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Production and Decomposition of Hydrogen Peroxide by Marine Phytoplankton

Dong-Beom Kim
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**PRODUCTION AND DECOMPOSITION OF
HYDROGEN PEROXIDE BY MARINE PHYTOPLANKTON**

by

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M.S. June 1988, Seoul National Univ., Korea

A Dissertation submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
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ABSTRACT

PRODUCTION AND DECOMPOSITION OF HYDROGEN PEROXIDE BY MARINE PHYTOPLANKTON

Dong-Beom Kim

Old Dominion University, 1993

Director: Dr. William M. Dunstan

H₂O₂ in seawater has complicated sources and sinks. The relative importance of biological regulation of H₂O₂ compared to other processes is not well understood. In addition, environmental factors affecting the biological regulation of H₂O₂ are largely unknown. Marine phytoplankton was examined for the kinetics of the production and decomposition of H₂O₂ in the dark. Effects of varying environmental factors such as light, temperature, salinity, nutrients, amino acids, trace metals and growth phase, were examined. H₂O₂ was determined with the scopoletin-fluorescence decay method.

Five out of 11 species produced H₂O₂, while all of the 11 species decomposed H₂O₂. The relative significance of these species for producing H₂O₂ decreased in the order of *Pleurochrysis carterae* > *Isochrysis galbana* > *Dunaliella tertiolecta* > *Tetraselmis levis* > *Emiliana huxleyi*, and for decomposing it in the order of *Synechococcus* sp. = *Skeletonema costatum* >> *Tetraselmis levis* > *Chaetoceros flexosus* > *Chaetoceros simplex* > *Isochrysis galbana* > *Thalassiosira oceanica* > *Amphidinium carterae* > *Pleurochrysis carterae* >

Emiliana huxleyi > *Dunaliella tertiolecta*. Coccoid or unialgal cells showed a tendency to produce H_2O_2 , whereas diatoms in chains were more likely to decompose H_2O_2 . Both the production and decomposition of H_2O_2 by these algae followed pseudo-first order reactions. The pseudo-first order rate constants related linearly to algal biomass. The biologically-mediated production and decomposition of H_2O_2 showed reaction rate constants (k) ranging from 0.0017 to 0.0072 ($\mu\text{g chl-a}\cdot\text{L}^{-1}$) $^{-1}\cdot\text{hr}^{-1}$ for the production of H_2O_2 and from 0.0242 to 0.0002 ($\mu\text{g chl-a}\cdot\text{L}^{-1}$) $^{-1}\cdot\text{hr}^{-1}$ for the decomposition of H_2O_2 . The studies on the rate kinetics suggested that marine phytoplankton regulates the H_2O_2 budget in surface oceans by mediating primarily decomposition of H_2O_2 rather than production of H_2O_2 .

The biological regulation of H_2O_2 was not strongly affected by physical environmental factors such as light, temperature and salinity. Among the tested factors, amino acids were the most influential factor enhancing the production of H_2O_2 . Inorganic nitrogen-limited conditions stimulated phytoplankton to produce more H_2O_2 per unit biomass. The production of H_2O_2 may be a result of amino acid utilization by nitrogen-starved phytoplankton. However, decomposition of H_2O_2 was not affected by the addition of amino acids. Nutrient effects on the decomposition rate constants were much more profound in coastal species than in oligotrophic species. In general, the biological production of H_2O_2 was small compared to photochemical production but could be significant in nitrogen-limited conditions whereas biological decomposition of H_2O_2 was more important than other removal processes. The results of pure culture studies

generally agreed with the results of the field studies. The oligotrophic Sargasso seawater showed biological production of H_2O_2 whereas the mesotrophic coastal water displayed predominantly decomposition of H_2O_2 . Biological production of H_2O_2 could occur mostly in inorganic nitrogen-limited conditions by a limited number of species whereas biological decomposition of H_2O_2 could remove H_2O_2 from most coastal waters by a large number of species. This study implied that nitrogen dynamics as well as phytoplankton species composition and their abundance are necessary to understand biological roles in H_2O_2 budget. The regulation of H_2O_2 by phytoplankton may also be related to the speciation of trace metals in ambient waters because of strong oxidizing/reducing properties of H_2O_2 .

DEDICATION

To my heavenly father, and
To my deceased father, Hyung-Soo Kim, who is in heaven

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TABLE OF CONTENTS

	Page
LIST OF TABLES	vi
LIST OF FIGURES	vii
CHAPTER	
1. GENERAL INTRODUCTION	1
2. PRODUCTION OF H ₂ O ₂ BY MARINE PHYTOPLANKTON	29
3. DECOMPOSITION OF H ₂ O ₂ BY MARINE PHYTOPLANKTON	63
4. PRODUCTION AND DECOMPOSITION OF H ₂ O ₂ IN OPEN OCEAN AND COASTAL WATERS	96
5. GENERAL SUMMARY AND CONCLUSIONS	137
BIBLIOGRAPHY	142
APPENDICES	
A. THE DETAILS ON THE MATERIALS AND METHODS	155
B. TIME COURSE DATA FOR H ₂ O ₂ NON-PRODUCERS	172
C. TIME COURSE PLOTS FOR H ₂ O ₂ DECOMPOSITION BY MARINE PHYTOPLANKTON	174

LIST OF TABLES

TABLE	Page
1.1. The concentrations of H_2O_2 found in various natural waters	8
1.2. H_2O_2 production by freshwater phytoplankton	18
1.3. H_2O_2 production by marine phytoplankton	19
2.1. List of 11 marine phytoplankton tested for H_2O_2 production	34
2.2. Summary of the kinetic parameters of H_2O_2 production by marine phytoplankton	42
3.1. Summary of the rate constants of H_2O_2 decomposition by marine phytoplankton	78
3.2. Comparison of k values with k values from other studies	82
3.3. Estimated time required for the decomposition of H_2O_2 in the coastal and open ocean	83
4.1. Summary of the physical parameters at stations 1, 4 and 23	111
4.2. Summary of the kinetic parameters of the production or decomposition of H_2O_2 at stations 1, 4 and 23	122
4.3. Summary of control experiments	125
4.4. Phytoplankton species compositions at stations 1, 4 and 23	133

LIST OF FIGURES

FIGURE	Page
1.1. A diagram showing sources and sinks of H_2O_2	5
1.2. Typical depth profiles of H_2O_2 in surface oceans ..	10
1.3. Diel variation of H_2O_2 in surface waters	11
2.1. Control experiments of H_2O_2 production	39
2.2. Kinetic curves of H_2O_2 production	41
2.3. Production of H_2O_2 with different biomass	46
2.4. Effect of light intensity on the production of H_2O_2	48
2.5. Effect of temperature on the production of H_2O_2 ...	50
2.6. Effect of salinity on the production of H_2O_2	52
2.7. Effect of amino acids on the production of H_2O_2 ...	54
2.8. Production kinetics as a function of amino acid ..	55
2.9. Effect of nutrient concentration on the production of H_2O_2	58
2.10. Effect of growth phase on the production of H_2O_2 ..	60
3.1. Control experiments of H_2O_2 decomposition	72
3.2. Toxic effects of H_2O_2 on the growth curves of two marine phytoplankton	74
3.3. Kinetic curves of H_2O_2 decomposition	75
3.4. H_2O_2 decomposition as a function of H_2O_2 concentration	80
3.5. Effect of light intensity on the decomposition of H_2O_2	85
3.6. Effect of temperature on the decomposition of H_2O_2	87

3.7.	Effect of salinity on the decomposition of H ₂ O ₂ ...	89
3.8.	Effect of nutrient concentration on the decomposition of H ₂ O ₂	90
3.9.	Effect of growth phase on the decomposition of H ₂ O ₂	93
4.1.	Map showing locations of the studied areas	102
4.2.	Horizontal distribution of temperature, salinity, density, <i>in vivo</i> fluorescence, and nutrients	108
4.3.	Depth profiles of density and <i>in vivo</i> fluorescence at the three selected stations	110
4.4.	Depth profiles of H ₂ O ₂ at stations 1, 4 and 23	113
4.5.	Horizontal distribution of H ₂ O ₂	115
4.6.	Kinetic curves obtained from the incubation experiment at station 1	117
4.7.	Kinetic curves obtained from the incubation experiment at station 4	119
4.8.	Kinetic curves obtained from the incubation experiment at station 23	121
4.9.	Depth profiles of nitrate, nitrite, and ammonium	128
4.10.	Depth profiles of chlorophyll-a, and chlorophyll-a vs. phaeopigment	130

Chapter 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Aquatic organisms can affect water chemistry by mediating diverse geochemical processes. Biological regulations of hydrogen peroxide (H_2O_2) by phytoplankton and bacteria have been observed (Zepp *et al.*, 1987b; Cooper *et al.*, 1989; Cooper and Zepp, 1990; Cooper *et al.*, 1992). Redox chemistry of the numerous elements in natural waters is affected by H_2O_2 due to its strong oxidizing/reducing properties. H_2O_2 also has toxic impacts on many biological processes. Therefore, the regulation of H_2O_2 concentration may have great consequences for the organisms. However, the production and decomposition of H_2O_2 by phytoplankton have been studied rarely.

Although the major source of H_2O_2 is believed to be photochemical formation (Fig. 1.1) (Zika, 1980, 1981; Cooper and Zika, 1983; Zepp *et al.*, 1987a; Cooper *et al.*, 1988), the importance of the biological roles as sources and sinks of H_2O_2 cannot be neglected (Cooper *et al.*, 1992). H_2O_2 can be produced biologically as a result of the Mehler reaction (Mehler, 1951a, b; Robinson and Gibbs, 1982; Robinson, 1988), photorespiration (Hall *et al.*, 1974), aerobic respiration (Baldwin, 1963; Forman and Boveris, 1982; Pera, 1983; Grufferty and Condon, 1983), cell-surface redox enzymes (Palenik and Morel, 1990a, b), or the complex interaction between endosymbiotic algae and their animal host (Dyken and Shick, 1982). In addition, H_2O_2 can be decomposed biologically by catalase or peroxidase in plants (Zepp *et al.*, 1987b;

Moffett and Zafiriou, 1990). The biological contribution of H_2O_2 to the surface oceans has not been clearly explained (Fig. 1.1) although there are some results suggesting the importance of biological production and decomposition. Phytoplankton in natural waters can produce H_2O_2 in the presence of light (Van Baalen, 1965; Stevens *et al.*, 1973; Patterson and Myers, 1973; Zepp *et al.*, 1987b) or in the absence of light (Stevens *et al.*, 1973; Palenik *et al.*, 1987; Palenik, 1989; Palenik and Morel, 1990a, b). The production of H_2O_2 in the light was observed in a number of freshwater phytoplankton. As for marine phytoplankton, however, there is only one study on the production of H_2O_2 by them in the dark (Palenik, 1989). Therefore, to substantiate the importance of H_2O_2 production by marine phytoplankton, it is necessary to study more of the common species and the environmental factors affecting production of H_2O_2 .

