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ABSTRACT

APPLICATION OF HYDROGEN PEROXIDE AS BROWN TIDE BLOOM CONTROL AGENT

by
Varunpreet Randhawa

Harmful algal blooms (HABs) are growing problem across the globe. One such HAB that's recurring in the coastal waters of New York and New Jersey since mid-1980s, and has been reported in other countries recently, is brown tide bloom. The causal organism is a minute pelagophyte (cell diameter ~ 2µm) *Aureococcus anophagefferens*. The brown tide bloom has been responsible for the severe ecological damage and economic loss (e.g. shellfishery) in the affected areas. This research focuses on finding a method to control brown tide blooms and explores the potential of natural chemical biocide hydrogen peroxide (H_2O_2) as a reagent to serve the purpose. Studies on laboratory cultures were carried out in order to determine the effectiveness of H_2O_2 at eradicating *A. anophagefferens*. Then, the safety (to non-target organisms) and selectivity of H_2O_2 treatment was tested by exposing a dozen common marine phytoplankton species to H_2O_2. This was followed by a study exploring the applicability of H_2O_2 treatment in the field and finally, the effects of algal growth phase and biomass on treatment efficacy were tested. The kinetics of H_2O_2 decomposition were examined in parallel. The dissertation discusses the possible action of H_2O_2 on algal cells based on cell wall structure, cell size, growth phase and algal cell density, factors potentially affecting the treatment efficacy and realistic scenario of H_2O_2 application.

APPLICATION OF HYDROGEN PEROXIDE AS BROWN TIDE BLOOM CONTROL AGENT

by
Varunpreet Randhawa

A Dissertation
Submitted to the Faculty of
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APPROVAL PAGE

APPLICATION OF HYDROGEN PEROXIDE AS BROWN TIDE BLOOM CONTROL AGENT

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I dedicate my dissertation work to my loving family, my parents and my parents-in-law, especially my mother, who always encouraged me.

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CHAPTER 1

INTRODUCTION

1.1 Objective

This research focuses on the development of a method for the control of brown tide blooms caused by an algae *Aureococcus anophagefferens* and exploring its applicability in field. The proposed method involves the use of chemical hydrogen peroxide (H_2O_2) to remove living cells of *A. anophagefferens*. H_2O_2 has been proven to be effective at controlling blooms of cyanobacteria *Microcystis aeruginosa*(Matthijs et al., 2012). Among other chemicals used to remove harmful dinoflagellates are sodium hypochlorite (NaOCl)(Jeong et al., 2002) and copper sulfate (CuSO4)(Rounsefell and Evans, 1958). The major concerns arising from the use of any chemical in field are its impact on environment and selectivity towards target organism(CENR, 2000).

H_2O_2 has been chosen in this study due to: a) its natural occurrence in sunlit water bodies, b) environmentally benign nature because of rapid decomposition into water and oxygen, c) ease of application, and d) being cost effective.

The objectives of this research are:

1. To examine the effectiveness of H_2O_2 to inhibit growth of *Aureococcus anophagefferens* and alga's physiological response to H_2O_2 exposure.
2. To examine the safety and selectivity of H_2O_2 addition by comparing growth response of *A. anophagefferens* and other marine algal species to H_2O_2.
3. To determine the applicability of method in field.
4. To determine the kinetics of H_2O_2 degradation in algal culture medium.
5. To examine the dependence of H_2O_2 action on algal growth phase and biomass, if any.

1.2 Background Information

1.2.1 Harmful Algal Blooms

An algal bloom is the rapid increase in the algae population in an aquatic system. These blooms result when algal growth and removal processes are unbalanced(Gobler et al., 2005). In case where these blooms pose a threat to the survival of other organisms and/or negatively impact human activities, they are referred to as Harmful Algal Blooms (HABs). The HABs exert severe ecological and sometimes economical effects. The ecological effects attributed to HABs have been categorized(Zingone and Oksfeldt Enevoldsen, 2000) as:

- *Risk to human health*: Algal toxins reaching humans through the consumption of vectors like fish and shell fish. These toxins cause neurological and/or gastro-enteric symptoms some of which may be fatal.
- *Impact on marine resources and ecosystems*: Large prolonged blooms can reduce light penetration to the bottom water, causing significant decrease in submerged aquatic vegetation which serve as nurseries for fish and shell fish. Hence negatively affecting marine invertebrates and cultivated fish.
- *Impact on recreational use of coastal areas*: These blooms discolor water, produce repellent odors, and in some cases produce foam and mucilage which can severely limit the use of coastal areas for recreational purposes.

Apart from these effects, HABs have been responsible for large scale economic losses, due to mass mortalities of farmed fish and other commercially important marine invertebrates(Gobler et al., 2005).

1.2.2 Brown Tide Blooms

Among a vast number of harmful algal blooms affecting the water bodies worldwide, is brown tide bloom. The causal organisms have been identified as *Aureococcus anophagefferens* and *Aureoumbra lagunensis*(DeYoe et al., 1997). Only the former has

been found in NY/NJ coastal water(Gobler et al., 2005), and is the focus of this research. The latter is associated with brown tides in Texas coastal waters(Muhlstein and Villareal, 2007). The brown tide bloom was first noticed in Narragansett Bay of Rhode Island, Long Island and Peconic Bays of New York, and Barnegat Bay of New Jersey. Since its first observation in 1985, the bloom has been found to recur in New York, New Jersey and later expanded to Delaware (Little Assawoman Bay), Maryland, Virginia (Chincoteague Bay)(Gobler et al., 2005). These blooms are not restricted to US coastal waters but expanding to other parts of world as well, an example being South Africa(Probyn et al., 2001). Brown tide blooms have been categorized based on cell density as Category 1 (< 35000 cell/ml), Category 2 (35000-200,000 cell/ml) and category 3 (> 200,000 cell/ml) blooms(Gastrich et al., 2004).

1.2.3 Characteristics of *Aureococcus anophagefferens*

Aureococcus anophagefferens is a coccoid, non-motile unicellular microalga (cell diameter ~ 2µm) and has been placed in class Pelagophyceae based on pigment composition and molecular phylogenetic studies(DeYoe et al., 1997), which was further supported by sterol composition analysis(Giner and Boyer, 1998). Ultrastructure of *A. anophagefferens* cell shows the lack of cell wall, instead a diffuse layer of polysaccharides was observed(Sieburth et al., 1988a). *A. anophagefferens* shows both heterotrophic and autotrophic mode of nutrition, giving it advantage over strictly autotrophic phytoplankton(Dzurica et al., 1989);(Mulholland et al., 2002). *A. anophagefferens* has been shown to survive under conditions of prolonged darkness and resume growth upon exposure to light, which could be an important factor in brown tide bloom spreading to new sites e.g., Saldanha Bay, South Africa, through ballast water

transport(Popels et al., 2007). Although its large photosynthetic antenna and relatively low quota of photoprotective pigments makes it more susceptible to photoinhibition, the same features provide it the ability of growing under low light conditions(MacIntyre et al., 2004). In a recent study, *A. anophagefferens* genome was shown to have 62 genes encoding for Light Harvesting Complex (LHC) proteins, which is 1.5 – 3 times more than six competing eukaryotic phytoplankton species sequenced - this feature likely enhances its adaptation to low light conditions in turbid estuaries(Gobler et al., 2011). This microalga can grow at tempratures ranging from 0 to 25° C, but the optimum temprature for growth of this alga lies in range 20 – 25° C(Cosper et al., 1989).

1.2.4 Brown Tide Bloom Dynamics

Multiple factors have been implicated as initiators of brown tide blooms caused by *Aureococcus anophagefferens*. These factors can be broadly classified as physical, chemical and biological factors(Bricelj and Lonsdale, 1998).

1.2.4.1 Physical Factors. Blooms in mid 1980s followed unusual drought periods with very low rainfall levels(Cosper et al., 1987), so reduced estuarine flushing rates resulting in increased salinity have been typicallly associated with the onset of brown tide blooms(Bricelj and Lonsdale, 1998).

Aureococcus anophagefferens is able to grow at wide range of tempratures (0 - 25° C) although optimum temprature for growth is 20 - 25°C(Cosper et al., 1989) which coincides with that in late spring when brown tide blooms fluorish(Bricelj and Lonsdale, 1998). The persistence and even initiation of brown tides during fall or early winter has been observed too(Gobler et al., 2002b). The ability to grow at much lower tempratures

like 5°C, although at lower growth rates(Cosper et al., 1989) allows seed populations to overwinter in mid-Atlantic waters(Bricelj and Lonsdale, 1998).

A. anophagefferens is able to maintain high growth rates at low irradiances owing to the possession of cellular carotenoids which allow to harvest light over a wide range of wavelengths(MacIntyre et al., 2004). This characteristic helps this alga fluorish in turbid waters.

1.2.4.2 Chemical Factors. Brown tides occur when dissolved inorganic nitrogen (DIN) levels are low(Gobler and Sañudo-Wilhelmy, 2001) which limits the growth of other phytoplankton species, including diatoms(Keller and Rice, 1989), and when dissolved organic nitrogen (DON) and dissolved organic matter (DOM) are high(Laroche et al., 1997). Several investigators have reported that *A. anophagefferens* cultures grow at the same rate using DIN or urea (Pustizzi et al., 2004), the outbreak of brown tide is due to its ability to use DON when DIN levels drop thus outcompete other phytoplankton(Gobler et al., 2005). *A. anophagefferens* can also use carbon from organic sources like amino acids(Mulholland et al., 2002). Hence, mixotrophic mode of nutrition provides added advantage to this alga as compared to co-occuring strict autotrophic phytoplankton(Dzurica et al., 1989).

A. anophagefferens fluorishes in shallow enclosed estuaries, which have higher level of trace metals (Russell Flegal and Sañudo-Wilhelmy, 1993), (Breuer et al., 1999). *A. anophagefferens* has a large and absolute requirement for the trace element selenium (Se), while the other competing phytoplankton species either don't require it (e.g. *Synechococcus sp.*) or, have lower Se requirement for growth (e.g. *Thalassiosira pseudonana, Phaeodactylum tricornutum*)(Gobler et al., 2011). Wang et al(Wang et al.,

2012) also found *A. anophagefferens* cultures growth to be substantially enhanced by environmentally relevant but elevated concentrations of Ni as compared to the control without Ni addition. Additionally, *A. anophagefferens* was found to be more resistant to elevated Cu concentrations than competing *Synechococcus sp*. This observation is in accordance with recent findings that *A. anophagefferens* genome is enriched with genes encoding for molybdenum (Mo), copper (Cu), nickel (Ni) and selenium (Se) containing enzymes(Gobler et al., 2011). Therefore, the abundantly available trace metals in shallow and enclosed estuaries potentially promote *A. anophagefferens* growth by satisfying its metal requirement in metalloenzymes, but at the same time, inhibit some of the competing phytoplankton species(Wang et al., 2012).

1.2.4.3 Biological Factors. The initiation of monospecific brown tide bloom may be the outcome of competition between *A. anophagefferens* and other similar sized phytoplankton and bacteria(Gobler et al., 2005). One factor favorable to *A. anophagefferens* could be its mixotrophic mode of nutrition. When inorganic nutrient supply is lower, the growth of autotrophic species like *Synechococcus sp.* would be reduced(Waterbury et al., 1986) which opens a niche for brown tide initiation(Laroche et al., 1997). The development and persistence of brown tides may reflect failure of normal grazing by zooplankton(Bricelj and Lonsdale, 1998). While a limited number of protozoa are known to graze on *A. anophagefferens*(Caron et al., 1989)(Caron et al., 2004), the grazing pressure on this alga reduces as the protozoa efficiently consume other picoplankters of similar size(Sherr et al., 1986). Moreover, *A. anophagefferens* secretes algal toxin its extracellular layer or within cytoplasm, suggested as a dopamine-

mimetic(Gainey and Shumway, 1991), may retard the grazers' feeding activity(Buskey and Hyatt, 1995);(Bricelj and Lonsdale, 1998).

The infection and subsequent lysis of *A. anophagefferens* cell by viruses(Sieburth et al., 1988a)(Gastrich et al., 1998) may affect brown tide dynamics. But on the other hand, viruses may promote brown tides by affecting the composition of microbial communities and regenerating DOM(Gobler et al., 2005).

1.2.5 Need to Control Brown Tides

Because of its annual occurrence and increasing expansion to new regions, the brown tides have become one of the major environmental problems. The negative impacts caused by these blooms are presented below, which necessitates the development of direct control method.

1.2.5.1 Disruption of Ecological Balance. The brown tide blooms have been listed among few Ecosystem Disrupting Algal Blooms (EDABs), which disturb the transfer of energy and nutrients to the higher trophic levels, hence negatively impact the ecosystem structure and function(Sunda et al., 2006).

*A. anophagefferen*s is responsible for feeding cessation and starvation of juvenile hard clam *Mercenaria mercenaria*(Bricelj et al., 2001)(Bricelj and MacQuarrie, 2007), blue mussels *Mytilus edulis*(Tracey, 1988) and bay scallops *Argopecten irradians*. Since *A. anophagefferens* affects some organisms more adversely than others, chronic bloom occurrence can change the species composition or biodiversity in an ecosystem(Gobler et al., 2004).

The severe light attenuation caused by brown tide leads to significant loss of eelgrass beds (*Zostera marina*)(Cosper et al., 1987) because of relatively higher light

requirement for the survival of eelgrass(Dennison et al., 1993). Survival of eelgrass is important because it serves as nursery habitat and predator refuge for many benthic invertebrates e.g. bay scallops(Pohle et al., 1991).

A. anophagefferens bloom period also coincides with spawning, larval development and juvenile growth of many commercially important bivalves and threatens reproductive success and early recruitment(Bricelj and Lonsdale, 1998).

1.2.5.2 Enormous Economic Loss. Brown tides exert substantial monetary effects on regional economies. The extensive failure of bay scallops in Peconic and Gardiner bays of Long Island in 1985 caused annual dockside value of scallops to drop to $27,000 in 1986 as compared to $ 2 million in early 1980s. Chronic occurrence of brown tides have been associated with decline in hard clam fishery as well. Diminishing hard clam production prompted Blue Point shell fish company to go out of business in 2001(Gobler et al., 2005). The monetary loss is further exacerbated by perturbing coastal environment and costs of conducting bloom monitoring programs(Hoagland et al., 2002).

1.2.6 Management of Brown Tide Blooms

Management options for HABs includes prevention (reducing extent and incidence before initiation of bloom), control (stopping occurrence of bloom) or mitigation (minimizing risk to ecosystem and human health)(CENR, 2000).

Despite its huge economic and ecological effects, the research on management of brown tides is limited. The clay flocculation approach has been explored for direct control of these blooms, but it showed only limited success in removing *A. anophagefferens* cells(Sengco et al., 2001), (Yu et al., 2004). Moreover, the application of clay poses threat to the survival of benthic organisms, by reducing their food quality,

filter feeders' filtration rates, and causing growth and reproduction reduction in scallops and clams(Shumway et al., 2003).

Other possible control options include using chemicals, flocculants and biological control (for example, grazers and lytic viruses)(CENR, 2000).

This dissertation is focused on the management of brown tides by controlling them, using a natural chemical biocide hydrogen peroxide at very low dose. The systematic approach towards the development of the method is described in the following chapters.

CHAPTER 2

APPLICABILITY OF HYDROGEN PEROXIDE IN BROWN TIDE CONTROL - CULTURE AND MICROCOSM STUDIES

2.1 Introduction

Harmful algal bloom (HAB) is a growing global problem, and in recent decades it has been seen increasing not only in the bloom incidences, but also in harmful species, affected areas, impacted fisheries resources and economic losses(Anderson, 1997, Anderson, 2009, Hallegraeff, 1993). Among the physical, chemical and biological methods, only clay flocculation/sedimentation is well explored with reported field application successes in some Asian countries(Pan et al., 2006, Sengco and Anderson, 2004). Chemical methods have not been actively explored for algal bloom control, partly because of concerns on environmental acceptability of the chemicals, and the potential side effects on other organisms.(Anderson, 1997, Anderson, 2009, Boesch et al., 1997) The goal of this study is to explore the capability of one of the natural chemical biocide, hydrogen peroxide (H_2O_2), to effectively yet safely control brown tide algal blooms. The localized occurrence of brown tides with the identifiable possible initiation sites in some of the brown tide prone bays, for example Peconic and Shennecock Bay of Long Island (Anderson, 1997), (Gastrich et al., 2004), makes the direct brown tide control possible.

Hydrogen peroxide (H_2O_2) is naturally produced in sunlit surface sea water from photochemical reaction of dissolved organic matter, oxygen and trace metals (Moffett and Zafiriou, 1990);(Palenik and Morel, 1988). Reported quantum yield ranged from 4200 to 2.1 µmol H_2O_2 mol^{-1} photons at < 340 nm light(O'Sullivan et al., 2005), and in mid-summer day H_2O_2 was reported to potentially accumulate at 30-59 nM hr^{-1} for

several hours in 0.2 µm filtered seawater in Naragansett Bay, Rhode Island(Miller et al., 1995). In the coastal marine recreational water of Southern California, summer dry season concentrations of H_2O_2 averaged 122±38 nM(Clark et al., 2010). Steigenberger(Steigenberger and Croot, 2008) reported surface H_2O_2 ranged from 21 to 123 nM along a meridional transect in the eastern Atlantic Ocean. Rain water also contains H_2O_2 ; up to 57 µM, approximately 2 mg L^{-1}, were observed in wet deposition in Bermuda Atlantic Time Series Stations (Avery Jr et al., 2005). On the other hand, H_2O_2 is rapidly decomposed into water and oxygen, via enzyme reactions mediated by microorganisms, trace metal catalysis, halide, organic carbon and direct photochemical loss (Avery Jr et al., 2005)·(Drábková et al., 2007a)·(Petasne and Zika, 1997)·(Yuan and Shiller, 2001).

Hydrogen peroxide has been commonly used in industry, agriculture, aquaculture and environmental and personal hygiene applications for its oxidizing and microbiocidal properties. In environmental field, it has been used in drinking water and wastewater treatment(Barrington and Ghadouani, 2008, Barrington et al., 2011, Schrader et al., 1998) (including cyanobacteria toxin removal(Bandala et al., 2004, Cornish et al., 2000, Liu et al., 2003)) *in situ* chemical remediation and to enhance *in situ* bioremediation of contaminated water, soil and sediment(Ravikumar and Gurol, 1994). In aquatic environment, it has been approved for freshwater aquaculture usage as an antifungal and antibacterial therapeutic agent for the prevention and control of mortalities associated with external fungal infections (saprolegniasis) in cultured fish and fish eggs(Meinertz et al., 2008, Schmidt et al., 2006). H_2O_2-based products, such as sodium percarbonate, has been used as aquatic herbicides and microbiocide to manage algae, cyanobacteria, fungi

and microorganisms in water (e.g. Massachusetts(MSDEP, 2010) and New York State(NYSDEC, 2005)). Cyanobacteria were found more sensitive to H_2O_2 than eukaryotic phytoplankton(Barroin and Feuillade, 1986, Drábková et al., 2007a). Low dose hydrogen peroxide has been shown successful in controlling a cyanobacteria bloom and microcystin toxin caused by *Planktothrix agardhii.*(Matthijs et al., 2012) In the study of Matthijs et al. a special dispersal device (water harrow) was used to disperse H_2O_2 to the lake at 2 mg L^{-1} , it resulted in more than 99% reduction of bloom organism of *Planktothrix agardhii* and algal toxin microcystin within a few days, while the eukaryotic phytoplankton, zooplankton, and macro-fauna remained largely unaffected(Matthijs et al., 2012).

This study was carried out to examine the effectiveness of hydrogen peroxide in brown tide bloom control, and the safety of its application to other and possibly co-occurring phytoplankton species. Firstly, a dose was established that effectively removed laboratory cultures of brown tide alga *A. anophagefferens*; then the H_2O_2 susceptibility of *A. anophagefferens* with eleven other marine phytoplankton species was compared. Next, the response of the natural phytoplankton community to H_2O_2 exposure was examined by analyzing photosynthetic pigments of the algal community using High Performance Liquid Chromatography (HPLC). The results provided an initial assessment on the effectiveness and safety of hydrogen peroxide in brown tide bloom control.

2.2 Materials and Methods

2.2.1 Algae Species and Culture Conditions

Phytoplankton species investigated in this study were obtained from Provasoli-Guillard National Centre for Marine Phytoplankton (CCMP; now NCMA) and included the brown tide bloom alga *Aureococcus anophagefferens* (Aa), five diatoms *Phaeodatylum tricornutum* (Pt), *Minutocellus polymorphus* (Mpo), *Thalassiosira pseudonana* (Tp), *Skeletonema costatum* (Sc), and *Thalassiosira weissflogii* (Tw), two green algae *Micromonas pusilla* (Mp) and *Dunaliella tertiolecta* (Dt), two dinoflagellates *Amphidinium carterae* (Ac) and *Prorocentrum micans* (Pm), and two Prymnesiophytes *Isochrysis galbana* (Ig) and *Emiliania huxleyi* (Eh) (Table 2.1).

2.2.2 H_2O_2 Exposure and Quantification of Algal Growth Response

Hydrogen peroxide (Fluka-95321, TraceSELECT®, 30% W:W, 9.8 M, kept in refrigerator and in dark) was first diluted with sterile culture medium to 10 mM (~0.03%), then immediately added to the mid- or late-exponential phase cultures. Upon gentle mixing, the cultures (50 mL in 250 mL polycarbonate bottles) were returned to growth chamber for incubation. The nominal H_2O_2 addition was 0, 0.8, 1.6, 3.2 and 6.4 mg L^{-1} for *A. anophagefferens*, and 0, 1.6 and 6.4 mg L^{-1} for other algal species. The nominal H_2O_2 concentrations were confirmed with 10 - 15 % error using spectrophotometric method of Lu et al(Lu et al., 2011), where H_2O_2 reacted with p-nitrophenylboronic acid stoichiometrically at pH 9 producing p-nitrophenol of 19400 cm^{-1} M^{-1} absorptivity. Cultures without H_2O_2 addition served as controls. Cultures were in triplicates for *A. anophagefferens*, and duplicates for other species.

Table 2.1 Marine Phytoplankton Species Used in the Study, Listed Group Wise and in the Order of Increasing Cell Size

Species	Class	Cell Size[e] (µm)	CCMP strain number	Features
Aureococcus anophagefferens	Pelagophyceae	2-4	1984	Small spherical non-motile cell, no cell wall but a diffuse polysaccharide layer(Sieburth et al., 1988b)
Micromonas pusilla[a]	Prasinophyceae	2-3 * 2-4	1545	Small green flagellate with organic scales, naked cell(Sym and Pienaar, 1993)
Dunaliella tertiolecta[a]	Chlorophyceae	6-9	1320	Green flagellate, no cell wall but mucilage layer(Levy et al., 2007)
Phaeodactylum tricornutum[b]	Bacillariophyceae	2-4 * 12-14	1327	Pennate diatom, weakly siliceous cell wall(Levy et al., 2007)
Minutocellus polymorphus[b]	Coscinodiscophyceae	3-6	499	Centric diatom, siliceous cell wall(Levy et al., 2007)
Thalasiossira pseudonana[b]	Coscinodiscophyceae	4-5 * 4-6	1335	Centric diatom, siliceous cell wall
Skeletonema costatum[b]	Coscinodiscophyceae	4-5 * 4-6	2092	Centric diatom, siliceous cell wall(Gélabert et al., 2004)
Thalassiosira weissflogii[b]	Coscinodiscophyceae	10-12 * 12-22	1336	Centric diatom, siliceous cell wall(Gélabert et al., 2004)
Isochrysis galbana[c]	Prymnesiophyceae	2-4 * 4-6	1323	Flagellate, no cell wall(Liu and Lin, 2001);(Zhu and Lee, 1997)
Emiliania huxleyi[c]	Prymnesiophyceae	4-6 * 4-8	374	Coccolith absent[e]
Amphidimium caterae[d]	Dinophyceae	9-13 * 12-18	1314	Dinoflagellate, thecal plate absent(Tomas, 1997)
Prorocentrum micans[d]	Dinophyceae	25-30 * 35-45	1589	Dinoflagellate, cellulose thecal plate present(Tomas, 1997)

[a]Green algae, [b]Diatoms, [c]Prymnesiophytes, [d]Dinoflagellates. [e]https://ncma.bigelow.org/strain information

Parameters reflecting algal growth were monitored typically at 0 hr (immediately before adding H_2O_2), 3 hr, and then daily up to 1 week after H_2O_2 addition. The *in vivo*

fluorescence (IVF) was monitored using Turner Design's Trilogy Fluorometer (excitation 485 nm, emission 685nm, band width 50nm). Cell density was measured by Coulter Counter Multisizer 3(with 70 µm aperture tube, Beckman). Total chlorophyll a (chl a)was measured with GF/F filtration, 90% acetone extraction and UV-VIS spectrophotometer (Agilent 8453) quantification using Jeffery and Humphrey's trichromatic method following EPA method 446.0.

2.2.3 Statistical Analysis

Throughout the study, Minitab 16 Software Package was used in statistical analysis with probability $p<0.05$ being accepted as statistically significant. Two-sample t-tests were used to compare the control cultures and the H_2O_2 treated cultures. Paired t-tests and/or ANOVA (analysis of variance) were used to compare temporal variation of the cultures. Exponential growth or log-linearity was determined with regression analysis. Regression was also used to determine the correlation between percent inhibition and both cell size and H_2O_2 dose.

2.2.4 Microcosm Study

Natural seawater was collected from Barnegat Bay, NJ, which is prone to summer brown tide, by pumping surface seawater into 20-L carboy (pre-cleaned with detergent and acid) from a boat on July 28, 2010. (The work does not involve endangered or protected species). Collected seawater was kept in a cooler and stored in cold room (4°C) for processing next day. The upper Barnegat Bay summer phytoplankton typically consists of small coccoidal picophytoplankton compex (< 5 µm) *Aureococcus-Chlorella-Nannochloris-Synechococcus* complex, other small flagellates of 3-7 µm size

(chrysophyte e..g. *Calycomonas oval*, prasinophyte e.g. *Micromonas sp*.), prymnesiophytes such as *Chrysochromulina sp*., diatoms of small or large sizes (e.g. *Minutocellus polymorphus, Thalassiosira sp., Cyclotella sp*.), and small numbers of larger species (> 15 µm) e.g. euglenophyte *Euglena sp*., and raphidophyte *Heterosigma carterae* (Olsen and Mahoney, 2001).

