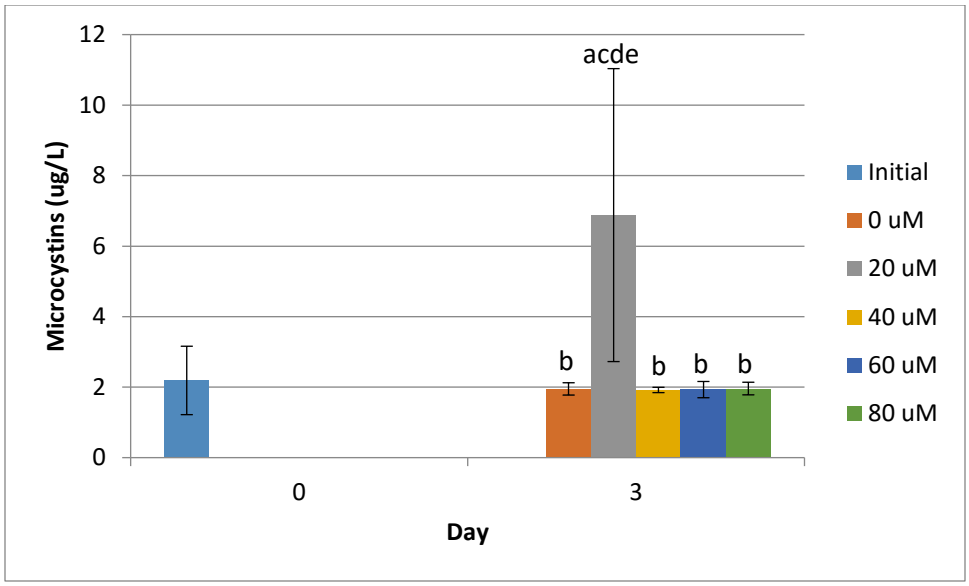


Figure 1.9 Concentrated *Microcystis* bioassay total (intracellular + extracellular) microcystins concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

### 3.5 Phytoplankton biomass

Chlorophyll *a* is representative of phytoplankton biomass. For each bioassay, chl *a* was measured fluorometrically and through HPLC. In the natural assemblage bioassay, initial fluorometric chl *a* was very high, at 274.9 µg/L (Figure 1.10). Although high, these chlorophyll concentrations are not unheard of in Meiliang Bay. In August of 2004, chl *a* concentrations reached well above 400 µg/L (Liu *et al*., 2011). Nevertheless, typical chl *a* concentrations are lower. The average whole water column, depth integrated chl *a* value in northwestern Lake Taihu during the summers of 2009 and 2010 was 192.1 ± 359.5 µg/L (Otten *et al*., 2012). A long-term study found that the average annual chl *a* concentrations in Lake Taihu from 1995 to 2003 ranged from 17.4 to 54.2 µg/L (Zhang *et al*., 2007). Annual averages are consistently lower than summer chl *a* concentrations because blooms are seasonal, with maximal chl *a* concentrations occurring in the summer and minimal concentrations occurring in the winter.

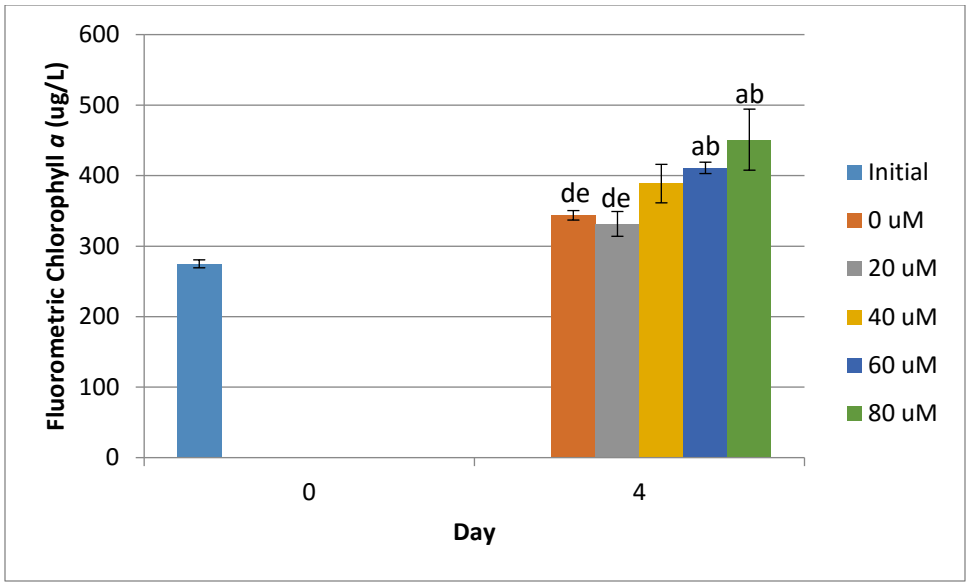


Figure 1.10 Natural assemblage bioassay fluorometric chlorophyll *a* concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

Chl *a* increased in the untreated control and all HOOH treatments during the bioassay. The final chl *a* concentration in the control was 343.7 µg/L. The final chl *a* concentrations in the HOOH treatments ranged from 331.5 to 451.0 µg/L, increasing with increasing HOOH concentration. Cyanobacterial chl *a* is expected to decrease upon HOOH addition (Samuilov *et al*., 1999; Dziallas and Grossart, 2011). However, since HOOH selectively inhibits cyanobacterial growth at lower concentrations than other phytoplankton groups (Stratford *et al*., 1984; Barroin and Feuillade, 1986; Schrader *et al*., 1998; Drábková *et al*., 2007a,b; Weenik *et al*., 2015), the increase in chl *a* may occur as the inhibition of cyanobacteria reduces competition and allows for enhanced growth of other phytoplankton groups. The effects of HOOH on phytoplankton community composition are discussed in detail in the following section.

Initial fluorometric chl *a* concentration in the concentrated *Microcystis* bioassay was much lower than in the natural assemblage bioassay, at 14.6 µg/L (Figure 1.11). The chl *a*

concentration was artificially lowered through the filtering procedures used to remove all phytoplankton before the *Microcystis* surface scum was re-added. Chl *a* concentrations increased in the untreated control and in all HOOH treatments by the final day of the bioassay. The untreated control had a final concentration of 33.1 µg/L. The 20 µM HOOH treatment had the highest chl *a* concentration, at 43.0 µg/L. Chl *a* decreased with increasing HOOH concentration, with the lowest concentration in the 80 µM treatment: 19.8 µg/L. However, there were no significant differences between mean values of treatments. The effects of HOOH on phytoplankton biomass differed amongst bioassays, likely resulting from the altered phytoplankton community composition in the concentrated *Microcystis* bioassay.

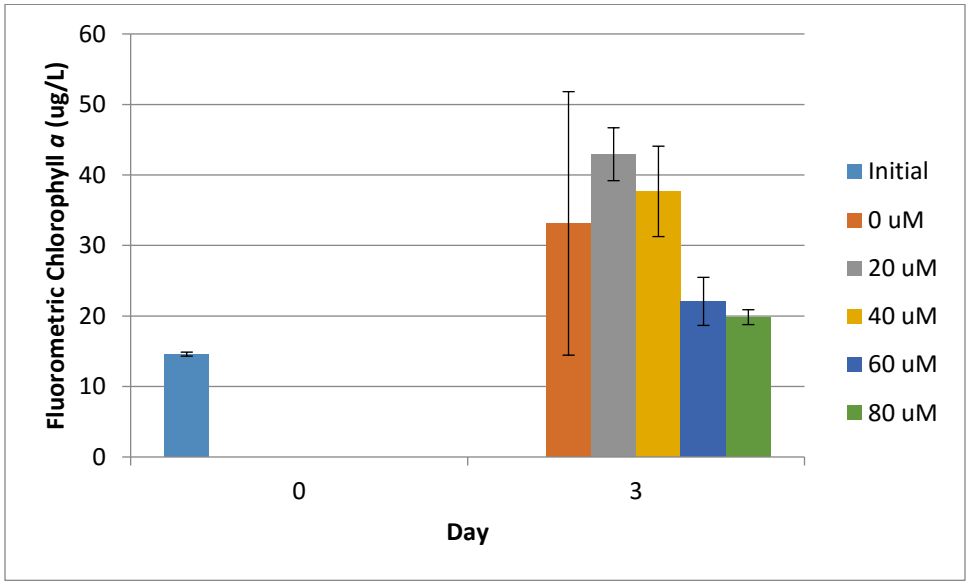


Figure 1.11 Concentrated *Microcystis* bioassay fluorometric chlorophyll *a* concentrations in HOOH treatments on initial and final days of the bioassay. One-way ANOVA ($p<0.05$) indicated no statistically significant differences between mean values. Error bars represent one standard deviation (n=3).

### 3.6 Phytoplankton community composition

#### *3.6.1 Total chlorophyll* a

In addition to fluorometric measurement, chl *a* was measured by HPLC. In the natural assemblage bioassay, initial HPLC chl *a* was measured as 211.5 µg/L (Figure 1.12). Final chl *a*

concentrations ranged from 246.5 to 280.4 µg/L, with no significant differences among treatments. Chl *a* values measured by HPLC were consistently lower than when measured by fluorometry, despite inclusion of degradation pigment chlorophyllide *a* in total chl *a* concentrations. This occurs because fluorometry does not distinguish between different chlorophyll species or derivatives (Meyns *et al*., 1994). Upon removal of chl *a* (by separation of pigments on the HPLC column and separate collection of chl *a* and all remaining pigments), a sample will still measure significant concentrations of "chl *a*" by the fluorometric method. This occurs when other compounds with fluorescent properties similar to chl *a* are present (Gieskes and Kraay, 1983).

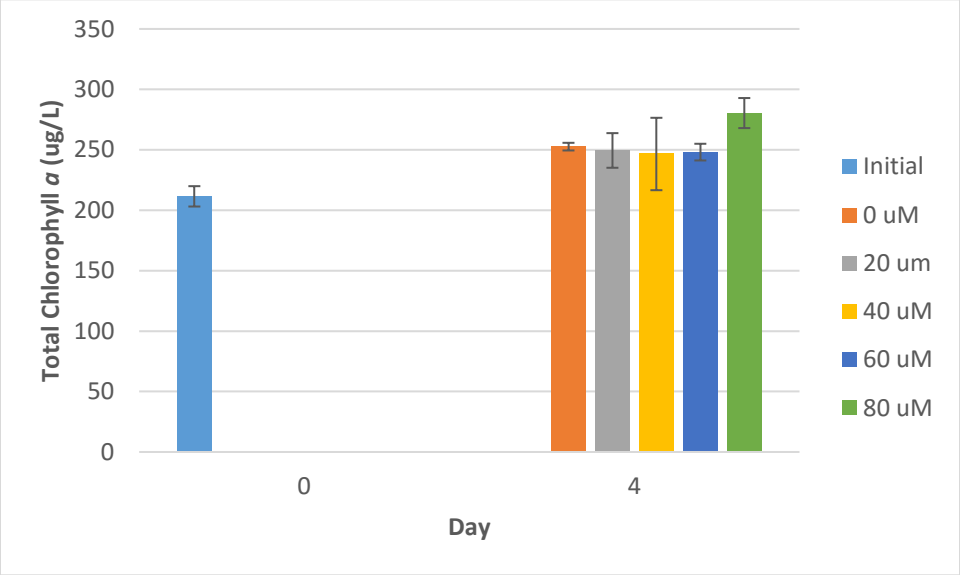


Figure 1.12 Natural assemblage bioassay HPLC total chlorophyll *a* (chlorophyll *a* + degradation pigment chlorophyllide *a*) concentrations in HOOH treatments on initial and final days of the bioassay. One-way ANOVA ($p<0.05$) indicated no statistically significant differences between mean values. Error bars represent one standard deviation (n=3).

The same trend was seen in the concentrated *Microcystis* bioassay HPLC chl *a* measurements, with all values lower than those obtained by the fluorometric method. Initial chl *a* was 9.8 µg/L (Figure 1.13). Final chl *a* concentrations ranged from 13.2 to 36.6 µg/L. All HPLC data from the concentrated *Microcystis* bioassay could not be tested for statistical significance

because only a single sample was collected per treatment due to volume constraints. However, the HPLC chl *a* measurements indicate the same relationship between chl *a* and HOOH concentration exhibited in the fluorometric chl *a* results.