The studies on the biological production of H_2O_2 may be hampered by the biological decomposition of H_2O_2 . Among the pathways of decomposition of H_2O_2 in natural waters, the biologically-mediated pathway seemed to be more important (Cooper and Zepp, 1990; Moffett and Zafiriou, 1990) than photolysis (Draper and Crosby, 1981), free radical oxidation and metal-catalyzed decomposition (Draper and Crosby, 1984). Biological decomposition of H_2O_2 is probably mediated by catalase or peroxidase enzymes which function to keep H_2O_2 from building up to toxic levels within cells (Fridovich, 1975b). H_2O_2 decomposition was suggested as a detoxification process.

However, the exact role of H_2O_2 decomposition by phytoplankton remains unknown and the results on the toxicity of H_2O_2 to marine phytoplankton have not been reported. Zepp *et al.* (1987b) have shown that several green and blue-green algae mediated the decomposition of H_2O_2 . Cooper *et al.* (1992) found H_2O_2 decomposition by two pure cultures of bacteria. H_2O_2 decay was also observed in estuarine waters (Szymczak and Waite, 1988), in several freshwater samples and soil suspensions (Cooper and Zepp, 1990), in San Francisco Bay water (Pamatmat, 1988), and in the coastal water of Vineyard Sound (Moffett and Zafiriu, 1990). However, to date, no studies on the decomposition of H_2O_2 by specific species of marine phytoplankton has been reported.

Because studies on the rates of H_2O_2 production and decomposition of marine phytoplankton are quite limited, the potential importance of the role of biology in the budget of H_2O_2 in the surface ocean is unknown (Fig. 1.1). In particular, the decomposition rates of H_2O_2 by specific marine phytoplankton have never been studied. Also, the production rates of H_2O_2 by specific marine phytoplankton are lacking. Marine phytoplankton and diel changes of H_2O_2 in the surface oceans, and factors affecting their activities have never been explained. Therefore, this study focuses on the kinetics of the production and decomposition of H_2O_2 , the controlling factors of H_2O_2 production and decomposition by marine phytoplankton, and their roles in the diel variation of H_2O_2 and the budget of H_2O_2 in the surface ocean.

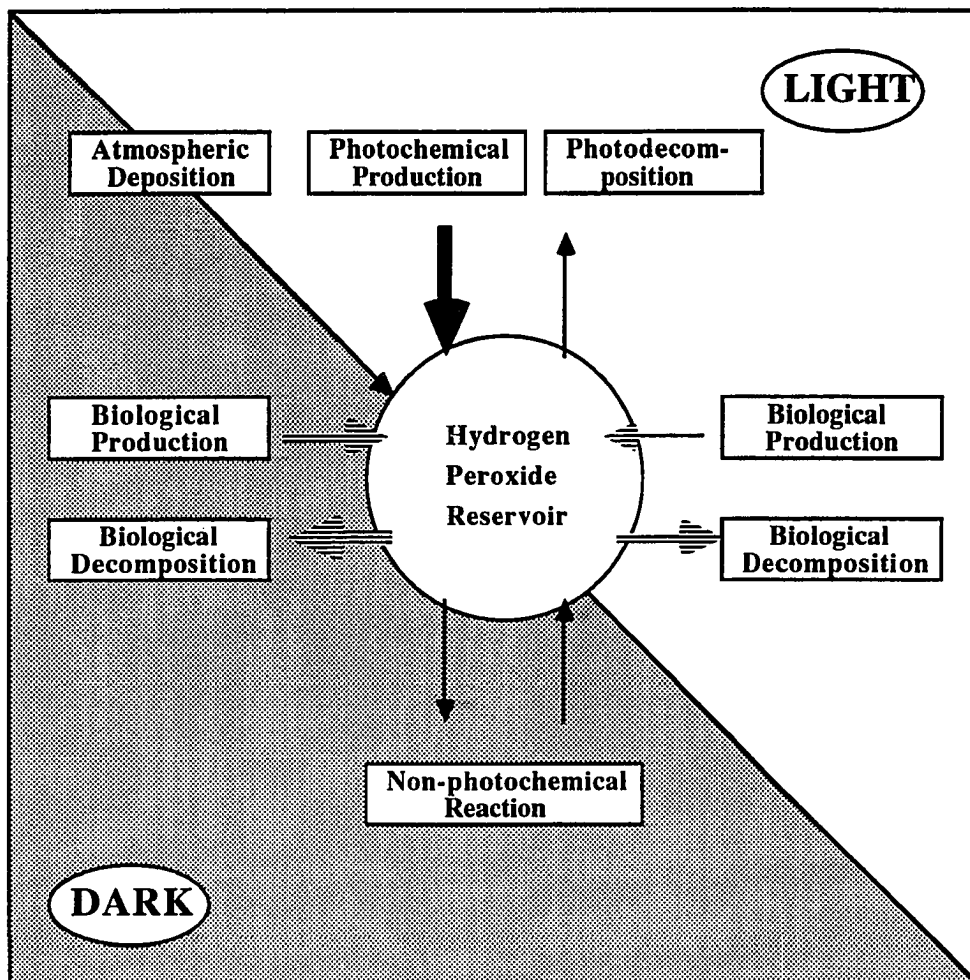


Fig. 1.1. A diagram showing sources and sinks of H_2O_2 in surface oceans. The thickness of arrow indicates the relative importance in controlling H_2O_2 budget. The shadowed arrows are uncertain in their importances.

1.2. HYPOTHESES

1. Marine phytoplankton significantly regulates H_2O_2 budget in surface oceans.
2. Nutrient, especially nitrogen, effects on the biological regulation of H_2O_2 are more important than physical factors.

1.3. SPECIFIC OBJECTIVES

The specific objectives for this study are to

1. determine whether a diel variation of H_2O_2 occurs even in the absence of phytoplankton.
2. determine whether the production of H_2O_2 is a general characteristic of marine phytoplankton.
3. determine whether the decomposition of H_2O_2 is a general characteristic of marine phytoplankton.
4. determine whether the production and decomposition of H_2O_2 by marine phytoplankton are controlled by light.
5. determine whether the production and decomposition by phytoplankton alone explains the diel variation of H_2O_2 in the surface oceans.
6. determine which environmental factors affect the production and decomposition rates of H_2O_2 .

1.4. LITERATURE REVIEW

1.4.1. THE DISTRIBUTION AND DYNAMICS OF H₂O₂

Since Van Baalen and Marler (1966) found H₂O₂ in seawater, H₂O₂ has been ubiquitously observed in various natural waters (Table 1.1): rainwater (Kok, 1980; Zika et al., 1982), freshwater (Sinel'nikov, 1971; Draper and Crosby, 1983a; Cooper et al., 1989; Cooper and Lean, 1989), ground water (Cooper and Zika, 1983; Holm et al., 1987), estuarine water (Szymczak and Waite, 1988) and seawater (Van Baalen and Marler, 1966; Zika, 1980; Zika et al., 1985a, b; Palenik and Morel, 1988; Johnson et al., 1989). The concentration of H₂O₂ in the surface ocean ranges between 10¹ to 10² nanomolar. Coastal waters commonly display a higher concentration of H₂O₂. The level of H₂O₂ increases toward the shore probably due to photooxidation of terrestrial humic material (Zika et al., 1985a). Estuarine water seems to have variable concentrations. River and lake water can reach a fairly high concentration (Sinel'nikov, 1971; Cooper and Lean, 1989). The highest concentration of H₂O₂ was found in rainwater with ranges of 10⁰ to 10¹ micromolar (Kok, 1980; Cooper and Lean, 1989). Ground water and upwelling water show a much lower concentration of H₂O₂ probably due to the lack of organic material and light (Holm et al., 1987).

Maximum concentration of H₂O₂ occurs typically at the sea surface but decreases exponentially to the detection limit

Table 1.1. The concentrations of H₂O₂ found in various natural waters.

References	H ₂ O ₂ conc (nM)	Areas
Van Baalen & Marler, 1966	100 - 160	Gulf of Mexico
Moffett & Zika, 1983	120 - 160	Gulf of Mexico
Zika et al., 1985a	90 - 140	Gulf of Mexico
Zika et al., 1985b	8 - 50	Peru Upwelling Area
Johnson et al., 1989	90 - 150	Western Mediterranean Sea
Miller & Kester, 1988	140	Sargasso Sea
Palenik & Morel, 1988	130 - 160	Sargasso Sea
Moffett & Zafirion, 1990	120	Coast, Vineyard Sound
Van Baalen & Marler, 1966	14 - 170	Coast, Texas
Zika et al., 1985a	100 - 240	Coast, Gulf of Mexico
Zika, 1980	80 - 210	Biscayne Bay, Florida
Miller & Kester, 1988	25 - 115	Narragansett Bay
Szymczak & Waite, 1988	35 - 100	Port Hacking Estuary
Sinel'nikov, 1971	1300 - 3200	Volga River, Russia
Cooper et al., 1989	100 - 200	Lakes: Erie, Jacks, Ontario
Cooper & Lean, 1989	200 - 400	Lakes: Jacks, Ontario
Holm et al., 1987	4 - 45	Ground Water
Kok, 1980	170 - 1590	Rain, California
Zika et al., 1982	10000 - 70000	Rain, Florida
Cooper & Lean, 1989	1300 - 34000	Rain, Ontario

toward the bottom of the euphotic zone (Fig. 1.2). Although sunlight is believed to play a major role in the formation of H_2O_2 in the surface water, there seems to be some other factors creating variable and dynamic profiles of H_2O_2 . Such factors may include the *in situ* biological formation and removal of H_2O_2 , vertical mixing, precipitation, etc (Zika *et al.*, 1985b; Johnson *et al.*, 1989; Cooper and Lean, 1989).

Diel variation of the concentration of H_2O_2 occurs in surface waters (Fig. 1.3) (Palenik and Morel, 1988; Cooper and Lean, 1989). The highest concentrations are observed in the mid to late afternoon and the lowest concentrations in the early morning (Zika *et al.*, 1985a; Palenik and Morel, 1988; Szymczak and Waite, 1988). The amplitude of the diel variation is about 20 % of the maximal concentration in the open ocean (Zika *et al.*, 1985a; Palenik and Morel, 1988). The diel cycle pattern is more pronounced in coastal waters than in open ocean waters. Lake water also shows the prominent diel variation of H_2O_2 (Cooper and Lean, 1989). Zika *et al.* (1985a) believed that the greatest variation of H_2O_2 in coastal waters was a result of the abundant transition metals and organic compounds (Zika *et al.*, 1985a). Seasonal variation of H_2O_2 has not been reported.