In order to test cell size dependence, if any, of H_2O_2 action, a fraction of the collected natural seawater passed through 5 µm PTFE membrane filters (Millipore) under low vacuum pressure (< 5 psi). Unfiltered and filtered seawater microcosms were set up by distributing 450 ml of seawater into 500 mL polycarbonate bottles (sterilized), and subsequently inoculating 35000 cell mL^{-1} cultured *A. anophagefferens* (to mimic medium density brown tide bloom) (Gastrich et al., 2004). H_2O_2, freshly prepared in sterile Aquil at 10mM, was added at 1.6 and 6.4 mg L^{-1}. The controls (microcosm without H_2O_2 addition) and 1.6 mg L^{-1} H_2O_2 treatments were in triplicates and those with 6.4 mg L^{-1} were not replicated. Microcosms were incubated in the growth chamber as in culture study.

Total phytoplankton growth, *A. anophagefferens* cell density, and phytoplankton community composition were analyzed at 0 hr (immediately before H_2O_2 addition), and then daily up to 72 hrs of H_2O_2 addition. Total phytoplankton growth was analyzed via *in vivo* chlorophyll fluorescence and total chl a. Changes in phytoplankton community composition were tracked via accessory pigment analysis: 19'-butanoyloxyfucoxanthin (19'-bf) for brown tide alga *A. anophagefferens* (Trice et al., 2004), fucoxanthin (fuco) for diatoms and chrysophytes, chlorophyll b (chl b) for chlorophytes and prasinophytes, peridinin for dinoflagellates, and zeaxanthin for cyanobacteria respectively (Andersen et

al., 1996);(Tester and Mahoney, 1995). *A. anophagefferens* cell density was further estimated from a standard curve that correlated the number of cultured *A. anophagefferens* cells used in pigment analysis and the resulting chromatographic peak area of 19'-bf.

Accessory pigment analysis followed Heukelem and Thomas' HPLC method (Van Heukelem and Thomas, 2001). Briefly, 75 mL of microcosm water sample was filtered through GF/F (nominal pore size 0.7 µm, 25 mm, Whatman, vacuum pressure <5 psi) and the filters were immediately preserved in liquid nitrogen. During analysis, the filters were put in 2-mL 90% acetone in amber micro-centrifuge vials (VWR 20170-084) and sonicated in ice-cold water bath for 30 minutes (Ultrasonic FS-28, Fischer Scientific) to extract the pigments. The acetone extracts were centrifuged and supernatants were filtered through 0.45 µm PTFE syringe filters (4 mm, Pall Life Sciences) for HPLC analysis. These operations were carried out in dim light to minimize photo-degradation of pigments. A Waters HPLC system equipped with Waters 2690 separation module, refrigerated auto sampler, Eclipse XDB C8 column (150 * 4.6 mm, 3.5 µm, Agilent) and guard column, column heater, Waters 486 UV-VIS absorbance detector set at 450 nm, and SRI Model 333 USB Chromatography Data System, was used in pigment analysis following Heukelem and Thomas (Van Heukelem and Thomas, 2001). Pigments were identified by comparing retention times of the standards, including chlorophylls a, b, β-carotene (Sigma Aldrich), and fucoxanthin (DHI group, Denmark). Other pigments were identified by comparison with the culture extracts from *A. anophagefferens*, *Amphidinium carterae*, *Synechococcus sp*. and *Dunaliella tertiolecta* for 19'-bf, peri, zea and chl b respectively.

2.3 Results

2.3.1 Effect of Hydrogen Peroxide on *A. anophagefferens* Growth

Hydrogen peroxide at 1.6 mg L^{-1} effectively inhibited *A. anophagefferens* growth as was observed with *in vivo* chlorophyll a fluorescence, total chl a, and cell density. The inhibition was transient at 0.8 mg L^{-1} but was lasting at $\geq$ 1.6 mg L^{-1} H_2O_2 (Figure 2.1a). The starting cultures were in the late exponential phase of growth, with IVF averaged 2565±35 (relative fluorescence unit, 15 cultures), total chl a 41.5±1.2 µg L^{-1}, and cell density 1.98±0.4 million cell mL^{-1}. The control cultures experienced 4.5-fold log linear increase in IVF during 1-week incubation (Ln F = 7.87 + 0.20t, where F is IVF, t is time in days, R^2 = 0.989, p=0.000), but the cultures with 0.8 mg L^{-1} H_2O_2 addition had a decrease in IVF nearly 50% at 24-hr (p = 0.001) which then rebounded at a rate comparable to that of control cultures (p = 0.975). More importantly, the cultures with 1.6, 3.2 and 6.4 mg L^{-1} H_2O_2 addition showed 98%, 99.7% and 99.7% (p = 0.000) decrease in IVF at 24-hr and re-growth was not observed over 1 week (Figure 2.1a). Total chl a in these cultures (with 1.6, 3.2, 6.4 mg L^{-1} H_2O_2) decreased more than 90% ($p \leq 0.002$) at 24-hr, and were below detection limit at 1-week (Figure 2.1b). In the cultures with 1.6 mg L^{-1} H_2O_2 addition, cell density decreased log- linearly (Log cell/L = 9.06-0.138t, where t is time in days, p = 0.000) from nearly 2 to 0.5, 0.34 and 0.16 million cells mL^{-1} at 24-hr, 48-hr and 1-week respectively, and in the cultures with 3.2 and 6.4 mg L^{-1} H_2O_2 addition, it decreased > 90% to below 0.19±0.2 million cells mL^{-1} at and after 24-hr (Figure 2.1c).

Medium effective concentration (EC_{50}) was calculated from IVF, based on logistic model $R = \frac{K}{1+e^{n(\log C - \log EC_{50})}}$ where R is growth response (% inhibition in IVF

relative to control cultures), K is the growth response of control (K = 1), C is H_2O_2 concentration (mg L^{-1}), EC_{50} is medium effective concentration. The resulting EC_{50}s for *A. anophagefferens* were 1.39±0.06 mg L^{-1} at 3-hr, and 0.91±0.00 mg L^{-1} at 24-hr and remained at this level during 1-week incubation.

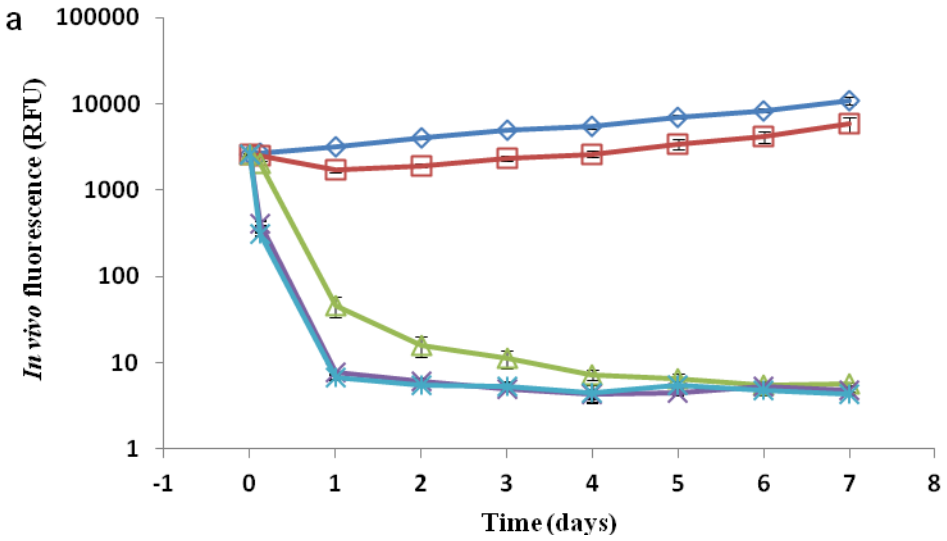

Figure 2.1 Response of *Aureococcus anophagefferens* cultures to the H_2O_2 addition at 0 (control, ◊), 0.8 (□), 1.6 (Δ), 3.2 (x) and 6.4 (*) mg L^{-1} H_2O_2. a) *In vivo* chlorophyll a fluorescence. b) Total chlorophyll a. c) Cell density. Vertical error bars represent standard deviations of triplicate cultures.

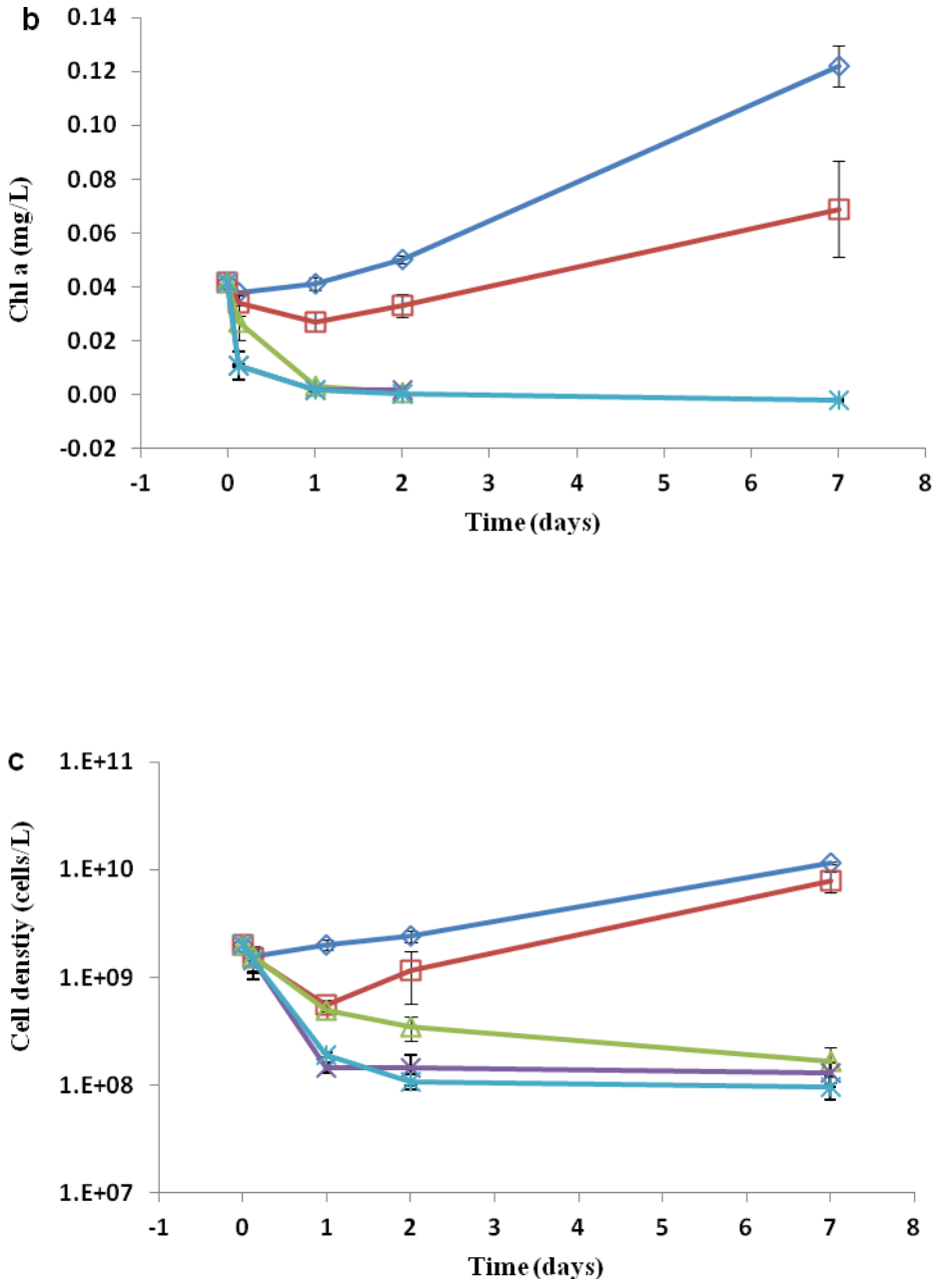

Figure 2.1 (Continued) Response of *Aureococcus anophagefferens* cultures to the H_2O_2 addition at 0 (control, ◊), 0.8 (□), 1.6 (Δ), 3.2 (x) and 6.4 (*) mg L-1 H_2O_2. a) *In vivo* chlorophyll a fluorescence. b) Total chlorophyll a. c) Cell density. Vertical error bars represent standard deviations of triplicate cultures.

Overall, *A. anophagefferens* growth response suggested that H_2O_2 at 1.6 mg L^{-1} or higher concentrations effectively removed high density A. *anophagefferens* within 24-hr

of exposure (> 90% in total chl a), and re-growth was not observed during the 1-week incubation.

2.3.2 Growth Response of Other Phytoplankton Species to H_2O_2 Exposure

Because effective (> 90% in total chl a) *A. anophagefferens* removal was accomplished by 1.6 mg L^{-1} H_2O_2 treatment, comparisons on survival and re-growth of *A. anophagefferens* with 11 other marine phytoplankton species (5 diatoms, 2 green algae, 2 prymnesiophytes and 2 dinoflagellates) were carried out, by exposing the growing or grown cultures of similar *in vivo* chlorophyll fluorescence (thus similar initial biomass) to 1.6 mg L^{-1} H_2O_2.

Among these 11 phytoplankton species, only *Micromonas pusilla* (*Mp*) (a very small green alga, class of Prasinophyceae) was eradicated by 1.6 mg L^{-1} H_2O_2. The IVF of *Mp* decreased > 90% within 24-hr of 1.6 mg L^{-1} H_2O_2 addition, and remained low during the 1-week observation, similar to *A. anophagefferens* tested in parallel (Figure 2.2a). A toxic dinoflagellate, *A. carterae* (*Ac*) had an overall 17% lower growth rate as compared to its control cultures ($p = 0.018$, Figure 2.2e).

The growth of rest 9 species i.e., the diatoms *Pt, Tp, Sc, Mpo* and *Tw* (Figure 2.2b), the other green algae *Dt* (Figure 2.2c), the prymnesiophytes *Ig* and *Eh* (Figure 2.2d), and the dinoflagellate *Pm* (Figure 2.2e), were not affected by 1.6 mg L^{-1} H_2O_2 addition, as compared to respective controls ($p > 0.05$, two-sample t-tests on growth rates for *Pt, Ig, Dt* and *Pm*, which grew exponentially with and without H_2O_2 addition, and two sample t-tests on IVF at each time point for *Mpo, Tp, Sc, Tw, Eh* which didn't grow exponentially during 1-week observation. The exponential growth status was determined with log-linearity regression analysis with $r \geq 0.88$, $n = 4$, $p < 0.05$).

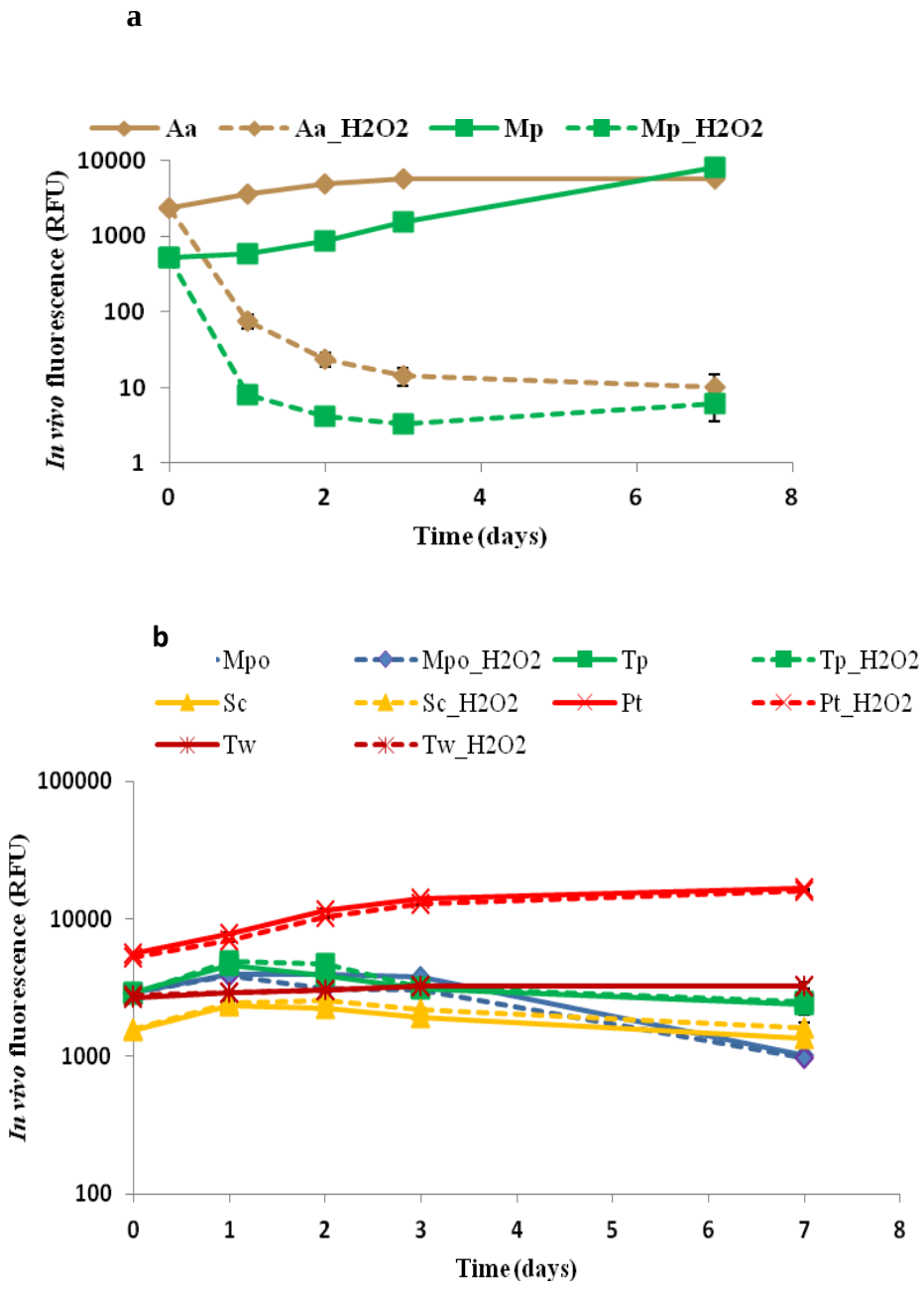

Figure 2.2 Variation of *in vivo fluorescence* of 12 marine phytoplankton species upon H_2O_2 addition at 0 (control, solid line) and 1.6 mg L^{-1} (treatment, dashed line). a) *Aureococcus anophagefferens* (*Aa*) and prasinophyte *Micromonas pusilla* (*Mp*). b) Diatoms *Phaeodactylum tricornutum* (*Pt*), *Minutocellus polymorphus* (*Mpo*), *Skeletonema costatum* (*Sc*), *Thalassiosira pseudonana* (*Tp*) and *Thalassiosira weissflogii* (*Tw*). c) Chlorophyte *Dunaliella tertiolecta* (*Dt*). d) Prymnesiophytes *Isochrysis galbana* (*Ig*) and *Emiliania huxleyi* (*Eh*). e) Dinoflagellates *Amphidinium carterae* (*Ac*) and *Prorocentrum micans* (*Pm*). Vertical error bars are deviations between duplicate cultures.

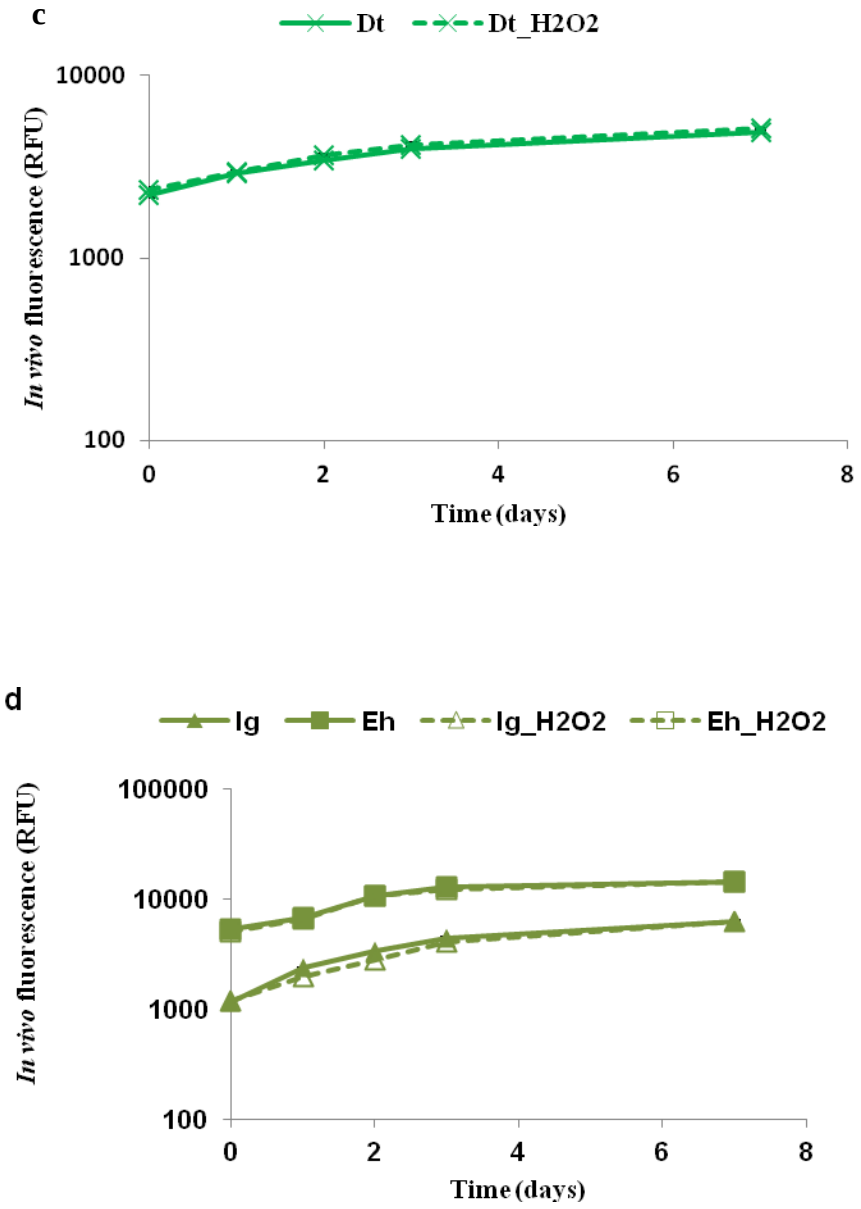

Figure 2.2 (Continued) Variation of *in vivo fluorescence* of 12 marine phytoplankton species upon H_2O_2 addition at 0 (control, solid line) and 1.6 mg L^{-1} (treatment, dashed line). a) *Aureococcus anophagefferens* (*Aa*) and prasinophyte *Micromonas pusilla* (*Mp*). b) Diatoms *Phaeodactylum tricornutum* (*Pt*), *Minutocellus polymorphus* (*Mpo*), *Skeletonema costatum* (*Sc*), *Thalassiosira pseudonana* (*Tp*) and *Thalassiosira weissflogii* (*Tw*). c) Chlorophyte *Dunaliella tertiolecta* (*Dt*). d) Prymnesiophytes *Isochrysis galbana* (*Ig*) and *Emiliania huxleyi* (*Eh*). e) Dinoflagellates *Amphidinium carterae* (*Ac*) and *Prorocentrum micans* (*Pm*). Vertical error bars are deviations between duplicate cultures.

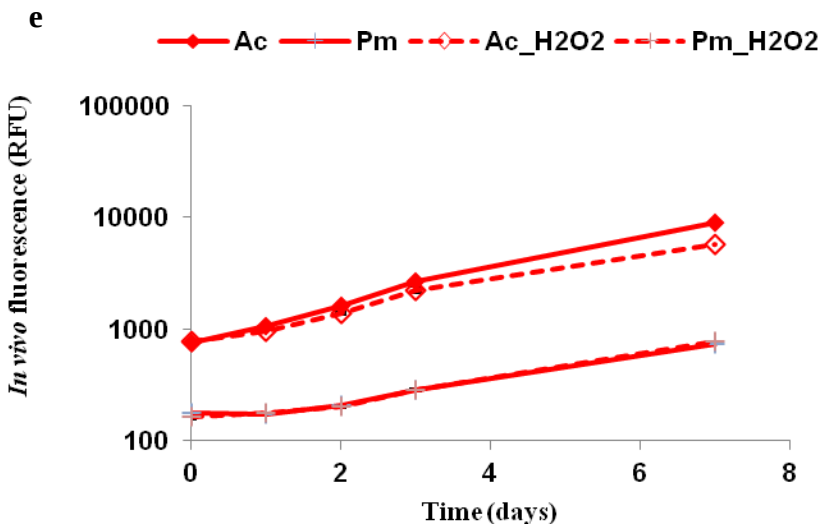

Figure 2.2 (Continued) Variation of *in vivo fluorescence* of 12 marine phytoplankton species upon H_2O_2 addition at 0 (control, solid line) and 1.6 mg L^{-1} (treatment, dashed line). a) *Aureococcus anophagefferens* (*Aa*) and prasinophyte *Micromonas pusilla* (*Mp*). b) Diatoms *Phaeodactylum tricornutum* (*Pt*), *Minutocellus polymorphus* (*Mpo*), *Skeletonema costatum* (*Sc*), *Thalassiosira pseudonana* (*Tp*) and *Thalassiosira weissflogii* (*Tw*). c) Chlorophyte *Dunaliella tertiolecta* (*Dt*). d) Prymnesiophytes *Isochrysis galbana* (*Ig*) and *Emiliania huxleyi* (*Eh*). e) Dinoflagellates *Amphidinium carterae* (*Ac*) and *Prorocentrum micans* (*Pm*). Vertical error bars are deviations between duplicate cultures.