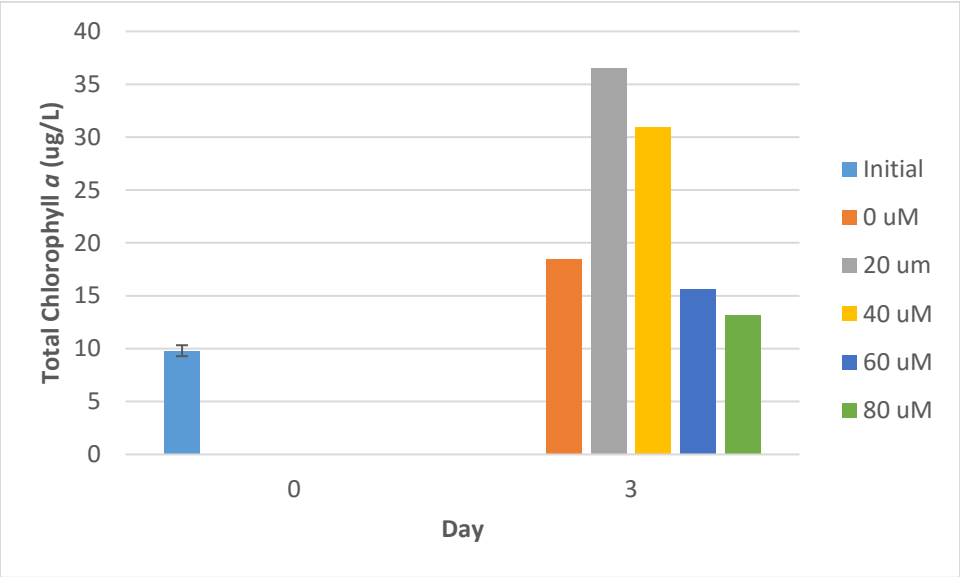


Figure 1.13 Concentrated *Microcystis* bioassay HPLC total chlorophyll *a* (chlorophyll *a* + degradation pigment chlorophyllide *a*) concentrations in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

*3.6.2 Zeaxanthin*

Zeaxanthin is a photoprotective carotenoid found in cyanobacterial cytoplasmic membranes (MacIntyre *et al*., 2002). Zeaxanthin concentrations serve as a proxy for cyanobacterial biomass. In the natural assemblage bioassay, initial zeaxanthin concentration was 2.9 µg/L (Figure 1.14). On the final day of the bioassay, zeaxanthin concentrations were highest in the untreated control, at 5.2 µg/L, and decreased with increasing HOOH concentration. Zeaxanthin concentrations in the 40, 60, and 80 µM HOOH treatments fell below the initial value. The lowest concentration, in the 80 µM HOOH treatment, was 2.0 µg/L. It has been demonstrated previously that HOOH inhibits cyanobacterial growth (Samuilov *et al*., 1999;

Dziallas and Grossart, 2011). In a *Microcystis aeruginosa* culture ($5\times10^5$ cells/cm$^3$), 50% inhibition of photosynthetic yield ($F_V/F_M$) occurs at 7.9 µM HOOH. In other cyanobacteria including *Trichormus variabilis*, *Cyanobium gracile*, *Synechococcus nidulans*, and *Aphanothece clathrata*, 50% inhibition occurs between 10.3 and 51.2 µM HOOH (Drábková *et al*., 2007b).

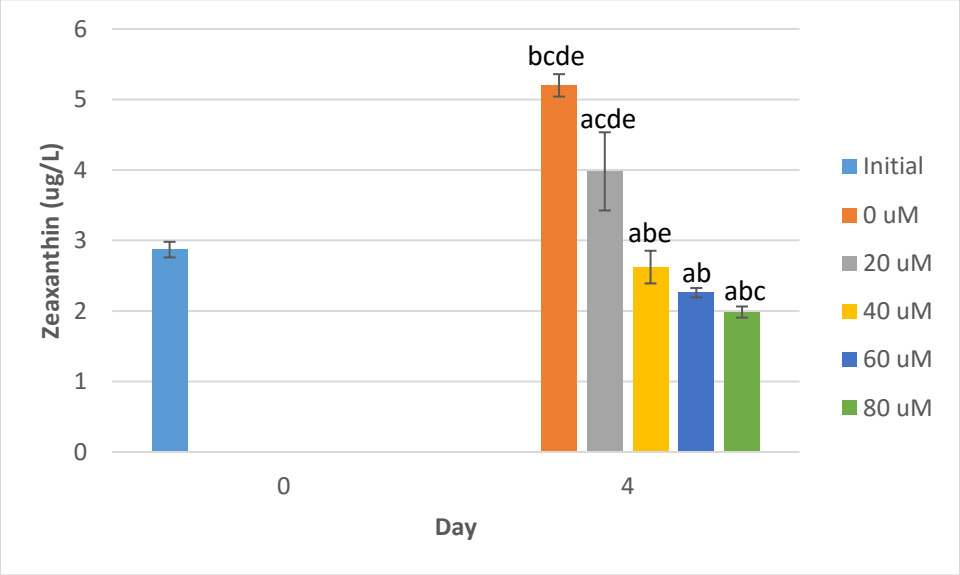


Figure 1.14 Natural assemblage bioassay HPLC zeaxanthin concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

In the concentrated *Microcystis* bioassay, the initial zeaxanthin concentration was 0.5 µg/L, and decreased in the untreated control and all HOOH treatments by the final day of the bioassay (Figure 1.15). The highest concentration occurred in the 40 µM HOOH treatment, 0.3 µg/L, and the lowest concentration occurred in the 80 µM treatment, 0.1 µg/L. Interestingly, zeaxanthin concentrations were higher in the 20 µM and 40 µM treatments than in the untreated control. The differences in response of cyanobacterial biomass to HOOH addition between the two bioassays illustrate the importance of determining the correct dosage for each bloom episode when using HOOH to suppress cyanobacterial growth. In the natural assemblage bioassay, 20

µM HOOH was sufficient to suppress cyanobacterial growth below control values. In the concentrated *Microcystis* bioassay, growth was not suppressed at 20 µM and 40 µM HOOH; 60 µM HOOH was required for suppression.

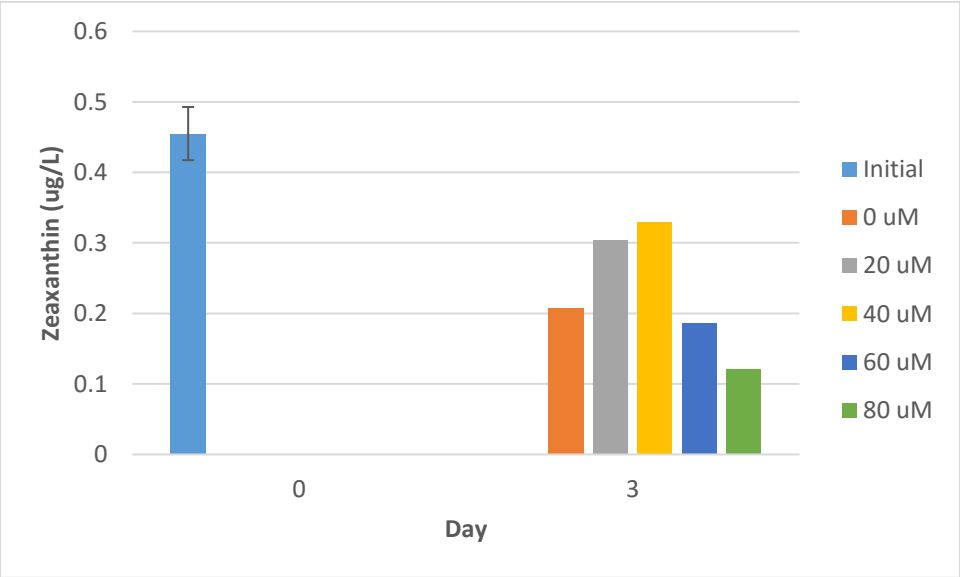


Figure 1.15 Concentrated *Microcystis* bioassay HPLC zeaxanthin concentrations in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

The minimum concentration of HOOH necessary to effectively reduce cyanobacterial biomass varies according to multiple factors, chief among them being phytoplankton biomass and community composition. Previous studies show that the necessary HOOH dosage varies considerably amongst blooms (Matthijs *et al*., 2012; Barrington *et al*., 2013). Higher biomass blooms necessitate higher HOOH concentrations due to enhanced biological degradation (Randhawa *et al*., 2013). Biological degradation may be especially rapid in blooms containing high eukaryotic phytoplankton biomass, since eukaryotic phytoplankton degrade ROS more effectively than cyanobacteria (Asada 1992; Shigeoka *et al*., 2002; Passardi *et al*., 2007). However, eukaryotic phytoplankton also produce greater concentrations of ROS (Weenik *et al*., 2015). Interestingly, despite higher total phytoplankton biomass and a higher proportion of

eukaryotic phytoplankton, cyanobacterial biomass was more dramatically inhibited by HOOH in the natural assemblage bioassay, possibly due to differences in the cyanobacterial assemblages of the two bioassays. Laboratory studies indicate that some cyanobacterial genera are more sensitive to HOOH than others. The $EC_{50}$ value for HOOH for *Microcystis aeruginosa* is 8 μM whereas the $EC_{50}$ value for another freshwater cyanobacterium, *Aphanothece clathrata*, is 51 μM. The $EC_{50}$ values of two other cyanobacterial species, *Cyanobium gracile* and *Trichormus variabilis*, are 34 and 10 μM, respectively (Drábková *et al*., 2007b). Although summer cyanobacterial assemblages in Lake Taihu are typically dominated by *Microcystis* (Chen *et al*., 2003b), other cyanobacterial species are undoubtedly present in varying proportions. Cyanobacterial biomass may have been more dramatically inhibited by HOOH in the natural assemblage bioassay because the cyanobacterial community contained a higher proportion of highly HOOH-sensitive cyanobacteria. Additionally, sensitivity of *Microcystis aeruginosa* to HOOH differs between toxic and non-toxic strains. Culture experiments have demonstrated a significantly higher reduction in *Microcystis* biomass upon HOOH addition in non-toxic strains as opposed to toxic strains (Dziallas and Grossart, 2011), possibly because cyanobacterial toxins protect against oxidative stress (Zilliges *et al*., 2011). In a separate study, non-toxic *Microcystis* was affected by 50 nM HOOH while 500 nM HOOH was required to affect toxic *Microcystis* (Leunert *et al*., 2014).

Other factors affecting the minimum dosage of HOOH required for cyanobacterial removal such as ultraviolet radiation (UVR) and PAR may have also played a role. UVR and PAR serve as catalysts to produce hydroxyl and hydroperoxyl radicals ($HO_2$) from HOOH, increasing the rate of cyanobacterial cell death (Samuilov *et al*., 2004). In wastewater stabilization ponds (WSPs), high levels of UVR have been shown to increase the cyanobacterial

removal efficiency of HOOH by an order of magnitude over laboratory conditions (Barrington *et al*., 2011).

*3.6.3 Fucoxanthin*

Fucoxanthin is a carotenoid found in approximately 100,000 fresh water and marine diatom species (Werner, 1977). Relative changes in diatom biomass can be estimated from fucoxanthin concentrations. In the natural assemblage bioassay, initial fucoxanthin concentration was 8.8 µg/L (Figure 1.16). Fucoxanthin increased in all HOOH treatments by the final day, ranging from 20.2 to 36.7 µg/L. Fucoxanthin concentrations were highest in highest HOOH concentration treatments. Although HOOH can inhibit diatom growth, inhibition of diatoms occurs at higher concentrations than inhibition of other phytoplankton groups, including cyanobacteria and chlorophytes (green algae). In a culture of the diatom *Navicula seminulum* ($2\times10^5$ cells/$cm^3$), 50% inhibition of photosynthetic yield occurs at 294.0 µM HOOH (Drábková *et al*., 2007b). During the bioassay, the selective suppression of other phytoplankton groups by HOOH allowed for enhanced diatom growth.

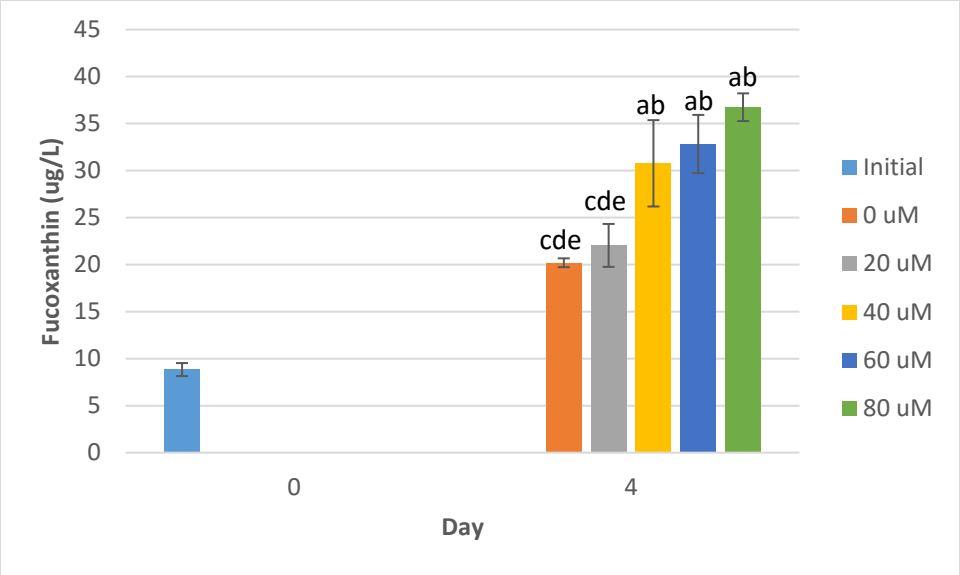


Figure 1.16 Natural assemblage bioassay HPLC fucoxanthin concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

The initial fucoxanthin concentration was very low in the concentrated *Microcystis* bioassay (0.1 µg/L; Figure 1.17), indicating successful removal of diatom biomass through filtration. Fucoxanthin concentrations increased in the untreated control and in all treatments. On the final day of the bioassay, the concentration was highest in the 20 µM HOOH treatment (11.6 µg/L) and lowest in the 60 and 80 µM treatments (1.7 and 1.9 µg/L, respectively). Unlike the natural assemblage bioassay, diatom growth was inhibited at high HOOH concentrations in the concentrated *Microcystis* bioassay.