1.4.2. THE BIOGEOCHEMICAL IMPORTANCES OF H_2O_2

H_2O_2 , an intermediate in the reduction of O_2 to H_2O , can be either a strong oxidant or a reductant with respect to the

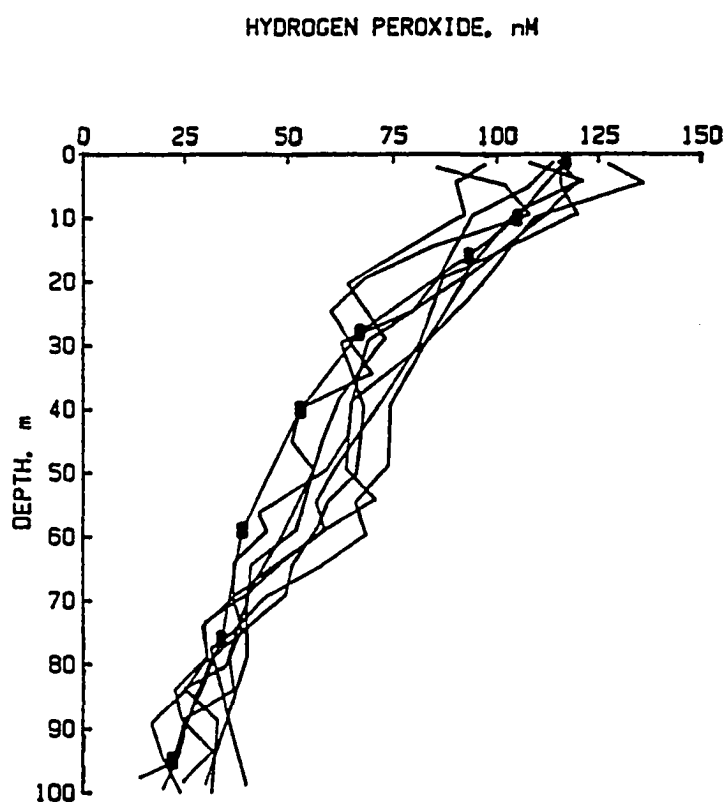


Fig. 1.2. Typical depth profiles of H_2O_2 in surface oceans (From Johnson et al., 1989). The line with marks is adapted from Zika et al. (1985a).

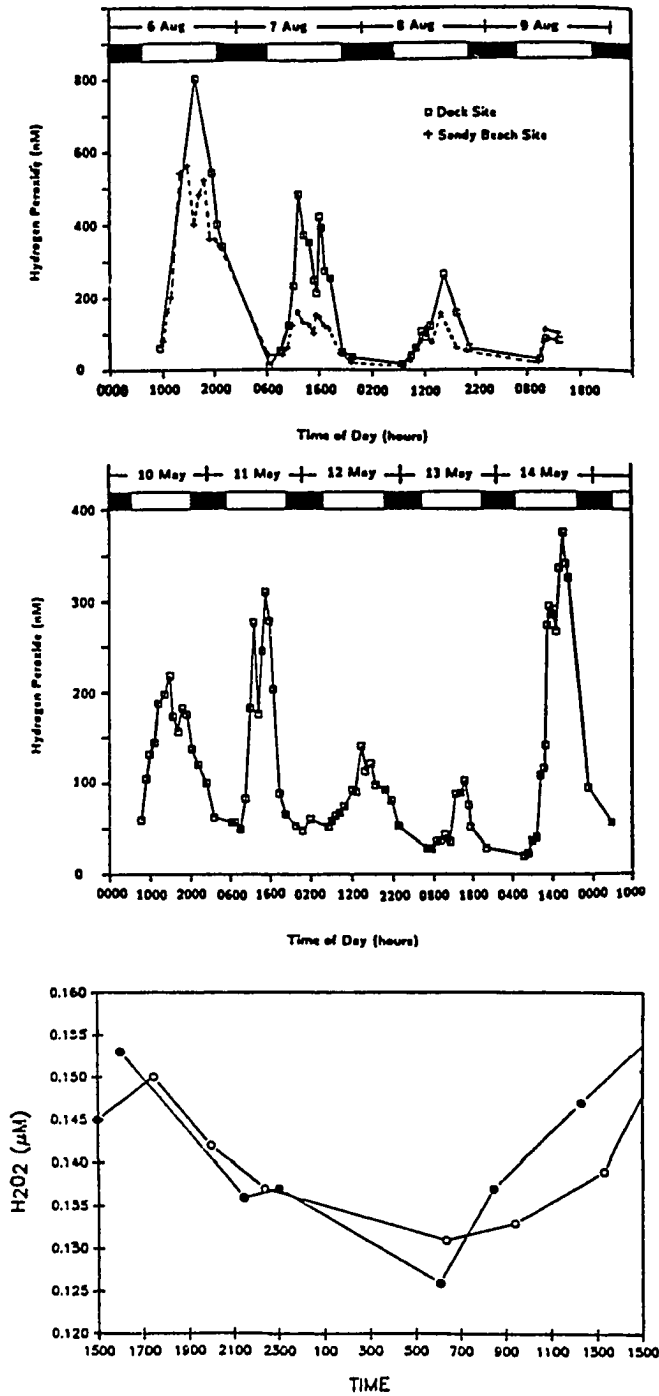


Fig. 1.3. Diel variations of H_2O_2 in eutrophic waters (a and b; From Cooper and Lean, 1989) and in oligotrophic waters (c; From Palenik and Morel, 1988).

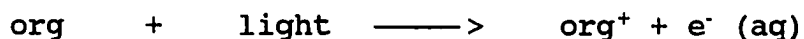
O_2/H_2O redox couple (Moffett and Zika, 1987b; Moffett and Zafiriou, 1990). As a result of its redox property, H_2O_2 may play an important role in the geochemistry of a number of elements. That is, it may affect the speciation and the fate of trace metals such as Fe^{2+} (Waite and Morel, 1984; Moffett and Zika, 1987a; Micinksi et al., 1990), Cr^{3+} (Pettine and Millero, 1990), Cu^{2+} (Moffett and Zika, 1983) and Mn^{4+} (Sunda et al., 1983). In addition, concentrations of H_2O_2 (20 - 300 nM) are much higher than those of the trace metals in surface seawater (e.g., Fe: 0.15-0.30 nM, Cu: 0.5-3.0 nM, Mn: 0.3-15.0 nM) (Szymczak and Waite, 1988). H_2O_2 is important in metal cycling in natural waters (McMahon, 1967, 1969; Sunda et al., 1983; Collienne, 1983), and also in the fate of pollutants in aquatic environments (Zepp and Schlotzhauer, 1983; Draper and Crosby, 1983a, 1984; Zepp et al., 1987b). In the atmosphere, H_2O_2 is important to the fate of both sulfur and nitrogen compounds (Heikes et al., 1988). H_2O_2 is considered a principal oxidant in the oxidation of sulfur dioxide (SO_2) to sulfuric acid (SO_4^{2-}), resulting in acid rain (Penkett et al., 1979; Calvert et al., 1985).

H_2O_2 , as a strong oxidant, could be a significant ecological variable affecting biological processes. Organisms may use H_2O_2 as a chemotactic agent (Haeder, 1984; Nultsch and Kumar, 1984). Organisms may control the toxicity/availability of the trace metals (Jones et al., 1987; Sunda, 1989; Bruland et al., 1991) by affecting their speciation with H_2O_2 . H_2O_2 may be associated with the nitrogen cycling by organisms in the

ocean (Palenik et al., 1990a, b). A trace amount of H_2O_2 may have far-reaching effects on the type or growth rate of organisms in the sea (Lucas, 1961; Van Baalen and Marler, 1966). In addition, membrane damage due to lipid peroxidation, mutagenicity, selective bacterial killing, and photobleaching of chlorophyll could occur as a result of the toxic effect of H_2O_2 (Iyer et al., 1961; Van Baalen, 1965; Ananthaswamy and Einstark, 1976; McCormick et al., 1976; Borg et al., 1978, 1981; Dixit et al., 1982; Kay et al., 1984; Lawlor, 1987). The toxicity effects could be much more profound if H_2O_2 is reduced to the hydroxyl radical ($HO\cdot$) by metal ions like Fe^{2+} , because $HO\cdot$ is an extremely reactive oxidant that attacks all cell molecules indiscriminately (Borg et al., 1978, 1981; Borg and Schaich, 1983).

1.4.3. PHOTOCHEMICAL PRODUCTION OF H_2O_2

Sunlight absorption by dissolved organic matter or humic substances can result in photochemical formation of H_2O_2 (Baxter and Carey, 1983; Cooper and Zika, 1983; Draper and Crosby, 1983b; Petasne and Zika, 1987; Cooper et al., 1988, 1989). In the presence of light, the dissolved constituents in seawater can act as electron sources in the reduction of oxygen, resulting in the formation of superoxide ion, O_2^- (Zika, 1981; Zafirou, 1983).





in which org is organic materials dissolved in seawater, org⁺ is an excited form of org, and e⁻ (aq) is hydrated electron. Subsequent disproportionation of O₂⁻ rapidly occurs producing H₂O₂ according to the reaction:



The production of H₂O₂ by sunlight may be proportional to the concentration of total organic carbon and the light intensity (Cooper and Zika, 1983; Petasne and Zika, 1987; Johnson *et al.*, 1989). Most of the solar energy absorbed by dissolved organic carbon has wavelengths between 300 and 500 nm (Zafiridou, 1983; Cooper *et al.*, 1989). Humic substances are found in most natural waters (Thurman, 1985), thus giving an implication of the importance of photochemical production of H₂O₂ during the day time.

1.4.3. BIOLOGICAL PRODUCTION OF H₂O₂

Phytoplankton could contribute to H₂O₂ production in surface water by excreting a large amount of dissolved organic matter during photosynthesis. Some of them are photochemically reactive organic carbon compounds, so called photosensitizers. These photosensitizers are the sources of the formation of H₂O₂ via sunlight.

Algae —> Photosensitizers —> H₂O₂ (in the sunlight)

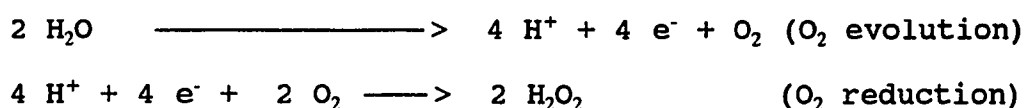
Among photosensitizers, riboflavin from marine phytoplankton is photochemically reactive and may be an important precursor of H₂O₂ formation (Dunlap and Susic, 1985; Mopper and Zika, 1987). Approximately 10 nmol H₂O₂ L⁻¹ was produced from every 1 nmol L⁻¹ riboflavin added to seawater (Mopper and Zika, 1987). In addition to riboflavin, indole and many other aromatic compounds can sensitize the formation of H₂O₂ in the presence of ultraviolet light (Chelala and Margolin, 1983). Draper and Crosby (1983a) and McCormick *et al.* (1976) reported that some solutes from biological origin, such as tryptophan, tyrosine and humic substances could generate H₂O₂ in sunlight. The distribution of these naturally-occurring photosensitizers in seawater and the stoichiometry between the precursors and H₂O₂ are poorly understood at the present time.

Phytoplankton can produce H₂O₂ directly in several ways. Production of H₂O₂ in the light by *Anacystis nidulans*, blue-green algae, was demonstrated by Van Baalen (1965) and Patterson and Myers (1973). According to the latter, NADPH, formed in excess by photosynthesis, combines with O₂ instead of CO₂ resulting in H₂O₂. However, they could find no electron transfer intermediates capable of reducing O₂ to H₂O₂ in the photosynthetic apparatus. Hence, they concluded that the peroxide production was the result of a small metabolic defect of *Anacystis nidulans*. Subsequently, Stevens *et al.* (1973)

found more than half of 38 axenic isolates of blue-green algae produced H_2O_2 , suggesting that H_2O_2 production is widespread among blue-green algae. However, some of them produced H_2O_2 even in the dark, which is contradictory to the photosynthetic production of H_2O_2 .