Exposure of these species to H_2O_2 at 6.4 mg L^{-1} revealed cell size dependent species sensitivity (Table 2.2). It was found that the smaller the cell size, the more sensitive it is to H_2O_2. For example, among the diatoms, with decreasing cell size (*Tw* > *Sc* > *Tp* > *Mpo* > *Pt*) the percent inhibition increased from non-inhibitive in *Sc* and *Tw* to more than 90% inhibition in *Pt* (Table 2.2). An empirical correlation was found statistically significant: % inhibition = 0.688-0.0561*cell diameter + 0.00262*H_2O_2/chl a ($p = 0.005$, $n = 12$, $R^2 = 0.69$) where inhibition is the average % inhibition at 24-hr, 48 hr and 72-hr upon 6.4 mg L^{-1} H_2O_2 addition as compared to the control, cell diameter is the mean cell size measured by Coulter Counter (μm), and H_2O_2/chl a is the weight ratio, mg

H_2O_2/mg chl a, at time zero. Since dinoflagellate *Pm* (45 µm) is substantially larger than rest of the species (1.1-11.05 µm), a correlation of 11 species excluding *Pm* was also established, % inhibition = 0.913-0.0952*cell diameter + 0.00224*H_2O_2/chl a ($p = 0.006$, $n = 11$, $R^2 = 0.72$), to reflect species sensitivity as a function of cell size and H_2O_2 dose. In this regression, the cell diameter alone was found to explain 40% of the variations.

Table 2.2 Mean Cell Diameter as Measured by Coulter Counter, and Percent Growth Inhibition (mean ± standard deviation) at 24-hr, 48-hr and 72-hr After the Addition of 6.4 mg L^{-1} H_2O_2, as Compared to Controls, of 12 Phytoplankton Species Tested

	Brown Tide Algae	**Green Algae**		**Diatoms**					**Prymnesio-phytes**		**Dinoflagellates**	
Species	Aa	Mp	Dt	Pt	Mpo	Tp	Sc	Tw	Ig	Eh	Ac	Pm
Cell diameter (µm)	1.83	1.4	5.5	3.4	3.5	3.8	6.6	11.05	3.6	3.7	9	45
% inhibition	99.8 ±0.2	99.4 ±0.4	1.3 ±8.1[a]	96.0 ±3.5	89.8 ±12.3	71.5 ±13.5	9.6 ±11.9[a]	2.6 ±2.6[a]	98.6 ±0.9	95.9 ±4.9	95.7 ±6.4	11.2 ±8.5

[a] The inhibitive effect is not statistically significant.

2.3.3 Phytoplankton Community Response to H_2O_2 Exposure in Microcosm Study

A. anophagefferens cell density was estimated at 27,900 ± 5,379 cell mL^{-1} (n = 2) in the original Barnegat Bay water samples, and at 54,150 ± 3,188 cell mL^{-1} in the testing microcosms before H_2O_2 addition (n = 12, laboratory cultured *A. anophagefferens* was added to the microcosms). In the control microcosms (i.e., no H_2O_2 addition), *A. anophagefferens* cell density increased 60% at 24-hr as compared to time zero ($p < 0.05$), and remained nearly constant at 24-hr, 48-hr and 72-hr ($p > 0.05$, one way ANOVA, for

both unfiltered and 5-µm filtered seawater microcosms), suggesting a developing brown tide bloom (Figure 2.3a).

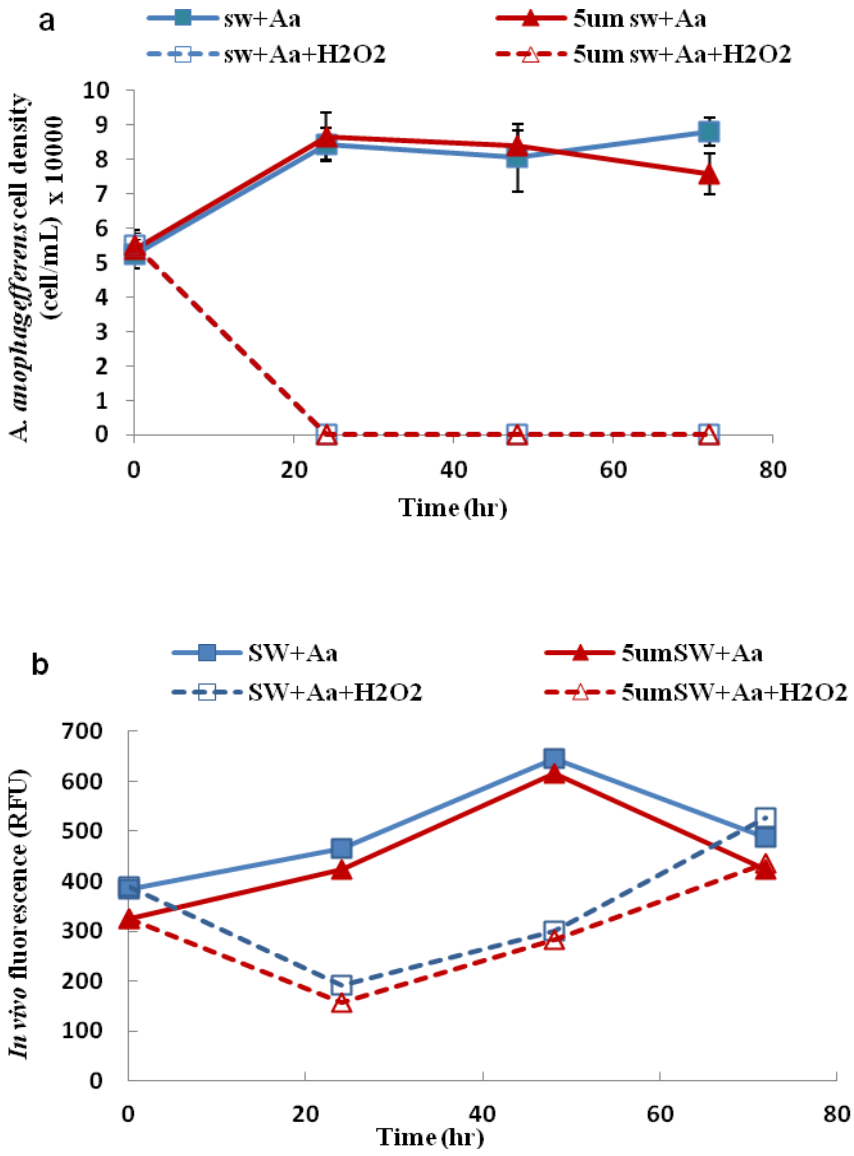

Figure 2.3 Variation in *Aureococcus anophagefferens* a) cell density, b) *in vivo* chlorophyll fluorescence, c) total chlorophyll a, in microcosms with 1.6 mg L^{-1} of H_2O_2 addition (treatment, dashed line) and without H_2O_2 addition (control, solid line). The microcosms were made of unfiltered (square symbols) and 5 µm filtered (triangle symbols) Barnegat Bay seawater amended with laboratory *A. anophagefferens* cultures. Vertical error bars represent standard deviations of triplicate microcosms.

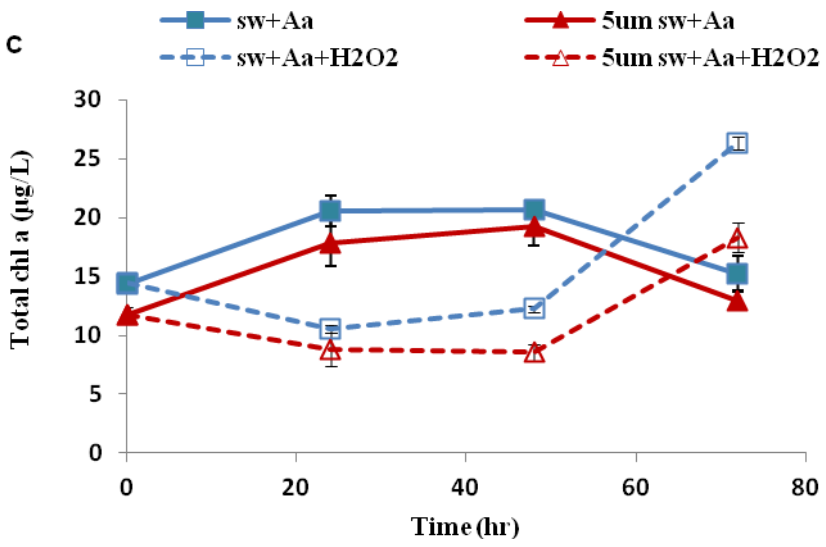

Figure 2.3 (Continued) Variation in *Aureococcus anophagefferens* a) cell density, b) *in vivo* chlorophyll fluorescence, c) total chlorophyll a, in microcosms with 1.6 mg L^{-1} of H_2O_2 addition (treatment, dashed line) and without H_2O_2 addition (control, solid line). The microcosms were made of unfiltered (square symbols) and 5 µm filtered (triangle symbols) Barnegat Bay seawater amended with laboratory *A. anophagefferens* cultures. Vertical error bars represent standard deviations of triplicate microcosms.

In microcosms with 1.6 mg L^{-1} H_2O_2 addition, *A. anophagefferens* was eradicated (~100%) at 24-hr, and its cell density remained low during the entire 72-hr study period, in both unfiltered and 5-µm filtered seawater microcosms (Figure 2.3a).

While *A. anophagefferens* was eradicated in the phytoplankton assemblage, total phytoplankton productivity remained robust after a transient suppression at 24-hr and 48-hr (Figure 2.3b). Specifically, after 1.6 mg L^{-1} H_2O_2 addition, the IVF was 50 – 60% lower than the controls at 24-hr and 48-hr ($p \leq 0.001$), but recovered, at 72-hr, to a level comparable to ($p = 0.333$, the 5 µm filtered microcosms,) or even higher ($p = 0.035$, the unfiltered microcosms) than those in the control microcosms (Figure 2.3b). The transient initial suppression and subsequent recovery was also evident from total chl a. Total chl a

was reduced 40 – 55% at 24-hr and 48-hr, as compared to the control, but recovered to exceed the control by 40% and 70% in 5 μm filtered and unfiltered microcosms, respectively ($p < 0.01$) (Figure 2.3c).

The response of the rest of the phytoplankton assemblage to 1.6 mg L^{-1} H_2O_2 treatment was estimated from algal marker pigments, fucoxanthin for diatoms and other chrysophytes, chl b for green algae, peridinin for dinoflagellates and zeaxanthin for cyanobacteria (Table 2.3). In the unfiltered seawater microcosms, fucoxanthin was reduced (by 1.6 mg L^{-1} H_2O_2) by 55% at 24-hr ($p = 0.006$) and 44% at 48-hr ($p = 0.000$), but subsequently recovered and exceeded the controls by 104% at 72-hr ($p = 0.003$); chl b was reduced approximately 55% at 24-hr and 48-hr ($p < 0.01$) then recovered to a level not significantly different from the controls ($p = 0.267$) (Table 2.3). In the 5 μm filtered seawater microcosms, fucoxanthin was reduced about 58% at 24-and 48-hr ($p < 0.01$) then exceeded the controls by 66% at 72-hr ($p = 0.000$); chl b was exceeded by the control by 54 - 67% at 24-hr and 48-hr ($p < 0.01$) and then to a less degree of 17% at 72-hr ($p = 0.018$) (Table 2.3). Peridinin and zeaxanthin were not always detected; but where they were quantifiable in all triplicates, peridinin seemed to be reduced by 34% at 24-hr then recovered to a level comparable to the control at 48-hr in unfiltered seawater microcosms, and zeaxanthin was still reduced approximately 48% and 55% at 72-hr ($p < 0.05$) in unfiltered and 5 μm filtered seawater microcosms, respectively (Table 2.3). Altogether, above results suggest transient reduction but robust re-growth of diatoms and green algae in both pico, nano- and micro-phytoplankton communities.

Table 2.3 Percent Decrease of the Marker Pigment Concentrations (Mean ± Standard Deviation of Triplicate Microcosms, and the Probability) Treated with 1.6 mg L^{-1} H_2O_2, as Compared to the Control Without H_2O_2 Addition, in Microcosms with Unfiltered and 5-μm Filtered Seawater

	Fucoxanthin	**Chlorophyll b**	**Peridinin**	**Zeaxanthin**
Microcosms with unfiltered seawater				
0 hr	2±3 % (0.248[a])	3±4% (0.452)	NA	15±5% (0.023)
24 hr	55±2% (0.006)	53±2% (0.007)	34±5% (0.010)	17±12% (0.136)
48 hr	44±1% (0.000)	58±1% (0.001)	18±18% (0.292)	NA
72 hr	-104[b]±3% (0.003)	8±9% (0.018)	NA	48±8% (0.012)
Microcosms with 5 μm filtered seawater				
0 hr	4±4% (0.224)	0±6% (0.939)	22±15% (0.289)	0±9% (0.978)
24 hr	60±7% (0.224)	54±6% (0.003)	NA	NA
48 hr	57±5% (0.002)	67±2% (0.003)	NA	NA
72 hr	-66[b]±5% (0.000)	17±4% (0.018)	NA	55±4% (0.010)

[a]Values in parenthesis are probabilities of the two sample t-tests; $p < 0.05$ suggests the percent change is statistically significant.

[b]A negative percentage value indicates pigment concentrations were greater in treatment microcosms than controls.

NA indicates pigment quantification might not be accurate as at least one of the triplicates went below detection limit.

Cell size dependent action of H_2O_2 was also evident, as the phytoplankton groups in 5 μm filtered seawater microcosms (dominated by pico-phytoplankton) experienced slightly greater percent inhibition and/or less robust recovery as compared to that in unfiltered seawater microcosms (which was a mix of pico-, nano- and micro-phytoplankton) (Table 2.3, and Figure 2.3b, and c).

A limited numbers of microcosms were exposed to 6.4 mg L^{-1} H_2O_2. As expected, 6.4 mg L^{-1} H_2O_2 completely removed 19'-bf pigment during the 72-hr study period, suggesting *A. anophagefferens* eradication. Chl a, fucoxanthin, chl b, peridinin were still exceeded by the controls at 72-hr by 64 - 71%, 93 - 97%, 5 - 28% and 49%, respectively and zeaxanthin went below detection limit during 24 – 72 hr (data not shown).

2.4 Discussion

This study presents strong evidence, from culture and microcosm studies, that low dose of H_2O_2 (e.g., 1.6 mg L^{-1}) effectively removes more than 90% of brown tide alga *Aureococcus anophagefferens* even at peak bloom density, without devastating most of the other potentially coexisting phytoplankton species and the natural phytoplankton community. Mechanisms of the differential susceptibility among phytoplankton and a realistic application scenario are discussed below.

2.4.1 Differential Phytoplankton Susceptibility to Hydrogen Peroxide

A. anophagefferens demonstrated sensitivity similar to those of cyanobacteria. For example the EC_{50} were 0.3 – 0.4 mg L^{-1} for *Microcystis aeruginosa* and *Trichormus variabilis* and were between 1.1 and 1.7 mg L^{-1} for *Synechococcus nidulans* and two other cyanobacteria species on photosynthetic yield over 3-hr H_2O_2 exposure, (Drábková et al., 2007c) in comparison with the EC_{50} of 1.39 mg L^{-1} on *in vivo* fluorescence based growth inhibition of *A. anophagefferens* over 3-hr exposure. Cyanobacteria has been shown to be more sensitive than most eukaryotic phytoplankton,(Barrington and Ghadouani, 2008, Barroin and Feuillade, 1986, Drábková et al., 2007a, Drábková et al., 2007c) partly because of their prokaryotic photosynthetic apparatus.(Drábková et al.,

2007c) The high sensitivity of *A. anophagefferens* to H_2O_2 may be attributed to both its small cell size and the fact that it lacks cell wall. *A. anophagefferens* has only a diffuse layer of polysaccharide external to plasma membrane.(Sieburth et al., 1988b) As a result, H_2O_2 can easily penetrate into the cell, and damage the cell by producing hydroxyl radicals which inhibit the photosynthetic electron transfer and the photosynthetic activity (Drábková et al., 2007c) among other effects.

The cell size dependent H_2O_2 sensitivity is likely caused by both the surface area to volume ratio being a function of cell size, and the non-selective reactivity of H_2O_2 and hydroxyl radical (·OH) product. The ·OH, a product of H_2O_2 interacting with ferrous ion via Fenton reactions (Walling, 1975) (Fe^{2+} may be formed via extracellular Fe(III) reduction (Shaked et al., 2005)), is much more reactive than H_2O_2. It reacts with a wide range of organic compounds (alcohols, esters, unsaturated organics, aromatics, chlorinated hydrocarbons etc.) very rapidly with a second order reaction rate constant in the range of 10^7 -10^9 $M^{-1}S^{-1}$.(Ravikumar and Gurol, 1994, Dorfman and Adams, 1973) The ·OH potentially initiates radical chain reactions such as hydrogen abstraction and double bond addition, causing damage to bio-molecules and cellular components on cell membrane, chloroplast (Drábková et al., 2007a, Drábková et al., 2007c) and DNA (Imlay and Linn, 1988), eventually leading to oxidative stress and cell death. In terms of total cell surface area, cultures of the same biomass concentration but smaller cell size, as compared to larger-cell cultures, would have greater total surface area exposed to H_2O_2 and ·OH. As a result, the damage would be more extensive.

Other than cell size, the cellular structure, particularly the presence of rigid cell wall, may contribute to a species' H_2O_2 resistance. For example, the silica-mucilage cell

wall of the diatoms (Gélabert et al., 2004, Hecky et al., 1973) may have given *Sc* and *Tw* their H_2O_2 resistance (diameter 6.6 and 11.05 μm, respectively not affected by 6.4 mg L^{-1} H_2O_2), as compared to the athecate dinoflagellate *Ac* of similar cell size (diameter 9 μm, but showed > 90% inhibition by 6.4 mg L^{-1} H_2O_2) (Table 2.1 and 2.2). The prymnesiophyte *Ig* lacks cell wall (and is often used in aquaculture for easy assimilation by larval animals because of *Ig*'s small cell size and lack of tough cell wall),(Liu and Lin, 2001, Zhu and Lee, 1997) while *Eh* (CCMP 374) is non-coccolith forming strain (https://ncma.bigelow.org/, strain information); and both are more sensitive to H_2O_2 (3.6 and 3.7 μm, respectively showed > 95% inhibition by 6.4 mg L^{-1} H_2O_2) than the diatoms of similar cell size e.g. *Mpo* (3.5 μm, 90% inhibition) and *Tp* (3.8 μm, 72% inhibition) (Table 2.1 and 2.2). The rigid cell wall, if present, potentially provides a first layer of defense against H_2O_2 and $HO\cdot$, and allowing them to be inactivated before reaching the more sensitive and biologically critical components of cell membrane and organelles.

Likewise, Jeong et al.(Jeong et al., 2002) found log-log relationship between the mortality rate of aquatic organisms (red tide dinoflagellates, diatoms, ciliates, copepods, fin-fish, brine shrimp and shellfish) and organisms' bio-volume, when exposed to hypochlorite, another small molecule oxidizing biocide. They further reported that diatoms were more resistant to hypochlorite than dinoflagellates even if they were smaller than the dinoflagellates in their study. This was attributed to siliceous cell wall and the large vacuole volume of diatom cells.(Jeong et al., 2002)

Hydrogen peroxide may be inactivated and detoxified by organisms' reactive oxygen species scavenging systems. Once H_2O_2 penetrates into a cell, reactive oxygen species scavenging systems would decompose and inactivate the excessive amounts of

H_2O_2.(Asada, 1999, Noctor et al., 2000) Glutathione peroxidase, ascorbate peroxidase,(Hossain and Asada, 1984) catalase,(Wolfe-Simon et al., 2005) and various antioxidants such as ascorbate,(Noctor et al., 2000) glutathione (Ahner et al., 2002) and even mycosporine and mycosporine-like amino acids (Garcia-Pichel and Castenholz, 1993, Neale et al., 1998) likely participate in H_2O_2 and HO· scavenging. This, along with the capability of the cell to repair and replace the damaged bio-molecules and cellular components, permits the culture to re-grow, as was observed in both the culture and the microcosm studies at relatively low H_2O_2 exposure.

2.4.2 A Consideration of Realistic Scenario

Ex situ treatment of coastal algal bloom could be very costly given the enormous large size of water to be treated. For example if a bay water of 5×5 km^2 with a depth of 1 m (2.5×10^{10} L or 6500 million gallons of water) is to be treated *ex situ* within 5 days, it would require a treatment capacity of 1300 million gallon per day (mgd), nearly 4 times that of Passaic Valley Sewage Commissioner, New Jersey, which is among the largest wastewater treatment facilities in Eastern US. Further, if the water is to be retained for 1 day before disposal, it would need 1300 million gallon reactor, representing an enormous size.

In situ treatment should be more feasible, although challenges exist. To treat brown tide bloom at 1.6 mg L^{-1} H_2O_2, one challenge is to disperse the concentrated H_2O_2 stock solution homogenously into seawater with minimal localized high concentration. In our study the stock solution (30% H_2O_2) was diluted 1000-fold before adding to the cultures. This dilution scheme requires an addition of 4.8-mm water containing 0.03% H_2O_2 to bloom water of 1-m depth (or 4.8 μm of 30% H_2O_2); and the wave action or a

mixing device makes the additional 200-fold dilution to arrive at 1.6 mg L^{-1}. The rate of chemical dispersal facilitated by wave action should be examined ahead of time, if possible, using hydrologic tracer chemicals such as sulfur hexafluoride (SF_6). (Busenberg and Plummer, 2010) In Lake Koetshuis cyanobacterial bloom control Matthijs et al. achieved nearly homogenous dispersal at 2 mg L^{-1} H_2O_2 using 10% H_2O_2 stock solution.(Matthijs et al., 2012) The mixing scheme was that computer controlled pumps (operated from a small boat) mixed 10% hydrogen peroxide stock solution with lake water at 500-fold dilution, which was then directly dispersed into the lake using a water harrow to obtain additional 100-fold dilution at 2 mg L^{-1}.(Matthijs et al., 2012) The homogeneity was verified within 3-hrs of dispersal.(Matthijs et al., 2012) Thus, this challenge is potentially solvable.

A second concern in *in situ* treatment is whether the efficacy of the treatment observed in the culture and microcosm studies is transferable in field applications, and further, what environmental factors may lead to variable success of bloom control. Since the OH radical is believed to be the active chemical species that attacks and kills the cells during H_2O_2 treatment, environmental factors that affect H_2O_2 decay and OH radical production likely also influence the efficacy of the treatment. Among other factors, Fe^{2+}/Fe^{3+} and UV radiation could be important, as they would promote the conversion of H_2O_2 into OH radical through Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$, $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$)(Fenton, 1894) and photolysis reaction ($H_2O_2 + h\gamma = 2\ OH\cdot$) (Crittenden et al., 1999), respectively. In this study, Fe^{3+} was well controlled at 6.6×10^{-19} M by using 100 µM EDTA and 8.3 µM total iron, and no Fe^{2+} was externally added.(Price et al., 1988/1989) In comparison, in natural seawater Fe^{3+} and total

dissolved iron (Fe_T) vary over a range of 1 – 2 orders of magnitude. For example, Peconic Estuary (an urban estuary in Long Island Sound, NY) had Fe_T at 10 – 100 nM (Breuer et al., 1999) and 28 - 237 nM averaging 114 ± 81 nM, and Fe^{3+} at 1.1×10^{-20} - 4.7×10^{-19} M averaging $1.3x10^{-19}$ M, where salinity ≥ 26 parts per thousand.(Gobler et al., 2002a) Wu et al. reported 0.45 - 6.2 nM Fe_T in northwest Atlantic coastal water from the continental slope to near the mouth of Delaware Bay (6.2 nM). (Wu and Luther Iii, 1995) Oceanic surface seawater has lower Fe_T, typically at 0.02 – 1 nM. (Wu and Luther Iii, 1995) Fe_T and Fe^{2+}/Fe^{3+} were not measured in microcosm sample taken from Barnegat Bay, NJ, for this study, but they could be similar to those of Peconic Estuary, based on reports of Fe_T in river water that discharges to these estuaries, e.g. 926 µg L^{-1} in Metedeconk River in Aug. 2003 (DeLuca et al., 2003) and > 300 µg L^{-1} during 1960 – 1997 in rivers that discharge to Barnegat Bay, in comparison to a similar level of 400 µg L^{-1} in Peconic River for Peconic Estuary. (Breuer et al., 1999). In both culture and seawater microcosm studies, more than 90% *A. anophagefferens* decline was achieved despite potentially up to 10-fold difference in Fe^{3+} concentrations in two types of media, suggesting limited variation in Fe^{3+} concentration and limited influence in shaping the outcome of bloom control in coastal water. Natural radiation (both UV and PAR, photosynthetically active radiation) likely enhance the treatment efficacy in the field. Barrington et al. examined laboratory-, mesocosm- and full-scale trials of H_2O_2 treatment for cyanobacterial bloom control in waste stabilization pond; they found significant synergistic effect of H_2O_2 addition with environmental factors (e.g. radiation) in the field trials. (Barrington et al., 2011) On the other hand, H_2O_2 may be consumed by excessive non-target materials such as dissolved humic substances leading to decrease of treatment

efficacy. Quantifying the natural fluctuation of H_2O_2 and OH radical concentrations and carrying out small-scale field trials with a range of H_2O_2 addition would likely be informative to assess the potential outcome of the full-scale field application.

In terms of environmental risk of hydrogen peroxide in natural waters, Schmidt et al. reviewed and summarized that fish and fish eggs are relatively tolerant of hydrogen peroxide, other vertebrates and mammals are much more tolerant than fish, and that microorganisms and zooplankton in aquatic ecosystem are generally less tolerant than fish or other vertebrates.(Schmidt et al., 2006). They determined that $H_2O_2 \leq 0.7$ mg L^{-1} in receiving water is not a significant threat to organisms, environmental and public health (and to be used a criterion for water quality). (Schmidt et al., 2006) Indeed the reported toxicological data (Meinertz et al., 2008, Gaikowski et al., 1999, Schmidt et al., 2006, Boettcher et al., 1997, Zhang et al., 2008)show that 1.6 mg L^{-1} H_2O_2 and transient exposure (as H_2O_2 decays rapidly in natural waters) should exert little harm to zooplankton,(Meinertz et al., 2008) shellfish and shellfish larvae,(Boettcher et al., 1997, Zhang et al., 2008) fish eggs, fingerling fish and fish of cold, cool and warm water.(Gaikowski et al., 1999, Schmidt et al., 2006) Lake Koetshuis cyanobacteria bloom control with 2 mg L^{-1} H_2O_2 also evidenced relatively low environmental risk of this treatment to aquatic organisms, as the eukaryotic phytoplankton (including green algae, cryptophytes, chrysophytes and diatoms), zooplankton and macrofauna remained largely unaffected by the treatment.(Matthijs et al., 2012) This study further evidenced relatively low risk of H_2O_2 treatment to marine phytoplankton in that a) phytoplankton cultures of $\geq$ 2 – 3 µm cell size were largely unaffected, and b) in natural seawater microcosms the diatom and the green algae were only transiently suppressed and total chlorophyll a

recovered and exceeded that of the control by 72-hr of H_2O_2 treatment. Therefore, although some eukaryotic phytoplankton could be eliminated by the H_2O_2 treatment at 1.6 mg L^{-1} (e.g. the target organism brown tide alga and the prasinophyte *Micromonas pusilla*), there would be very low risk for the majority eukaryotic phytoplankton, zooplankton, fish, larval fish, and other larger aquatic animals.