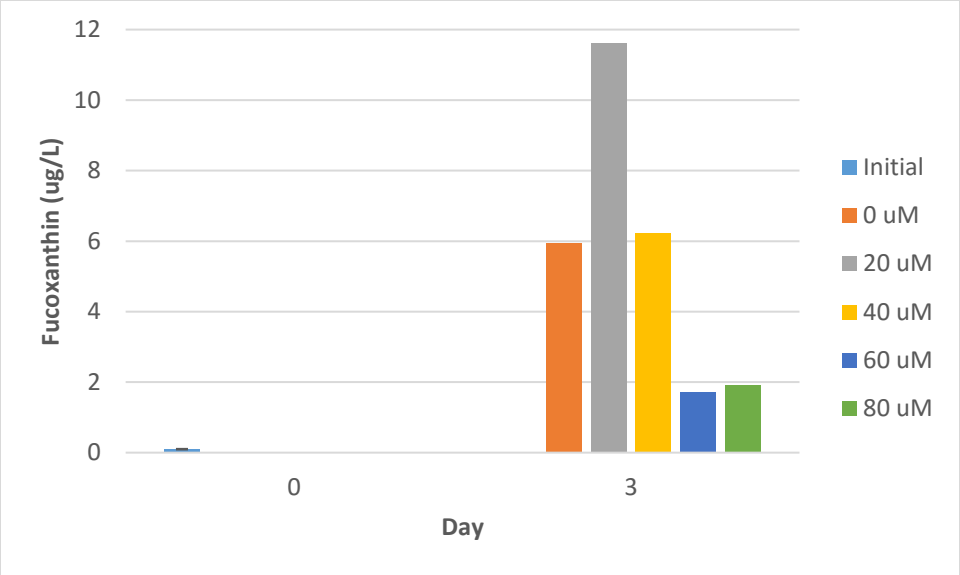


Figure 1.17 Concentrated *Microcystis* bioassay HPLC fucoxanthin concentrations in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

*3.6.4 Alloxanthin*

Alloxanthin is a carotenoid primarily found in cryptophytes. The initial alloxanthin concentration in the natural assemblage bioassay was 2.4 µg/L (Figure 1.18). Alloxanthin concentration increased in the untreated control and all treatments by the final day of the bioassay. The concentration was lowest in the untreated control and significantly higher in the 60 and 80 µM HOOH treatments (3.7 and 3.6 µg/L, respectively). The sensitivity of cryptophytes to HOOH has not been studied as thoroughly as cyanobacteria, diatoms, and green algae. Eukaryotes such as cryptophytes, diatoms, and green algae are generally less sensitive than prokaryotes such as cyanobacteria (Drábková *et al*., 2007b). In a whole lake HOOH addition study, cryptophyte biomass was unaffected by a 60 µM HOOH addition (Matthijs *et al*., 2012).

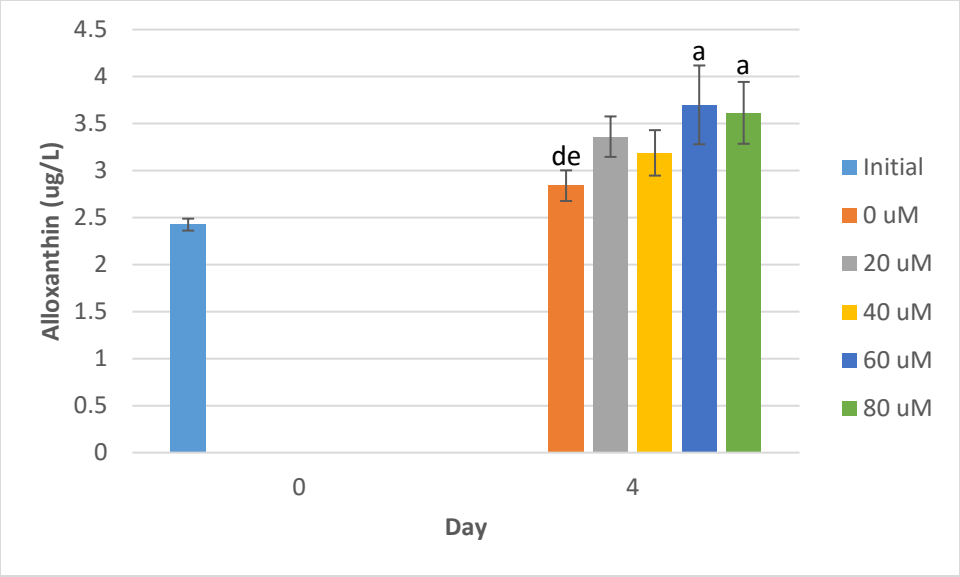


Figure 1.18 Natural assemblage bioassay HPLC alloxanthin concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

In the concentrated *Microcystis* bioassay, the initial alloxanthin concentration was 0.08 µg/L (Figure 1.19). In the untreated control, cryptophyte biomass remained stable throughout the bioassay, with a final alloxanthin concentration of 0.07 µg/L. Alloxanthin concentrations increased in the 20 and 40 µM HOOH treatments (0.14 and 0.15 µg/L, respectively) and decreased in the 60 and 80 µM treatments (0.03 µg/L in both).

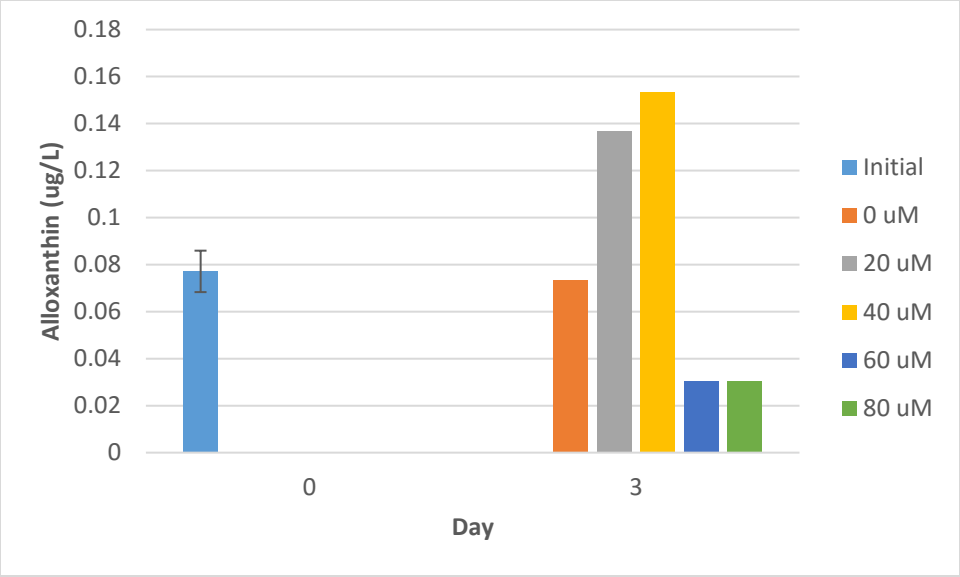


Figure 1.19 Concentrated *Microcystis* bioassay HPLC alloxanthin concentrations in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

*3.6.5 Chlorophyll* b

While chl *a* absorbs wavelengths of violet-blue and orange-red light, chlorophyll *b* (chl *b*) absorbs blue light. Among freshwater phytoplankton, it is primarily found in chlorophytes. In the natural assemblage bioassay, the initial chl *b* concentration was high: 72.2 µg/L (Figure 1.20). By the end of the bioassay, chl *b* had decreased in the untreated control and in all treatments except the 80 µM HOOH treatment. Chl *b* was lowest in the untreated control (37.5 µg/L) and increased with increasing HOOH concentration to 75.0 µg/L in the 80 µM HOOH treatment. In green algae cultures (*Pseudokirchneriella subcapitata*, *Scenedesmus quadricauda*, and *Chlamydomonas reinhardtii*; cell density $2\times10^5$ cells/cm$^3$), 50% inhibition of photosynthetic yield occurred at 182.9, 271.9, and 618.6 µM HOOH, respectively (Drábková *et al*., 2007b). HOOH concentrations in the bioassay were too low to significantly reduce growth of green algae. At high HOOH concentrations, selective suppression of other algal groups allowed for enhanced chlorophyte growth.

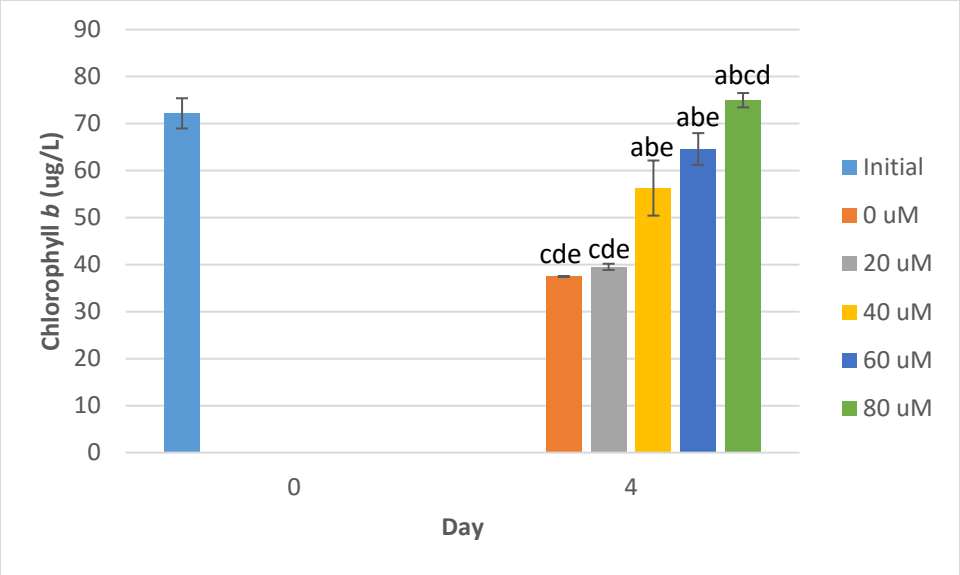


Figure 1.20 Natural assemblage bioassay HPLC chlorophyll *b* concentrations in HOOH treatments on initial and final days of the bioassay. Statistical significance by one-way ANOVA ($p<0.05$) and post-hoc comparison. Statistically different mean values represented by unique letters (a= significant difference from 0 µM, b= significant difference from 20 µM, c= significant difference from 40 µM, d= significant difference from 60 µM, e= significant difference from 80 µM). Error bars represent one standard deviation (n=3).

Initial chl *b* concentration was low in the concentrated *Microcystis* bioassay (0.3 µg/L; Figure 1.21), since the majority of chlorophyte biomass was removed by filtering. Chl *b* increased in the untreated control and in all treatments during the bioassay. On the final day of the bioassay, the untreated control had the lowest chl *b* concentration (1.3 µg/L) and the 40 µM HOOH treatment had the highest chl *b* concentration (56.3 µg/L). As discussed in section 3.2, P limitation occurred during both bioassays. Since green algae are not as strong competitors for P as cyanobacteria, diatoms, and cryptophytes (Holm and Armstrong, 1981; Grover, 1989; Baldia *et al*., 2007), P limitation during these bioassays may have reduced the growth of green algae relative to the other major phytoplankton groups in all HOOH treatments as well as the untreated control.