There are several known mechanisms of biological H_2O_2 production in plant cells in the light. In the glycolate to glyoxylate reaction, O_2 is taken up and H_2O_2 is formed. The presence of glycolate oxidase has been confirmed in many higher plants. However, it remains uncertain whether this mechanism could be important in the marine environment as a source of H_2O_2 because this newly-formed H_2O_2 is believed to be destroyed quickly by catalase before it damages the photosynthetic apparatus and because the glycolate oxidase enzyme is apparently more sporadic in occurrence in the algae (Hess and Tolbert, 1967).

Mehler (1951a, b) and subsequent workers (Mehler and Brown, 1952; Brown and Good, 1955; Good and Hill, 1955) reported that, in isolated chloroplasts, an uptake of O_2 and a simultaneous production of H_2O_2 was observed. It was found that H_2O_2 was the product of photoreduction of O_2 , not an intermediate in the evolution of O_2 resulting from photosynthetic processes.



It is well documented that H_2O_2 production via the Mehler reaction occurs in isolated chloroplasts. However, it is questionable whether an identical or similar generation of H_2O_2 occurs in intact chloroplasts in the untreated, normally growing algae. There is only one example of direct measurement of H_2O_2 production from intact cells (De Kouchkovsky, 1964). While most aerobic oxidases catalyze the reduction of oxygen to water in the respiratory process, aerobic dehydrogenase leads to the formation of H_2O_2 rather than H_2O (Baldwin, 1963). *Streptococcus mutans* released as much as 90 % of its oxygen uptake as H_2O_2 (Pera, 1983). *Streptococcus lactis* was found to accumulate H_2O_2 to concentrations reaching autoinhibitory levels (Grufferty and Condon, 1983). Production of H_2O_2 by marine bacteria has never been proved. H_2O_2 productions by some blue-green algae in the dark (Table 1.2) (Stevens et al., 1973) and by the membranes of *Anabaena variabilis* (Honeycutt and Krogmann, 1970) suggest that the production of H_2O_2 is not linked to a photosynthetic activity. Since then, studies have focused on the biological production of H_2O_2 in the dark. Palenik et al. (1987) observed H_2O_2 production by several cultures of phytoplankton in the dark (Table 1.3). According to them, nitrogen-depleted cells can effectively convert L-amino acids in surrounding waters to ammonium, H_2O_2 and α -keto acids by using L-amino acid oxidases (L-aa oxidases). The nitrogen starved cells subsequently take up ammonium from their own ammonium patch they had created.

Table 1.2. Freshwater phytoplankton tested for H₂O₂ production.
(From Stevens et al., 1973)

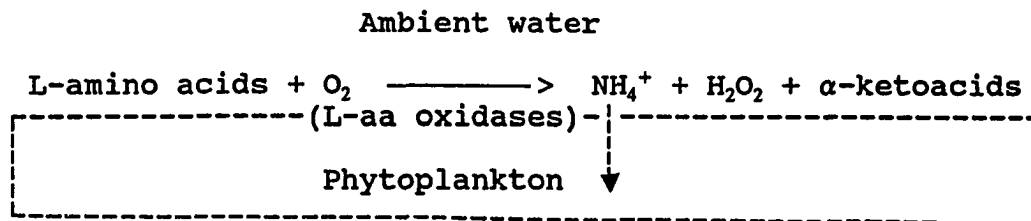
H ₂ O ₂ producers	Strain	Light*
<i>Anacystis nidulans</i>	TX-20	+
<i>Anabaena flos-aquae</i>	A-37	+
<i>Anabaena variabilis</i>		-
<i>Chloroglea fritschii</i>		-
<i>Coccochloris elabens</i>	Di, 17-A	+
<i>Eucapsis</i> sp.	0506	+
<i>Lyngbya lagerheimii</i>	Mont	+
<i>Microcoleus chthonoplastes</i>	BA-1	+
<i>Nostoc muscorum</i>	G	-
<i>Nostoc</i> sp.	TX-49	-
<i>Nostoc</i> sp.	0346, 0411	+
<i>Oscillatoria</i> sp.	SFO	+
<i>Plectonema terebrans</i>	Calsp-31, Jam-Fil	+
<i>Schizothrix calcicola</i>	TX-27	+
<i>Synechocystis</i> sp.	083	+
<i>Synechococcus</i> sp.	084	-
	A-1	+
	A-2	+
H ₂ O ₂ non-producers		
<i>Agmenellum quadruplicatum</i>	BG-1	
<i>Agmenellum quadruplicatum</i>	PR-6	
<i>Anabaena cylindrica</i>		
<i>Anacystis marina</i>	6, WH-20, AMH	
<i>Chlorella pyrenoidosa</i>	Emerson strain	
<i>Fischerella ambigua</i>		
<i>Gloeocapsa</i> sp.	0126	
<i>Microcystis aeruginosa</i>	0510	
<i>Nostoc</i> sp.	TX-50	
<i>Nostoc</i> sp.	G0161	
<i>Oscillatoria williamsii</i>		
<i>Phormidium</i> sp.		
<i>Schizothrix calcicola</i>	Man	
<i>Schizothrix mexicana</i>	0404	
<i>Synechocystis</i> sp.	0444	

* : with light (+); without light (-)

Table 1.3. Marine phytoplankton tested for H₂O₂ production.
(From Palenik, 1989)

H ₂ O ₂ producers	Strain	Light*
<i>Amphidinium carterae</i>	AMPHI	-
<i>Amphidinium operculatum</i>	AMPHID, Klebsii	-
<i>Pleurochrysis carterae</i>	COCCO II	-
<i>Prymnesium parvum</i>	PRYM	-
<i>Pymnesium calathiferum</i>	CHANG1	-
H ₂ O ₂ non-producers		
<i>Emiliana huxleyi</i>	12-1	
<i>Isochrysis galbana</i>	ISO	
<i>Micromonas pusilla</i>	DW8	
<i>Pavlova gyrans</i>	MPPAV	
<i>Tetraselmis</i> sp.	4B-9	
<i>Thalassiosira weissflogii</i>	ACTIN	

* : without light (-)



According to Palenik and Morel (1990a, b), L-aa oxidases are located at the cell surfaces because the H_2O_2 production can be completely inhibited by a surface-protein-labeling reagent. Therefore, the extracellular production of H_2O_2 in the dark in the nitrogen-depleted environment may be more important than the intracellular production of H_2O_2 in the light because it is possible that phytoplankton produce H_2O_2 as a by-product of L-aa oxidase activity and release it into the surrounding medium much more effectively. Palenik *et al.*, (1987) proposed that the biological turnover rate of H_2O_2 in the surface ocean is as important as the other processes. Palenik and Morel (1988) also found dark production of H_2O_2 in the Sargasso sea, which should be considered in the H_2O_2 dynamics and budget in the surface oceans. Moffett and Zafiriou (1990) measured biological production of H_2O_2 in a coastal water in the dark. There are various nonlight-dependent biological processes that may lead to H_2O_2 production in seawater (Forman and Boveris, 1982; Frimer *et al.*, 1983).

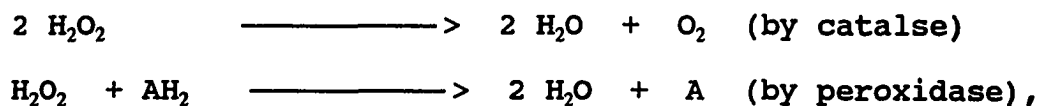
Although the importance of biological production of H_2O_2 has been amply suggested, it still remains largely unknown whether the biological source of H_2O_2 could be significant relative to other processes occurring in the surface ocean. Stevens *et al.*, (1973) implied that biological production of

H_2O_2 is widespread among algae whereas Palenik and Morel (1988) thought that it may not be so. At the present time, generalized information on the production of H_2O_2 by marine phytoplankton is very limited. Since Palenik *et al.* (1987) have reported H_2O_2 production by some marine phytoplankton, no one has supported or confirmed their results. Their field study in the Sargasso sea showed a dark production rate of up to $11 \text{ nM}\cdot\text{hr}^{-1}$, which is too high to be neglected. Further studies are needed.

1.4.5. BIOLOGICAL DECOMPOSITION OF H_2O_2

Biological decomposition of H_2O_2 is mediated by catalase or peroxidase enzymes. Catalase is a heme-containing tetrameric enzyme of 240 kDa whereas peroxidase is a monomeric heme-containing protein of 40 kDa. Catalase is found abundantly in plant tissue and most of its activity is associated with peroxisomes (Halliwell, 1982). Catalase activity does not appear to occur in the chloroplast (Whitehouse *et al.*, 1971, Van Ginkel and Brown, 1978). The catalase enzyme decomposes specifically H_2O_2 into O_2 and H_2O (Baldwin, 1963). Plants also contain abundant amounts of peroxidases that exhibit broad substrate specificity (Salin, 1987). Peroxidases are associated with cell walls, causing a lignification process and degrading indoleacetic acid (Abeles and Biles, 1991). These processes play a key role in growth and development (Salin, 1987). These two enzymes mediate the

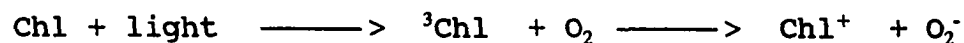
following reactions:



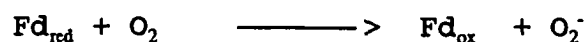
where AH_2 is a hydrogen donor molecule.

Molecular oxygen is not toxic, but its photochemical by-products, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\text{OH}\cdot$), are highly reactive and produce severe cellular damage, lipid peroxidation in membrane, viral inactivation, cellular toxicity, single strand breaks in DNA, chromosomal aberration (Frimer *et al.*, 1983; Fridovich, 1986), photobleaching of chlorophyll (Kay *et al.*, 1984), etc. Thus, the superoxide scavenger SOD, catalase and peroxidase were believed to be developed as a defense system for photosynthetic organisms against the threat of toxicity.

In photosynthesis, excited chlorophyll (^3Chl) donate energy to O_2 , producing superoxide by electron transfer (Lawlor, 1987). Photosystems can pass e^- to O_2 directly,



or via physiological intermediates such as ferredoxin (Fd),



Also as described earlier, O_2^- can be produced in the

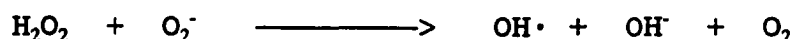
surrounding medium by light and dissolved organic matter (Org),



Because of its detrimental toxicity, O_2^- should be removed (Dahl *et al.*, 1987). Superoxide dismutases catalyze the disproportionation of two O_2^- molecules, to give H_2O_2 which is destroyed by catalase,



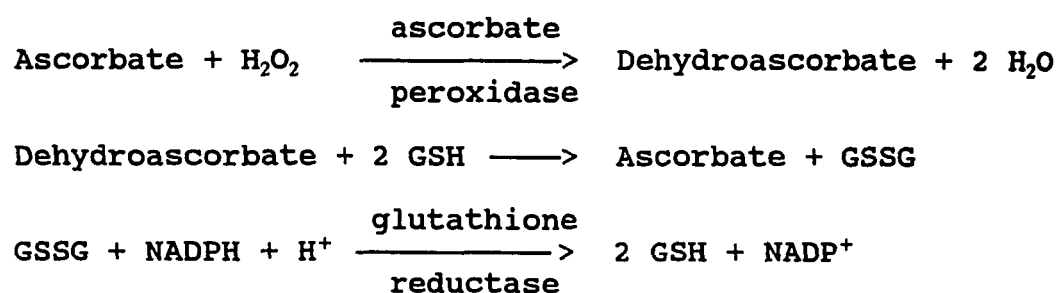
Thus, catalase is very important for preventing the accumulation of toxic H_2O_2 in the cells or the further reaction that produces hydroxyl radicals.