It is logical to think that aquatic bacterial community could be most sensitive to H_2O_2 treatment, as H_2O_2 is also used as a therapeutic agent in controlling aquaculture bacterial infection. (Schmidt et al., 2006) However bacteria sensitivity varies greatly and many factors including contact time, water quality and pH affect the inactivation of bacteria by H_2O_2 treatment. (Wolfe et al., 1989) Standardized bacterial culture tests showed that some bacteria had their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on the order of mg L^{-1} or higher. For example, 11 strains of 4 species of oral *Streptococci* had MIC and MBC of 3.2 – 14 mg L^{-1} and 3.5 – 28.2 mg L^{-1}, respectively (for an initial 10^6 – 10^7 colony forming units per mL of logarithmic growth phase, trypticase soy broth, and 24-hr anaerobic incubation).(García-Mendoza et al., 1993) MIC of 469 – 2500 mg L^{-1} were observed for cultures of *Bacillus subtilis*, *Bacillus stearothermophilus*, *Escherichia coli*, *Staphylococcus aureus Enterobacter cloacae*, *Serratia marcescens* and *Acinetobactev calcoaceticus*(for an initial $\geq 10^6$ CFU mL^{-1}, in tryptic soy broth, 24-hr incubation) (*E. coli* had MIC of 2505 mg L^{-1}). (Penna et al., 2001) Baldry compared the bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid and found that H_2O_2 to be a poor bactericide but bacteriostatic effect was achieved at and above 0.15 mM (5.1 mg L^{-1}) (Baldry, 1983). Studies on marine bacteria were fewer; MIC was

reported at 10 – 41 mg L^{-1} for four marine bacteria *Vibrio alginolyticus, V. harveyi, V. parahaemolyticus* and *V. vulnificus* in Mueler Hinton Broth and were 0.6 – 2.4 mg L^{-1} in 1.5% NaCl solution.(Srisapoom et al., 1999) Laboratory incubation of seawater (with 10 time lower bacterial abundance as compared to the *in situ* abundance) showed that natural background levels of H_2O_2 (0.1 - 1.35 µM, 3.4 - 46 µg L^{-1}) could cause bacterial production reduction to 25% of its control over 24-hr, and induce 1 – 3.4% lysogenic (due to virus) bacterial mortality. (Weinbauer and Suttle, 1999) Xenopoulos and Bird reported (for a humic lake in Quebec, Canada) that bacterial productivity was consistently suppressed by 3.4 mg L^{-1} H_2O_2 and was variably suppressed (dependent on the date and time of observation) at the exposure of as low as 0.0034 mg L^{-1}.(Xenopoulos and Bird, 1997) Therefore, some marine bacteria species could be very sensitive, but others such as *E. coli* very resistant. Nevertheless, bacteria are expected to inoculate from surrounding waters and quickly reproduce and repopulate the treated water once H_2O_2 is sufficiently dissipated after treatment. Thus, long lasting impacts are not expected.

Cyanobacteria community could be most severely affected by H_2O_2 treatment, as was seen in the field work of Matthijs et al. where 2 mg L^{-1} H_2O_2 eradicated cyanobacterial bloom of *Planktothrix agardhii* in the freshwater lake(Matthijs et al., 2012) . However, cyanobacterial sensitivity to H_2O_2 also varied substantially among the species. For example, EC_{50} for photosynthetic yield (3-hr H_2O_2 exposure) were 0.3 – 0.4 mg L^{-1} for *Microcystis aeruginosa* and *Trichormus variabilis* but were 4-fold higher at 1.1 - 1.7 mg L^{-1} for *Synechococcus nidulans* and two other cyanobacteria species.(Drábková et al., 2007c) Schrader et al. reported a lowest complete inhibitory concentration (LCIC) of 100 µM for sodium carbonate peroxide (equivalent to 32.5 µM

or 1.1 mg L^{-1} H_2O_2) for off-flavor producing cyanobacteria *Oscillatoria cf. chalybea* but it was 10-fold higher (equivalent to 325 µM or 11 mg L^{-1} H_2O_2) for *Anabaena sp* LP691. (Schrader et al., 1998) Kay et al. reported more than 85% bleach of chlorophyll in cyanobacteria *Raphidiopsis* sp. after 48-hr exposure to 200 µM H_2O_2 at 90 µmol m^{-2} s^{-1} but no significant bleach was observed in *Anabaena sp* under the same condition. (Kay et al., 1984) Observing substantial species variability in H_2O_2 tolerance among cyanobacteria, Schrader et al. concluded that H_2O_2-based algaecide "may be less disruptive to the aquatic ecosystem by not eliminating all of the cyanophytes so quickly as to create low dissolved oxygen levels which could stress and kill fish". (Schrader et al., 1998) In this seawater microcosm study the cyanobacterial population was not completely eliminated, but reduced about 50% by 72-hr of H_2O_2 addition (as compared to the control without H_2O_2 addition). Furthermore, the most sensitive cyanobacteria species, though eliminated by H_2O_2 treatment, likely get re-inoculated from surrounding water via natural water exchange, while the more resistant ones would persist and repopulate the treated water.

Hydrogen peroxide is rapidly decomposed into water and oxygen (Avery Jr et al., 2005, Drábková et al., 2007a, Petasne and Zika, 1997, Yuan and Shiller, 2001) and does not produce persistent chemical residues. Cooper et al.(Cooper et al., 1994) observed the half-life of hydrogen peroxide in lake water at 4.4, 4.7, 6.4, 19.1 and 58.7 hr in unfiltered, 64 µm filtered (zooplankton removed), 12 µm filtered (large algae removed), 1 µm filtered (small algae removed), and 0.2 µm filtered (bacteria removed) waters, respectively. Matthijs et al. found that hydrogen peroxide applied to Lake Koetshuis at 2 mg L^{-1} was degraded to 0.7 mg L^{-1} after one day and to below detection limit (~ 0.1 mg

L^{-1}) after two days.(Matthijs et al., 2012) Thus, it's expected that the concentration of H_2O_2, if applied at 1.6 mg L^{-1} to natural seawater, to be reduced to the natural background level within a couple of days due to decomposition and simultaneous dilution. The proposed concentration is indeed quite low as compared to a) the proposed receiving water quality criterion of 0.7 mg L^{-1},(Schmidt et al., 2006) b) the natural occurrence in rain water at e.g. 5 – 25 μM H_2O_2 in North Carolina (Mullaugh et al., 2011) or up to 57 μM (~ 2 mg L^{-1}) at Bermuda Atlantic Time Series Stations,(Avery Jr et al., 2005) and, c) up to hundreds nM background existence in surface seawater.(Clark et al., 2010, Steigenberger and Croot, 2008) The decomposition of H_2O_2 potentially improves water quality by oxygenating the water column, facilitating dissolved organic matter degradation,(Häkkinen et al., 2004) and even inactivating algal bloom toxins.(Barrington and Ghadouani, 2008) Further, in natural waters, UV-light could have synergistic effect with H_2O_2,(Drábková et al., 2007a) thus, even lower dose than 1.6 mg L^{-1} H_2O_2 may be effective in brown tide bloom control.

2.5 Conclusion

Harmful algal bloom has become an increasingly important issue to the economic development and environmental sustainability, such that it demands both direct control methods and long term prevention measures. This study evidences, from culture and microcosm studies, that 1.6 mg L^{-1} H_2O_2 effectively removes brown tide even at peak bloom intensity, and the other phytoplankton of $> 2 – 3$ μm cell size remain largely unaffected by the treatment. Thus, hydrogen peroxide is proposed as a natural and environmentally friendly agent potentially useful for brown tide control.

CHAPTER 3

EFFECT OF AUREOCOCCUS ANOPHAGEFFERENS BIOMASS ON ITS INACTIVATION BY HYDROGEN PEROXIDE: CULTURE STUDY AND EMPIRICAL MODEL

3.1 Introduction

The excessive growth of the Pelagophyte *Aureococcus anophagefferens* produces a harmful algal bloom known as brown tide. The brown tide is a recurring problem in several coastal bays in northeastern U.S., South Africa, and China (Gobler and Sunda, 2012, Zhang et al., 2012). Because of its algal toxin(s) and light attenuation, the brown tides cause substantial economic and ecological damage, for example, the declining of shellfishery and submerged aquatic vegetation in Long Island bays, NY (Gobler et al., 2005). The brown tide may be controlled by reducing nutrient runoff, increasing seawater flushing (Gobler and Sunda, 2012), and direct removal of the blooming alga using physical, chemical and biological agents (Boesch et al., 1997). The latter, although does not fundamentally solve the problem, may provide a temporary relief from the harmful impacts of the algal blooms. Randhawa et al. (2012) found that the brown tide alga *A. anophagefferens* was very sensitive to hydrogen peroxide (H_2O_2), and that treating microcosms of developing *A. anophagefferens* bloom with 1.6 mg L^{-1} hydrogen peroxide (final concentration) eradicated the bloom without substantial adverse effects on the co-occurring phytoplankton groups. This study was initiated to assess the impact of initial cell density of *A. anophagefferens* on hydrogen peroxide dosage and contact time requirements for effective bloom control, and provide an empirical mathematical model to describe *A. anophagefferens* inactivation by H_2O_2.

Generally considered as environmentally friendly, hydrogen peroxide has the potential to be used an effective chemical agent to control certain harmful algal blooms in natural waters. As was shown in cyanobacteria bloom control, hydrogen peroxide effectively and selectively inhibited the growth of the target cyanobacteria with low or no effects on other phytoplankton groups (Barrington and Ghadouani, 2008, Barrington et al., 2011, Barroin and Feuillade, 1986, Drábková et al., 2007a, Drábková et al., 2007b, Drábková et al., 2007c). Successful field applications were also reported, for example, cyanobacterial bloom of *Planktothrix agardhii* and its microcystin toxin were removed by low doses of hydrogen peroxide at 2 mg L^{-1} at more than 99% efficiency within a few days, while the eukaryotic phytoplankton, zooplankton and other macrofauna were largely unaffected (Matthijs et al., 2012). In terms of dosage requirement for effective bloom control Barrington et al. (2008) provided a ratio of 0.003 g H_2O_2 per µg chlorophyll a as a lowest H_2O_2 dosage to achieve effective removal of phytoplankton biomass in waste stabilization pond. There is a need to develop empirical models to describe the decline or inactivation of target alga as a function of key variables, for example, H_2O_2 dose, contact time, and other important factors.

In water treatment, empirical models are often used to characterize the target microorganism's response to a disinfectant, and to guide the disinfection design. The simplest empirical model is Chick–Watson model, which describes the rate of inactivation of a microorganism being dependent upon the concentration of the disinfectant and the contact time.

$$dN/dT = -k\, C^n N$$

$$\text{Ln } (N/N0) = -kC^{n}t \quad (3.1)$$

where N, N_0 are target microbial density at time t and time zero, C is the disinfectant concentration, and n, k are empirical constants. k is also known as Chick-Watson coefficient of specific lethality, n is the coefficient of dilution, when viewed from the semi-log plot of $\text{Ln}(N/N_0)$ against concentration or time. A more complicated model was Hom's model which considers a relationship between disinfectant concentration and time (Haas and Joffe, 1994 and reference therein).

$$dN/dT = -k\, C^{n}\, m\, t^{m-1}\, N$$

$$\text{Ln } (N/N0) = -k\, C^{n}t^{m} \quad (3.2)$$

where k, n and m are empirical constants; and when m = 1, Hom's model becomes Chick-Watson model; when both n =1, m = 1, it becomes the simplified Chick-Watson model .

In this study, Hom's model was applied to describe *A. anophagefferens* inactivation by H_2O_2, and assessed the effect of initial cell density on the decomposition of H_2O_2 and the inactivation of the target alga. *A. anophagefferens* cultures of a range of initial cell densities were treated with H_2O_2. The inactivation of *A. anophagefferens* and the decomposition of H_2O_2 were determined; mathematical relationships between effective inactivation, initial concentration of hydrogen peroxide and time were established. Results potentially facilitate understanding the behavior of *A.*

anophagefferens in H_2O_2 treatment, and may benefit the designing of *A. anophagefferens* control.

3.2 Materials and Methods

3.2.1 Algal Strain and Culturing

The brown tide alga *Aureococcus anophagefferens* (CCMP 1984) was obtained from Provasoli-Guillard National Centre for Marine Phytoplankton (NCMA), Maine, USA. The alga was maintained in polycarbonate bottle (Nalgene®) and in artificial seawater media Aquil (excluding Si), which was prepared and sterilized according to Price et al. (1988/1989), and was used as culture media throughout the study. Culture vessels were washed with soapy-water, rinsed with tap water then distilled water, soaked in 1 N HCl overnight, then rinsed with plenty of Milli-Q water, and dried in HEPA filtered air stream. The media and the culture were handled in HEPA filtered laminar flow hood. The alga was cultured in a diurnal growth chamber (VWR), which was set at 19±1° C and illuminated with 120 µE m^{-2} s^{-1} from four 40 W cool white fluorescence bulbs at 12 hr: 12 hr light-dark cycle.

3.2.2 Preparation of stock Culture

Aureococcus anophagefferens stock culture was started in a 1-L polycarbonate bottle by inoculating 10 mL grown (early stationary phase) culture in fresh culture media. The growth of the stock culture was monitored on daily basis for *in vivo* chlorophyll fluorescence and cell density. The *in vivo* fluorescence was measured with a Turner Designs' Trilogy Fluorometer, which was equipped with an optical block for *in vivo* chlorophyll a measurement at excitation 485 nm, emission 685 nm, and band width 50

nm. Cell density was obtained from Coulter Counter (Multisizer 3) equipped with a 70-µm aperture tube.

3.2.3 Test Culture and Exposure to Hydrogen Peroxide

A. anophagefferens stock culture of early stationary growth phase and 8.5×10^6 cell mL^{-1} was used in the exposure tests. Aliquots of the stock culture were added to fresh media in 5-mL sterile glass culture tubes (12×75 mm, polyethylene caps, pre-cleaned with the same protocol as described in "Algal strain and culturing") to get initial cell density of 0.09, 0.19, 0.25, 0.38, 0.50, 0.75, 1.0 and 1.5×10^6 cell mL^{-1} , respectively. Upon gentle mixing, the *in vivo* fluorescence (IVF) of the test cultures were measured by inserting the culture tubes directly into Trilogy Fluorometer to obtain IVF at time zero, i.e., IVF_0.

Then hydrogen peroxide was added to the test cultures. The working solution of hydrogen peroxide (10 mM) was freshly prepared by ~ 1000-fold dilution of the stock solution (Fluka-95321, *Trace*SELECT®, 30% w:w, 9.8 M, kept in refrigerator and in dark) with sterile culture medium. The working solution was immediately added to the test cultures to achieve the target nominal concentrations of H_2O_2 of 0, 0.8, 1.2 and 1.6 mg L^{-1}. The nominal concentrations were confirmed (to be within 10-15% deviation) with a chromatic method, where H_2O_2 reacts with p-nitrophenylboronic acid (p-NPBA) stoichiometrically at pH 9 producing p-nitrophenol (p-NP) with molar absorptivity of 19400 cm^{-1} M^{-1} at 406 nm (Lu et al., 2011).

The culture tubes were held in clear polycarbonate racks, and upon H_2O_2 addition and gentle mixing, the test cultures were returned to the growth chamber for incubation. The test cultures were prepared in duplicates. The growth or inactivation of the alga was monitored by measuring *in vivo* fluorescence at 1, 3, 6, 12, 24, 48, 72 and 168 h after

H_2O_2 addition, i.e. IVF_t. The inactivation of *A. anophagefferens* was determined from the ratio of IVF_t to IVF_0.

3.2.4 Decay of Hydrogen Peroxide in Culture Media

To determine the decomposition of hydrogen peroxide as a function of initial alga cell density, four replicate test cultures was prepared in glass culture tubes (similarly prepared as described in "Test culture exposure to hydrogen peroxide") with initial cell density of 0.04 - 1.5 $\times 10^6$ cell mL^{-1}. Two of these four replicate cultures were used to prepare 0.2-µm filtrate by passing the cultures through 0.2-µm membrane filters. H_2O_2 was added to the cultures and the 0.2-µm filtrates at 1.6 mg L^{-1} (nominal concentration). Immediately after mixing (within 15 min) and every 3 hours afterward (till 24-hr) H_2O_2 concentrations were determined in the cultures and the 0.2-µm filtrates of the cultures.

The chromatic method of Lu et al. (2011) was used to quantify H_2O_2 in culture media. To measure H_2O_2 in culture media, 1 mL culture media was filtered through 0.2-µm syringe filter (4 mm diameter, nylon membrane, polypropylene housing), 0.75 mL of the filtrate was added to equal volume 2 mM p-nitrophenylboronic acid (p-NPBA) in 150 mM bicarbonate buffer at pH 9. After 20 min reaction at room temperature, the absorbance of the color product p-nitrophenol (p-NP) was determined at 406 nm (Agilent 8453 spectrophotomter). There were little or no interfering substances in the culture filtrates as when the samples were pre-treated (room temperature 24 hours) with 100 U/mL catalase (Sigma C1345) before reaction with p-NPBA**,** the absorbance at 406 nm was comparable to that of the blank (fresh Aquil). The absorbance at 406 nm was converted to concentration using an external calibration curve of 6.25 – 50 µM H_2O_2 in

Aquil media (e.g. A = 0.0085 C, n = 5, R^2 = 0.995, where A is absorbance, C is H_2O_2 concentration in µM).

3.2.5 Statistical Analysis

Statistical analysis was conducted with Minitab 16 Software Package with probability $p < 0.05$ being accepted as statistically significant. Non-linear regression uses Gauss-Newton algorithm with tolerance level of 10^{-5}.

3.3 Results

3.3.1 Effective Inactivation of *A. anophagefferens*

Initial *in vivo* fluorescence of the test cultures (IVF_0) ranged from 2600 to 160 RFU (relative fluorescence unit); it correlated with initial cell density (B_0) at $B_0 = 586.6\times IVF_0$ (R^2 = 0.99, n = 8). The test cultures with H_2O_2 addition declined substantially relative to the control cultures (no H_2O_2 addition) during 1-week observation (Figure 3.1). In most cases the *in vivo* fluorescence (IVF) was no more than 6% of the controls by 24-hr; and no re-growth were observed during 1-week observation (Figure 3.1). One exception was found in the test cultures with the highest initial cell density (1.5×10^6 cell mL^{-1}) and the lowest H_2O_2 addition (0.8 mg L^{-1}), where despite of an initial rapid decline, substantial re-growth was observed by 168-hr (Figure 3.1) In these cultures, IVF_{24h} (IVF at 24 hr) were about 240 – 280 RFU, and the re-growth after 48-hr was 0.57 d^{-1}, similar to that of healthy *A. anophagefferens* cultures, e.g., 0.54 ± 0.03 d^{-1} (n = 5). Additional culture tests with this alga and H_2O_2 addition showed that when IVF_{24h} was reduced to less than 20 ± 2 within 24 hr, re-growth was not likely to happen over 1-week. IVF_{24h} of 10 RFU was used as a target value for effective inactivation of the alga.

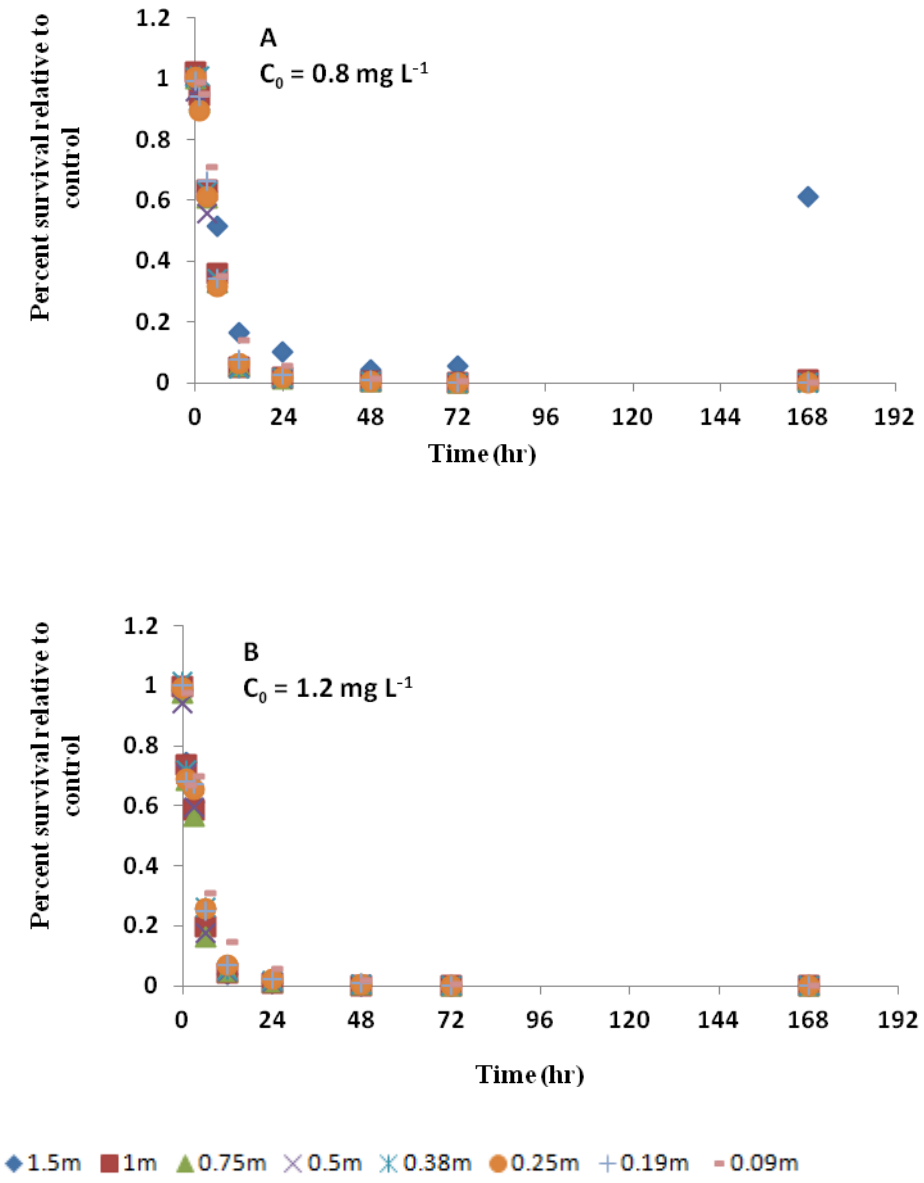

Figure 3.1 Percent survival of *A. anophagefferens* relative to control (no H_2O_2 addition) over 1-week experimental time period. Initial concentration of H_2O_2, C_0, were 0.8 mg L^{-1} (A), 1.2 mg L^{-1} (B) and 1.6 mg L^{-1} (C). Initial cell density, B_0, ranged from 0.09 to 1.5 million cells ml-1 shown as different legends on each panel, i.e., 1.5m, 1m...0.09 million cell mL^{-1}.

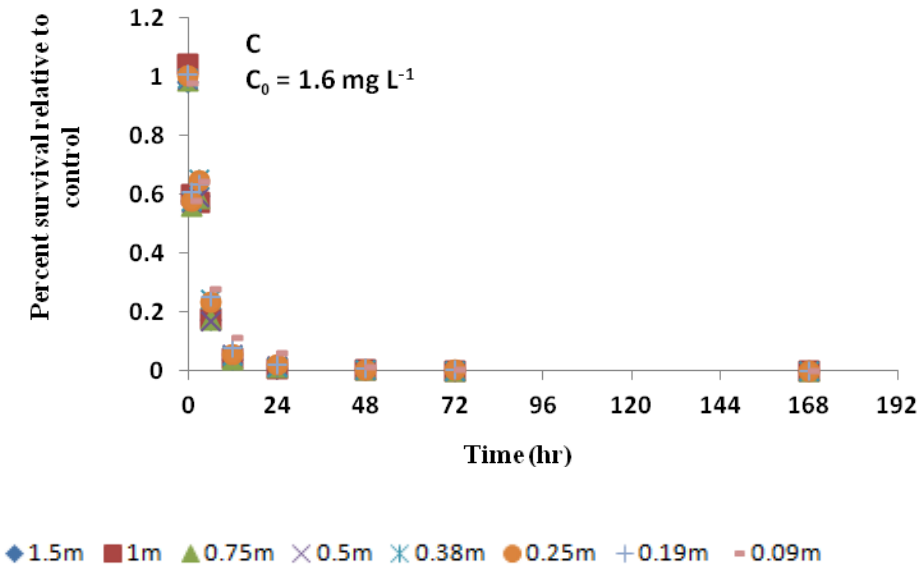

Figure 3.1 (Continued) Percent survival of *A. anophagefferens* relative to control (no H_2O_2 addition) over 1-week experimental time period. Initial concentration of H_2O_2, C_0, were 0.8 mg L^{-1} (A), 1.2 mg L^{-1} (B) and 1.6 mg L^{-1} (C). Initial cell density, B_0, ranged from 0.09 to 1.5 million cells ml-1 shown as different legends on each panel, i.e., 1.5m, 1m...0.09 million cell mL^{-1}.