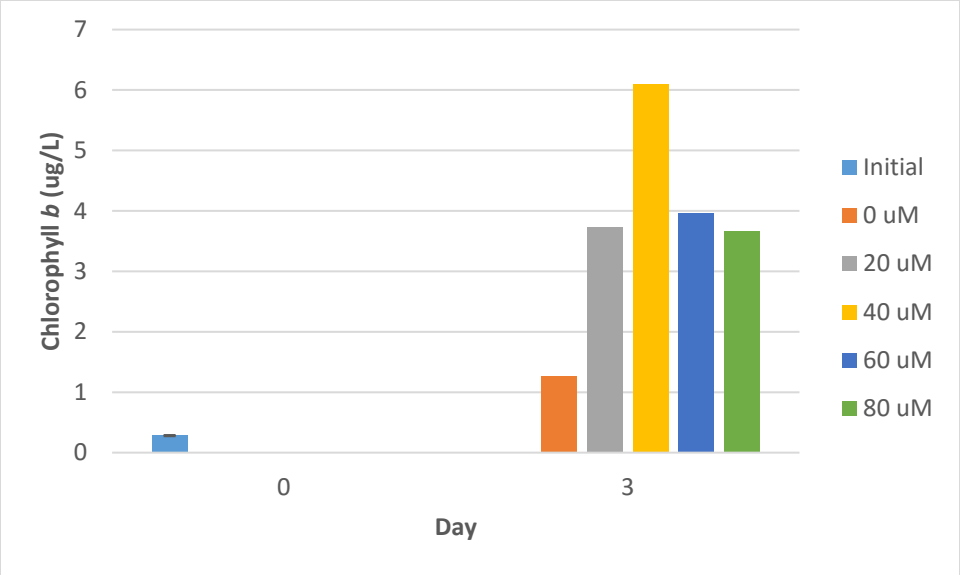


Figure 1.21 Concentrated *Microcystis* bioassay HPLC chlorophyll *b* concentrations in HOOH treatments on initial and final days of the bioassay. Data were not tested for statistical significance, since a single sample was collected per treatment (except at initial measurement) due to volume constraints. Error bars represent one standard deviation (n=3).

*3.6.6 ChemTax*

Analysis of HPCL results by ChemTax allows for determination of the relative importance of the major phytoplankton groups throughout the bioassays. Monthly monitoring data from 1992 through 2012 indicates that 31 genera of phytoplankton representative of six phyla have been identified in Lake Taihu. Four of these six phyla constitute 98% of total phytoplankton biovolume: Cyanobacteria, Chlorophyta, Bacillariophyta, and Cryptophyta (Deng *et al*., 2014). ChemTax was used to analyze the relative contribution of these four phyla to total chl *a*.

The natural assemblage bioassay was initially dominated by chlorophytes (89%; Figure 1.22). Cyanobacteria, diatoms, and cryptophytes contributed 6%, 3%, and 2% of total phytoplankton biomass, respectively. In Lake Taihu, phytoplankton dominance is primarily controlled by nutrients and water temperature (Ke *et al*., 2008; Deng *et al*., 2014). Chlorophyte blooms are most likely to occur in the spring, when nutrient (total N and total P) concentrations

are maximal (Deng *et al*., 2014). Unlike cyanobacteria, chlorophytes have a high demand for nutrients due to their high growth and loss rates (Reynolds, 1988). In the summer, warm water temperatures favor *Microcystis* blooms (Paerl and Huisman, 2008). Cyanobacterial growth rates increase at high temperatures, providing a competitive advantage over other phytoplankton. Additionally, warming of surface waters enhances vertical stratification. Under conditions of minimal vertical mixing, buoyant cyanobacteria rise to the surface and form dense blooms which limit light availability to non-buoyant phytoplankton (Jöhnk *et al*., 2008).

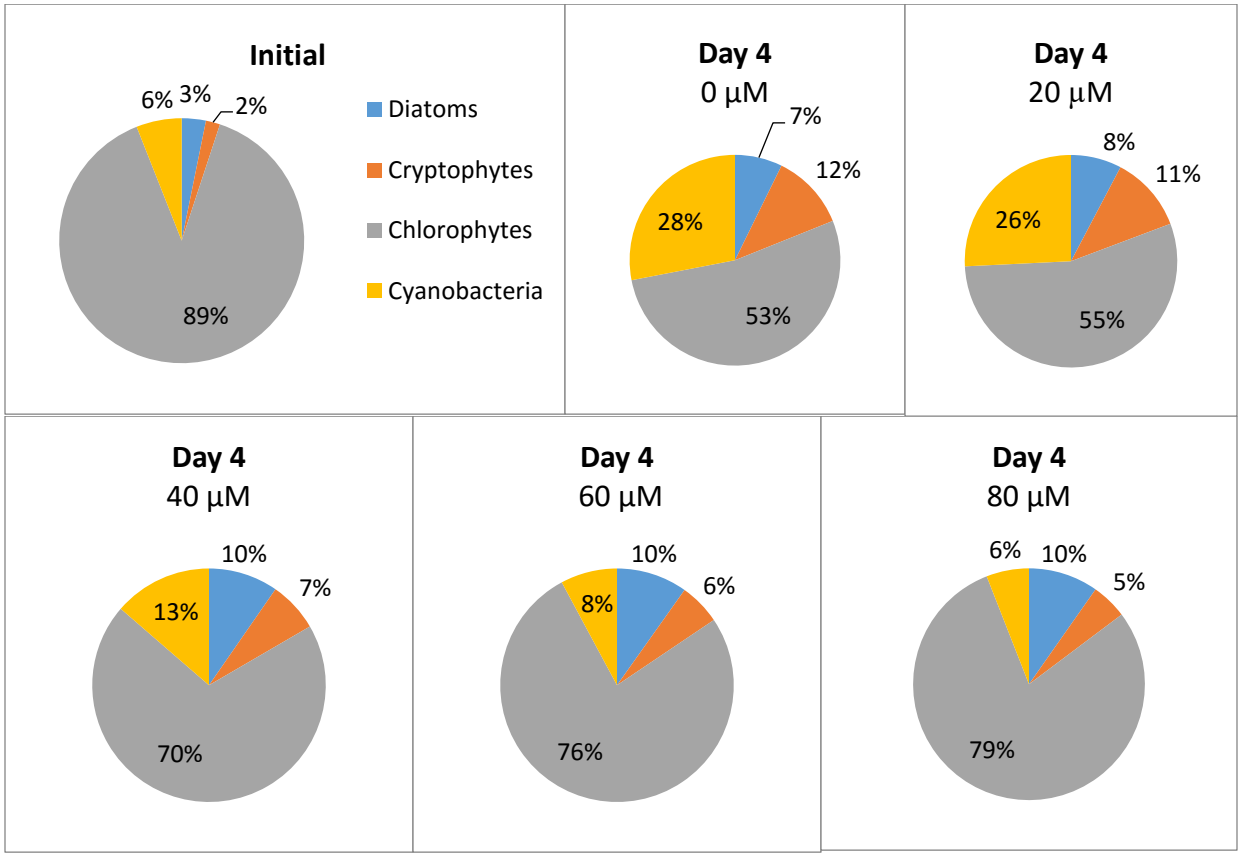


Figure 1.22 Natural assemblage bioassay contributions of four major freshwater phytoplankton classes to total chlorophyll *a* on the initial and final days of the bioassay calculated from HPLC data using the ChemTax program.

On the final day of the natural assemblage bioassay, the untreated control and all HOOH treatments remained dominated by chlorophytes. However, the relative contribution of

chlorophytes increased in higher HOOH treatments, from 53% in the control to 79% in the 80 μM treatment. Diatoms also showed a slight increase in relative abundance at higher HOOH concentrations, increasing from 7% in the control to 10% at 40, 60, and 80 μM HOOH. Cyanobacterial and cryptophyte relative contributions, on the other hand, decreased at high HOOH concentrations. Cyanobacteria decreased from 28% in the untreated control to 6% in the 80 μM treatment and cryptophytes decreased from 12% to 5%. Phytoplankton community composition dynamics illustrate the selective suppression of cyanobacteria by HOOH. In this bioassay, 40 μM HOOH reduced the relative contribution of cyanobacteria by over 50% compared to the control. The reduction in cyanobacterial biomass allowed for enhanced growth of eukaryotic algae, including chlorophytes and diatoms.

Multiple hypotheses have been proposed to explain why cyanobacteria exhibit higher sensitivity to HOOH than other phytoplankton (Drábková *et al*., 2007b). In prokaryotes such as cyanobacteria, the light-harvesting complexes are located outside of the thylakoid membrane in the cytoplasm, which makes them highly accessible to externally added chemicals. In eukaryotes such as algae and diatoms, the enclosed chloroplasts provide some measure of protection. Additionally, cyanobacteria primarily utilize catalase or catalase-peroxidase as cytoplasmic HOOH detoxification enzymes. Ascorbate peroxidase, which is used by eukaryotic phytoplankton to rapidly degrade HOOH before it reaches the photosynthetic apparatus, is not found in cyanobacteria (Asada 1992; Shigeoka *et al*., 2002; Passardi *et al*., 2007). Eukaryotic algae likely require ascorbate peroxidase for more efficient degradation of ROS because they generate higher concentrations of ROS. In eukaryotic algae, $O_2^-$ is formed during the Mehler reaction (Mehler, 1951) and converted into HOOH by SOD (Latifi *et al*., 2009). In

cyanobacteria, $O_2^-$ formation is prevented by flavoproteins (Helman *et al*., 2003; Allahverdiyeva *et al*., 2011, 2013), resulting in lower levels of oxidative stress (Weenik *et al*., 2015).

As intended, the concentrated *Microcystis* bioassay was initially dominated by cyanobacteria (57%; Figure 1.23). Although in this case manufactured through water filtration and re-addition of *Microcystis* surface scum, a *Microcystis*-dominated phytoplankton bloom is common during the summer in Lake Taihu. At the beginning of the bioassay, chlorophytes, cryptophytes, and diatoms contributed 24%, 17%, and 2% of total phytoplankton biomass, respectively. While the relative contribution of diatoms was similar in both bioassays (3% and 2%), the initial contribution of cryptophytes was higher (17% versus 2%) and that of chlorophytes was lower (24% versus 89%) in the concentrated *Microcystis* bioassay. In other words, while the relative contribution of *Microcystis* was successfully increased, the relative contributions of the other phytoplankton groups were also altered in proportion to their initial contributions in the natural assemblage bioassay. This may be partially due to natural shifts in the Lake Taihu phytoplankton community during the time between water collections for the two bioassays (7 days). Additionally, the filtering and surface scum addition process used to increase the relative contribution of *Microcystis* in this bioassay likely unintentionally altered the relative contributions of the other major phytoplankton groups.

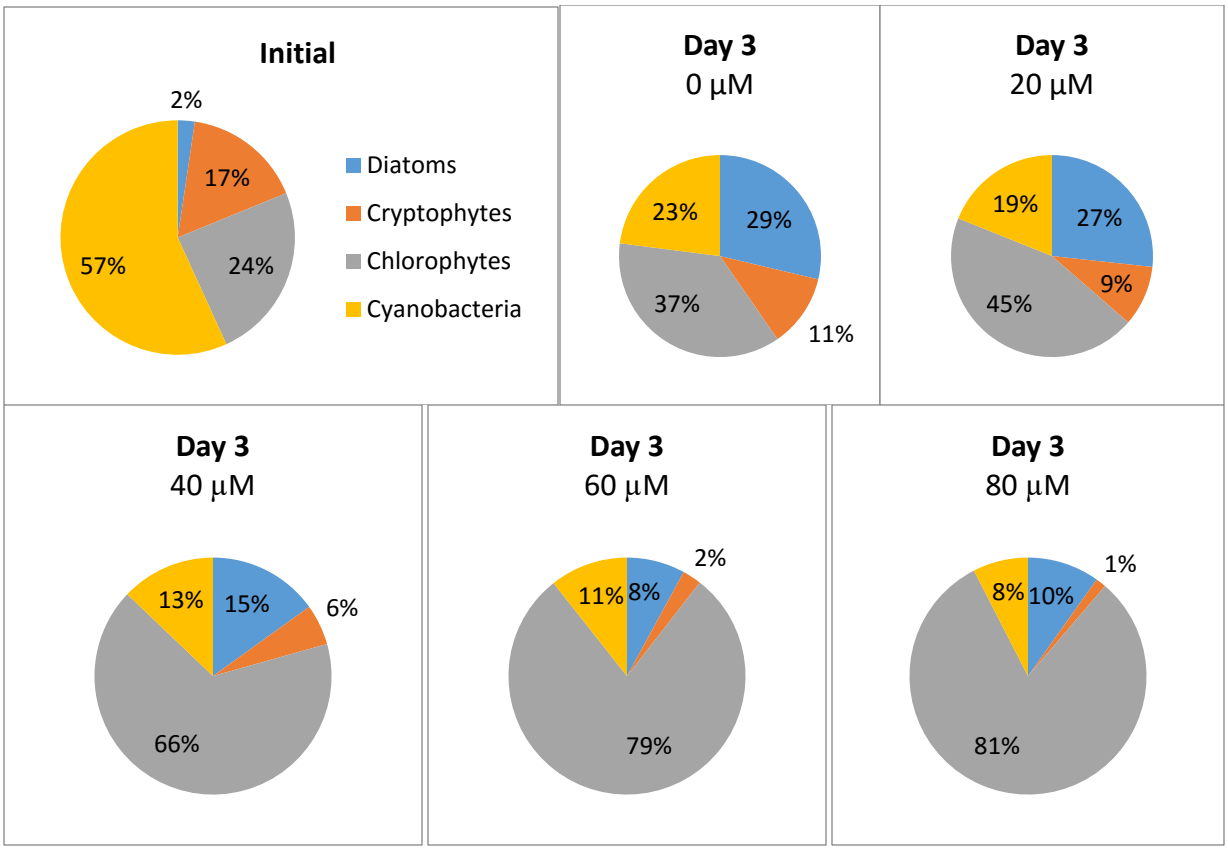


Figure 1.23 Concentrated *Microcystis* bioassay contributions of four major freshwater phytoplankton classes to total chlorophyll *a* on the initial and final days of the bioassay calculated from HPLC data using the ChemTax program.