This reaction is presumably metal catalyzed and referred to as the Haber-Weiss reaction (Haber and Weiss, 1934). The hydroxyl radical is an extremely indiscriminate oxidant, damaging most biological materials such as enzymes, small metabolites, nucleic acids or membranes. The destructive and mutagenic potential of the hydroxyl radical in biological systems is enormous.

Among peroxidase-mediated decomposition of H_2O_2 , the ascorbate cycle is of major importance to the chloroplast, in

which ascorbate is oxidized and then reduced (Eltner and Kramer, 1973; Foyer and Halliwell, 1976; Groden and Beck, 1970). In this system ascorbate, which is found in chloroplasts at around 50 mM, is oxidized to dehydroascorbate by ascorbate peroxidase. The dehydroascorbate is then reduced by glutathion (Halliwell, 1982). The glutathion reduction is achieved by NADPH-glutathion reductase. The reaction can be visualized as follows:



in which GSH is a reduced glutathione and GSSG is an oxidized glutathione. As a result of this cycle, H_2O_2 is removed by photosynthetically generated NADPH.

There are other types of non-enzymatic removal of toxic oxygen species. Endogenous small molecules can play a role in the removal of toxic oxygen species. Examples of the low-molecular weight compounds are hydroquinones and carotenes (Salin, 1987). But the significance of their role in removing H_2O_2 remains largely unknown.

In seawater, as mentioned earlier, the diel variation of H_2O_2 occurs, showing maximum concentration in the afternoon and minimum concentration in the early morning. To this day, most work has focused on the sources of H_2O_2 ; thus we lack information on the sinks of H_2O_2 in the absence of light.

There are several pathways accounting for the sinks of H_2O_2 in natural waters such as photolysis, peroxidase/catalase - mediated decomposition, free radical oxidation catalyzed by some metals, and chemical decomposition (Zepp *et al.*, 1987b; Moffet and Zafiriou, 1990; Cooper *et al.*, 1992). The relative significance of these various decomposition processes in natural waters has not been determined yet. However, biological decomposition of H_2O_2 was suggested to be more important than other processes (Cooper and Lean, 1989; Moffett and Zafiriou, 1990; Cooper *et al.*, 1992). The biological decomposition processes stated above occur inside cells in which decomposing enzymes are located. According to Frimer *et al.* (1983), H_2O_2 can diffuse far away from its site of production due to its relative inertness, and can cross the lipid bilayer membrane without affecting the cell wall's integrity. Subsequently, H_2O_2 transported or diffused into cells can be readily decomposed by catalase or peroxidase. There is no evidence that cells release enzymes into surrounding medium to decompose H_2O_2 . Moffett and Zafiriou (1990) reported decomposition of H_2O_2 by catalase and peroxidase, and proposed that the removal of H_2O_2 was mediated by organisms living in the water. Pamatmat (1988) also measured catalase activity of organisms in San Francisco Bay water. The catalase activity is measured by the disappearance of H_2O_2 or the accumulation of O_2 from the added H_2O_2 . Peroxidase-mediated decomposition of H_2O_2 can be measured, as shown in the equation, by monitoring the disappearance of

hydrogen donor molecules such as p-anisidine after adding H_2O_2 in the dark (Cooper and Zepp, 1990). Peroxidase systems are of particular interest because many important pollutants can function as hydrogen donors (Moffett and Zafirou, 1990). Johnson *et al.* (1989) observed dark decay of H_2O_2 in the western Mediterranean seawater. The dark decay life time of H_2O_2 in the oceans estimated by Plane *et al.* (1987) was on the order of hours to weeks, 4 days as an average.

Biological decomposition of H_2O_2 probably by algae and/or bacteria may be responsible for the decline of H_2O_2 at night in freshwater systems (Cooper *et al.*, 1989; Cooper and Zepp, 1990). The dark decay life times estimated from these works ranged from several hours to several tens of hours. There is only work by Zepp *et al.* (1987b) with pure cultures of freshwater algae in a defined lab condition. They found decay of H_2O_2 in the algal suspension but not in the supernatant portion, which meant that the decay was mediated by algae. The overall decay rate equation was second order and is as follows:

$$\text{Rate} = k_1 \cdot [H_2O_2] = k_{\text{bio}} \cdot C_a \cdot [H_2O_2],$$

where k_1 is a pseudo-first order rate constant, k_{bio} is a second-order rate constant, and C_a is a concentration of chlorophyll-a. According to them, the second-order rate constants ranged from 0.18×10^{-3} to 8.8×10^{-3} (mg chl a) $^{-1} \cdot h^{-1}$ and the half life of H_2O_2 was 1.5 hours on average, indicating that

freshwater algae decompose H_2O_2 very efficiently. Cooper and Zepp (1990) demonstrated the decay kinetics of H_2O_2 in natural freshwater, which can be described by a pseudo-first order rate law. Recently, decomposition of H_2O_2 by two pure bacteria were investigated by Cooper *et al.*, (1992). Both bacteria were efficient in removing H_2O_2 and the overall rate law was the same as that followed by freshwater algae. No report has been made on the systematic investigation of decomposition of H_2O_2 by marine algae or by marine bacteria. An assumption for decomposition experiments with dark bottles is that there is no significant production in the dark. Decomposition studies in the absence of light are much easier than in the light. Bottle incubation can also prevent decay losses of H_2O_2 from diffusive (mixing) losses. Biological decomposition of H_2O_2 may provide a clue to the excess oxygen found in the dark bottles during measurement of the primary production using the O_2 method. After incubation the oxygen concentration in the dark bottles often turned out to be higher than before incubation. This is probably due to activity of catalase, which decompose H_2O_2 to O_2 and water (Pamatmat, 1988). It is certain that many other workers have obtained similar results but ignored or excluded them on the assumption that they were erroneous (Dugdale and Wallace, 1960). According to Zika (1980), the greater diel variation in coastal waters may be the result of a higher decay rate caused by higher concentrations of transition metals and dissolved organic materials. However, this proposal has not been demonstrated.

Szymczak and Waite (1988) found, from the incubation of two different estuarine waters, that one sampling station showed decomposition but the other station showed none over 8 hours. At present, factors controlling the decomposition of H_2O_2 in seawater are unclear.

Chapter 2

PRODUCTION OF H₂O₂ BY MARINE PHYTOPLANKTON

2.1. INTRODUCTION

Hydrogen peroxide (H_2O_2) is found ubiquitously in the surface waters of the hydrosphere (Zika *et al.*, 1982; Cooper and Zika, 1983; Zika *et al.*, 1985a; Palenik and Morel, 1988; Johnson *et al.*, 1989; Moffett and Zafirou, 1990). It is well known that, under certain conditions, H_2O_2 can act either as an oxidizing or a reducing agent (Moffett and Zafirou, 1990). Recent studies also indicate that the oxidation-reduction reactions between H_2O_2 and some trace metals in the sea may have profound effects on the speciation of these metals, such as iron (Waite and Morel, 1984; Moffett and Zika, 1987a; Millero and Sotolongo, 1989), copper (Moffett and Zika, 1983, 1987b; Sharma and Millero, 1989) and chromium (Pettine and Millero, 1990). The speciation of these metals have been shown to be important in determining their biogeochemistry and their availability and/or toxicity to marine organisms (Sunda, 1989).

The main source of H_2O_2 in surface waters is generally believed to be the photochemical reaction between sunlight and dissolved organic materials (Cooper and Zika, 1983; Draper and Crosby, 1983a; Petasne and Zika, 1987). Thus, the maximum concentration of H_2O_2 is usually found at the surface during the day time (Zika *et al.*, 1985a, b). However, according to Johnson *et al.*, (1989), the vertical profiles of H_2O_2 decrease at a slower rate than that of light profiles. Sometimes, increased concentrations of H_2O_2 were found at subsurface

layers (Zika *et al.*, 1985a). In addition, even at night, some areas maintain a relatively high level of H_2O_2 , such as 80 nM in the Sargasso sea (Palenik and Morel., 1988). Thus, biological production of H_2O_2 has been suggested as a potential, important source of H_2O_2 .

Photosynthetic production of H_2O_2 by blue-green algae and several other freshwater phytoplankton was demonstrated by Patterson and Myers (1973), Stevens *et al.* (1973) and Zepp *et al.* (1987b). Recently, the production of H_2O_2 in the dark by several marine phytoplankton was observed by Palenik *et al.* (1987). Their results implied that H_2O_2 production has an important relationship with nitrogen cycling in the oceans. In addition, Palenik and Morel (1988) measured a significant production of H_2O_2 in the dark in the Sargasso sea. Moffett and Zafiriou (1990) also measured biological production of H_2O_2 in the coastal water of Vineyard Sound.

However, rates of the production of H_2O_2 by specific species of marine phytoplankton have not been determined in most of the commonly found species (Palenik *et al.*, 1987). In spite of the potential importance of the biological production of H_2O_2 , the roles of marine phytoplankton in the H_2O_2 budget have yet to be quantified. This study investigated the importance of the dark production of H_2O_2 by marine phytoplankton by determining production rates of H_2O_2 and factors affecting the rates. Eleven marine phytoplankton were studied for their H_2O_2 production rates. Five out of 11 species produced H_2O_2 in the dark. The addition of amino acids

was the most influential factor among several tested variables stimulating H₂O₂ production.

2.2. MATERIALS AND METHODS

Production of H_2O_2 was studied in the cultures of species obtained from the Provasoli-Guillard Center for Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, McKown Point, West Boothbay Harbor, Maine 04575, U.S.A.). The names of the tested phytoplankton are listed in Table 2.1. These cover some of the major types of marine phytoplankton such as *Skeletonema costatum* and *Synechococcus* sp. The algal cultures were grown in batch cultures at 23 °C, 12:12 light:dark cycle and under light intensity of $75 \mu E \cdot m^{-2} \cdot s^{-1}$. Culture media, f_{20} , were prepared from the filtered Sargasso seawater according to Guillard and Ryther (1962).

In vivo fluorescence was monitored so that cells were harvested at the late-log growth phase. After being conditioned in the dark for 2 - 3 hours, the cells were transferred to disposable sterile centrifuge tubes and then concentrated by centrifugation usually at 1000 rpm and for 5 minutes. The supernatant was carefully decanted and the remaining concentrated cells were washed with fresh medium of f_{20} (no addition of trace metals) and then recentrifuged. The centrifuged cells were quantitatively transferred into incubation bottles with f_{20} medium. The dilution factor achieved by the above steps was about 10^4 times, which may reduce the possible introduction of H_2O_2 in the stock culture solution. To achieve a very low background level of H_2O_2 in the medium, 0.2- μm filter-sterilized Sargasso seawater was

Table 2.1. List of 11 marine phytoplankton used for H₂O₂ studies.