3.3.2 IVF Decline Fits First Order Kinetics

During the initial 24-hr of H_2O_2 exposure, the decline of IVF fit pseudo first order kinetics for a given initial cell density (B_0, B_0 = 0.09 – 1.5×10^6 cell mL^{-1}) and H_2O_2 addition (C_0, 0.8 - 1.6 mg L^{-1}), that is, Ln (IVF_t/IVF_0) = - k_{ivf} t, where t is time, k_{ivf} is rate constant (h^{-1}), and IVF_t and IVF_0 are *in vivo* fluorescence at time t and zero (Figure 3.2). It was also observed that the rate constant of IVF decline, k_{ivf}, increased with initial cell density B_0 as k_{ivf} = a + b Ln(B_0) (where a and b are empirical constants); for example, the rate constant during the initial 24 hours, $k_{ivf\text{-}24h}$ = 0.0334 Ln(B_0) – 0.269 (Figure 3.3). k_{ivf} also decreased with time (Figure 3.3), which was likely due to the decomposition of H_2O_2 over time.

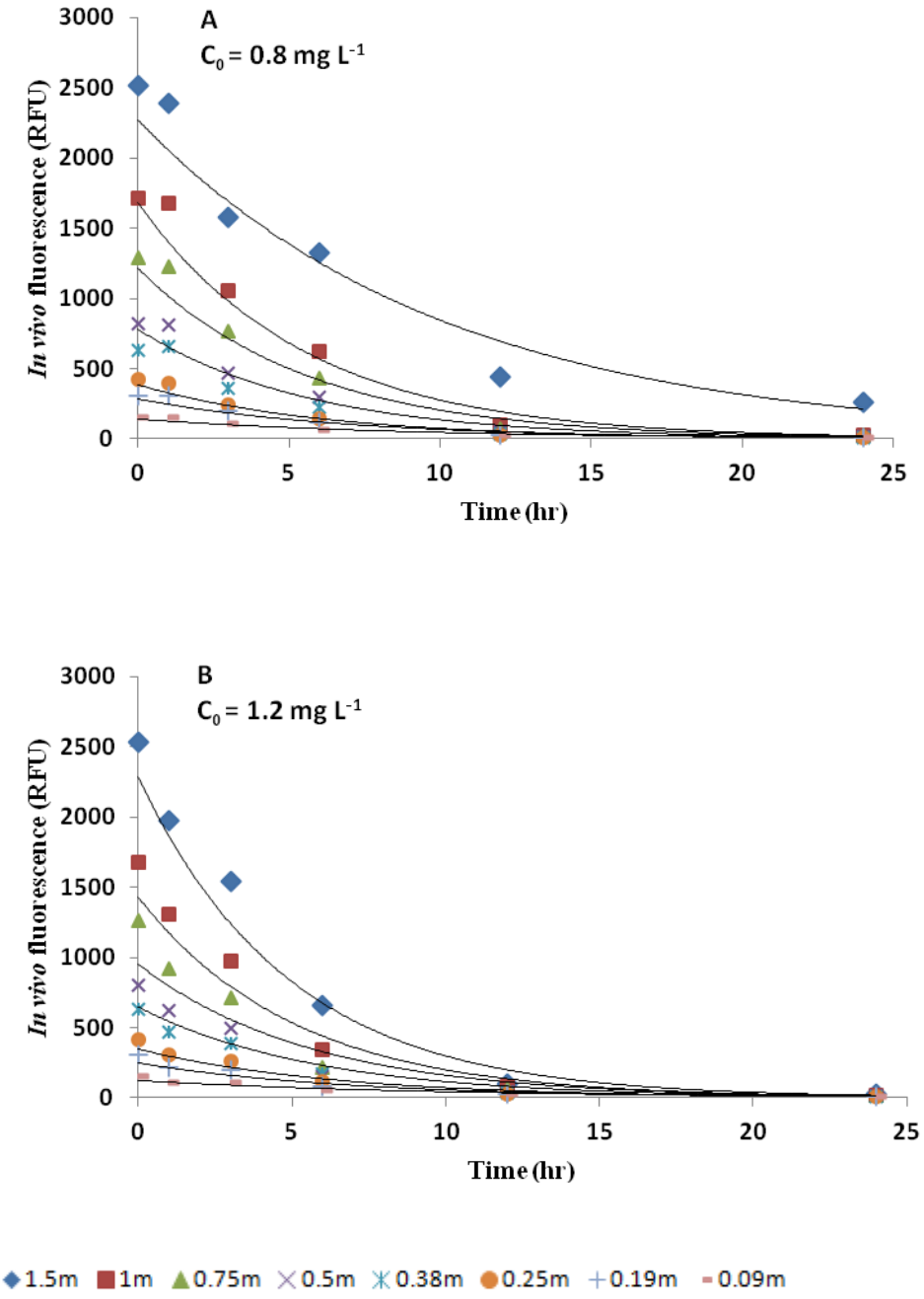

Figure 3.2 Exponential decrease of *in vivo* fluorescence over 24 hours of H_2O_2 addition at nominal initial concentration of 0.8 mg L^{-1} (A), 1.2 mg L^{-1} (B), and 1.6 mg L^{-1} (C). Curves on each panel indicate different starting biomass concentration, (showing those from experiment 2 only, for clarity). Legends indicate the initial cell density (million cell mL^{-1}).

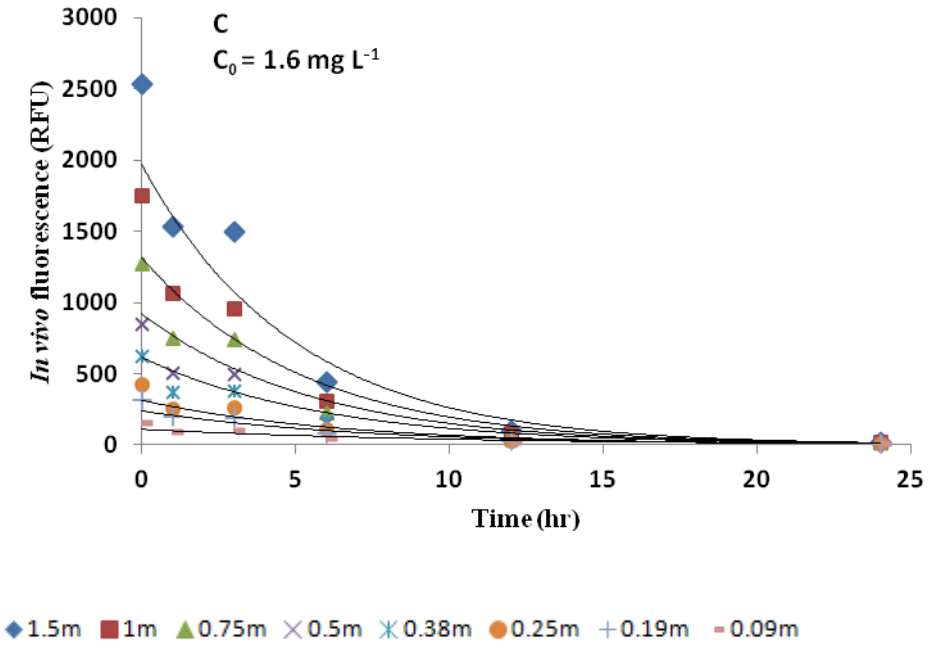

Figure 3.2 (Continued) Exponential decrease of *in vivo* fluorescence over 24 hours of H_2O_2 addition at nominal initial concentration of 0.8 mg L^{-1} (A), 1.2 mg L^{-1} (B), and 1.6 mg L^{-1} (C). Curves on each panel indicate different starting biomass concentration, (showing those from experiment 2 only, for clarity). Legends indicate the initial cell density (million cell mL^{-1}).

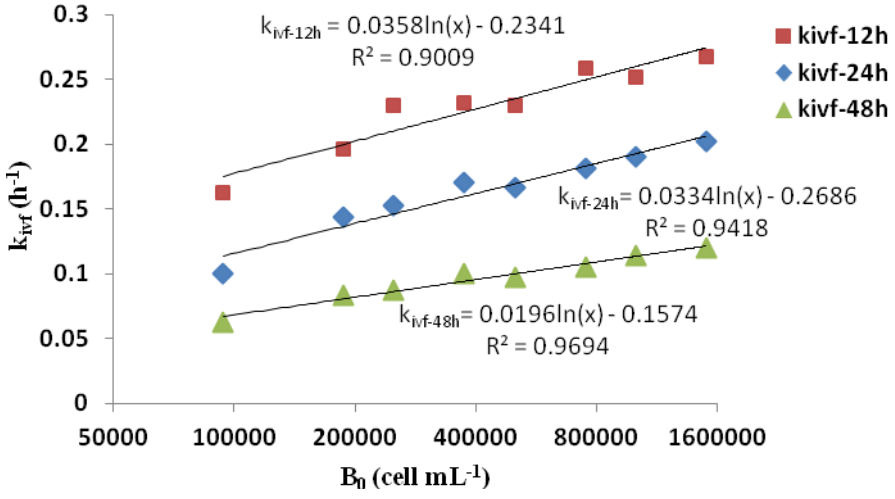

Figure 3.3 The pseudo first order rate constant of *in vivo* fluorescence decline, k_{ivf} (h^{-1}) during the initial 12-hr (■), 24-hr (♦), and 48-hr (▲) for 1.6 mg L^{-1} H_2O_2 addition.

3.3.3 Hydrogen Peroxide Decomposition

The decomposition of H_2O_2 fits pseudo-first order reaction kinetics in the culture media and the 0.2-µm filtrate of the media during the 24-hr observation (the regression probability *p* ranged from 0.000 to 0.02, n = 4 – 9 depending on how fast H_2O_2 was reduced to < 0.27 mg L^{-1}, the experimental quantification limit according to the standard curve). The rate constants ranged from 1.3 to 3.9 d^{-1} in culture media containing 0.04 – 1.5×10^6 cell mL^{-1} and an initial H_2O_2 addition at 1.6 mg L^{-1} (Figure 3.4), which suggests that 70 – 98% H_2O_2 was decomposed within 24-hr under these conditions. It was also shown that the majority of the decomposition was mediated by *A. anophagefferens* cells, because H_2O_2 decay rate decreased with decreasing cell density, and was substantially lower (averaging 0.74 ± 0.11 d^{-1}, n = 12) in the 0.2-µm filtrate of these cultures (Figure 3.4).

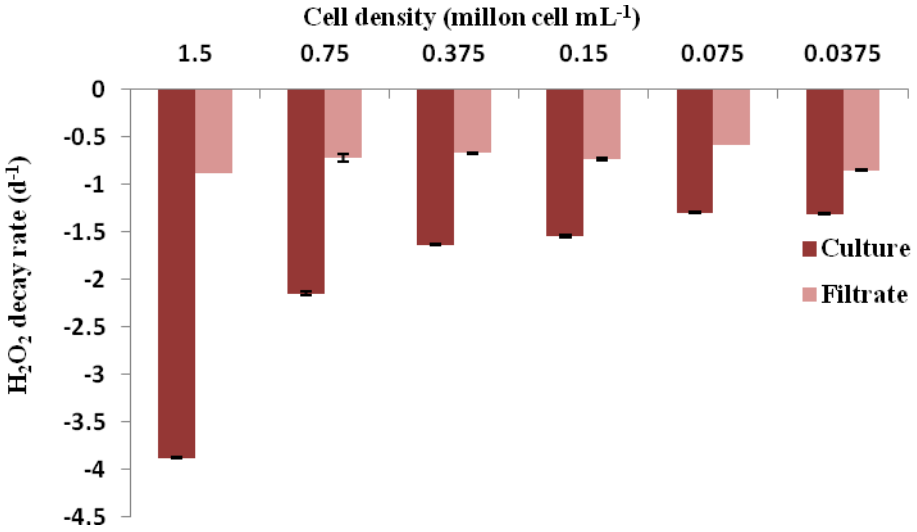

Figure 3.4 First order H_2O_2 decay rate constant in cultures and the 0.2-µm filtrate of the cutlures with 0.04 – 1.5 million cell mL^{-1} and initial nomimal H_2O_2 addition of 1.6 mg L^{-1}. Rate constant were based on the residual H_2O_2 concentration in the media measured every 3-hr until 24-hr or the residual H_2O_2 reaching limit quantification (~ 0.27 mg L^{-1}), whichever reached first. Error bars are deviations between duplicate cultures.

3.3.4 Empirical Model Development

Since both H_2O_2 decomposition and *A. anophagefferens* IVF declining occurred most substantially within 24-hr of H_2O_2 addition, the initial 24-hr were focused on to build the empirical kinetics model for *A. anophagefferens* inactivation by H_2O_2. Total 120 data sets (after averaging the duplicate cultures) were used in modeling; they were from 8 nominal initial cell density levels × 3 H_2O_2 concentration levels × 5 time points, all satisfying criteria $C_0 > 0$ mg L^{-1}, 24 h ≥ t > 0 h, and $IVF_t > 10 \pm 2$ (for reliable IVF reading in the test culture tubes). Four forms of empirical models were produced :

$$Ln(IVF_t/IVF_0) = - kC_0 t \qquad k = 0.175 \pm 0.006 \tag{3.3}$$

$$Ln(IVF_t/IVF_0) = - kC_0^n t \qquad k = 0.183 \pm 0.006,\ n = 0.586 \pm 0.104 \tag{3.4}$$

$$Ln(IVF_t/IVF_0) = - kC_0^n t^m \qquad k = 0.181 \pm 0.014,\ n = 0.587 \pm 0.107,\ m = 1.01 \pm 0.045 \tag{3.5}$$

$$Ln(IVF_t/IVF_0) = -k^*(Ln(B_0))C_0^n t^m \qquad k^* = 0.014 \pm 0.001,\ n = 0.583 \pm 0.105,\ m = 1.01 \pm 0.044 \tag{3.6}$$

where IVF_t/IVF_0 is the ratio of *in vivo* fluorescence of the culture at time t and zero, and $Ln(IVF_t/IVF_0)$ is proxy of the alga inactivation, while k, n, m and k* are empirical constants. Here Equations 3.3 – 3.5 were Chick-Watson, and Hom's models. Equation 3.6 was modified from Hom's model by including initial algal cell density as a variable. All four models produced reasonable estimation on the empirical constants (having relatively low standard error on the estimation), and were not substantially different from one and another for the common constants, i.e., k, n and m. As can be seen in Figure 3.5, the model predictions on IVF_t/IVF_0 matched with experimental observation reasonably well. The 4th model, Equation 3.6, had slightly smaller standard error of regression as compared to others three (Table 3.1), and this model had the algal biomass

as a variable along with C_0 and t, it was used for further estimation on H_2O_2 dose requirement for effective inactivation of the alga.

Table 3.1 Models on *A. anophagefferens* Inactivation by Hydrogen Peroxide

Model	Empirical constant (estimate ± standard error)	Standard error of regression
$Ln(\frac{IVF_t}{IVF_0}) = -kC_0 t$	$k = 0.175 \pm 0.006$	S = 0.0866
$Ln(\frac{IVF_t}{IVF_0}) = -kC_0{}^n t$	$k = 0.183 \pm 0.006$ $n = 0.586 \pm 0.104$	S = 0.0818
$Ln(\frac{IVF_t}{IVF_0}) = -kC_0{}^n t^m$	$k = 0.181 \pm 0.014$ $n = 0.587 \pm 0.107$ $m = 1.006 \pm 0.045$	S = 0.0821
$Ln(\frac{IVF_t}{IVF_0}) = -(k^* Ln(B_0))C_0{}^n t^m$	$k^* = 0.014 \pm 0.001$ $n = 0.583 \pm 0.105$ $m = 1.006 \pm 0.044$	S = 0.0806

C_0 is initial nominal concentration of H_2O_2 addition (mg L^{-1}), t is time (hr), B_0 is initial cell density of the alga (cell mL^{-1}), IVF_t/IVF_0 is the ratio of the *in vivo* fluorescence at time t and time zero. k, n, m and k* are empirical constants.

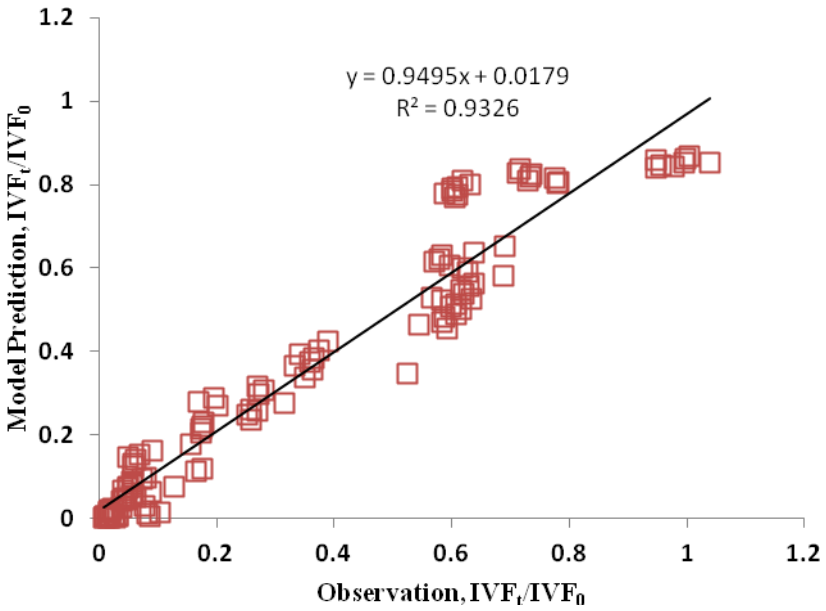

Figure 3.5 Comparison on IVF_t/IVF_0 from the experimental observation and the model prediction based on B_0, C_0 and t (1 – 24 h).

3.3.5 Application of the Model

The empirical model, Equation 3.6, was used to estimate the required H_2O_2 dose to reduce *A. anophagefferens* from an initial cell density of 0.2 – 1.5×10^6 cell mL^{-1} (category 3 brown tide bloom (Gastrich et al., 2004)) to achieve IVF_t of 10 RFU within 24-hr. IVF_t of 10 RFU was chosen as it likely represents effective inactivation of *A. anophagefferens* with low probability of re-growth over 1-week. The model result showed that the required dosage would be between 0.9 and 2.1 mg L^{-1} for B_0 = 0.2 – 1.5×10^6 cell mL^{-1} and t = 8 – 24 h (Figure 3.6). The higher the initial cell density and the shorter the target time period, a higher H_2O_2 dosage would be required (Figure 3.6), however a much higher dosage H_2O_2 is not recommended as it may adversely affect other sensitive biota in aquatic system (Gaikowski et al., 1999, Meinertz et al., 2008). Similarly this model can also be used to estimate the time it would take to reduce *A.*

anophagefferens density at a given H_2O_2 dose. For example, to reduce *A. anophagefferens* by 90% at given dose between 1 – 2 mg L^{-1} and initial biomass concentration of 0.2 – 1.5×10^6 cell mL^{-1} the model estimated a time period of 7.5 – 13.5 hr (Figure 3.7).

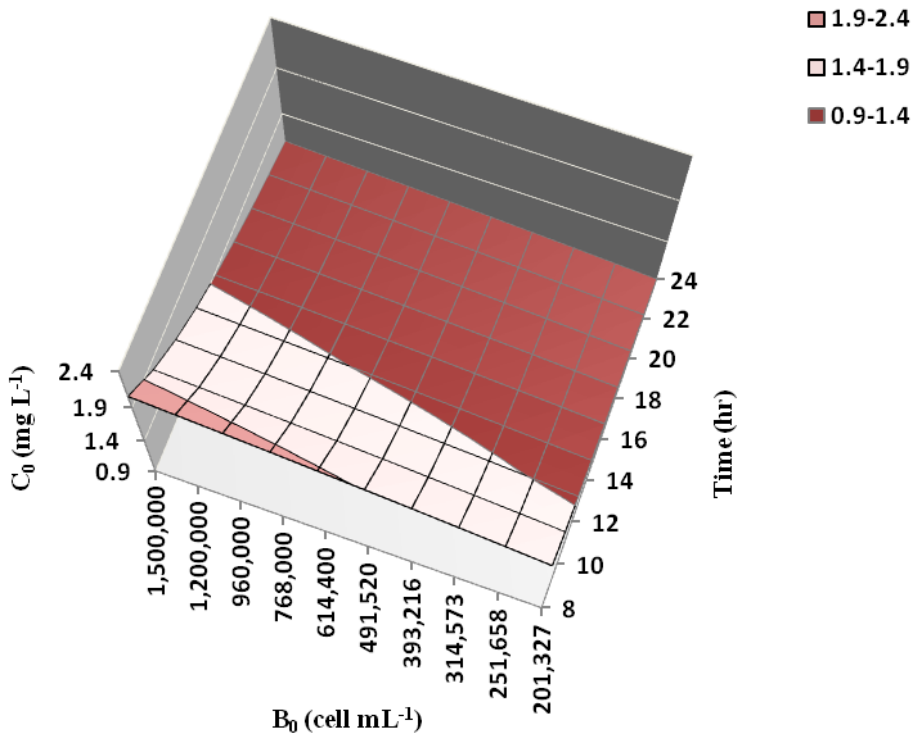

Figure 3.6 Model prediction on the initial H_2O_2 concentration C_0 (mg L^{-1}) required to reduce *A. anophagefferens in vivo* fluorescence to IVF_t = 10 (equivalent to cell density of 5866 cell mL^{-1}) at various Category 3 initial cell density B_0 (0.2 – 1.5 million cell mL^{-1}) and specified time period (8 – 24 hr).

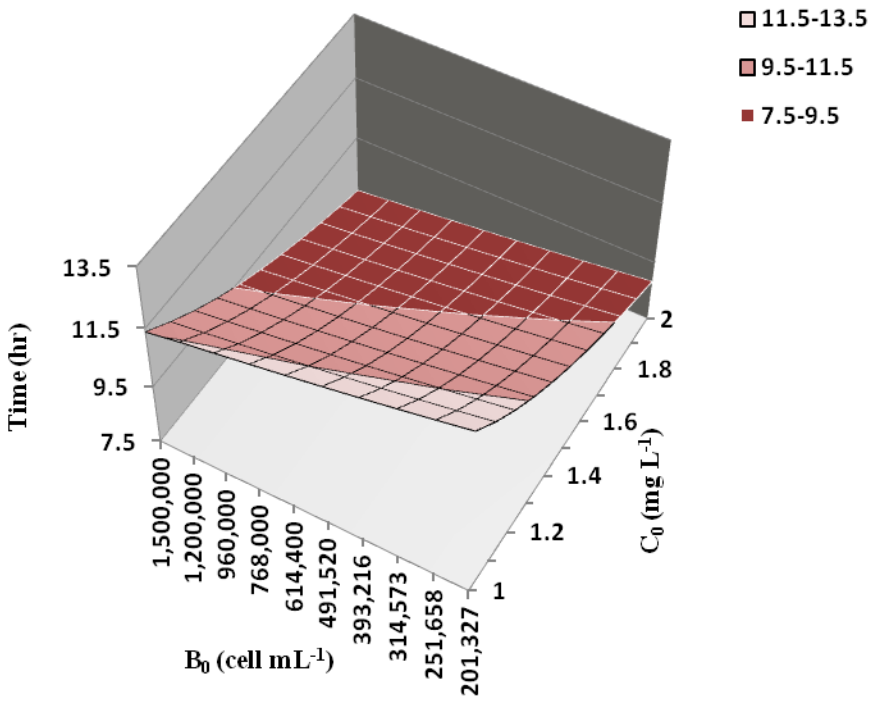

Figure 3.7 Model prediction on the time it would take to achieve 90% decrease of *A. anophagefferens in vivo* fluorescence at various initial cell density B_0 (0.2 – 1.5 million cell mL^{-1}) and initial H_2O_2 concentration C_0 (1 – 2 mg L^{-1}).

3.4 Discussion

In model application, IVF_t ($t \leq 24$ h) of 10 RFU was used arbitrarily as a criterion of effective inactivation of the target organism *Aureococcus anophagefferens*. Although laboratory culture tests did show that a rapid reduction of IVF from IVF_0 of hundreds or thousands RFU to 20 or less, no re-growth would be observed over 1-week, a mechanistic justification was not available. It's speculated that it may be related to the reduction and/or elimination of quorum sensing substances for bloom development if present, and/or the sensitizing of the remaining cells (potentially stressed but not dead yet) to additional stresses which eventually killed them.

In the final model (Equation 3.6) Ln (IVF_t/IVF_0) = - $k^*(Ln(B_0))C_0^n t^m$ = -0.014 Ln (B_0) $C_0^{0.583}$ $t^{1.006}$, the empirical constant *n* had relatively large standard error of 0.1. This is probably because only three levels of H_2O_2 additions were tested in the study, limiting further improving of this empirical constant. Attempt to expand the number of levels and the concentration range of H_2O_2 additions may improve this model further. However the narrow range of H_2O_2 dose, (i.e. 0.9 – 2.1 mg L^{-1}), predicted to be effective in inactivating *A. anophagefferens* of category 3 bloom density (highest observed in coastal bays), probably would not make such an attempt productive.

The use of $k^*(Ln(B_0))$ in Equation 3.6 was apparently supported by the observation that the rate constant of IVF decline was a function of $Ln(B_0)$ as shown in Figure 3.3, it also conforms to Haas and Jeffe's model (Equation 3.7) where Haas and Jeffe (1994) used a dimensionless "efficiency factor", η, to correct the loss of disinfectant residual over time.

$$Ln(N/N_0) = - k C_0^n t^m \eta \qquad (3.7)$$

In the original Haas and Jeffe's model, η was a function of disinfectant first order decay rate constant k':

$$\eta = \frac{m}{(nk't)^m}\gamma(m, nk't)$$

$$\gamma\ (m, nk't) = \int_0^z \exp(-z) z^{m-1} dz$$

$$z = nk't$$

for which Haas and Jeffe (1994) gave an approximate solution for η as

$$\hat{\eta} = \left[\frac{1-\exp(-\frac{z}{m})}{\frac{z}{m}}\right]^{m}$$

However, this approximation did not produce a fit for the data in this study (having unreasonable estimation of the empirical constants, and large standard error of regression etc.), probably because the disinfectant decay rate constant was not a constant but varied with *A. anophagefferens* cell density as observed in Figure 3.4.