The final relative contribution of cyanobacteria decreased in the untreated control and in all HOOH treatments. As seen in the natural assemblage bioassay, the relative contribution of cyanobacteria decreased with increasing HOOH concentration, from 23% in the untreated control to 8% in the 80 µM treatment. At 60 µM HOOH, the cyanobacterial contribution was reduced by over 50% from the control value. Cryptophyte growth was also suppressed by high doses of HOOH. Cryptophytes comprised 11% of total phytoplankton biomass in the untreated control and only 1% in the 80 µM HOOH treatment. These results differ from a whole lake study in which cryptophyte biomass was unaffected by a 60 µM HOOH addition (Matthijs *et al*., 2012). Although HPLC chl *b* pigment analysis indicates that chlorophyte biomass was reduced in

the 60 and 80 μM HOOH treatments, the relative contribution of chlorophytes increased with increasing HOOH concentration, from 37% in the control to 81% in the 80 μM treatment. While diatom growth was enhanced at high HOOH concentrations in the natural assemblage bioassay, the opposite was seen in the concentrated *Microcystis* bioassay. Diatom relative contribution decreased from 29% in the control to 10% in the 80 μM HOOH treatment. The decrease in the relative contribution of diatoms most likely was not directly HOOH treatment related.

## 4. Conclusions

In recent years, HOOH has been employed as an algicide to combat harmful cyanobacterial blooms. HOOH is preferable over traditional algicides such as aluminum and copper sulfate because it rapidly decays into $O_2$ and water and does not result in the accumulation of harmful heavy metal by-products (Cooper and Zepp, 1990; Matthijs *et al*., 2012; Fan *et al*., 2013). The HOOH approach has met with success in Lake Koetshuis, a small lake in the Netherlands plagued by *Planktothrix agardhii* blooms (Matthijs *et al*., 2012) and at the Water Corporation's Merredin wastewater treatment plant in Australia, in a WSP with high cyanobacterial biomass (Barrington *et al*., 2013). HOOH is effective as an algicide because cyanobacterial growth is inhibited at concentrations lower than those affecting most eukaryotic phytoplankton and zooplankton. Thus, the key to employing HOOH as an algicide is determining the minimum dose at which the bloom is terminated, while eukaryotic phytoplankton and zooplankton are minimally affected. Here, two bioassays were performed in Lake Taihu, China examined the effect of HOOH concentrations ranging from 20 to 80 μM on phytoplankton community composition.

The natural assemblage bioassay demonstrated that HOOH effectively reduces cyanobacterial biomass in Lake Taihu water samples, allowing for enhanced growth of other,

more desirable phytoplankton groups such as diatoms and chlorophytes. In the natural assemblage bioassay, cyanobacterial biomass decreased by 56% and the relative contribution of cyanobacteria to total phytoplankton biomass was reduced by 71% following 4 daily 60 μM HOOH additions. In the concentrated *Microcystis* bioassay, although community composition data could not be tested for significance, the relative contribution of cyanobacteria was reduced by 52% following 3 daily 60 μM HOOH additions. In contrast, Matthijs and colleagues found that a single 60 μM HOOH addition reduced the cyanobacterial population by 90% after 3 days (Matthijs *et al*., 2012). These bioassay indicate that a HOOH addition higher than 80 μM would be required to reduce cyanobacterial biomass by 90% in Lake Taihu. This variability illustrates the importance of determining the required HOOH concentration for bloom control on a case-by-case basis. Given the variability in bloom biomass and phytoplankton community composition between bloom episodes, microcosm and mesocosm bioassay studies are highly recommended prior to whole lake HOOH application.

In the natural assemblage bioassay, HOOH addition did not significantly reduce total phytoplankton biomass. Chlorophytes, diatoms, and cryptophytes grew best at high HOOH conditions, where cyanobacterial biomass was lowest. Eukaryotic phytoplankton use ascorbate peroxidase to degrade ROS more effectively than cyanobacteria and are less sensitive to ROS due their internal cellular structure (Asada 1992; Shigeoka *et al*., 2002; Passardi *et al*., 2007). At high HOOH concentrations, eukaryotic phytoplankton likely capitalized on reduced competition for light and/or nutrients due to inhibited cyanobacterial growth. Although nutrients were added to prevent nutrient limitation, P was limiting in all treatments by the end of the bioassays and light limitation may have occurred in low HOOH treatments with high cyanobacterial biomass. Buoyant cyanobacteria form dense surface blooms which can reduce light availability to non-

buoyant phytoplankton species through shading (Huisman *et al*., 2004). In the concentrated *Microcystis* bioassay, on the other hand, diatom and cryptophyte growth appears to have been inhibited in 60 and 80 µM HOOH treatments, although this data could not be tested for statistical significance. Since diatoms have demonstrated a high tolerance for HOOH (Drábková *et al*., 2007b), it is unlikely that HOOH directly inhibited diatom growth. Cryptophyte sensitivity to HOOH has not yet been studied in detail. Nevertheless, as eukaryotic phytoplankton, cryptophytes likely have a higher tolerance for HOOH than prokaryotic cyanobacteria.

In addition to the major phytoplankton groups studied here, the effects of HOOH on zooplankton and the bacterial community should be considered. For two zooplankton species, *Daphnia carinata* and *Moina* sp., the highest HOOH concentrations at which survival is not significantly different from the control were 88.2 and 44.1 µM, respectively (Reichwaldt *et al*., 2012). However, a whole lake study demonstrated no significant effect on *Daphnia* and *Diaphanosoma* spp. at 60 µM HOOH (Matthijs *et al.,* 2012). Prokaryotic heterotrophic production (rates of protein synthesis) in natural samples from Lac Cromwell was inhibited by HOOH additions as low as 3.8 nM. Upon addition of 100 nM HOOH, prokaryotic heterotrophic production was inhibited by 40%. Such a large reduction in bacterial production may significantly alter dynamics of competition, C flow and nutrient cycling (Xenopoulos and Bird, 1997).

When dealing with toxin-producing cyanobacterial species, controlling toxin concentrations is an important aspect of bloom management. Although the addition of HOOH causes lysing of cyanobacterial cells and the release of intracellular microcystins into the water column, HOOH increases microcystin degradation rates, somewhat ameliorating this effect (Ross *et al*., 2006). For example, in a whole lake treated with 60 µM HOOH, water column microcystin

concentrations declined sharply after a time lag of 2 days following HOOH addition (Matthijs *et al*., 2012). This study found a measurable increase in microcystin concentrations only at 20 µM HOOH, possibly resulting from a combination of heightened production due to HOOH-induced oxidative stress and minimal enhancement of degradation rates by the relatively low concentration of HOOH. In cases of blooms successfully managed by low doses of HOOH, managers should be particularly mindful of the effect on microcystin release into ambient waters.

Long-term effects on toxin concentrations should also be considered. *Microcystis* toxic potential (the ratio of toxic to non-toxic cells) may be altered by HOOH addition. Toxic cells generally grow better under high HOOH concentrations than non-toxic cells, possibly due to the role of microcystin in protection against oxidative stress (Dziallas and Grossart, 2011). Since HOOH concentrations are added to whole lakes in dosages sufficient to remove most but not all *Microcystis* cells, there is a strong possibility that HOOH addition selectively removes non-toxic cells. Thus, subsequent blooms in a lake treated with HOOH may have a higher toxic potential. Future studies might determine the effect of HOOH on toxic versus non-toxic *Microcystis* using qPCR assays to quantify total *Microcystis* cells (c-phycocyanin gene equivalents) and toxic *Microcystis* cells (microcystin synthetase E gene equivalents). At this time, no multi-year studies have been performed detailing the long-term effects of HOOH as an algicide.

These bioassays did not address the primary limitation to the use of HOOH as an algicide, namely how to apply HOOH homogenously to the entire water body. To date, HOOH addition has only been attempted in small lakes and ponds. In a WSP, HOOH pumped in from an upwind corner chosen to maximize wind mixing. Wind-driven mixing was effective for horizontal distribution but a steep thermocline prevented mixing at depth (Barrington *et al.,* 2013). To achieve a more homogenous distribution, Matthijs and colleagues designed a ‘water

harrow,' which is used on a small boat and injects HOOH evenly at depths up to 6 m (Matthijs *et al*., 2012). No methods of HOOH application have been proposed for large lakes such as Lake Taihu. The necessity of frequent application may pose another logistical difficulty. Barrington and colleagues (2013) found that phytoplankton biomass increased to pre-HOOH addition levels after 3 weeks, although Matthijs and colleagues measured low biomass for 7 weeks (2012). Variation in duration is likely due to variability in the extent of the initial reduction of cyanobacterial biomass and flushing rates, particularly in the case of rapid inflow of cyanobacteria-dominated water to the lake or pond system.

Despite these remaining questions, this study supports previous findings demonstrating the effectiveness of HOOH as an algicide. The natural assemblage bioassay demonstrates that, at the appropriate dosage, HOOH addition successfully reduces cyanobacterial biomass without inhibiting eukaryotic phytoplankton. Furthermore, in this study, HOOH-induced cell lysing did not significantly increase microcystin concentrations above untreated control values at HOOH doses above 20 μM, and in some cases concentrations were significantly reduced (natural assemblage bioassay, 80 μM HOOH treatment). Although dual nutrient reduction strategies are the best option for long-term overall improvements to water quality, HOOH application is recommended as a viable short-term strategy for small, shallow lakes. For best results, the two strategies should be used in conjunction. Since nutrient reduction is a slow process, HOOH application can be used in the meantime to rapidly reduce cyanobacterial biomass with minimal consequences to the ecosystem. Over time, nutrient reduction can be used to control eutrophication so that algicides are no longer required.

# CHAPTER 2: DIEL VARIABILITY IN SURFACE HYDROGEN PEROXIDE CONCENTRATIONS IN EUTROPHIC LAKE TAIHU, CHINA

## 1. Introduction

HOOH addition bioassays performed in Meiliang Bay, Lake Taihu (discussed in Chapter 1) demonstrate the effects of high concentrations of HOOH on phytoplankton community composition. As seen in previous laboratory and field studies, HOOH selectively inhibits cyanobacterial growth, reducing competition and allowing other major phytoplankton groups such as diatoms and chlorophytes to effectively compete (Drábková *et al*., 2007a,b; Barrington *et al*., 2008, 2013; Matthijs *et al*., 2012). HOOH is naturally produced in lacustrine ecosystems, albeit at concentrations orders of magnitude lower than those required for effective use as an algicide (Zafiriou *et al*., 1984; Cooper *et al*., 1988). Nevertheless, natural concentrations likely affect phytoplankton community composition and toxic potential (Dziallas and Grossart, 2011; Paerl and Otten, 2013; Leunert *et al*., 2014). To date, although several studies have measured lacustrine HOOH concentrations (e.g. Cooper *et al*., 1988; Cooper and Lean, 1989; Cooper *et al*., 1989; Herrmann, 1996; Wilson *et al*., 2000; Häkkinen *et al*., 2004; Richard *et al*., 2007; Rusak *et al*., 2010), only one study has determined background levels of HOOH during a cyanoHAB (Cory *et al*., 2016). Bloom HOOH concentrations may vary significantly from typical surface lacustrine HOOH concentrations due to the enhanced role of biological sources and sinks during a dense cyanoHAB.

## 1.1 Hydrogen peroxide sources in freshwater systems

### *1.1.1 Abiotic sources*

Traditionally, photochemical production has been considered the primary source of HOOH in natural waters (Cooper *et al*., 1988; Draper and Crosby, 1983; Cooper and Zika, 1983). Upon absorption of UVR and visible radiation, chromophoric dissolved organic matter (CDOM) enters an excited triplet state. Excited DOM reduces DO to $O_2^-$, which disproportionates to HOOH:

$$O_2 + \text{electron donor} \xrightarrow{h\nu} O_2^- + \text{electron donor}^+$$

$$2O_2^- + 2H^+ \longrightarrow HOOH + O_2.$$

Rates of photochemical production are dependent on solar irradiance, temperature, and DOM concentrations (Kieber *et al*., 2003). Apparent quantum yield (note: apparent quantum yield is measured per incident photon, whereas quantum yield is measured per absorbed photon) for the photochemical production of HOOH decreases exponentially with increasing wavelength. Thus, the majority of HOOH production occurs due to photons in the UV-B (280-320 nm) and UV-A (320-400 nm) ranges as opposed to the visible range (400-700 nm). Apparent quantum yield decreases with decreasing temperature, thus rates of photochemical HOOH production are higher at higher temperatures (Miller, 2000). Finally, HOOH production increases non-linearly with increasing dissolved organic carbon (DOC) content (Cooper *et al*., 1988). This non-linearity occurs because the chromophoric fraction of DOC increases with increasing DOC concentrations in lake water (Scully and Lean, 1994).