Class	Species Name	Strain
1. Bacillariophyceae	<i>Skeletonema costatum</i>	SKEL
	<i>Chaetoceros simplex</i>	BBSM
	<i>Chaetoceros flexosus</i> ⁺	
	<i>Thalassiosira oceanica</i>	13-1
2. Dinophyceae	<i>Amphidinium carterae</i>	AMPHI
3. Prymnesiophyceae	<i>Pleurochrysis carterae</i>	COCOII
	<i>Isocrysis galbana</i>	ISO
	<i>Emiliana Huxleyi</i>	BT-6
4. Prasinophyceae	<i>Tetraselmis levis</i>	PLATY1
5. Chlorophyceae	<i>Dunaliella tertiolecta</i>	DUN
6. Cyanophyceae	<i>Synechococcus</i> sp.	DC2

⁺ : isolated from the Sargasso sea surface water
(75 °W, 32 °N)

used instead of autoclaved incubation medium because autoclaving results in a high background H_2O_2 due to chemical reactions under high temperature and pressure (Marler and Van Baalen, 1965; Cooper et al., 1992). H_2O_2 produced by autoclaving was often more than 150 nM. Sargasso seawater was filtered through an A/E type glass fiber filter and aged in the dark to decay residual H_2O_2 . This water was refiltered through a 0.2- μ m Nucleopore filter with the sterilized units covered from light. The concentration of H_2O_2 in the water obtained in this way was lower than the detection limit of 5 nM.

Preparation of controls : Control samples were routinely prepared by filtering cell suspensions from the incubation bottles through a GFF filter and then a 0.45- μ m Millipore filter with low pressure (< 10 mm Hg). An additional control experiment was performed. That is, an 1- μ m filtrate of the cell suspension was tested for any bacterial production of H_2O_2 because the stock culture was not completely axenic. Killed cells with mild heating at 60 °C for 30 minutes were also tested for any H_2O_2 production.

H_2O_2 determination : H_2O_2 analysis was performed using the scopoletin-horseradish peroxidase (HRP) method (Andreae, 1955; Holm et al., 1987). The method measures the fluorescence decay of scopoletin by the peroxidase mediated decomposition of H_2O_2 . A Perkin Elmer 650-10S fluorescence spectrophotometer

was used. HRP and H₂O₂ working standard solutions were prepared fresh each day. For each medium or culture studied, separate standard curves were determined. The detection limit of the method was about 5 nM. Replicate analyses on the same samples were within $\pm 5 \%$ throughout the experiments.

Chlorophyll-a determination : Chlorophyll-a was used as an index of biomass. Cell suspensions from the incubation bottles were filtered onto glass fiber filters. The filters were ground with a homogenizer and pigments were extracted with 90 % acetone before fluorometric measurement with a Turner Design Fluorometer. The concentration of chlorophyll-a and phaeopigment-a were calculated using the method of Strickland and Parsons (1972).

Data treatment : Hyperbolic types of observed H₂O₂ production were approximated by pseudo-first order kinetics using the following equation modified from Falkowski (1983),

$$P_t = (P_0 - P_{\max}) \cdot e^{-k \cdot Ca \cdot t} + P_{\max}$$

in which P₀ is a H₂O₂ concentration at time 0, P_t is a net production of H₂O₂ at time t, Ca is a chlorophyll-a content, P_{max} is an asymptotic concentration of H₂O₂, and k is a rate constant in the unit of (μg chl-a · L⁻¹)⁻¹ · hr⁻¹. P_{max} was obtained by averaging several data points on the maximum production level where the cellular production of H₂O₂ no longer

increased. P_{\max} values may be underestimated due to the decomposition of H_2O_2 after the period of quasi-steady state. The above equation was rearranged to obtain a second-order rate constant,

$$k = t^{-1} \cdot Ca^{-1} \cdot \ln \{ (P_{\max} - P_0) \cdot (P_{\max} - P_t)^{-1} \}$$

P_{\max} and k values were used as parameters representing production curves.

2.3. RESULTS AND DISCUSSION

2.3.1. Biological production of H_2O_2 and its kinetics

The production of H_2O_2 by 11 pure cultures of marine phytoplankton (Table 2.1) were tested with the HRP-scopoletin method. The production of H_2O_2 in the phytoplankton samples and in the control bottles were compared with each other to show the production is due to biological processes.

Control experiments : The possibility of H_2O_2 production by factors other than phytoplankton were tested. A preliminary test with incubation of medium alone (f_{20} without addition of trace metals) in the dark showed no production of H_2O_2 within the incubation period. The incubation of filtrates ($0.45 \mu m$ and $1 \mu m$) of the cell suspension of *Pleurochrysis carterae* in the dark did not produce H_2O_2 significantly (Fig. 2.1). Therefore, chemical production of H_2O_2 by the added nutrients and the dissolved organic materials released by phytoplankton as well as bacterial production were not important in this study. In addition, killed cells and live cells were incubated in the medium in the dark (Fig. 2.1). The bottle with the killed cells produced an insignificant amount of H_2O_2 compared to the one with the live cells. The results of the control experiments indicate that the factors other than the living phytoplankton in the incubation bottle were not important in the production of H_2O_2 in the dark.

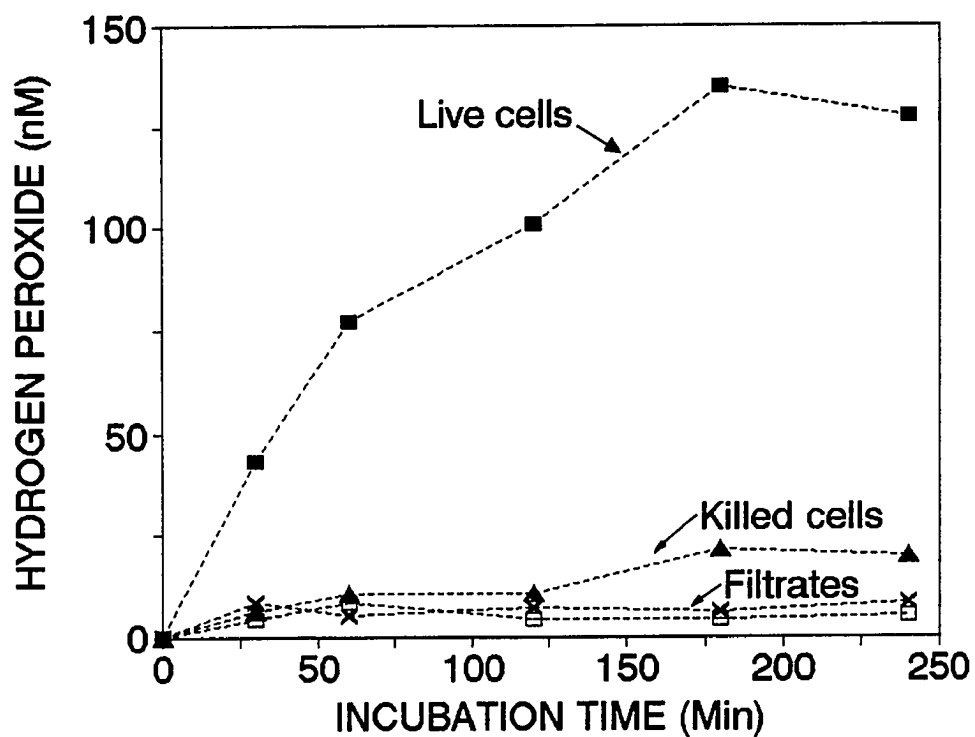


Fig. 2.1. The control experiment for H_2O_2 production in the dark by using cell suspensions of *Pleurochrysis carterae*. Killed cells were prepared by mild heating at $60\text{ }^\circ\text{C}$ for 30 min. ■ represents a $0.45\text{-}\mu\text{m}$ filtrate of the suspension and x represents an $1\text{-}\mu\text{m}$ filtrate of the suspension.

Production of H₂O₂ by phytoplankton : The surveyed 11 species were categorized into two groups, producers (five species) and non-producers (six species) (Fig. 2.2). Among the producers, *Pleurochrysis carterae* was the most important species yielding the largest production rate constant and the highest P_{max} per µg chl-a (Table 2.2). *Pleurochrysis carterae* is a coccolithophorid of about 12 µm in diameter. The common characteristics of the 5 producers was that they are species of coccoid or unicellular type. Two of them are > 10 µm in size (*Tetraselmis levis*, *Pleurochrysis carterae*) and the remaining three are smaller. Stevens et al. (1973) found that five out of 38 species of green algae and cyanobacteria produced H₂O₂ in the dark. Palenik (1989) also found that five out of 11 marine phytoplankton produced H₂O₂.

Non-producers included *Skeletonema costatum*, a common coastal species, and *Synechococcus* sp., a common oceanic species in oligotrophic areas. Most of the non-producers were diatoms of the filamentous type except one dinoflagellate and one cyanobacterium. These species did not seem to contribute significantly to the biological production of H₂O₂ in surface oceans.

Coccolithopores are important in oligotrophic waters. These are the members of the nanoplankton (< 20 µm), which in general are poorly described. Many of the nanoplankton are flagellates or coccoid cells which belong to a small size fraction, the ultraplankton (those cells passing through a 3-µm filter). They are members of several different algal