Overall, this study provided an empirical model to describe *A. anophagefferens* inactivation by H_2O_2 as a function of initial cell density, dosage and time. Since the model was based purely on laboratory cultures, thus it is more of a description of the alga's characteristics to H_2O_2 treatment than a practical guide to field application. Nevertheless, the relative narrow range of H_2O_2 dose (0.9 – 2.1 mg L^{-1}) to achieve effective inactivation (and potential 1-week no re-growth) for a wide range of initial alga cell density (0.2 – 1.5 million cell mL^{-1}) within 24-hr suggests an environmentally safe low concentration of H_2O_2 likely capable of achieving effective brown tide bloom control. The discussion on the considerations on realistic scenarios, such as uniform dispersion of H_2O_2 in field, effects of the treatment on bacteria and other marine biota, and potential effects of iron concentration on treatment efficiency, may be found in Randhawa et al (2012).

CHAPTER 4

EFFECT OF ALGAL GROWTH PHASE ON *AUREOCOCCUS ANOPHAGEFFERENS* SUSCEPTIBILITY TO HYDROGEN PEROXIDE

4.1 Introduction

Brown tide bloom caused by Pelagophyte *Aureococcus anophagefferens* adversely impacts shellfishery and ecosystem, leading to the decline of several shellfish species of economic values and the decline of submerged aquatic vegetation (Gobler et al., 2005, Gobler and Sunda, 2012). Brown tide alga was found very sensitive to hydrogen peroxide exposure as compared to many other phytoplankton species (Randhawa et al., 2012). It encourages a method of brown tide control by administering low doses of hydrogen peroxide (Randhawa et al., 2012). This study aims to examine *A. anophagefferens*' susceptibility to hydrogen peroxide as affected by the alga's physiological status of exponential and stationary growth, thus improve on the timing and dose of brown tide control using hydrogen peroxide

In the field of harmful algal bloom control, hydrogen peroxide has been proposed and used as an environmentally friendly chemical agent to selectively remove harmful cyanobacteria and their toxins (Barrington and Ghadouani, 2008, Barrington et al., 2011, Barroin and Feuillade, 1986, Drábková et al., 2007a, Drábková et al., 2007b, Drábková et al., 2007c). A recent field study of Matthijs et al. found that upon dispersing H_2O_2 at 2 mg L^{-1} to a Netherland lake the developing *Planktothrix agardhii* (cyanobacteria) bloom and the cyanobacterial toxin was eliminated at more than 99% removal efficiency within a few days of H_2O_2 application, while the eukaryotic phytoplankton, zooplankton and other macro-fauna remained largely unaffected(Matthijs et al., 2012).

Barrington et al. also showed that low doses of hydrogen peroxide effectively reduce cyanobacteria fraction and microcystins in waste stabilization ponds, in the mean time eukaryotic phytoplankton increased while the zooplankton population was likely not affected (Barrington et al., 2013, Reichwaldt et al., 2012).

It is currently believed that Fenton reaction leading to the production of free hydroxyl radicals is the basis of hydrogen peroxide action (Linley et al., 2012). This reaction leads to oxidation of DNA (by forming ferryl radical from reacting with DNA associated iron(Imlay and Linn, 1988)), oxidation of proteins (on cysteine or selenocysteine residues of selected proteins (Kim et al., 2000) and oxidation of membrane (Baatout et al., 2006) and other cellular components *in vivo*, leading to cell death. Although it is commonly believed that hydrogen peroxide action on living cells would be non-selective, the vastly different microbial cellular characteristics, such as intracellular organization and the inherent antioxidant capability, have been shown to have very different sensitivity to hydrogen peroxide. For example, Penna et al. reported a minimum inhibitory concentration range of 469 – 2500 mg L^{-1} for different bacterial Gram positive and negative species (Penna et al., 2001). Baldry showed substantial difference on bactericidal, fungicidal and sporicidal properties of hydrogen peroxide (Baldry, 1983). Cyanobacteria *Microcystis aeruginosa* was shown to be about 10 times more sensitive to H_2O_2 than a green alga and a diatom, possibly owing to different organization of cellular components within the cells (Drábková et al., 2007a, Drábková et al., 2007c). Brown tide alga's small cell size and the lack of rigid cell wall may have contributed to its great sensitive to hydrogen peroxide (comparable to many cyanobacteria species) (Randhawa et al., 2012). Hence, although the mechanism of H_2O_2

action may be non-selective, different and species specific sensitivity could be utilized to selectively remove the sensitive *A. anophagefferens,* while preserving the less sensitive ones. In addition, *A. anophagefferens'* susceptibility to hydrogen peroxide may be influenced by its metabolic and physiological status, as there was evidence that microorganisms' biocide target sites and their available abundance are plastic and vary with organism's metabolic and physiological status (Denyer and Stewart, 1998, Russell, 2003). The latter may have practical implications in brown tide bloom control, as brown tide tends to develop in natural waters analogous to batch cultures demonstrating exponential and stationary growth phases and likely different metabolic and physiological status.

An example of *A. anophagefferens* bloom development in coastal bays may be found in the work of Gobler et al (2011). During 2007 – 2009 Quantuck Bay (NY) *A. anophagefferens* started to increase late spring, then increased rapidly (approximately exponentially) during late May - June which accompanied rapid decrease in total dissolved nitrogen nutrient, reaching monospecific peak in summer around late June or July, which lasted for a week or longer, then the bloom progressed to decline (Gobler et al., 2011), demonstrating distinct exponential, stationary and decline phases. The physiochemical and biological conditions of the bay also resemble a batch culture condition, particularly, the limited water exchange and retarded grazer activities during bloom development (Gobler et al., 2005, Gobler et al., 2011). Thus, the brown tide bloom development could be quite comparable to a batch culture evolving from exponential phase to stationary or decline phases, and the cells at different growth phases are likely of

different metabolic and physiological status, which may lead to different susceptibility to H_2O_2 treatment.

The goal of this study is to examine if *A. anophagefferens'* growth phase, exponential versus stationary phase, affects its susceptibility to hydrogen peroxide treatment. Batch culture of *Aureococcus anophagefferens* at stationary phase was diluted with fresh media to produce test cultures of the same cell density as exponential phase cultures. Test cultures were then subjected to H_2O_2 treatment. The growth and physiological changes in *A. anophagefferens* and the decay of H_2O_2 were characterized. Results produced a comparison on the effect of algal cell growth phase on its H_2O_2 susceptibility. Knowledge on this would improve field trials of brown tide control with H_2O_2 by refining the dose and timing of H_2O_2 addition; it may also shed light on other harmful algal bloom control with H_2O_2.

4.2 Materials and Methods

4.2.1 Algal Strain and Culturing

The brown tide alga *Aureococcus anophagefferens* (CCMP 1984) was obtained from Provasoli-Guillard National Centre for Marine Phytoplankton (NCMA), Maine, U.S.A. Artificial seawater media Aquil (excluding Si) was prepared and sterilized according to Price et al.(Price et al., 1988/1989), and was used as culture media throughout this study. Cultures were maintained in polycarbonate bottle in a growth chamber (VWR Signature^{TM} model 2015) with four Philips 40 W cool-white fluorescent lamps producing about 120 µE m^{-2} s^{-1} illumination, under 12 hr: 12 hr light-dark cycle and 19±1° C. Polycarbonate bottles (Nalgene®) were used as culture vessels and they were carefully

cleaned by washing with detergent, rinsing with plenty of distilled water, soaking in 1-N HCl acid overnight, rinsing with plenty of Milli-Q water, and finally drying in HEPA filtered air stream. Media and cultures were handled in HEPA filtered laminar flow hood.

4.2.2 Preparing Test Cultures of Exponential and Stationary Growth Phase

Stock cultures of *A. anophagefferens* were started with 3-L media in 2-L and 1-L polycarbonate bottles. On the 3rd-day of culturing, approximately 250 mL mid-exponential phase stock culture were transferred to ten 500-mL polycarbonate bottles, and they were made into test cultures of exponential growth phase directly. On the 8th -day of stock culturing, the stationary phase stock culture was diluted 5.4-fold with fresh media, and 250 mL was transferred to another ten 500-mL polycarbonate bottles to make test cultures of stationary phase. Test cultures were made in duplicates for five levels of H_2O_2 addition. The resulting test cultures had similar initial cell density of 1.63 ± 0.11 x10^6 cell mL^{-1} (n = 20) as measured with Coulter Counter Multisizer 3 (equipped with a 70 μm aperture tube, Beckman Coulter).

4.2.3 Hydrogen Peroxide Dosing and Quantification

As soon as the test cultures were prepared, hydrogen peroxide (H_2O_2) was added at nominal concentration of 0, 0.4, 0.8, 1.2 and 1.6 mg L^{-1}. To do this, hydrogen peroxide (Fluka-95321, *Trace*SELECT®, 30% w:w, 9.8 M, kept in refrigerator and in dark) was first diluted about 1000-fold to 10 mM with sterile Aquil medium, then immediately added to the test cultures. Actual concentrations of H_2O_2 in test cultures were quantified following Lu et al. (Lu et al., 2011). Briefly, culture samples were centrifuged at 10000 rpm at 4^{o}C for 5 min, and 1-mL the supernatant was transferred to microcentrifuge tubes.

H_2O_2 in the supernatant reacted with p-nitrophenylboronic acid at pH 9 with 0.15 M bicarbonate buffer, which produces p-nitrophenol (experimental molar absorptivity around 17,000 $M^{-1}cm^{-1}$ at 406 nm, Aligent 8453) proportional to the molar concentration of hydrogen peroxide.

4.2.4 Assess Algal Growth and Physiological Changes

The growth responses of the test cultures were assessed with temporal variation of *in vivo* chlorophyll a fluorescence (IVF), and "snapshots" of 6-h total chlorophyll a (chl a) and 24-h cell density upon H_2O_2 treatment. IVF was obtained from Turner Designs' Trilogy Fluorometer (equipped with an optical block for *in vivo* chlorophyll a measurement, excitation 485 nm, emission 685 nm, band width 50 nm) at time 0, 2, 4, 6, 12, 24, 48 and 168h (1-week). IVF was further used to calculate the rate of culture increase or decrease from $IVF_t = IVF_0 \exp(\mu t)$, where IVF_t and IV_0 are IVF at time t and zero, μ is specific rate of growth (positive μ) or decline (negative μ), t is time in days or hours. Cell density (cell mL^{-1}) was measured by Coulter Counter Multisizer 3 (equipped with a 70 μm aperture tube, Beckman Coulter). Total chlorophyll a (chl a, μg L^{-1}) was determined upon GF/F filtration (nominal pore size 0.7 μm), 90% acetone extraction, and UV-Vis spectrophotometric quantification (Agilent 8453) using Jeffery and Humphrey's trichromatic equation, following EPA method 446.0.

Algal cell's physiological changes were assessed with temporal variation in photosynthetic quantum yield (F_v/F_m), and a "snapshot" of 6-h total intracellular non-protein thiol content. F_v/F_m was measured at 0 (immediate before H_2O_2 addition) and 2, 4, 6, 12, 24, 48 and 168 h of H_2O_2 addition. It was obtained from Satlantic Fluorescence Induction and Relaxation System (FIRe, using single flash turnover mode) upon 15-min

dark incubation of the culture samples. Fv/Fm is the maximum photosynthetic quantum yield of photosystem II (PSII); it reflects the overall health status of the algal cells (Baker, 2008, Gorbunov and Falkowski, 2004, Gorbunov et al., 1999, Kolber et al., 1998).

At 6-hr of H_2O_2 exposure, total intracellular non-protein thiols, predominantly glutathione, was also quantified to produce a snapshot on the effect of H_2O_2 addition on cell's major antioxidant and ROS (reactive oxygen species) scavenger(Pompella et al., 2003, Sies, 1999). Intracellular non-protein thiols were extracted from the algal cells and were quantified upon derivatization with monobromobimane. Briefly, the algal cells were collected on GF/F filters under low vacuum (< 5 psi), and the non-protein thiols (predominately glutathione (Ahner et al., 2002)) were extracted into 1 µM glutathione, which was freshly prepared in 10 mM methanesulfonic acid and served as a reductant to minimize the oxidation of the target thiols during sample processing. The extraction was achieved upon denaturing the cells at 70^{o}C for 2-min and subsequently 30-min sonication in a water bath (0^{o}C, Ultrasonic FS-28, Fischer Scientific). A 0.8 mL of the extract was derivatized by adding 84 µL of borate-DTPA buffer (of pH 9, made of 100 mM borate and 10 mM diethylenetriamene pentaacetate, DTPA), and 3 µL of 50 mM monobromobimane (in acetonitrile) which tags the thiol to form strongly fluorescent thiol-bimane conjugate. The reaction was terminated after 10-min by adding 25 µL of 800 mM methanesulfonic acid (which brings the pH to about 3). The fluorescence was measured with a fluorometer (Perkin Elmer, LS 55) set at excitation 380 nm and emission 470 nm and 2.5 µm slit size. The concentration of total non-protein thiol was calculated

from external standard curve of glutathione using procedure blank (i.e. GF/F filter unloaded with cells but processed as a culture sample was used as a blank).

4.2.5 Assess Hydrogen Peroxide Decomposition

The decomposition of hydrogen peroxide in the test cultures of exponential and stationary phases were assessed by quantifying residual H_2O_2 in media at 0 (immediately after adding H_2O_2), 2, 4 and 6-hr of H_2O_2 addition (all in the light period of incubation). In parallel to the culture study the decomposition of 1.6 mg L^{-1} H_2O_2 in fresh Aquil media (no algal culture) was also measured.

In addition decomposition of 1.6 mg L^{-1} H_2O_2 was further tested in 0.2 µm filtrate of the exponential and stationary cultures in order to distinguish media- and cell-mediated H_2O_2 decomposition. This was because the filtrate of stationary test cultures, although had been diluted 5.4-fold with fresh Aquil, and the filtrate of the exponential phase cultures, might differ substantially in dissolved organic matters or other components that affect the effective concentration of H_2O_2 to react with and inactivate *A. anophagefferens* cells. To do this, additional test cultures of exponential and stationary phases were prepared as before. Aliquots of the test cultures were passed through 0.2-µm syringe filters to obtain cell free filtrate. Then H_2O_2 was added at initial concentration of 1.6 mg L^{-1}. The decomposition of H_2O_2 was examined by quantifying residual H_2O_2 in these cultures and filtrate over 6 hours.

4.2.6 Statistical Analysis

Statistical analysis was conducted with Minitab 16 Software Package with probability $p < 0.05$ being accepted as statistically significant unless otherwise stated. ANOVA (analysis

of variance) was used to compare different treatments. In regression analysis (linear or non-linear), data sets from the duplicate cultures were first averaged, then, fit with the regression model to produce the estimate and the standard error of the estimate. Non-linear regression uses Gauss-Newton algorithm with tolerance level of 10^{-5}.

4.3 Results

Figure 4.1 shows the growth curve of the stock culture of *A. anophagefferens* that was used in preparing the test cultures of exponential and stationary phases. *A. anophagefferens* cells that were made into exponential and stationary phase test cultures were having 0.65 d^{-1} and 0.02 d^{-1} growth rates, and maximum quantum yield of photosystem II of 0.53 (healthy) and 0.37 (stressed) respectively.

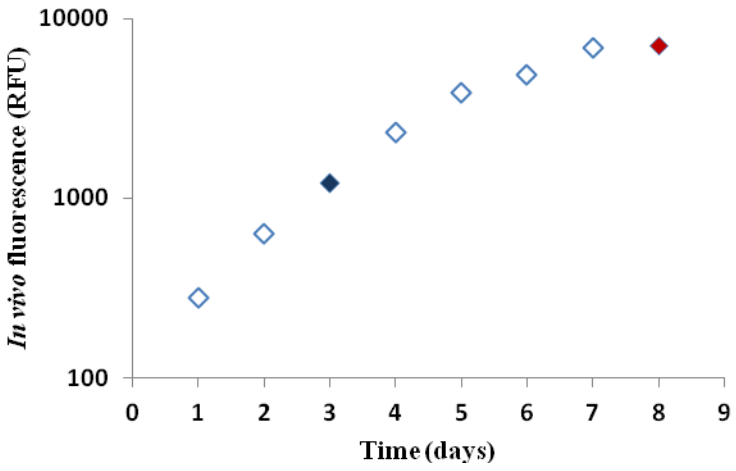

Figure 4.1 Growth curve of the stock culture of *A. anophagefferens*. On the 3rd and 8th day of culturing, the stock culture was used to prepare the test cultures of exponential and stationary phase, respectively.

Different growth rates and quantum yields confirm different metabolic and physiological status of the exponential and stationary cultures. The initial cell density of the test cultures averaged 1.63 ± 0.11×106 (n = 20).

4.3.1 Growth Responses

The overall growth response of the test cultures are shown in Figure 4.2 as temporal variation of *in vivo* chlorophyll fluorescence (IVF) over 1-week. Cultures with 1.2 and 1.6 mg L^{-1} H_2O_2 addition reached more than 90% reduction in IVF within 12-h exposure and showed no significant re-growth during 1-week observation. In contrast, cultures received lower H_2O_2 addition either did not decline (0 and 0.4 mg L^{-1} H_2O_2) or declined but did not reach 90% reduction at 12 h (0.8 mg L^{-1} H_2O_2); they had robust re-growth during 1-week observation (Figure 4.2).

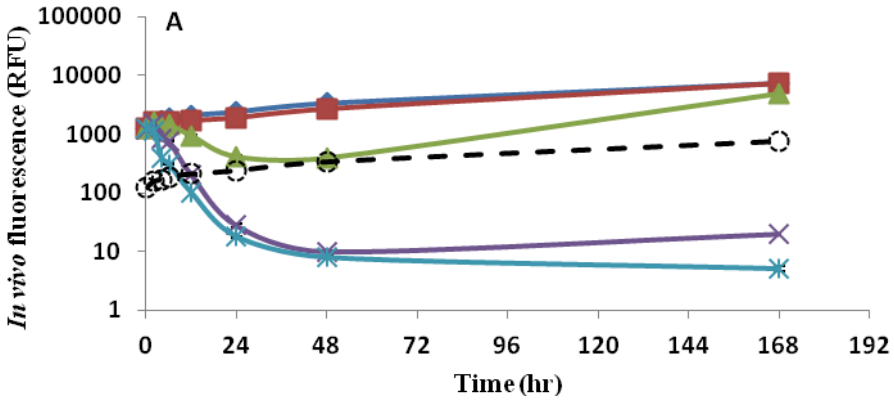

Figure 4.2 Temporal variation of *in vivo* fluorescence of the test cultures of (A) exponential and (B) stationary growth phase, exposed to H_2O_2 at 0, 0.4, 0.8, 1.2 and 1.6 mg L^{-1}. The dotted lines indicate IVF values of the 10% of control cultures. Error bars are deviations between duplicate cultures.

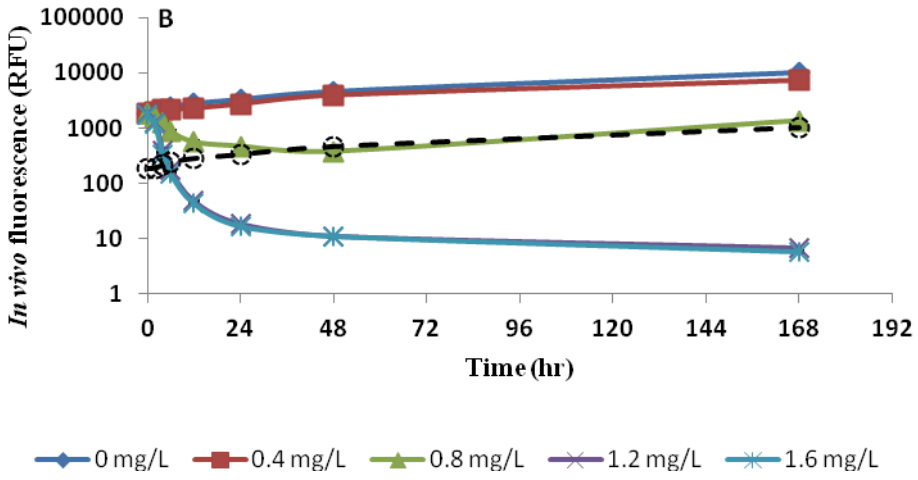

Figure 4.2 (Continued) Temporal variation of *in vivo* fluorescence of the test cultures of (A) exponential and (B) stationary growth phase, exposed to H_2O_2 at 0, 0.4, 0.8, 1.2 and 1.6 mg L^{-1}. The dotted lines indicate IVF values of the 10% of control cultures. Error bars are deviations between duplicate cultures.

Further, the decline of IVF during 0 – 12 h of H_2O_2 addition followed first order kinetics in cultures with 1.2 and 1.6 mg L^{-1} H_2O_2 (Figure 4.2). The rate constants were 0.32 ± 0.003 h^{-1} in stationary cultures, but were only 0.16 ± 0.005 and 0.22 ± 0.001 h^{-1} (for 1.2 and 1.6 mg L^{-1} H_2O_2 addition, respectively), i.e. 30 – 50% lower in exponential cultures ($p \leq 0.016$, two sample t-test).

Two-way ANOVA on growth responses (percent relative to control) against growth phase and H_2O_2 concentration suggests significant effect (p = 0.000) of phase on growth response for IVF at 2, 4, 6, and 12-h, total chl a at 6-h, and cell density at 24-h (Figure 4.3). To further compare the effect of algal growth phase on its susceptibility to H_2O_2, EC_{50} (medium effective concentration) was estimated for IVF-4h, IVF-6h, IVF-12h, chl a-6h and cell density-24h. (EC_{50} was not estimated for IVF-2h, because percent inhibition was no more than 50% even at the highest H_2O_2 addition.) Logistic model

$R = \frac{K}{1+e^{n(\log C - \log EC_{50})}}$ was used, where R is the growth response relative to the control (without H_2O_2 addition), K is the growth response of the control (K = 1), C is nominal initial concentration of H_2O_2 (mg L^{-1}), *n* and EC_{50} are indicative of susceptibility, and are the slope and the medium effective concentration, respectively. Results show that EC_{50} values for IVF-4h, IVF-6h, IVF-12h, chl-6h and cell density-24h were lower by 20 – 35% (paired t-test, p = 0.003) and the slopes were higher by 20 - 110% (p = 0.000) in stationary cultures as compared to exponential cultures (Table 4.1). Both indicate substantially greater susceptibility of stationary phase cells to H_2O_2 exposure.

Using the resultant logistic models, EC_{90}s were estimated, H_2O_2 concentration that would cause 90% growth reduction relative to control. In the exponential phase cultures EC_{90} ranged 1.4 – 2.4 mg L^{-1} for IVF-4h, IVF-6h and IVF-12h, but they were 40% lower (1.0 – 1.5 mg L^{-1}) in stationary phase cultures. This difference was also statistically significant (paired t-test, p = 0.039).

Table 4.1 The Slope and EC_{50} from Logistic Modeling of the Growth Response Against Nominal Initial Concentration of H_2O_2

	IVF-4h	IVF-6h	IVF-12h	Chl a-6h	Cell density-24h
Exponential phase					
Slope	6.71±1.54	8.01±2.25	7.37±1.33	2.35±0.47	2.50±0.58
EC_{50}	1.15±0.07	1.07± 0.08	0.70±0.05	1.27±0.14	0.99±0.11
R	0.979	0.972	0.990	0.968	0.960
Stationary phase					
Slope	9.26±2.04	9.80±0.97	9.89±0.50	4.22±0.72	5.29±0.79
EC_{50}	0.88±0.05	0.70±0.02	0.57±0.01	0.94±0.07	0.67±0.05
R	0.990	0.998	0.999	0.983	0.988

The errors represent standard error from the model fitting of the averaged growth responses from duplicate cultures, which were larger than the deviation between duplicates.

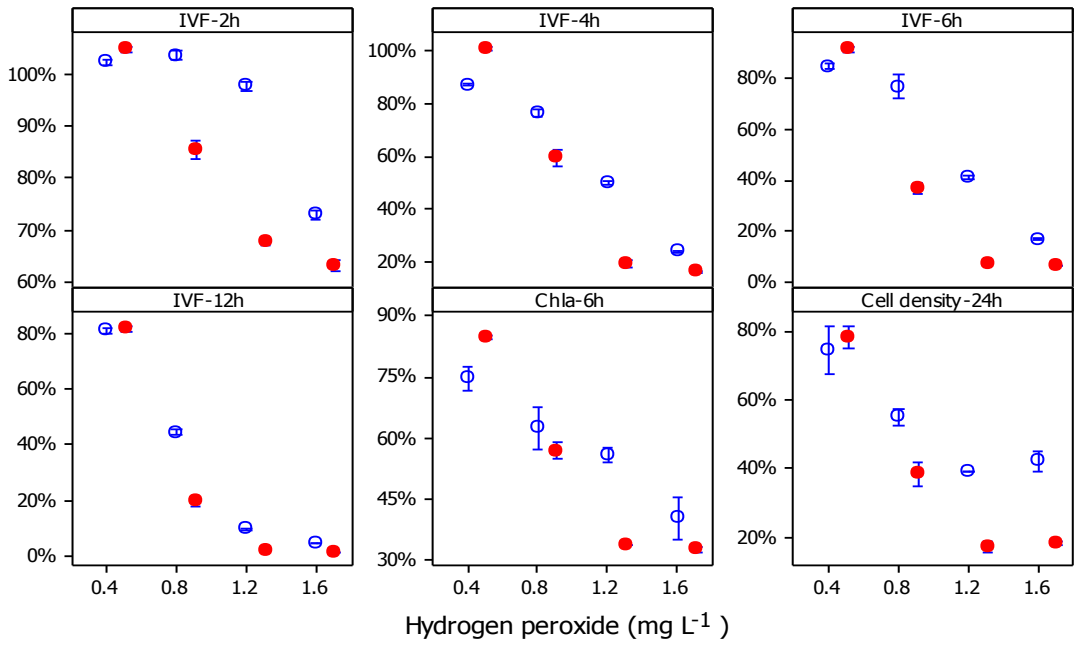

Figure 4.3 Interval plot of the growth responses (percentage relative to the controls) for IVF-2h, IVF-4h, IVF-6h, IVF-12h, chl a-6h and cell density-24h in EP (○) and SP (●)cultures exposed to 0.4, 0.8, 1.2 and 1.6 mg L^{-1} H_2O_2. The error bars are propogated error of the precentage response from the duplicate cultures.