In the atmosphere, both gas-phase and aqueous HOOH are generated above the troposphere. Gas-phase HOOH is formed when $HO_2$ formed through photochemical processes dismutates:

$$2\ HO_2^- \text{ ------> } HOOH + O_2^-$$

Gas-phase HOOH diffuses into the surface layer of natural waters (Vione *et al*., 2003). Wet deposition of HOOH also plays an important, and frequently episodic, role as a source of HOOH in freshwater systems. Rainwater HOOH concentrations are orders of magnitude higher than typical surface freshwater HOOH concentrations. Additional wet deposition occurs in the form of snow and glacial melt (Kieber *et al*., 2003).

*1.1.2 Biotic sources*

Although abiotic production has traditionally been considered the primary method of HOOH production, recent evidence indicates that biotic sources may outweigh abiotic sources in some cases. During a dense bloom, the fraction of UV light absorbed by CDOM is likely reduced, limiting photochemical production of HOOH (Dixon *et* al., 2013). Intracellular HOOH is produced as a byproduct of aerobic cellular metabolism (Imlay, 2003). Although biological organisms possess mechanisms to manage intracellular HOOH, when production exceeds removal capabilities, HOOH, which has similar membrane permeability to water, diffuses out of the cell. HOOH is produced intracellularly because the enzymes engaged in aerobic metabolism are designed to transfer single electrons to a substrate, and thus most also have the ability to reduce $O_2$ if and when it is encountered. High $O_2$ concentrations near the photosynthetic apparatus lead to HOOH production through the Mehler reaction, in which $O_2$ is reduced to $O_2^-$ upon donation of an electron. $O_2^-$ is then converted to HOOH by SOD, and HOOH is converted to water by ascorbate peroxidase (Mehler, 1951; Asada, 1999). The autoxidation of respiratory dehydrogenases, which use flavin cofactors, provides an example. Without the interference of $O_2$, flavin cofactors become reduced upon accepting a hydride anion from an organic substrate, and subsequently transfer the electron to a secondary redox functional group. When $O_2$ is

present, however, it can accept the electron to generate $O_2^-$ and flavosemiquinone, which bond to form a peroxy adduct. When the bond is broken, the bonding electron pair is split unevenly between the products (heterolytic cleavage), releasing HOOH (Imlay, 2003).

HOOH is also generated within cells through the photorespiration pathway, when Rubisco catalyzes a wasteful $O_2$-fixation reaction instead of the intended $CO_2$-fixation reaction. When the $O_2$-fixing reaction occurs, one of the enzymes involved in processing the glycolate byproduct generates HOOH (Imlay, 2003). Additionally, under stressful conditions, a primary electron acceptor may be unavailable within a cell, which results in ROS-generated $O_2$-reduction (Latifi *et al*., 2005).

**1.2 Hydrogen peroxide sinks in freshwater systems**

*1.2.1 Abiotic sinks*

Abiotic HOOH removal occurs through photochemical degradation through the Fenton reaction:

$$Fe^{2+} + HOOH \text{ ------> } Fe^{3+} + OH\cdot + OH^-$$

In the Fenton reaction, HOOH is heterolytically cleaved by ferrous iron, resulting in ferric iron, hydroxide ions, and $OH^-$. In natural waters, Fe(III) is photochemically reduced to Fe(II), and the Fenton reaction is limited by the availability of Fe(II) and thus the rate of Fe(III) reduction. Therefore, the Fenton reaction is particularly important in natural waters with elevated Fe(II) concentrations (Fenton, 1894).

*1.2.2 Biotic sinks*

Biotic sinks are the primary removal pathway for HOOH in freshwater systems (Kieber *et al*., 2003). Bacteria disproportionate HOOH to $O_2$ and water. Abiotically, this reaction occurs at a slow rate. However, membrane permeable HOOH diffuses into cells where biological

organisms, including algae and bacteria, employ enzymes such as catalases and peroxidases to speed the reaction. Peroxidases are inferior to catalases at scavenging HOOH because peroxidases require a reductant (substrate), and therefore can turn over only as quickly as the reductant can be supplied, while catalases remove HOOH at near diffusion-controlled rates (Kieber *et al*., 2003; Latifi *et al*., 2009). Additionally, bacteria and fungi release extracellular SODs and catalases, either actively or upon cell lysis (Eremin *et al*., 2000). Biological decay of HOOH is positively correlated with microbial biomass (Marsico *et al*., 2015).

## 1.3 The role of hydrogen peroxide in freshwater systems

### *1.3.1 Inhibition of phytoplankton and bacterioplankton productivity*

ROS such as HOOH cause oxidative damage to DNA, proteins, and lipids. ROS have the ability to fragment the DNA ribose sugar backbone and modify the nucleotide bases (He and Häder, 2002). In proteins, side chains and polypeptide backbones may be oxidized, forming a carbonyl group (Halliwell and Gutteridge, 2007). Lipids are damaged via radical propagation. In the example of ROS damage to the fatty acid side chains of membrane lipids (as previously stated in the Chapter 1 Introduction), a radical cleaves a H from a C and water is formed. The C is left with an unpaired electron and reacts with $O_2$ to form a peroxyl radical, which cleaves a H from a nearby side chain. In this manner, free radicals convert other molecules into free radicals, creating a chain reaction. Eventually, two radicals meet and each contributes its unpaired electron to form a covalent bond, and radical formation ends (Halliwell and Gutteridge, 2007; Latifi *et al*., 2009). Thus, through intracellular damage, HOOH inhibits photosynthesis. In a study of heterotrophic bacteria in the subtropical Atlantic, 100 to 1000 nM HOOH was shown to decrease prokaryotic heterotrophic production (rates of protein synthesis) by 36 to 100% (Baltar *et al*., 2013). In a study of bacterial productivity in a Canadian lake, a 100 nM HOOH addition

reduced production by up to 40% (Xenopoulos and Bird, 1997). In phytoplankton, photosynthetic yield significantly decreases upon HOOH addition (Drábková *et al*., 2007).

*1.3.2 Redox chemistry in natural waters*

HOOH plays an important role in changes in speciation of biologically important trace metals, including copper and iron. Iron may be limiting to phytoplankton growth, particularly in high nutrient low chlorophyll regions of the open ocean, and copper is toxic (Kieber *et al*., 2003). In natural waters, HOOH oxidizes Fe(II) and Cu(I) and reduces Fe(III) and Cu(II). Bioavailability of these trace metals is believed to be linked to their redox properties. In surface seawater, reaction with HOOH may be the most important oxidation pathway for Fe(II) (Moffett and Zika, 1987). In the photo-Fenton reaction, photochemically produced Fe(II) reacts with HOOH, producing the strongly oxidizing hydroxyl radical (Fenton, 1894; Zepp *et al*., 1992):

$$Fe^{3+} + H_2O \text{ ---}h\nu\text{---> } Fe^{2+} + HO\cdot + H^+$$

$$Fe^{2+} + HOOH \text{ ------> } HO\cdot + Fe^{3+} + OH^-$$

The reaction of HOOH with Cu(I) is much slower, and the most important oxidation pathway for Cu(I) is oxidation by $O_2$ (Moffett and Zika, 1987).

## 1.4 Hydrogen peroxide concentrations and depth distributions in freshwater and marine systems

*1.4.1 Freshwater systems*

HOOH concentrations in freshwater systems are highly variable (Häkkinen *et al*., 2004; Mostofa *et al*., 2013). Zafiriou and colleagues (1984) reviewed HOOH concentrations in several lakes and determined a range of 100 to 7000 nM, with typical concentrations falling at the lower end of the range. Similarly, Cooper and colleagues (1988) present a compilation of freshwater HOOH concentrations from three individual studies which each collected a range of HOOH concentrations for a given lacustrine environment, including the Volga River region in Russia, a

separate reservoir in Russia, and the southeastern USA. The HOOH concentrations range from 90 to 3200 nM. Mostofa and colleagues (2013) suggest that lacustrine concentrations range from 10 to 1300 nM in the USA and Canada (Mostofa *et al*., 2013).

Freshwater HOOH depth profiles typically exhibit a maximum near the surface and decrease with depth throughout the epilimnion, with a fairly uniform distribution below the epilimnion (Cooper *et al*., 1989). Although HOOH is primarily generated through light-dependent abiotic and biotic processes, high HOOH concentrations may be found at depths of minimal light penetration. This HOOH likely mixed down from the surface or was biologically produced (Cooper *et* al., 1989; Vermilyea *et al*., 2010; Cory *et al*., 2016). For this reason, the deeper the epilimnion, the deeper high HOOH concentrations are found. Accordingly, surface HOOH levels are lowered through dilution in lakes with a deeper epilimnion, resulting in a more gradual change in HOOH concentrations with depth. However, in cases of high biological dark production by phytoplankton and bacteria, little variation between surface and bottom HOOH concentrations may be seen (Cory *et al*., 2016). HOOH depth profiles demonstrate significant diel variability in surface waters, with concentrations greatly increasing from midday through the afternoon, while HOOH levels exhibit much less temporal variation at depth, as might be expected (Häkkinen *et al*., 2004).

*1.4.2 Marine systems*

HOOH concentrations in marine environments are lower than freshwater environments, typically around 14 to 290 nM (Zafiriou *et al*., 1984). In a study performed by Cooper and colleagues (1988), marine HOOH concentrations were determined in Texas coastal waters, the North Atlantic, Biscayne Bay, the Gulf of Mexico, Bahama Bank, and the Peru coast and offshore. HOOH concentrations ranged from 18 to 190 nM.

In marine environments, HOOH depth profiles typically contain a surface maximum relative to deeper waters (Zika *et al*., 1985). In the mixed layer, HOOH levels either remain relatively uniform due to mixing or decrease slightly with depth. Below the pycnocline, however, a rapid decrease in HOOH concentrations occurs. As in freshwater environments, diel variability in HOOH is significant in surface waters, where HOOH reaches maximum concentrations in the afternoon and minimum concentrations before dawn. The photic zone reaches greater depths in marine environments than lake environments. Consequently, photochemical HOOH production occurs at greater depth (Zika *et al*., 1985; Cooper *et al*., 1989).

### 1.5 Study goals

HOOH studies have been performed in several lakes over the past decades (e.g. Cooper *et al*., 1988; Cooper and Lean, 1989; Cooper *et al*., 1989; Herrmann, 1996; Wilson *et al*., 2000; Häkkinen *et al*., 2004; Richard *et al*., 2007; Rusak *et al*., 2010). However, studies of HOOH concentrations during phytoplankton blooms are very limited (Cory *et al*., 2016), and none have been performed in Lake Taihu, China. Because phytoplankton are involved in both HOOH production and decay, HOOH concentrations in a cyanobacteria bloom-dominated lake may differ significantly from concentrations in non-bloom-dominated lakes. In this study, diel measurements of HOOH were taken over the course of 4 days to assess diel variability and the range of surface HOOH concentrations in eutrophic Lake Taihu, China.

## 2. Materials and Methods

### 2.1 Study site and sample collection

The study site, Lake Taihu, is described in detail in Chapter 1, Section 2.1. Samples were collected in duplicate from the highly eutrophic Meiliang Bay at TLLER on June 27$^{th}$ through June 30$^{th}$ of 2014. Surface samples were collected in a plastic bucket and were filtered through

0.2 μM Supor membrane filters into 4 mL amber vials. Samples were transported in the dark on ice for immediate analysis at TLLER. The TLLER dock was chosen as a sampling location to minimize the time between sample collection and HOOH analysis (transportation time: 3 minutes).

**2.2 Amplex UltraRed fluorometric method for hydrogen peroxide determination**

HOOH concentrations were determined using a fluorometric method described by Shaked and colleagues (2010) based on the protocol developed by Zhou and colleagues (1997). In this technique, horseradish peroxidase catalyzes a reaction between HOOH and Amplex UltraRed to produce the fluorophore resorufin. Since HOOH and Amplex UltraRed react in 1:1 stoichiometry, the concentration of HOOH is determined from the increase in fluorescence as resorufin is produced. HOOH standard additions are used to back-calculate the concentration of HOOH in the sample using the slope of the linear regressions ($R^2 > 0.99$) and the x-intercept. Error at each timepoint was calculated as one standard deviation.

*2.2.1 Reagents*

Reagent grade chemicals were used. Nanopure ultrapure water was stored in the dark for at least 7 days prior to usage to allow for degradation of contaminating HOOH. All reagents were stored in the dark to prevent HOOH formation. Because the Amplex UltraRed (Invitrogen) is highly light sensitive, the procedure was carried out under dark conditions. A 1 M standard HOOH solution was prepared by diluting 30% (w/w) HOOH in Nanopure water. The exact concentration was determined spectrophotometrically using UV absorbance at 240 nm ($\varepsilon$=38.1 $M^{-1}$ $cm^{-1}$). Lower concentration standards (to 2 μM) were prepared by serial dilution of the 1 M standard in nanopure $H_2O$.