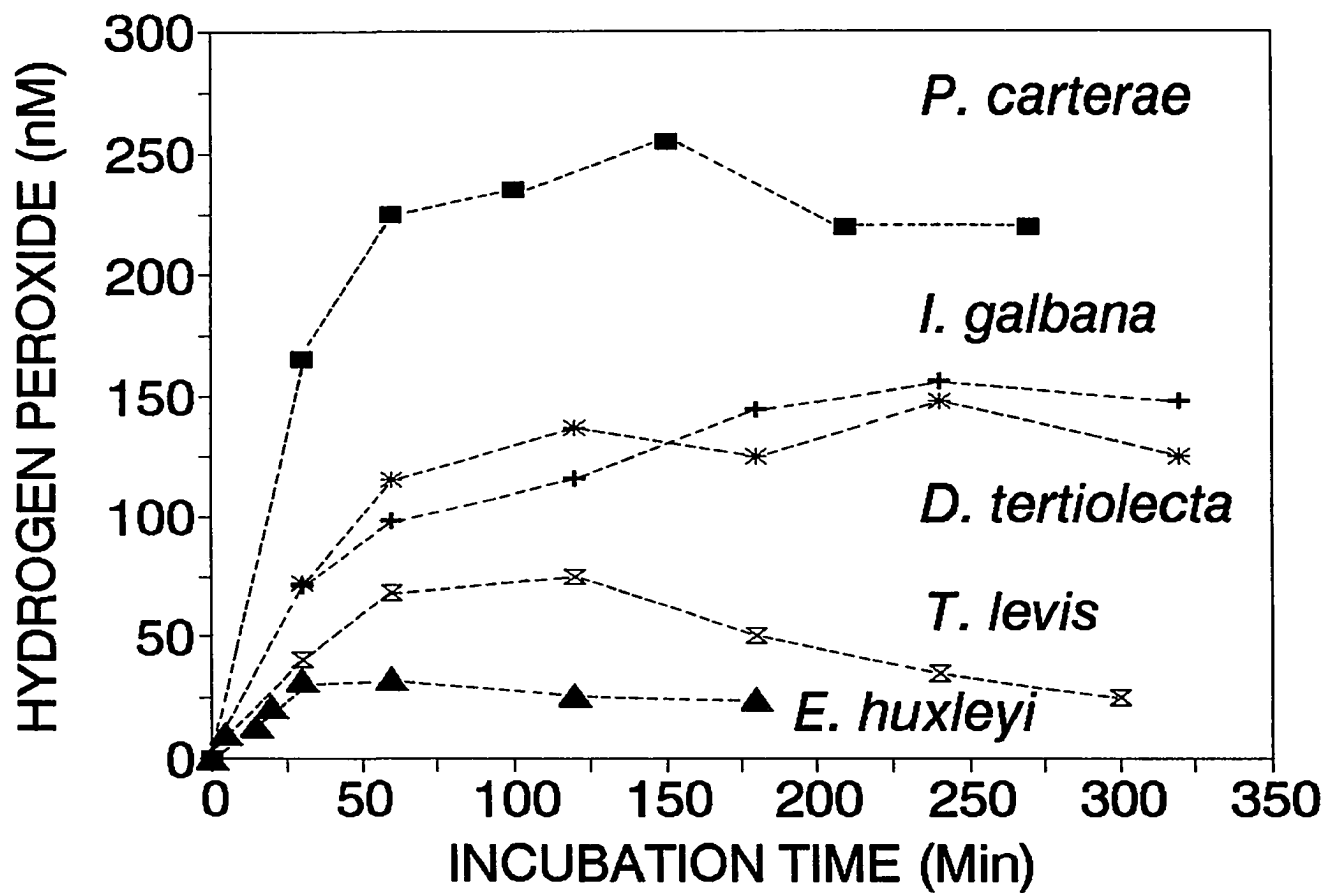


Fig. 2.2. Dark production of H₂O₂ by five cultured marine phytoplankton.

Table 2.2. Summary of the kinetic parameters for the H₂O₂ production by marine phytoplankton. P_{max} and t_{0.5} were estimated for open and coastal oceans by using these parameters.

Species	Class	P _{max} (nM)	k (μg chl-a·L ⁻¹ ·hr ⁻¹)	P _{max} /chl-a (nmol/μg·chl-a)	
H₂O₂ producers					
<i>Pleurochrysis carterae</i>	Prymnesiophyceae	231	0.0072	0.66	
<i>Isochrysis galbana</i>	Prymnesiophyceae	148	0.0024	0.32	
<i>Dunaliella tertiolecta</i>	Chlorophyceae	134	0.0020	0.15	
<i>Tetraselmis levis</i>	Prasinophyceae	71	0.0056	0.20	
<i>Emiliana huxleyi</i>	Prymnesiophyceae	28	0.0017	0.12	
		<i>P. carterae</i>		Average of 5 species	
		P _{max} (nM)	T _{0.5} (hr)	P _{max} (nM)	T _{0.5} (hr)
OPEN OCEAN	(1-5 μg chl-a·L ⁻¹)	0.7-3.3	18 - 88	<1 -1.5	188-38
COASTAL OCEAN	(10-50 μg chl-a·L ⁻¹)	6.6- 33	1.8-8.8	3 - 15	4- 19

H₂O₂ non-producers include

Skeletonema costatum (Bacillariophyceae), *Chaetoceros simplex* (Bacillariophyceae),
Chaetoceros flexosus (Bacillariophyceae), *Thalassiosira oceanica* (Bacillariophyceae),

classes, including Chrysophyceae, Prasinophyceae and Prymnesiophyceae. They are mentioned here because many produced substantial amounts of H_2O_2 . These small algae are present in all environments at densities of $10^6 - 10^7$ cells \cdot L $^{-1}$, and often dominate the deep chlorophyll maximum in oceanic areas (Glover *et al.*, 1986). Thus, representatives of the Chrysophyceae, Prasinophyceae and Prymnesiophyceae may be important sources of H_2O_2 in areas they dominate. Since the abundance of these small phytoplankton is usually at least two orders of magnitude higher than their larger counterpart, size differences (which are also approximately two orders of magnitude) are compensated. H_2O_2 production from oligotrophic regions found by Palenik and Morel (1988) may be mainly from this size class of phytoplankton.

A somewhat evident general taxonomic pattern of H_2O_2 producers indicates that biological production of H_2O_2 is species-specific and may be seasonal and regional in nature and highly dependent on the numerical abundance of key species. A consideration of phytoplankton species composition and succession must be included in any area for the study of H_2O_2 production in the oceans. H_2O_2 levels may vary temporally and spatially, depending on the species composition of the flora. A general information on the phytoplankton distribution and succession coupled with this taxonomic survey of the H_2O_2 production allows to understand H_2O_2 distribution patterns in the oceans.

Kinetics of H₂O₂ production : The production of H₂O₂ increased hyperbolically with time (Fig. 2.2). At the onset of the experiment, production of H₂O₂ occurred quickly and continued at a rapid rate for 30 min to one hour, then slowed down turning to a steady state or decreasing pattern. This pattern is similar to the results of Patterson and Myers (1973) and Stevens *et al.* (1973). They also found the initial burst of H₂O₂ production and then steady state after the burst. The same pattern also appeared in Palenik (1989) and in Moffett and Zafiriou (1990). *Dunaliella tertiolecta*, *Pleurochrysis carterae*, and *Isochrysis galbana* maintained the steady states for two to three hours whereas *Tetraselmis levis* showed non-steady state with the loss of H₂O₂. Probably, this is a result of decomposition of H₂O₂ by *Tetraselmis levis* after the H₂O₂ production reached its P_{max}.

P_{max} and k values based on pseudo-first order reactions are two important kinetic parameters in determining production curves (Table 2.2). P_{max} values could be affected either by environmental factors or by physiological status. Patterson and Myers (1973) observed P_{max} values increased with light intensity to which green algae are exposed. Production rate constants were of a smaller order than decomposition rate constants (chapter 3), which is the indication of less importance of the biological production of H₂O₂ than the biological decomposition of H₂O₂. As comparison to the biological production, photochemical production of H₂O₂ by dissolved organic material seemed to be much important.

Biomass and production of H₂O₂ : Production of H₂O₂ by *Pleurochrysis carterae* was measured at several different concentrations of biomass (Fig. 2.3). The rate of H₂O₂ production was related linearly to algal biomass (chlorophyll-a). High P_{max} and k values were observed with high biomass. That is,

$$d[H_2O_2]/dt = k \cdot [\text{biomass}] \cdot (P_{\max} - [H_2O_2])$$

in which k is a second-order rate constant. Zepp et al. (1987b) also found a linear relationship between biomass and production rates from *Chlamydomonas* sp. Assuming 50 μg·L⁻¹ of chlorophyll-a and k values of 0.001 μg chl-a·L⁻¹·hr⁻¹ in coastal oceans, the half-life time for approaching P_{max} can be as short as 13 hours. However, assuming the averaged P_{max} (normalized to biomass) value is up to 1 nmol·(μg chl-a)⁻¹, the maximum concentration of H₂O₂ after 13 hours can be only up to 50 nM. This indicates that the production of H₂O₂ by marine phytoplankton in normal growth conditions could be a minor source of H₂O₂ in the surface oceans. Cooper et al. (1992) also asserted that biological production of H₂O₂ under normal condition is insignificant.

2.3.2. Factors affecting the production of H₂O₂

Effect of light intensity : *Pleurochrysis carterae* was tested for effects of light on the production of H₂O₂ under the

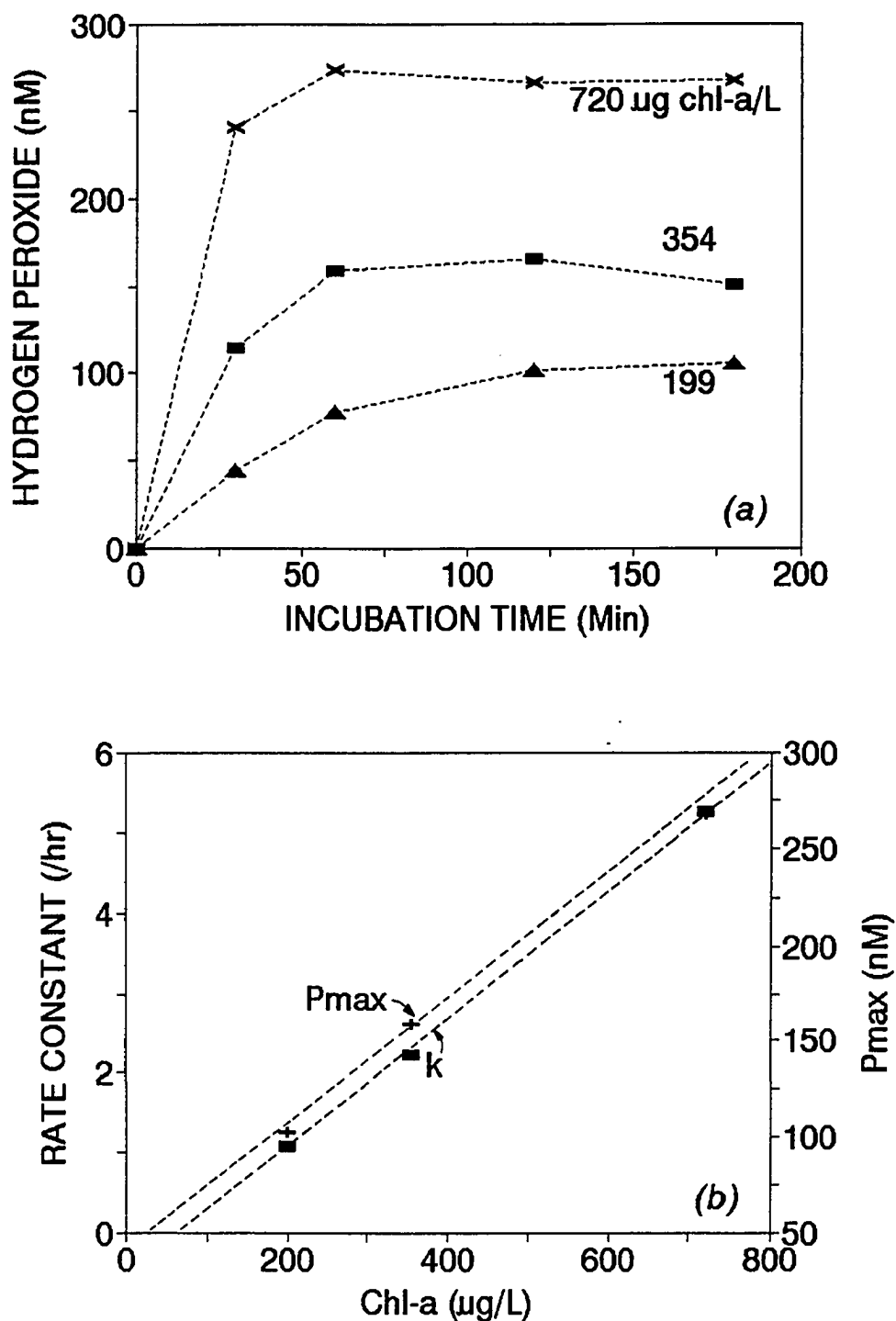


Fig. 2.3. Dark production of H_2O_2 by *Pleurochrysis carterae* as a function of biomass (μg chlorophyll-a/L). (a) Kinetic curves. (b) Kinetic parameters vs. chl-a concentration.

varying light intensities from 0 to 183 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. *Pleurochrysis carterae* exposed to a higher light intensity resulted in a higher level of P_{max} (460 nM) as opposed to the cells in the dark (270 nM) (Fig. 2.4a). However, the k values were smaller in the light-exposed samples than in the samples in the dark (Fig. 2.4b), which implied higher efficiency of H_2O_2 production in dim light. The filtrate sample exposed to light also produced H_2O_2 due to dissolved organic materials released by the cells. The difference between unfiltered and filtered samples exposed to the same light intensity of 183 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ well-matched with the amount of H_2O_2 produced in the dark, which implies that *Pleurochrysis carterae* can produce H_2O_2 independently of light. Moffett and Zafiriou (1990) also found that the biological production of H_2O_2 is light-independent. In fact, because excretion of the dissolved organic materials by the cells continues during the incubation period, photochemical production of H_2O_2 can be higher than observed. Therefore, production of H_2O_2 by *Pleurochrysis carterae* under light (low k) could be even less than that produced in the dark. Fig. 2.4b shows that phytoplankton produced H_2O_2 more effectively (high k) in the dark than in the light. This result agrees with others that H_2O_2 production by phytoplankton may be important in a dimly-lit water column (Palenik and Morel, 1988; Moffett and Zafiriou, 1990). Zepp et al. (1987b) thought that the low efficiency in the light could be a result of the destruction or suppression of the H_2O_2 -decomposing enzymes by the intense light.