4.3.2 Physiological Responses

Overall, physiological responses of the test cultures to H_2O_2 addition were reflected in temporal variation of maximum quantum yield of photosytem II (F_v/F_m, Figure 4.4). Specifically, in the exponential phase cultures, F_v/F_m values were above 0.50 at 0 and 0.4 mg L^{-1} H_2O_2 addition (averaging 0.54 ± 0.02, 0 – 48 h, healthy physiological status), except at 1-week (168 h) where F_v/F_m reached 0.40 likely because of entering late exponential phase and/or approaching stationary phase after 1-week robust growth (Figure 4.4a). Mild physiological stress was observed at 0.8 mg L^{-1} H_2O_2 addition during 12 – 48 h, during which F_v/F_m reduced from 0.53 ± 0.02 (control cultures) to 0.46 ± 0.02 (p = 0.027, two sample t-test), but the stress status probably did not persist much longer and at 168 h F_v/F_m averaged 0.52 ± 0.00 (Figure 4.4a) which was consistent with robust

re-growth upon initial inhibition as observed in IVF. When exposed to higher H_2O_2 concentrations (1.2 and 1.6 mg L^{-1}), the exponential test cultures maintained healthy F_v/F_m value for 2 h then substantially reduced (to 0.35 at 4 h and no more than 0.1 at 6 – 168 h with 1.2 mg L^{-1}, and to zero at 1.6 mg L^{-1} H_2O_2 (Figure 4.4a).

In comparison, the stationary cultures had greater and more rapid stress responses (Figure 4.4b). The stationary phase test cultures had increased its maximum quantum yield from 0.37 of the inoculums to 0.48 upon transferring into fresh media and before H_2O_2 addition. They were having healthy F_v/F_m of above 0.5 (averaging 0.53 ± 0.01) in cultures with zero or lowest H_2O_2 addition during 2 – 48 h, but were reduced to 0.33 ± 0.02 at 1-week possibly due to nutrient limitation after 1-week of healthy growth (Figure 4.4b). Slight physiological stress was observed in stationary phase cultures at 0.8 mg L^{-1} H_2O_2 addition as early as 2 h, and during 2 – 48 h F_v/F_m averaged 0.40 ± 0.03, but it was eventually reversed with Fv/Fm averaging 0.54 ± 0.00 at 168 h (Figure 4.4b).

Further, H_2O_2 addition at 1.2 and 1.6 mg L^{-1} completely damaged photochemical capability of the stationary phase cultures (F_v/F_m zero) during entire observation period of 2 – 168 h (Figure 4.4b). Substantial reduction in maximum quantum yield at 1.2 and 1.6 mg L^{-1} H_2O_2 was consistent with substantial growth reduction (> 90%, relative to control in IVF) and no observable re-growth over 1-week.

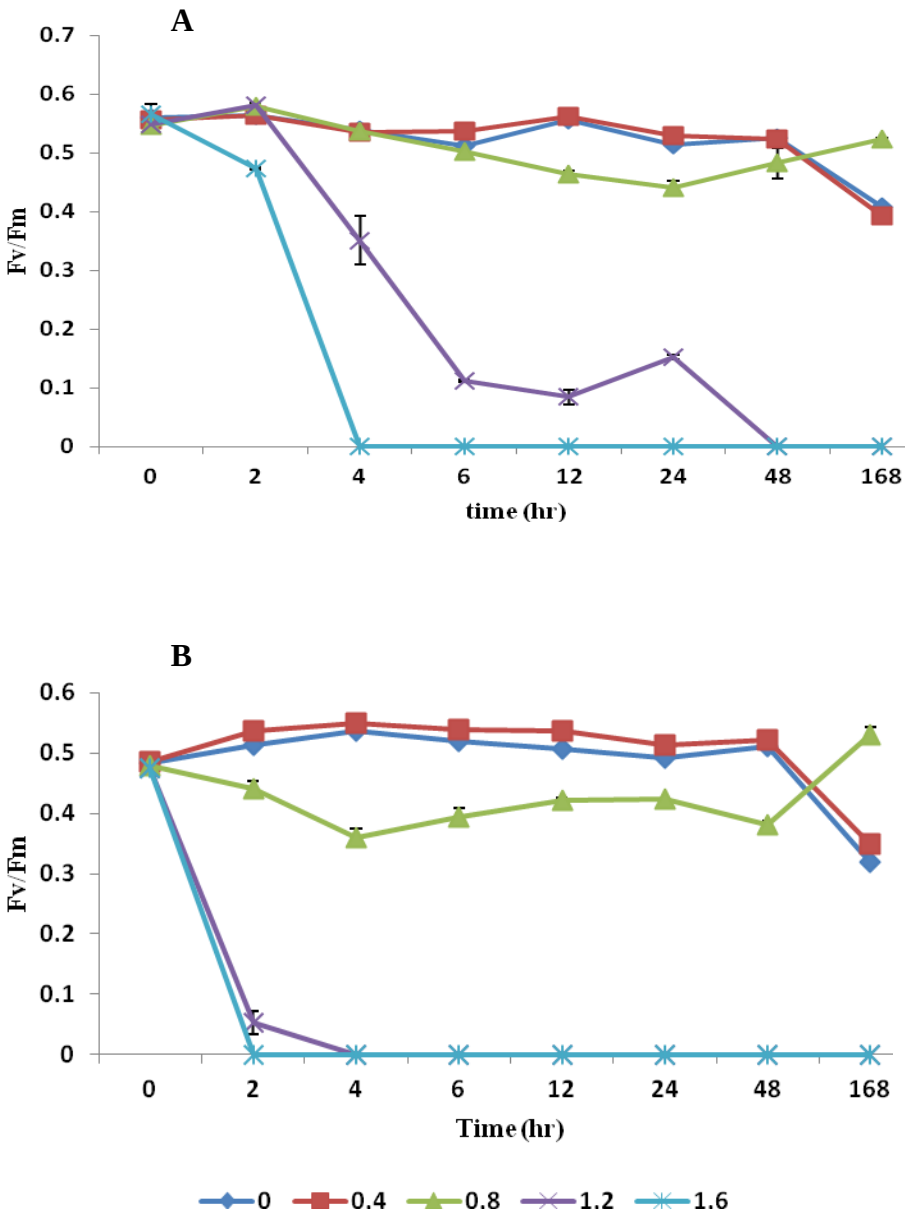

Figure 4.4 Temporal variation of maximum quantum yield of photosystem II (F_v/F_m) in exponential (a) and stationary phase (b) test cultures. Error bars are deviations between duplicate cultures.

A second measure of cells' physiological response to H_2O_2 addition was a snapshot of intracellular non-protein thiol concentration measured at 6-h of H_2O_2 addition. Total non-protein thiols were normalized either to the volume of the culture (nmol L^{-1} culture) as a measure of total amount of thiols in the cultures (Figure 4.5a), or

to total chl a measured at the same time point (µmol g^{-1} chl a) as biomass normalized non-protein thiol concentration (Figure 4.5b). In this snapshot, non-protein thiols were the highest at 0.8 mg L^{-1} H_2O_2 addition as compared to the other levels of H_2O_2 addition (Figure 4.5). Specifically, they were 2.5 and 1.3 times of the controls upon volume-normalization, and 4 and 2.3 times of the controls upon biomass normalization, for exponential and stationary cultures, respectively. Thus, 0.8 mg L^{-1} H_2O_2 addition stimulated intracellular non-protein thiol production, and the stimulation was substantially greater in exponential cultures than stationary cultures. Lower than 0.8 mg L^{-1}, non-protein thiols were not significantly different in the control cultures and cultures with 0.4 mg L^{-1} H_2O_2 addition (statistics available only for the stationary phase cultures), consistent with the no-effect observations in IVF and F_v/F_m at these concentrations. Higher than 0.8 mg L^{-1}, 1.2 and 1.6 mg L^{-1} H_2O_2 addition seemed to reduce cultures' total non-protein thiols (nmol L^{-1} cluture), but still enhanced biomass based non-protein thiols (µmol g^{-1} chl a) (Figure 4.5). Explicitly, non-protein thiols were not different from or 40% lower than the controls upon volume-normalization, but were still 80% higher than the controls upon biomass normalization, for exponential and stationary cultures, respectively (Figure 4.5).

4.3.3 Hydrogen Peroxide Decomposition

H_2O_2 concentration decreased log-linearly in the cultures (exponential and stationary), fresh Aquil, and the 0.2 µm filtrate of the cultures during the initial 6 h of incubation (e.g., Figure 4.6a, showing 1.6 mg L^{-1} H_2O_2 addition only). The log-linear decay was not significant in cultures with 0.4 mg L^{-1} H_2O_2 addition ($p > 0.1$), but they were statistically significant at $p \leq 0.1$ in cultures with 0.8 mg L^{-1} or higher H_2O_2 addition.

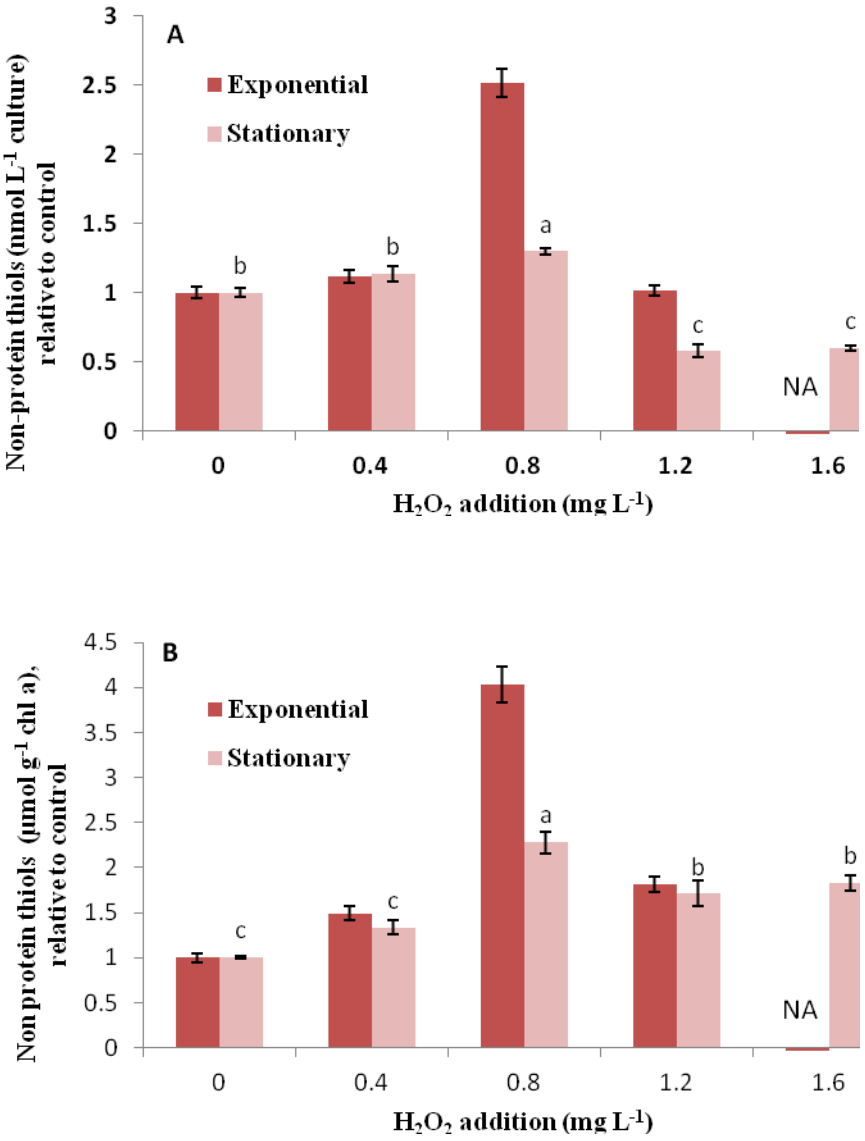

Figure 4.5 Relative amount of non-protein thiols in cultures with 6-hr of H_2O_2 addition as compared to the control (no H_2O_2 addition). Non-protein thiols were normalized to volume (nmol L^{-1} culture, A) and total chl a (B: µmol g^{-1} chl a). Error bars are deviations between duplicates in stationary cultures; the exponential phase cultures did not have replicate results and the error bars on them were estimated as 4% in A and 5% in B based on duplicate stationary cultures. NA: not available. Different letters (a, b, c) above the stationary cultures indicate statistically significant differences according to one way ANOVA with Fisher's multiple comparison.

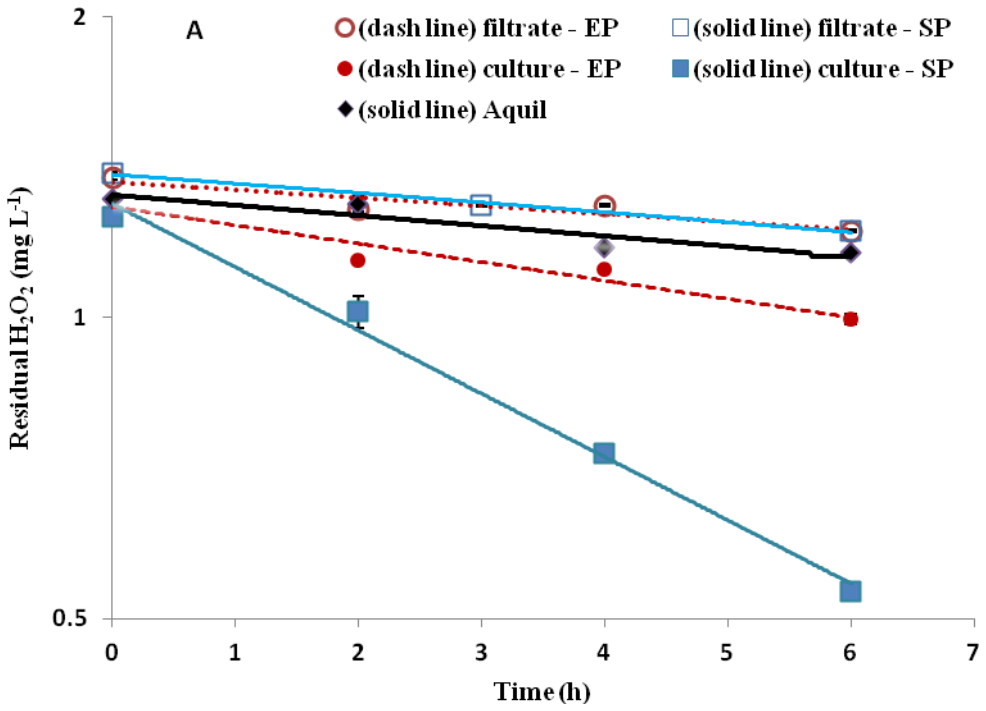

Figure 4.6 (a) Log-linear decrease of residual H_2O_2 concentration in fresh Aquil, exponential culture, stationary culture and its 0.2 µm filtrate, all with 1.6 mg L^{-1} H_2O_2 addition. Error bars are deviations between duplicate cultures. (b) H_2O_2 decay rate constant (h^{-1}) in exponential and stationary cultures with 0.8, 1.2 and 1.6 mg L^{-1} H_2O_2 addition, and in fresh Aquil with 1.6 mg L^{-1} H_2O_2. Error bars are standard error from model fit, as they are larger than the deviations between the duplicate cultures.

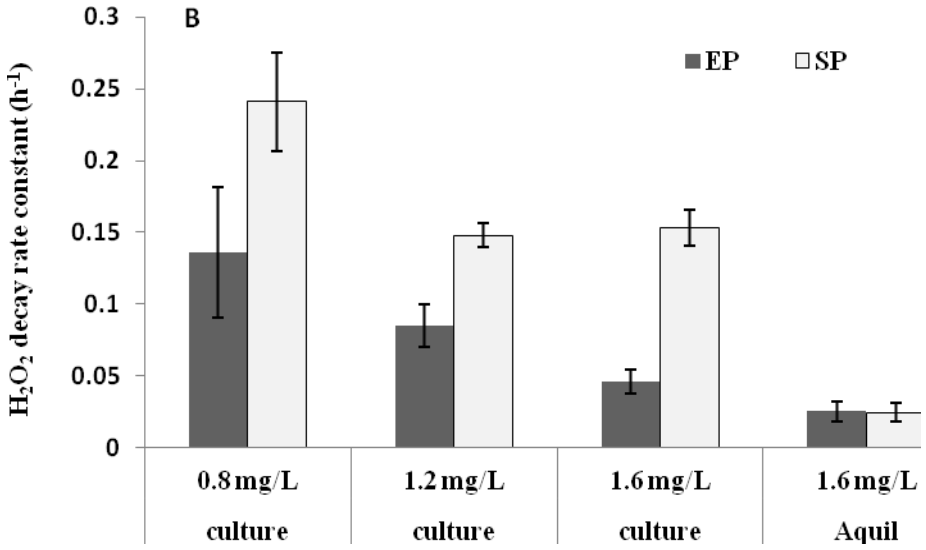

Figure 4.6 (Continued) (a) Log-linear decrease of residual H_2O_2 concentration in fresh Aquil, exponential culture, stationary culture and its 0.2 µm filtrate, all with 1.6 mg L^{-1} H_2O_2 addition. Error bars are deviations between duplicate cultures. (b) H_2O_2 decay rate constant (h^{-1}) in exponential (EP) and stationary (SP) cultures with 0.8, 1.2 and 1.6 mg L^{-1} H_2O_2 addition, and in fresh Aquil with 1.6 mg L^{-1} H_2O_2. Error bars are standard error from model fit, as they are larger than the deviations between the duplicate cultures.

H_2O_2 decay rate constants, k_{H2O2}, obtained from the slope of log-linear curve, were compared for exponential and stationary cultures (Figure 4.6b). In the exponential cultures k_{H2O2} decreased (linearly) with increasing H_2O_2 addition (p = 0.001, linear regression); but that was insignificant in stationary cultures (p = 0.354, ANOVA) where they averaged 0.18 ± 0.03 h^{-1} for 0.8 – 1.6 mg L^{-1} H_2O_2 addition (Figure 4.6b). Further, at the same level of H_2O_2 addition, k_{H2O2} was typically greater in the stationary cultures than the exponential cultures (Figure 4.6b). The difference was statistically significant at 1.6 mg L^{-1} H_2O_2 addition for a 2-fold greater k_{H2O2} in stationary than the exponential cultures (0.15 ± 0.01 versus 0.05 ± 0.01 h^{-1}, Figure 4.6b). At 1.6 mg L^{-1} H_2O_2 k_{H2O2} in fresh Aquil (0.03 ± 0.01 h^{-1}, n = 4) was about half of that in the exponential cultures (Figure 4.6b).

Moreover, H_2O_2 decay rate constants were substantially higher in the culture than in the 0.2 µm filtrate of the cultures. For example, while was H_2O_2 decomposed at 0.13 ± 0.01 h^{-1} in a stationary phase culture with nominal 1.5×10^6 cell mL^{-1} and 1.6 mg L^{-1} H_2O_2 addition, it was decreased at only 0.02 ± 0.00 h^{-1} in the 0.2-µm filtrate of this culture. In the filtrate of the exponential phase culture with 1.7 $\times10^6$ cell mL^{-1} and 1.6 mg L^{-1} H_2O_2 addition k_{H2O2} was also very low at around 0.02 ± 0.00 h^{-1}, and was comparable to that in fresh Aquil. Subtracting the contribution of the media to H_2O_2 decomposition, it appeared that 1.6 mg L^{-1} H_2O_2 was decomposed by 1.6 ± 0.1 $\times10^6$ cell mL^{-1} stationary phase cells at 0.11 – 0.12 h^{-1}, as compared to 0.02 – 0.03 h^{-1} by exponential phase cells under the same conditions. Thus, the decay of H_2O_2 in cultures was mediated mostly by algal cells and less by the media, and further, the stationary phase cells mediated several-fold greater H_2O_2 decomposition than exponential cells.

4.4 Discussion

This study examined the effect of different algal growth phase, thus physiological status, of brown tide alga *Aureococcus anophagefferens* on its susceptibility to hydrogen peroxide exposure. Different physiological status of the cells from exponential and stationary growth phase was indicated in different growth rate and photosynthetic quantum yield. The experiment was designed so as to minimize any effect arising from different initial cell density of *A. anophagefferens*, which had been shown to affect the alga's inactivation by H_2O_2 (Randhawa et al., 2013). The dilution used in making stationary phase test cultures not only reduced cell density to be comparable to the exponential phase test cultures, but also effectively reduced total dissolved organic matter and other H_2O_2 reactive chemical species in media (Myklestad, 2000), so that the media

itself had about the same contribution as the media of the exponential phase cultures or fresh Aquil to H_2O_2 decomposition. This supported an approach of comparing cultures for cells' susceptibility to H_2O_2.

In intact chloroplast, hydrogen peroxide and other reactive oxygen species (ROS) are naturally produced during photosynthesis; but they are rapidly scavenged at the site of generation so that they do not inactivate the enzymes, photosystem, or the scavenging system itself (Asada, 1999, Pospíšil, 2009, Pospíšil, 2012). It is remarkable that externally added hydrogen peroxide as low as 0.8 mg L^{-1} greatly increased intracellular levels of total non-protein thiols (which are effective ROS scavengers) (Sies, 1999) observable at 6-h of H_2O_2 addition (Figure 4.5). Exponential phase *A. anophagefferens* cells demonstrated substantially greater capacity for non-protein thiol up-regulation when challenged with H_2O_2, rendering greater protection and thus less damage (e.g., to the photosystem as observed in PSII quantum yield, Fv/Fm) by H_2O_2 addition as compared to the stationary cells.

Although intracellular non-protein thiol production system (and potentially other ROS scavenging systems too) was weaker in stationary than exponential phase cells, "cell-mediated" H_2O_2 decomposition were typically greater in stationary than exponential cultures (Figure 4.6). It was clear that the media (no algal cells) mediated only a minor fraction of H_2O_2 decomposition (e.g. 0.02 h^{-1}) in our study, and the majority of H_2O_2 was decomposed by reacting with the cells (e.g., more than 0.1 h^{-1} in stationary cultures). It's speculated that the weaker ROS scavenger systems in stationary phase cells lead to greater plasma membrane damage, hence incurred greater cell leakage and even cell burst by externally added H_2O_2 (cell density was more substantially reduced by H_2O_2 addition

in stationary cultures than exponential cultures, Figure 4.3). Consequent release of intracellular materials including catalase and other enzymes involved in H_2O_2 decomposition greatly promoted rapid H_2O_2 decomposition. The relationship between k_{H2O2} and H_2O_2 addition in the culture study was not explained with the current data precision but it could indicate Michaelis-Menten or other types of interactions.

Most evident from this study was that *A. anophagefferens* of stationary growth phase was more susceptible than exponential phase. This was observed from both growth response (e.g., initial rate of IVF decline upon H_2O_2 addition, EC_{50} and *n* on IVF-4, 6, 12 h, chl a-6h, and cell density-24h) and physiological changes (non-protein thiols and Fv/Fm). Stationary phase cells potentially would require substantially less H_2O_2 than exponential cells to attain the same levels of inactivation.

Altogether, this study provided some biochemical understanding on hydrogen peroxide interacting with *A. anophagefferens* cells; and the findings of different H_2O_2 susceptibility between stationary and exponential phase cells potentially improve field trials of brown tide bloom control using hydrogen peroxide.

CHAPTER 5

HYDROGEN PEROXIDE AND BROWN TIDE ALGA CONTROL IN BARNEGAT BAY WATER - FIELD ENCLOSURE STUDY

5.1 Introduction

The interest in environmentally friendly chemicals has been on the rise in recent years. One such chemical is hydrogen peroxide (H_2O_2), which decomposes rapidly into harmless products water and oxygen. It has been used as a biocide to control microorganism growth in environmental(Meinertz et al., 2008, Schmidt et al., 2006), medical and industrial fields. Application of H_2O_2 has been found of great potential in the control of harmful algal blooms. It has been tested and proven effective for the selective removal of harmful blooms caused by cyanobacteria in natural lakes(Matthijs et al., 2012) and in waste stabilization ponds(Barrington and Ghadouani, 2008). Recently its application in the removal of brown tide alga *Aureococcus anophagefferens* was also explored(Randhawa et al., 2012).

Although the effectiveness of H_2O_2 in controlling brown tide bloom has been proven in laboratory cultures and microcosms(Randhawa et al., 2012), the success of the method in field application could be influenced by other factors, especially the dynamics of hydrogen peroxide in natural waters and UV-H_2O_2 synergism on microbial inactivation. H_2O_2 is produced naturally in sunlit surfaces of water bodies(Cooper, 1988), which would add to any externally applied concentration. On the other hand, the decay of externally added H_2O_2 may be substantially enhanced by UV irradiance (Drábková et al., 2007a).

UV light has been shown to enhance microbial inactivation by H_2O_2 due to increased hydroxyl radical formation and photo-Fenton reaction(Drábková et al., 2007a).

The irradiance, especially the UV component, commonly applied under laboratory culture conditions is far below that of normal summer day light when brown tide tends to bloom. In addition, H_2O_2 is consumed by reacting with dissolved humic substances, and dissolved iron catalyzes H_2O_2 decomposition via Fenton reaction. These factors may decrease treatment efficacy of any given H_2O_2 addition. Owing to these and other potentially variable environmental conditions, a field trial is conducted to test the efficacy of H_2O_2 treatment in brown tide control under natural conditions.

In this study, field enclosure trials were conducted in Barnegat Bay, one of the New Jersey coastal bays affected by brown tides recurrence (Gastrich et al., 2004). Using quartz enclosures the dynamics of H_2O_2 was determined, and the effect of externally added 1.6 mg L^{-1} H_2O_2 on brown tide alga and other co-occurring phytoplankton were assessed through marker pigment analysis via HPLC.

5.2 Materials and Methods

5.2.1 Location and Time

Barnegat Bay water was accessed at Berkeley Island County Park, Ocean County, NJ (39° 52' 23" N 74° 07' 55" W). It was selected for the convenience of water access and low in immediate residential influence. The experiment was conducted on July 31, 2012 from 10 AM to 7 PM U.S. Eastern Time. It was partly cloudy till noon then sunny in the rest of the day. On this day, the Sun rose at 5:56 AM, solar noon was 1:05 and sun set 8:15 PM.

5.2.2 Set Up Field Enclosures

Three small enclosures were set up using UV-transparent open-ended quartz tubes of 150 cm length and 2.54 cm diameter. The quartz tubes were acid-cleaned (0.1 N HCl overnight and Milli-Q water rinse) in laboratory, and were further rinsed with the bay water on site. They were made to stand in water with the bottom 10 cm inserted into the sediments underneath the water column (further secured by string-tying to piers). The tubes enclosed approximately 500 mL bay water of 1-m water depth. The tubes were labeled as enclosure A, B, and C for different treatment: bay water alone, bay water with H_2O_2 addition, and bay water with *Aureococcus anophagefferens* and H_2O_2 addition, respectively.