A 80 mM sodium citrate stock solution was prepared for use as a reaction buffer by combining citric acid and sodium citrate with Nanopure water. 1 N sodium hydroxide (NaOH) was used to adjust the pH to 6.0. A horseradish peroxidase stock solution was prepared by adding 1 mL sodium citrate buffer to 32.47 mg horseradish peroxidase (308 units/mg). The working stock was stored frozen at -20 ºC. An Amplex UltraRed stock solution was prepared by adding 150 µL dimethyl sulfoxide (DMSO) to 1 vial of Amplex UltraRed. 150 µL horseradish peroxidase were combined with 150 µL Amplex UltraRed in 14.7 mL sodium citrate buffer to create a horseradish peroxidase/Amplex UltraRed working solution. The working solution was divided into 100 µL single-use aliquots and frozen at -20 ºC.

A catalase stock solution was prepared by dissolving 8 mg (3356 units/mg) catalase in 1 mL Nanopure water. The catalase stock solution was further diluted to a working solution by combining 50 µL catalase stock solution with 1 mL Nanopure water.

*2.2.2 Apparatus*

Fluorescence was measured on a Turner Designs Trilogy fluorometer. A custom filter set was designed for the application (excitation: 515-570 nm, emission: 590 nm). Excitation and emission wavelengths were chosen based upon the excitation and emission spectra of Amplex UltraRed.

*2.2.3 Procedure*

Samples were collected in dark glass containers and placed on ice for immediate analysis. A blank was prepared by combining 1 mL of sample with 20 µL catalase working solution. The blank was stored in the dark for 1 hour prior to analysis. Seven fluorometric glass minicells were required per sample: 3 to run samples in triplicate and 4 for standard additions. A volume of 200 µL of sample was pipetted into each minicell. A HOOH standard was added in increasing

concentrations to 4 minicells. Nanopure water was added to the minicells to maintain equal volume following the standard additions. A volume of 2 µL horseradish peroxidase/Amplex UltraRed working solution was added to each minicell. After waiting 2 minutes to allow the reaction to proceed to completion, the fluorescence was measured.

*2.2.4 Calculations*

The HOOH concentration in each sample was back-calculated, given the increase in fluorescence due to a known quantity of HOOH in each standard addition. The fluorometric response (minus the blank response) was plotted versus the concentration of the standard addition, where the concentration of the standard addition was calculated as:

$$C_{SA} = V_{Std} \cdot C_{Std} / V_{Minicell}$$

where

$V_{Std}$= volume of the standard added

$C_{Std}$= concentration of the standard

$V_{Minicell}$= volume in the minicell

Using the x-intercept from the plot, the concentration in the sample was calculated from the equation:

$$C_{Sam} = -(X_{Int}) \cdot V_{Minicell} / V_{Sam}$$

where

$X_{Int}$= x-intercept

$V_{Sam}$= volume in the sample

### 2.3 Phytoplankton biomass

To provide an estimate of phytoplankton biomass during the diel HOOH study, chl *a* was measured prior to the diel study, on June 22nd, 2014. Samples were analyzed following the protocol outlined in Chapter 1, Section 2.2.

### 2.4 Photosynthetically active radiation

PAR (400-700 nm) was measured at the water surface on an hourly basis at the TLLER weather station throughout the duration of the study using a spherical quantum sensor (LI-COR 192SA).

## 3. Results and Discussion

### 3.1 Diel variability in hydrogen peroxide concentrations

Diel variability in surface HOOH concentrations was studied during a summer phytoplankton bloom in Meiliang Bay, Lake Taihu. Weather during the study was cloudy on June 27th and sunny on June 28th, 29th, and 30th. Surface PAR (400-700 nm) at the TLLER weather station ranged from 0 to 2239.4 μmol/$m^2$/s during June 27th through June 30th of 2014 (Figure 2.1). The peak PAR value occurred at 13:00 on June 29th. HOOH concentrations in the lake surface waters exhibited a diel pattern, with higher values occurring during daylight hours and lower values at night (Figure 2.2). Hydrogen peroxide concentrations increased as solar radiation increased from early morning to midday. Following a midday peak, concentrations decreased to a minimum just prior to sunrise. The peak HOOH concentration, 168 ± 8 nM at 13:05 on June 29th, corresponds to the peak PAR value. The lowest HOOH concentration, 17 ± 6 nM, was measured just prior to sunrise (4:15) on June 28th. Similar patterns of diel cycling in HOOH concentrations have been demonstrated in multiple lakes (e.g. Cooper *et al*., 1988;

Cooper and Lean, 1989; Cooper *et al*., 1989; Herrmann, 1996; Wilson *et al*., 2000; Häkkinen *et al*., 2004; Richard *et al*., 2007; Rusak *et al*., 2010).

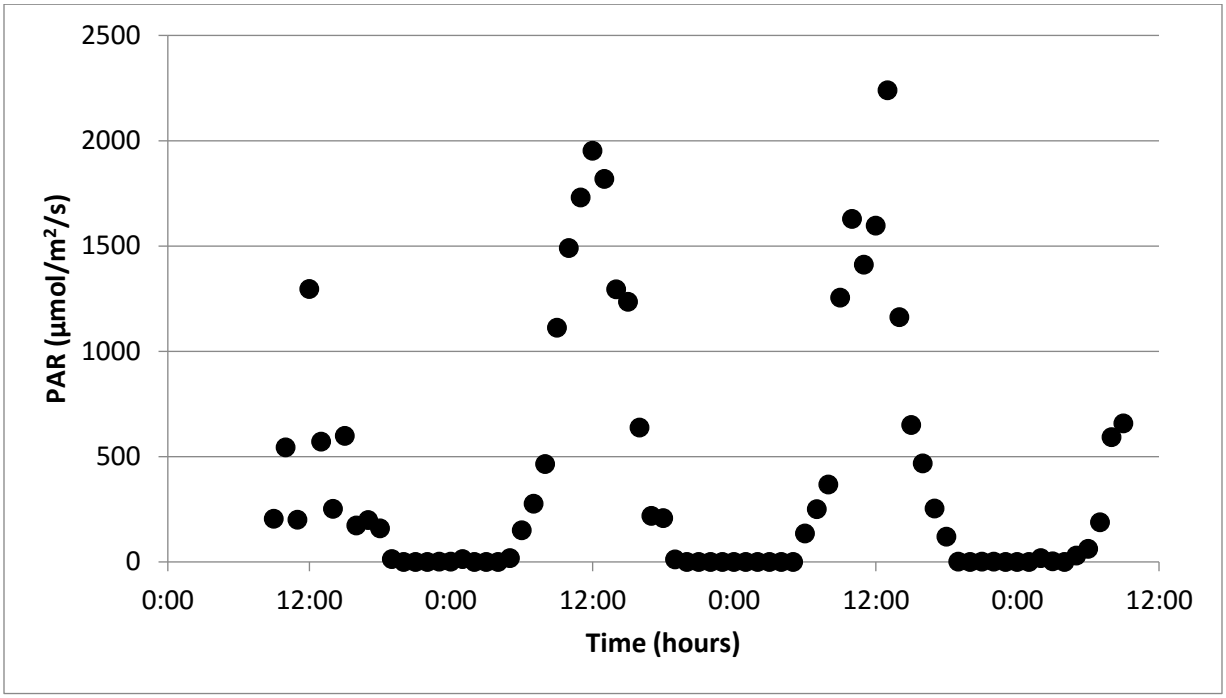


Figure 2.1 Surface atmospheric photosynthetically active radiation (PAR; 400-700 nm) at the TLLER weather station. 0:00 indicates midnight and 12:00 indicates noon.

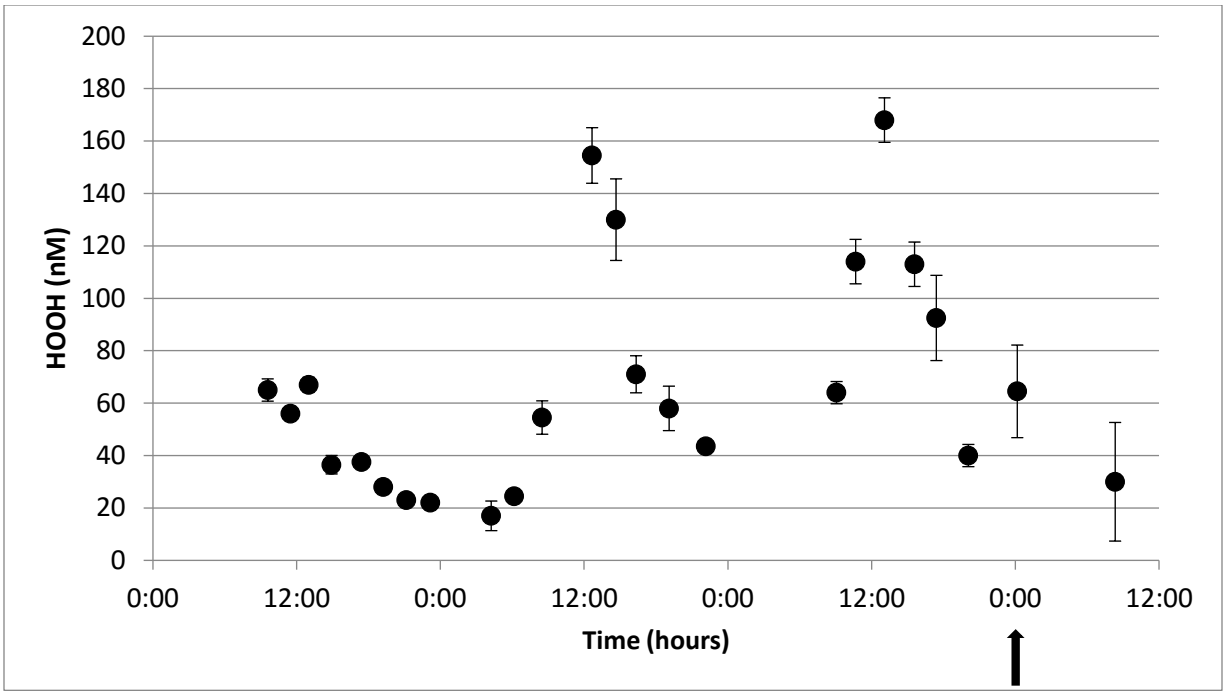


Figure 2.2 Mean surface HOOH concentrations in Meiliang Bay, Lake Taihu during June 27th through June 30th 2014. 0:00 indicates midnight and 12:00 indicates noon. The black arrow indicates episodic HOOH input through wet deposition (rain). Error bars represent one standard deviation (n=2).

Diel cycling in HOOH concentrations reflects the dependence of HOOH on light intensity. It has been suggested that diel cycling in HOOH concentrations indicates photochemical production as the primary source of HOOH in freshwaters (Cooper and Lean, 1989; Cooper and Zepp, 1990; Rusak *et al*., 2010). In filtered freshwater, HOOH is produced photochemically at rates of 0.1 to 1 μM/h (Richard *et al*., 2007). Rates of photochemical production are dependent on solar irradiance, temperature, and DOM concentrations (Kieber *et al*., 2003). The high solar irradiance, warm temperatures, and high DOM concentrations typical of Meiliang Bay in the summer would suggest high photochemical production, resulting in diel variation in HOOH concentrations (Chen *et al.*, 2003a,b; Zhang *et al*., 2011). While photochemical production of HOOH is a well-recognized contributor to diel cycling in HOOH concentrations in lacustrine environments (Cooper *et al*., 1988; Cooper and Lean, 1989; Cooper

*et al*., 1989; Herrmann, 1996; Wilson *et al*., 2000; Häkkinen *et al*., 2004; Richard *et al*., 2007; Rusak *et al*., 2010), other processes may play a role in diel HOOH patterns.

In Lake Taihu, photoautotrophic production of HOOH likely contributes to diel variability. Biological production of HOOH in eutrophic lakes occurs at rates up to 0.25 μM/h (Marsico *et al*., 2015). In a study of 38 cyanobacterial species, light-induced HOOH production occurred in 22 species and dark production occurred in 5 species (Stevens *et al*., 1973). Although biological HOOH production occurs under both light and dark conditions, light-dependent production contributes to diel patterns. Under high light conditions, HOOH is produced during photosynthesis when electron transport occurs more rapidly than electron consumption (Latifi *et al*., 2009). Light has been shown to enhance biological production of HOOH in the marine diatom *Thalassiosira weissflogii* (Milne *et al*., 2009). In culture studies, algae have been shown to produce 40 to 800 nM/h, dependent upon light intensity and algal cell abundance (Zepp *et al*., 1987).

Field studies indicate that non-photochemical HOOH production may be an equally important contributor to the total HOOH budget as photochemical production in some cases Vermilyea *et al*., 2010; Dixon *et al*., 2013). In an in-stream mesocosm study, for example, total HOOH production greatly exceeded abiotic photochemical production, likely due to the role of biological production (Dixon *et al*., 2013). Biological production may be especially important relative to photochemical production during a dense bloom. During a particle-rich bloom, the fraction of UV light absorbed by CDOM is likely reduced, limiting photochemical production of HOOH, while biological production is likely high due to high phytoplankton biomass (Zepp *et al*., 1987; Dixon *et al*., 2013). In Lake Erie cyanoHAB microcosm experiments, HOOH was biologically produced at a net rate of 30 ± 14 nM/h. In comparison, the rate of photochemical

production in the lake ranged from below 10 nM/h in October to approximately 150 nM/h in May and June (Cory *et al*., 2016). In Lake Erie, HOOH concentrations reached their maximum just prior to maximum chl *a* concentrations, further demonstrating the importance of biological production. Due to the high phytoplankton biomass in Lake Taihu at the time of this study, biological production of HOOH was likely an important factor in the total HOOH budget, with photoautotrophic production contributing to diel HOOH patterns.