H_2O_2 was added to the enclosure B and C. H_2O_2 (Fluka-95321, *Trace*SELECT®, 30% w:w, 9.8 M, kept on refrigerator and in dark) was freshly diluted to 10 mM with artificial seawater media Aquil, prepared according to Price et al. (1988/1989). Then 2.6 mL of the diluted H_2O_2 was added (from top of the enclosures, using a pipette) to achieve a nominal concentration of 1.8 mg L^{-1} H_2O_2. Water in the enclosure was immediately mixed by gently moving a looped semi-rigid polypropylene tubing (tubing diameter ~ 0.4 cm) up and down the enclosure for several times.

Laboratory cultured *A. anophagefferens* (CCMP 1984) was added to enclosure C. *A. anophagefferens* (CCMP 1984) was cultured as described before (Randhawa et al., 2012). Approximately 50 mL of *A. anophagefferens* stock culture (stationary phase, 6.5 million cell mL^{-1}) was added to increase *A. anophagefferens* cell density to about 0.65 million cell mL^{-1}, mimic a Category 3 intensity brown tide (Gastrich et al., 2004). After

adding *A. anophagefferens* the water was mixed again the same way as after H_2O_2 addition.

5.2.3 Sample the Enclosures

A sampler syringe, made of 30-mL syringe (BD, sterile) luer-lok connected with a long polypropylene tubing (pre-rinsed with bay water), was used to take water samples from each enclosure. The sampler was first rinsed by drawing in 20-mL water into syringe, disconnecting at the luer lock point, then rinsing the syringe and rejecting the water. The sampler was then used in actual water sampling for H_2O_2 and phytoplankton. When water samples of different depth were withdrawn, care was taken to minimize vertical mixing.

5.2.4 Determine Hydrogen Peroxide Production

H_2O_2 samples were collected from each enclosure at three water depths (0.1, 0.4, and 0.8 m) and four time points (10 AM, 1, 4, 7 PM). Typically the sampler syringe drew in ~ 15 mL water; the sampler syringe was disconnected from the sampler, and re-connected to a 0.2 µm syringe filter (25 mm, Pall Life Science, Acrodisc) via luer lok, and the filtrate was collected in 2 mL amber microcentrifuge tubes after being rinsed with 2 mL filtrate. H_2O_2 concentrations were determined following the method of Lu et al. (Lu et al., 2011). A 0.75 mL filtrate was mixed with equal volume p-nitrophenyl boronic acid (p-NPBA) solution (2 mM in 150 mM bicarbonate-carbonate buffer, pH 9) in another amber microcentrifuge tube. After 20 minutes reaction at ambient temperature, the samples were stored in ice for absorbance measurement in laboratory (It was pre-tested that the reaction product, p-nitrophenol, was stable over 12 hours at $\leq 4°C$). The absorbance was measured at 406 nm using Agilent 8453 UV-Vis spectrophotometer. Un-reacted water sample

filtrate was also collected and measured for absorption at 406 nm to account for the absorbance from CDOM (colored dissolved organic matter) in the water samples.

5.2.5 Phytoplankton Analysis

Phytoplankton samples were taken from enclosure A and C at 10 AM and 7 PM (before and after 9-hr H_2O_2 exposure in enclosure C, enclosure A serving as a control without H_2O_2 addition). Both enclosures were thoroughly mixed, and then water samples were taken at mid-depth for phytoplankton. Duplicate 55 mL water sample was used for accessory pigment analysis and duplicate 30 mL for total chl a analysis. The water samples were filtered through GF/F filters (Whatman, 25 mm diameter, nominal pore size 0.7 μm) held in a Swinney filter holder (Pall Life Science) to collect the particulates. The filters with collected particulates were immediately put into either cryogenic vials (for accessory pigment analysis) and plunged in a liquid nitrogen thermal flask or submerged in 1.5 mL of 90% acetone in 2-mL amber micro-centrifuge tube and stored in ice (for total chlorophyll a analysis). The samples were transported to the laboratory within 12 hours of collection.

In the laboratory, the accessory pigment samples were transferred into -80°C freezer for later processing, while total chlorophyll a samples were processed right away with sonication, centrifugation, UV-Vis spectrophotometric quantification of supernatant for total chlorophyll a as described before (Randhawa et al., 2012). The accessory pigments were analyzed with HPLC using Van Heukelem and Thomas method (2001). Chromatographic peak areas for 19'-butanoyloxyfucoxanthin (19'-bf), fucoxanthin, chlorophyll b (chl b) and lutein, dinoxanthin were recorded to track the temporal changes of brown tide alga *A. anophagefferens,* diatoms and other chrysophytes, chlorophytes,

and dinoflagellates and related groups, respectively, in the same way as was described in Randhawa et al. (2012).

5.2.6 Statistical Analysis

Statistical analysis was conducted with Minitab 16 Software Package with probability $p < 0.05$ being accepted as statistically significant. ANOVA (analysis of variance) was used to compare different depths, time points, and enclosures.

5.3 Results

5.3.1 Hydrogen Peroxide in Barnegat Bay

Temporal variation of H_2O_2 concentration is shown in Figure 5.1 for enclosure A (bay water alone), B (bay water with H_2O_2 addition) and C (bay water with H_2O_2 and *A. anophagefferens* additions) at different depths (0.1, 0.4 and 0.8 m, Figure 5.1 A, B, C), or upon depth average (Figure 5.1D). For each of the three enclosures, there was significant effect of time on H_2O_2 concentration (ANOVA, $p < 0.05$, Figure 5.1D). In enclosure A, H_2O_2 was 0.20 mg L^{-1} mid-morning at 10 AM and peaked at solar noon (1 PM) at 0.23 mg L^{-1} (thus a net production rate of 0.03 mg L^{-1} per 3-h), then decreased gradually to 0.13 mg L^{-1} around sunset 7 PM with maximum decrease occurring at 4 – 7 PM at 0.08 mg L^{-1} per 3 h. The depth variation of H_2O_2 concentration in enclosure A was quite small ($\leq$ 0.015 mg L^{-1}), and was not statistically significant according to two-way ANOVA (against time and depth) ($p = 0.064$ for depth). In enclosure B and C, hydrogen peroxide averaged 1.6 ± 0.03 mg L^{-1} (n = 6) upon dispersion in the enclosures (10 AM), and it did not change substantially at solar noon (1.56 ± 0.11 mg L^{-1}, n = 6), but decreased during 1 – 7 PM to 0.57 ± 0.12 mg L^{-1} (n = 6) at 7 PM with maximum decrease observed during 4

– 7 PM at 0.7 – 0.8 mg L^{-1} per 3-h. Between enclosure B and C there was no significant difference, except at 4 PM when H_2O_2 was slightly lower in C than in B (paired t-test p = 0.018). The depth variation of H_2O_2 concentration was significant in both enclosure B and C (p = 0.046 and 0.018, respectively), and were on average 15 – 16% lower at 0.8 m than 0.1 m during 1 – 7 PM.

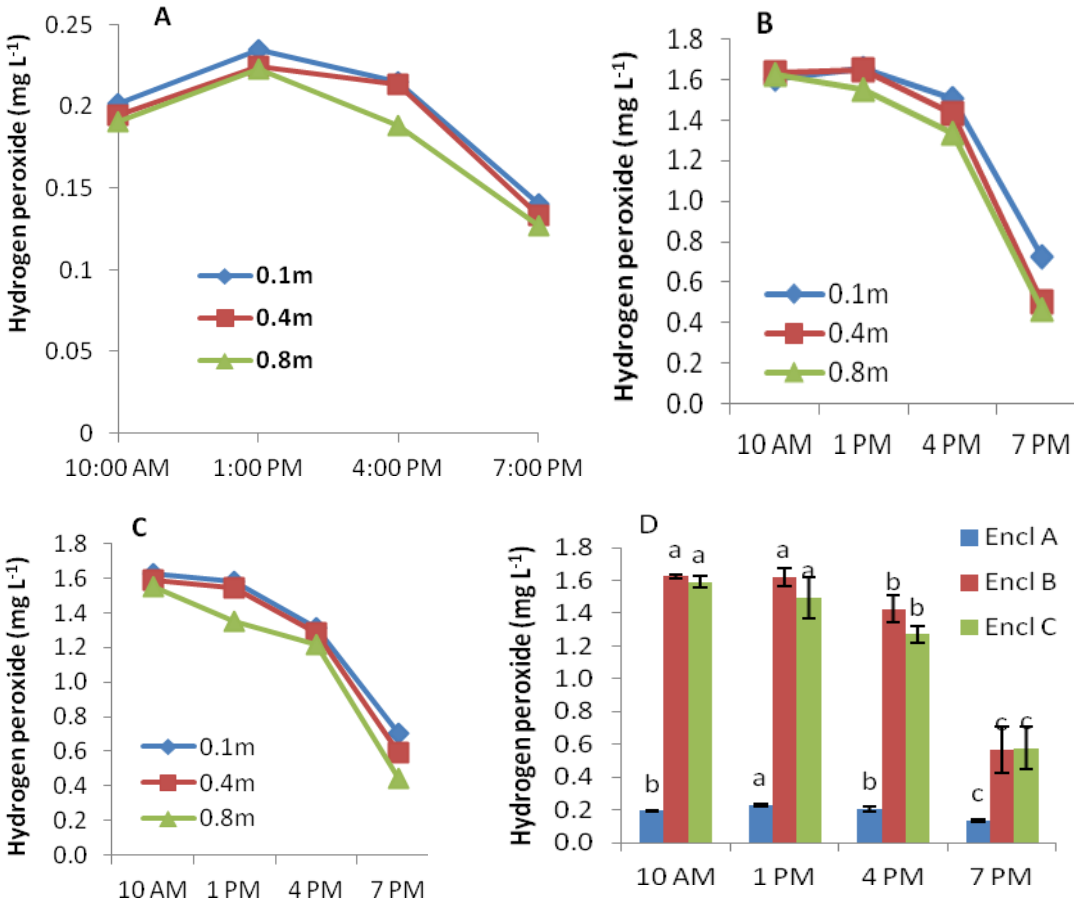

Figure 5.1 Temporal variation of hydrogen peroxide in enclosure A, B, C at 0.1, 0.4 and 0.8m water depth (A – C). Average H_2O_2 concentration (over depth) in these enclosures at different time points, different letters (a, b, c) indicate statistical difference from one way ANOVA with Fisher's multi-comparison (D).

5.3.2 Effect of H_2O_2 Exposure on Phytoplankton

Upon dispersing ~ 0.65 million cell mL^{-1} of *A. anophagefferens* to the enclosure C, total chl a increased by 11% as compared to enclosure A and B (p = 0.018, Figure 5.2). After 9-hr exposure to nominal 1.8 mg L^{-1} (and measured 1.6 mg L^{-1}) hydrogen peroxide, total chl a levels were not significantly different among three enclosures, although enclosure B and C with H_2O_2 addition on average was 18% lower than enclosure A (p = 0.052, Figure 5.2).

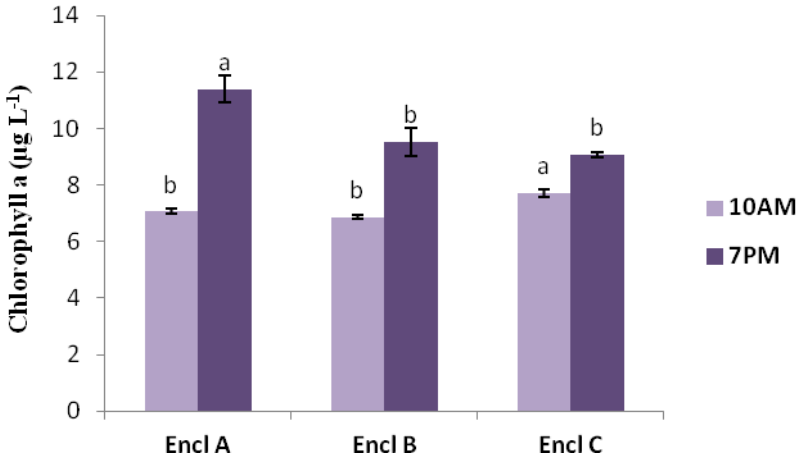

Figure 5.2 Total chlorophyll a in enclosure A, B and C at 10 AM (immediately after dispersing H_2O_2 and *A. anophagefferens* where applicable), and at 7 PM (end of the experiment). Letter a, b, c indicate significant difference by Fisher's multi-comparison, for 10AM data set ANOVA p = 0.018, but 7PM data set ANOVA p = 0.052.

The major accessory pigments identified in the phytoplankton samples include dinoxanthin (dino), fucoxanthin (fuco), chl b, lutein, suggesting substantial presence of dinoflagellates, diatoms, and green algae. *A. anophagefferens'* marker pigment 19-bf was identified only in samples from enclosure C after *A. anophagefferens* addition (10 AM). Figure 5.3 shows the percent change of algal pigments in enclosure A and C over 9-hr from 10 AM to 7 PM. The percent change in enclosure A serves as a control, while that

in enclosure C reflects the effect of 9-h H_2O_2 exposure on phytoplankton assemblage including that on the artificially created brown tide bloom (enclosure B was not sampled for algal pigment analysis). Two-way ANOVA showed significant difference between enclosure A and C, and among different pigments (p = 0.000 for both enclosures and pigments, no factorial interaction as indicated by p = 0.374). In both enclosure A and C, dinoxanthin and fucoxanthin increased more substantially than chl b, lutein or chl a (p = 0.000, one-way ANOVA, Figure 5.3). The increase was 32 – 36% for dino and fuco and only 13 – 17% for chl b, lutein and chl a in enclosure A; and they were 27 – 28% versus 9 – 12 % in enclosure C (Figure 5.3).

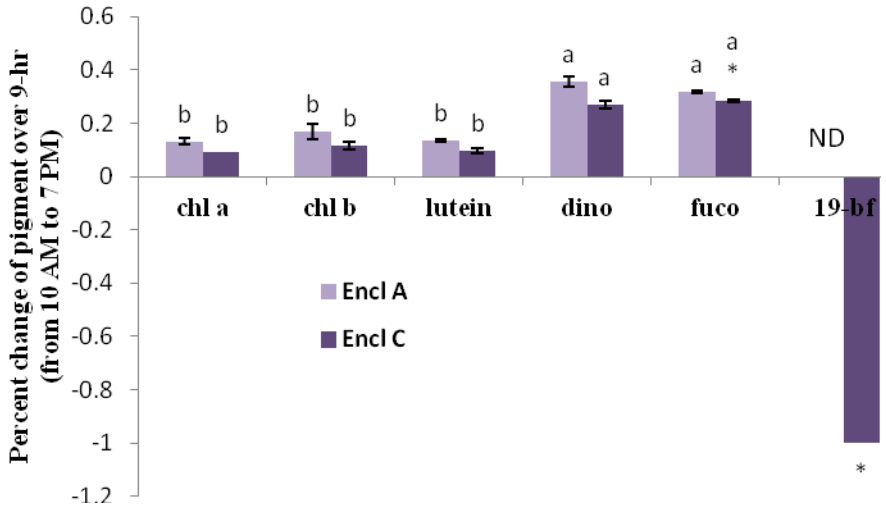

Figure 5.3 Percent increase of chl a, chl b, lutein, dinoxanthin (dino), fucoxanthin (fuco) and percent decrease of 19'- butanoyloxyfucoxanthin (19'-bf) at 7 PM as compared to 10 AM in enclosure A and C. * indicates significant difference between enclosure A and C for individual pigment ($p < 0.05$).

19'-bf was reduced by 100% in enclosure C, indicating complete eradication of the brown tide alga. Between enclosure A and C, enclosure C tends to have lower percent increase than A for chl a, b, lutein, dino and fuco (p = 0.000, paired t-test). When

individual pigment were compared, other than substantial difference in 19'-bf, the only statistically significant difference was that for fucoxanthin, which increased 32% in enclosure A versus 28% in C (p = 0.038, one way ANOVA).

5.4 Discussion

This study set out to test the dynamics of hydrogen peroxide in brown tide-prone natural waters and how this may affect the use of hydrogen peroxide in controlling brown tide bloom. It was shown that hydrogen peroxide increased from morning to solar noon then decreased from solar noon to sunset in unmodified seawater (enclosure A). This natural photochemical production of hydrogen peroxide was relatively small as compared to the amount of H_2O_2 addition in enclosure B and C for brown tide control; and the photochemical production did not cause any significant increase in H_2O_2 concentration in enclosure B and C even at solar noon, rather H_2O_2 decomposition was dominating (Figure 5.1).

The natural fluctuation of H_2O_2 as measured in enclosure A must be interpreted with care. Although the overall trend (increase in the morning, peak at solar noon, then decrease) agreed with the diurnal cycle of H_2O_2 observed in typical surface seawater, H_2O_2 concentration 0.2 mg L^{-1} (~ 5900 nM), and the net rate of H_2O_2 production during 10 AM to 1 PM (~ 0.03 mg L^{-1} over 3-hr, approximately 300 nM h^{-1}) were an order of magnitude higher than the high values reported for surface seawater. For example, H_2O_2 concentration in its diurnal pattern were of a few to several hundreds nM in surface seawaters (Clark et al., 2008), and H_2O_2 net photochemical production rate was on the order of tens nM h^{-1} e.g. 30 – 59 nM h^{-1} in the 0.2 μm filtrate of Narragansett Bay water, Rhode Island (Miller et al., 1995). Clark et al. (2008) did point out that H_2O_2 production

rates could be much higher if CDOM levels are high, which enhance photochemical production, as was observed in lakes and rivers with net H_2O_2 production rate of 81 – 2120 nM h^{-1} (Scully et al., 1996). The seawater examined in this study had a quite low absorbance at 406 nm (comparable to Milli-Q water on 10cm spectrophotometric cuvette) thus contributed little to interfere with absorbance measurement of the color product in hydrogen peroxide quantification; but it does not rule out relatively high level of CDOM in these water samples. However, the most important results from the H_2O_2 dynamics was that the environmental factors in the bay water did not promote a substantial increase in H_2O_2 concentration as compared to that was initially added, thus very low probability of overdosing because of H_2O_2 and natural environmental factor interaction.

Phytoplankton assemblage analysis showed that total phytoplankton biomass was increasing from 10 AM to 7 PM in untreated seawater, and percent increase was greater in dinoflagellates and diatoms than green algae. Similarly, in bay water with 0.65 million cell mL^{-1} *A. anophagefferens* and measured 1.6 mg L^{-1} H_2O_2 addition, total phytoplankton also increased from 10 AM to 7 PM, and the increases were not significantly different from that of untreated seawater for dinoflagellate and green algae, and was only 4% less in diatoms, while *A. anophagefferens* was eradicated by H_2O_2 addition (Figure 5.3). These results suggest a selective removal of *A. anophagefferens* from the phytoplankton assemblage, and were consistent with culture and microcosm study where *A. anophagefferens* was shown to be more sensitive to H_2O_2 than most species of other algal groups, and was preferentially eliminated while not substantially affecting the rest of the algal assemblage (Randhawa et al., 2012). Although any conclusions on the effect of H_2O_2 on cyanobacteria population could not be made

because zeaxanthin peak (marker pigment for cyanobacteria) could not be located precisely in chromatograms of the field samples, but, as discussed by Randhawa et al, the effect would vary widely with the species in case of cyanobacteria, and same would be the case with aquatic bacteria community (Randhawa et al., 2012).

It also appeared from this study that the externally added hydrogen peroxide could be lower at deeper depth than at surface when vertical mixing was excluded. The small diameter and rigid-wall quartz prevented natural wind or wave driven vertical mixing. An average 15 – 16% lower was observed at 0.8 m than 0.1 m during 1 PM – 7 PM in enclosure B and C, which could be from a greater photochemical production of H_2O_2 at surface water at 0.1 m, and/or from more rapid microbe-mediated H_2O_2 decomposition (Petasne and Zika, 1997) at 0.8 m as it was only ~ 0.2 m from the sediment layer, where bacterial activity maximum was expected as compared to the shallow water column. Depending on the depth H_2O_2 was decomposed to 0.4 - 0.7 mg L^{-1} by 7 PM (9-hr after H_2O_2 addition, Figure 5.1), a concentration considered to be safe to natural aquatic systems (Schmidt et al., 2006). It is speculated that wind or wave action promoting vertical mixing would have some additional effect on H_2O_2 decomposition; and water stratification may promote slightly greater *A. anophagefferens* inactivation on surface than in deeper water.

CHAPTER 6

CONCLUSION

This dissertation work set out to find a solution to one of the most pressing environmental problems of present times - the brown tide blooms, caused by algae *Aureococcus anophagefferens*. This final chapter reviews the research contributions of this dissertation, as well as discusses directions for future research.

6.1 Contributions

6.1.1 Provides an Effective Way to Control Brown Tides

The first and foremost significance of this dissertation is to suggest a method for brown tide control that works quickly and effectively. Even after 27 years of their first observation, there's still no method at hand to prevent, or even control these blooms. The results from this study can be applied to design the commercial scale application of H_2O_2 in controlling brown tide blooms. Specifically, this dissertation:

- Provides an initial assessment on the effectiveness of hydrogen peroxide at killing *A. anophagefferens* cells, and determines the lowest possible application dose.
- Puts forth an evidence for low adverse impacts of hydrogen peroxide application, on phytoplankton that co-occur with the brown tide alga. One major concern with the use of any chemical biocide in environment is its potential effect on the environment and non-target organisms. The effect on environment from hydrogen peroxide application would be minimal due to very low application dose and rapid decay into water and oxygen. This dissertation presented results on varying susceptibility of phytoplankton from different classes (potentially for having varying cellular characteristics). This dissertation also provides literature survey on the effect of H_2O_2 application on other micro- and macro-organisms.

6.1.2 Explores the Factors Affecting the Method Applicability

While H_2O_2 is effective at inactivating brown tide cells, there may be other factors at play. Two hypotheses were tested to explore these factors.

- Studies subjecting brown tide alga of exponential and stationary growth phase to H_2O_2 exposure have been carried out to examine alga's growth phase dependent peroxide sensitivity. This piece of work serves to increase our knowledge on variable sensitivity of algae cells to biocides, as affected by physiological status and it also gives insights on the mechanism of H_2O_2 action.
- The biomass has been examined as another potential factor influencing the method applicability. This study exposed *A. anophagefferens* of different cell densities. Based on observations, an empirical mathematical model is developed. This model could serve as a useful tool in designing the field trials, as it enables us to calculate the H_2O_2 dose required to eliminate the blooms of different density, within a target time period. Since *A. anophagefferens* cell density can be readily determined for field samples, e.g., using monoclonal antibodies, this model can be applied accordingly.

6.1.3 Determines the Practical Applicability of Method

The above mentioned studies were carried out in laboratory setting i.e. under controlled conditions (light exposure, temperature, availability of nutrients and trace metals). Because the conditions in field could be quite different, they may limit the application of H_2O_2. So, to determine effectiveness of method under natural conditions, the small enclosures in brown tide prone water body are treated with H_2O_2. Although it's a small scale trial, it serves to set a stage for further investigations.

Another important aspect considering the field application is - how to distribute hydrogen peroxide to the affected water body without creating concentration hotspots. The dissertation proposes against the pump and treat method for potentially enormous volume of water to be treated, but references a water harrow method, which according to literature produces an even distribution of H_2O_2.

6.1.4 Determines Hydrogen Peroxide Decay Kinetics in Culture Medium

H_2O_2 is known to degrade rapidly. So, part of dissertation focuses on studying the decay kinetics of H_2O_2, in both culture media and the cultures of different growth phases. The H_2O_2 dynamics in brown tide prone Barnegat Bay has also been examined. These studies evidence an important role of brown tide algae cells, and growth phase dependence of cells in mediating H_2O_2 decomposition, and quantified how fast externally added H_2O_2 may be dissipated in natural and culture conditions.

6.1.5 Other Potential Benefit

The stepwise approach used to develop algae control method undertaken in this dissertation may serve as guide to develop controls for other harmful algal blooms, e.g., cyanobacteria bloom.

6.2 Directions for Future Research

This dissertation could be considered as an initial study toward the development of a sophisticated approach to brown tide bloom control. Although this dissertation examined several key factors affecting the method and its success, there are still aspects to be examined to improve the method. The remainder of this chapter lays out some aspects for future research.

6.2.1 Explore Variable Sensitivity of Phytoplankton to H_2O_2

Eleven species of phytoplankton have been tested in this dissertation for their response to H_2O_2 exposure. Their differential sensitivity was described based on cellular characteristics. Although effect of cell size, cell wall etc. were thought to be most

important in different peroxide sensitivity, the additional hypotheses need to be tested to better understand the action of hydrogen peroxide on living cells. The future work may be focused on testing the effect on cell membrane permeability as it changes as a result of exposure to H_2O_2. The changes in lipid content and pigments (acting as antioxidants) upon H_2O_2 exposure may be assessed to further our understanding on factors causing variable cellular response to biocides.

6.2.2 Explore Differential Susceptibility to H_2O_2 at Different Growth Phases

This dissertation reveals that exponential phase cells of *A. anophagefferens* are less susceptible to H_2O_2 than stationary cells, and that H_2O_2 decay is faster in stationary phase cultures. It was speculated that weaker cellular antioxidant components of stationary phase cells led to greater cell membrane disruption and release of catalase and other cellular substances, leading to faster H_2O_2 decay. However, additional studies are required to confirm this speculation. It may include quantifying catalase in the culture medium of H_2O_2 exposed exponential and stationary phase cells. Microscopic studies could be done in order to check for any obvious changes in cell membrane in two types of cells when exposed to H_2O_2.

6.2.3 Practical Approach

As far as practical applicability of method is concerned, only a small scale field study has been carried out. Before the method can be proposed for commercial scale use, larger scale field trial is needed. The iron concentration and dissolved organic matter in natural water are the factors affecting H_2O_2 action and should be quantified in the water body to be treated. The effect of iron and organic matter on the applicability of H_2O_2 treatment of

brown tides may be carried out by chemical amendment experiment to alter iron and organic matter content in field and laboratory cultures, followed up examining the changes in brown tide control efficiency. Results from these additional studies would improve method application in field.

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