A week prior to the study, surface chl *a* was measured as 274.86 µg/L, indicative of the dense phytoplankton blooms which have occurred in Meiliang Bay every summer since the mid-1980's (Lai and Yu, 2006). As discussed in Chapter 1, such a high chl *a* concentration is not unheard of in hypereutrophic Meiliang Bay. In August of 2004, for instance, chl *a* concentrations reached well above 400 µg/L (Liu *et al*., 2011). However, this chl *a* value may be higher than the average Meiliang Bay chl *a* concentration at the time of sample collection due to the nearshore sampling location, since blooms tend to accumulate in calm nearshore waters. Regardless of exact chl *a* concentrations, a thick surface bloom was evident during the diel study, indicating high phytoplankton biomass (Figure 2.3). Thus, the diel patterns in HOOH peroxide concentrations in Lake Taihu likely occur due to a combination of photochemical production and photoautrophic production.

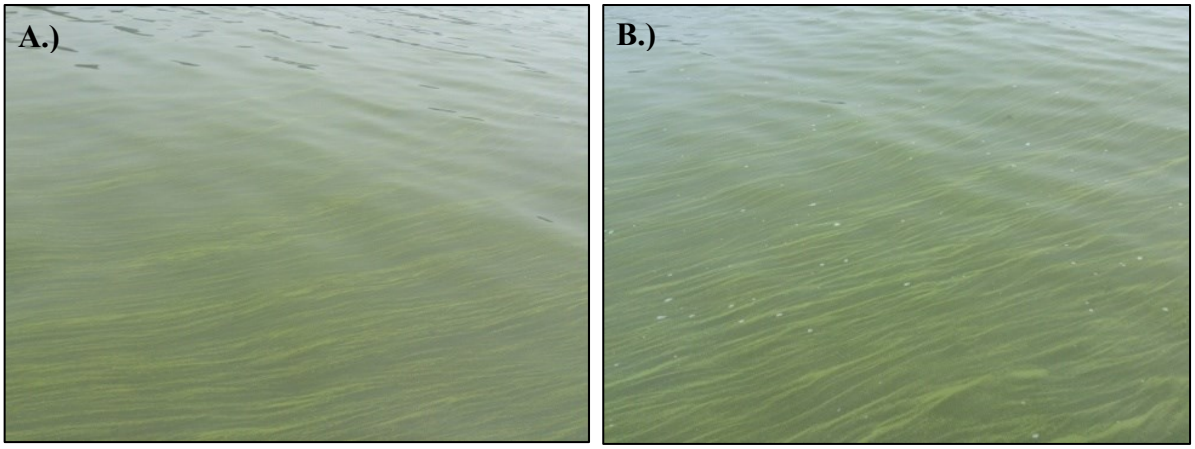


Figure 2.3 Surface scum in Meiliang Bay during diel HOOH study. A.) June 28th, 12:40 B.) June 29th, 15:35.

### 3.2 Range of hydrogen peroxide concentrations

Despite the likelihood of high biological HOOH production during this study, surface HOOH concentrations in Meiliang Bay during this study ranged from 17 ± 6 to 168 ± 8 nM, at the low end of the typical lacustrine range. Typical lacustrine HOOH concentrations range from 10 to 7000 nM (Zafiriou *et al*., 1984; Mostofa *et al*., 2013). Low HOOH concentrations do not necessarily suggest low rates of photochemical and/or biological HOOH production. Instead, the low values seen here are likely an effect of high degradation rates. HOOH decomposition is primarily biotic (Cooper *et al*., 1994). Under dark conditions, the half-life of HOOH in unfiltered freshwater is on the order of hours (Cooper and Zepp, 1990). In highly productive freshwaters, the half-life is typically less than 3 h (Richard *et al*., 2007; Mostofa *et al*., 2013). In a laboratory study of water from 10 oligotrophic lakes, the half-life of HOOH ranged from 1.4 to 58.2 h (Häkkinen *et al*., 2004). Upon removal of particles larger than 0.45 µm, little to no decay occurs (Cooper *et al*., 1989; Cooper and Lean, 1990). Decay rates are correlated with microbial biomass (Cooper *et al*., 1994; Marsico *et al*., 2015) and DOC concentrations (Herrmann, 1996).

The high phytoplankton and bacterial biomass seen in Lake Taihu during the summer bloom season suggest high rates of biological HOOH decay. High rates of biological decay have been measured in laboratory incubations of water from bloom-dominated Sloan's Lake, Colorado (Marsico *et al*., 2015). High biological production and decay occur in *Microcystis*-plagued Lake Erie, with biological HOOH production outweighing biological decay during daylight hours and vice versa overnight (Cory *et al*., 2016). Although daylight biological decay may not outweigh daylight biological production, peak decay rates coincide with peak HOOH concentrations during daylight hours (Morris *et al*., 2016). As discussed in Chapter 1, phytoplankton and bacteria remove ROS using antioxidant enzymes including SOD, peroxidase, and catalase (Cooper and Zepp, 1990). In oceanic samples, genes encoding bacterial and algal HOOH-degrading enzymes were most abundant during daylight hours (Morris *et al*., 2016). Catalase was responsible for 65 to 80% HOOH decay and peroxidase was responsible for 20 to 35% HOOH decay in laboratory incubations of water from Vineyard Sound, Massachusetts (Moffett and Zafiriou, 1990).

HOOH is also removed from the epilimnion through diffusive processes (mixing). Lake Taihu is polymictic, with minimal stratification due to its shallow depth and wind-driven mixing. In July of 2008, the change in temperature between surface and bottom layers was 0.3 ºC or less (Xu *et al*., 2010). In well-mixed lakes, HOOH produced by light-dependent processes in the surface layer is rapidly distributed throughout the water column (Cooper *et al*., 1989).

The maximum amplitude of the diel cycle was 137.5 nM on June 28$^{th}$. A greater change in amplitude may have occurred on June 29$^{th}$, given the peak HOOH concentration on that day, but HOOH concentrations were not measured just prior to sunrise that morning. Pronounced variation on a diel timescale reiterates the rapidity of HOOH formation and decay processes. The

maximum rate of HOOH accumulation (net formation given simultaneous formation and decay) in this study was 31.6 nM/h during the morning of June 29th. Rates of production and decay were not determined.

Episodic wet deposition (rain) of HOOH occurred once during the study. It rained for about an hour from 23:00 to 24:00 on June 29th. The quantity of rain and HOOH concentration of the rainwater were not measured. Without wet deposition, HOOH concentrations consistently decreased during the dark period (June 28th). Post rain input, however, HOOH concentrations increased from 40 nM at 20:05 to 64.5 nM a little over 4 hours later. Rainwater input is known to increase HOOH concentrations in surface ocean waters (Cooper *et al*., 1987; Kieber *et al*., 2001) and freshwaters (Cooper and Lean, 1989; Willey *et al*., 1996), since HOOH concentrations in rainwater are orders of magnitude higher than average surface water concentrations (Cooper *et al*., 1987). In shallowly-stratified ocean waters, rain input can increase HOOH concentrations by a factor greater than 10 (Cooper *et al*., 1987). In East Asia in particular, high levels of HOOH are found in rainwater, likely due to high levels of air pollution from rapid industrialization (Hua *et al*., 2008).

The relatively low nanomolar HOOH concentrations in Meiliang Bay in June of 2014 are unlikely to significantly affect phytoplankton community composition. Although cyanobacteria are affected by HOOH at lower concentrations than eukaryotic phytoplankton, cyanobacterial biomass is typically reduced at HOOH concentrations well above the natural concentrations measured here (Stratford *et al*., 1984; Barroin and Feuillade, 1986; Schrader *et al*., 1998, Drábková *et al*., 2007a,b; Weenik *et al*., 2015). In the bioassays discussed in Chapter 1, a significant reduction in cyanobacterial biomass occurred at the lowest HOOH dose tested (20 μM) in the natural assemblage bioassay, but 60 μM HOOH was required to reduce

cyanobacterial biomass in the concentrated *Microcystis* bioassay. However, natural concentrations may affect cyanobacterial toxic potential. In culture experiments, non-toxic *Microcystis* was affected by 50 nM HOOH, well within the range of concentrations measured here, while 500 nM HOOH was required to affect toxic *Microcystis* (Leunert *et al*., 2014). The heightened sensitivity of non-toxic strains to HOOH suggests that cyanobacterial toxins may play a role in intracellular protection against oxidative stress (Zilliges *et al*., 2011). Further studies are required to assess the extent to which natural cyanobacterial populations are affected by HOOH at naturally-produced concentrations. Heterotrophic bacteria are also affected by low levels of HOOH. In the subtropical Atlantic, 100 to 1000 nM HOOH was shown to decrease prokaryotic heterotrophic production by 36 to 100% (Baltar *et al*., 2013). Similarly, a 100 nM HOOH addition reduced prokaryotic heterotrophic production in a small Canadian lake by up to 40% (Xenopoulos and Bird, 1997).

**4. Conclusions**

HOOH concentrations in Meiliang Bay, Lake Taihu over the course of 4 days in June of 2014 ranged from 17 ± 6 nM to 168 ± 8 nM, at the low end of typical lacustrine concentrations (Zafiriou *et al*., 1984; Mostofa *et al*., 2013). The low HOOH concentrations were most likely due to high rates of biological decay, given the high phytoplankton biomass. HOOH concentrations exhibited the expected diel pattern, likely explained by a combination light-dependent abiotic and biotic sources of HOOH production, namely, photochemical production and photoautotrophic production. At this stage, the relative contributions of individual production and decay processes have not been assessed in Lake Taihu. In a study of HOOH concentrations during a cyanoHAB in Lake Erie, biotic sources and sinks of HOOH outweigh abiotic sources and sinks (Cory *et al*., 2016). Cory and colleagues (2016) hypothesized that photochemical

production of HOOH in Lake Taihu may be similar to photochemical production of HOOH in Lake Erie, since CDOM levels and sources are similar. While rates of photochemical production may in fact be similar, it's important to note that measured concentrations in Lake Erie are much higher. The maximum HOOH concentration measured here, 168 ± 8 nM, was almost an order of magnitude lower than the maximum HOOH concentration measured in Lake Erie from June to November of 2014 and 2015 (1570 ± 60 nM; Cory *et al*., 2016). Either overall production rates are lower in Lake Taihu than Lake Erie and/or decomposition rates are higher. These production and decay rates are affected by several interacting factors including: light intensity and wavelength, CDOM concentration and source, temperature, and phytoplankton and bacterial abundance and species composition (Zepp *et al*., 1987; Wetzel *et al*., 1992; Scully and Lean, 1994; Miller, 1998; Kieber *et al*., 2003; Samuilov *et al*., 2004; Marsico *et al*., 2015).

HOOH concentrations have been measured in natural waters since 1966 (Van Baalen and Marler, 1966). Nevertheless, to date, no studies have measured lacustrine concentrations over a long enough period to assess possible effects of ongoing climate change. The effects of global warming on bloom development have been given considerable attention, however. Warmer water temperatures and enhanced stratification favor bloom development (Paerl and Huisman, 2008). These conditions (high temperatures, high light) also favor HOOH production (Mostofa *et al*., 2009; Paerl and Otten, 2013; Cory *et al*., 2016). Additionally, the increase in frequency and intensity of rainfall events associated with climate change, which favors bloom development through nutrient input (Reichwaldt and Ghadouani, 2012; Paerl and Otten, 2013) provides another source of HOOH. As a result, HOOH concentrations may increase in the future. Given the higher sensitivity of non-toxic cyanobacterial species to oxidative stress (Dziallas and Grossart, 2011; Leunert *et al*., 2014), heightened concentrations of HOOH and other ROS may

select for toxic cyanobacterial species (Paerl and Otten, 2013). However, given the complexity of lacustrine HOOH dynamics, long-term changes in lacustrine HOOH concentrations cannot be accurately predicted without further study.

Although the data available on HOOH concentrations during cyanobacterial blooms is limited, production and decay of ROS such as HOOH is undoubtedly affected by the presence of a bloom. A more comprehensive study in Lake Taihu would be valuable in assessing temporal (seasonal, annual) and spatial variations in HOOH concentrations, in particular comparing times and locations at which blooms are and are not occurring. Furthermore, abiotic and biotic HOOH production and decay rates should be quantified to determine the relative importance of each. Future studies may also examine the effects of ambient HOOH concentrations on bloom toxic potential, possibly using qPCR to quantify toxic and non-toxic cell abundances in relation to HOOH concentrations.

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