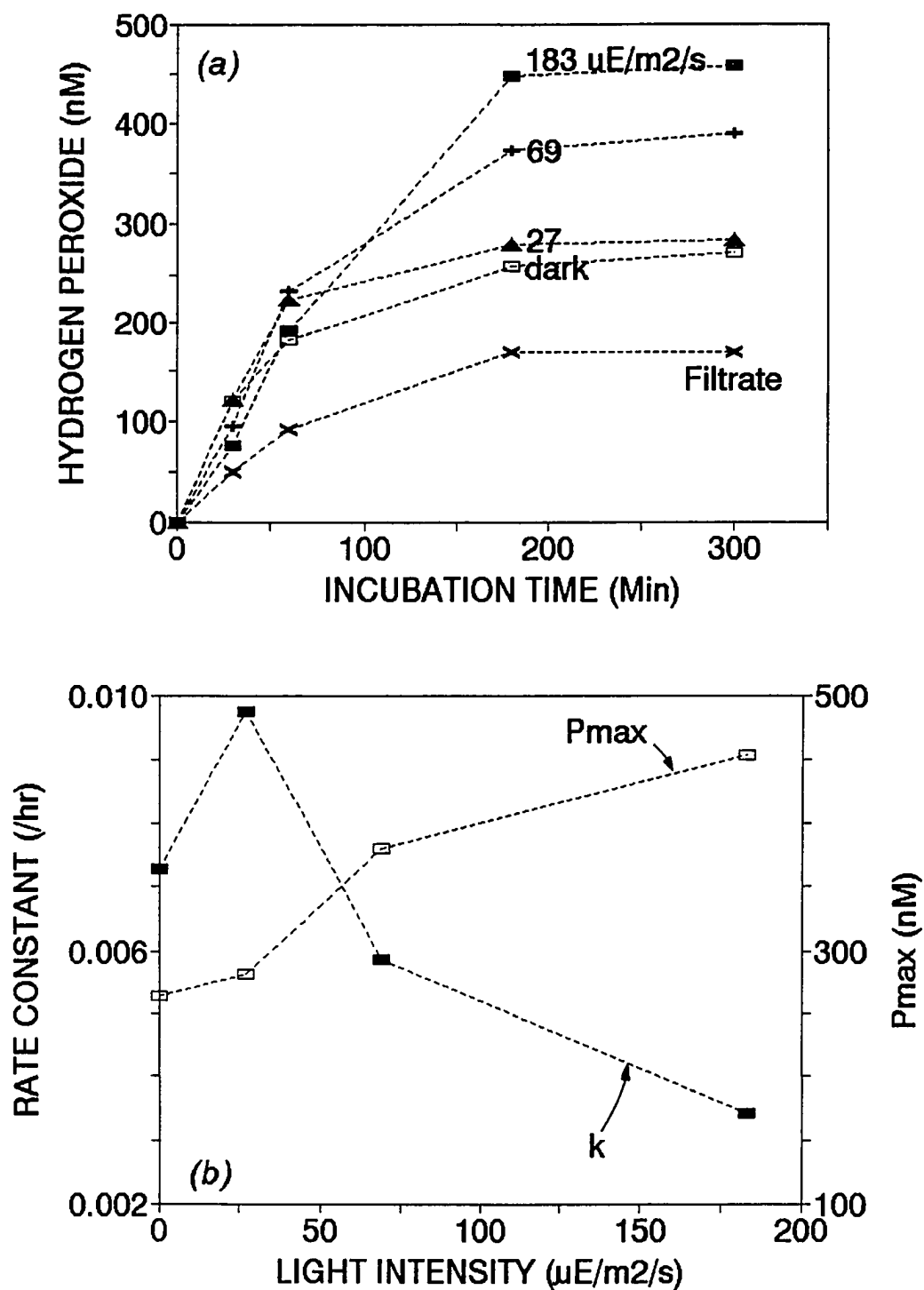


Fig. 2.4. Effect of varying light intensity on the production of H_2O_2 by *Pleurochrysis carterae*. Filtrate sample ($0.45\text{-}\mu\text{m}$) was exposed to $183 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. (a) Kinetic curves (b) Kinetic parameters vs. light intensity.

The filtrate of *Pleurochrysis carterae* exposed to two light intensities resulted in more H_2O_2 formation at the higher light intensity. This is in agreement with other reports on the linear relationship between the light intensity and dissolved organic materials. Thus, dissolved organic materials released by phytoplankton could make a significant contribution to the photochemical production of H_2O_2 under strong sunlight. However, the filtrate of *Skeletonema costatum* incubated under the same conditions did not produce any significant amount of H_2O_2 (see Appendix B). Thus, the indirect contribution of H_2O_2 by phytoplankton under sunlight can be more likely related to the types and amounts of the photosensitizers they release to the medium. In conclusion, the results of the experiment on the effect of light intensity suggest that phytoplankton produce more H_2O_2 in dim light and also that they produce H_2O_2 in an indirect way during photolysis of extracellular products in sunlight.

Effect of temperature : Effect of temperature on the production of H_2O_2 was tested with *Pleurochrysis carterae* at varying temperatures from 10 °C to 33 °C. Fig. 2.5 shows only a slight difference between samples. P_{max} and k values were not notably altered by the temperature change of more than 20 °C. This result opposes the notion that biological activity increases as temperature increases. Considering that 20 °C is a large temperature change compared to *Pleurochrysis carterae*'s narrow optimum growth range of 16 - 23 °C, the

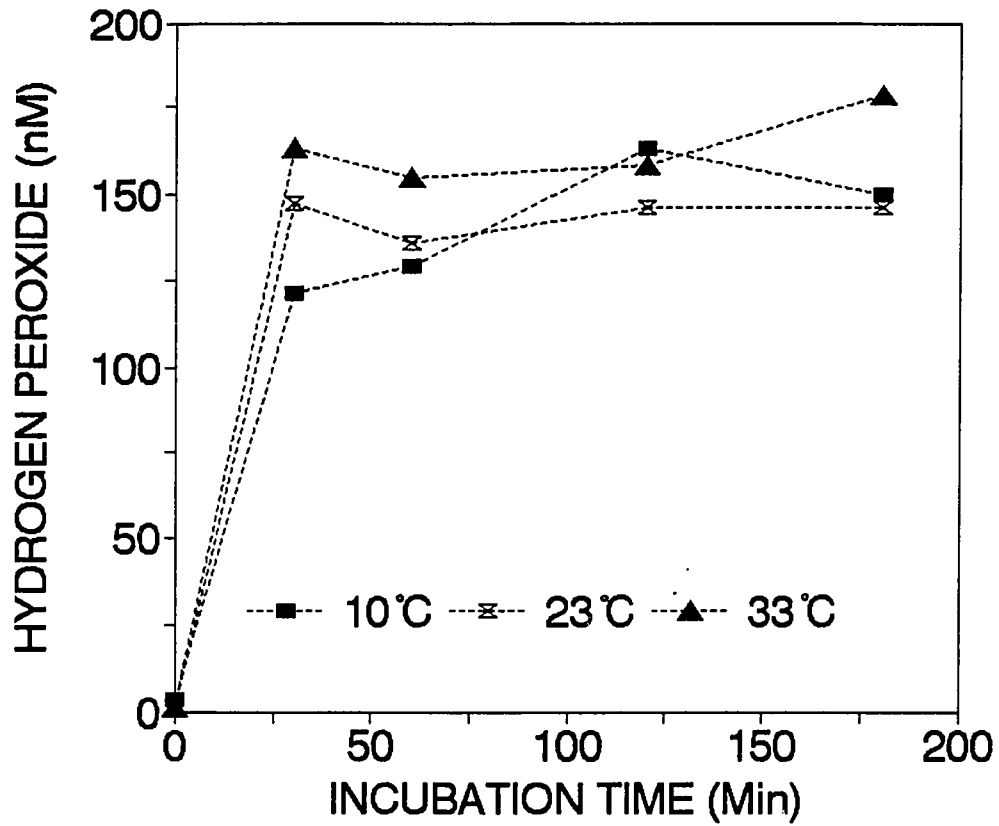


Fig. 2.5. Effect of varying temperature on the dark production of H_2O_2 by *Pleurochrysis carterae*.

production of H_2O_2 by *Pleurochrysis carterae* appeared independent of temperature.

Effect of salinity : Effect of salinity on the production of H_2O_2 was tested with *Pleurochrysis carterae* and *Dunaliella tertiolecta* grown in media of different salinities; 12, 25, 30 and 35 ppt. Fig. 2.6 shows the different P_{max} values between 12 ppt and 35 ppt. The difference in P_{max} between 12 ppt and 35 ppt was more than 100 nM of H_2O_2 . More production of H_2O_2 was associated with a higher salinity. *Dunaliella tertiolecta* resulted in a difference of about 30 nM of H_2O_2 between 12 ppt and 35 ppt. Both species can grow in quite broad ranges of salinity, 3 to 45 ppt for *Pleurochrysis carterae* and < 36 ppt for *Dunaliella tertiolecta* (Andersen et al., 1991). This result could be related to the more effective decomposition of H_2O_2 by phytoplankton at lower salinities (chapter 3). This study suggests that the production of H_2O_2 by phytoplankton is more important in open oceans (around 35 ppt) than in coastal oceans or near estuaries of much less salinity.

Effect of amino acids : Two amino acids, L-leucine and L-tyrosine, were added to *Pleurochrysis carterae* to examine their effect on the production of H_2O_2 . The cells were grown in media without addition of nitrogen sources to prepare nitrogen-limited cells. The concentrations of the amino acids used in this experiment ranged from 0 to 2 μM , which are

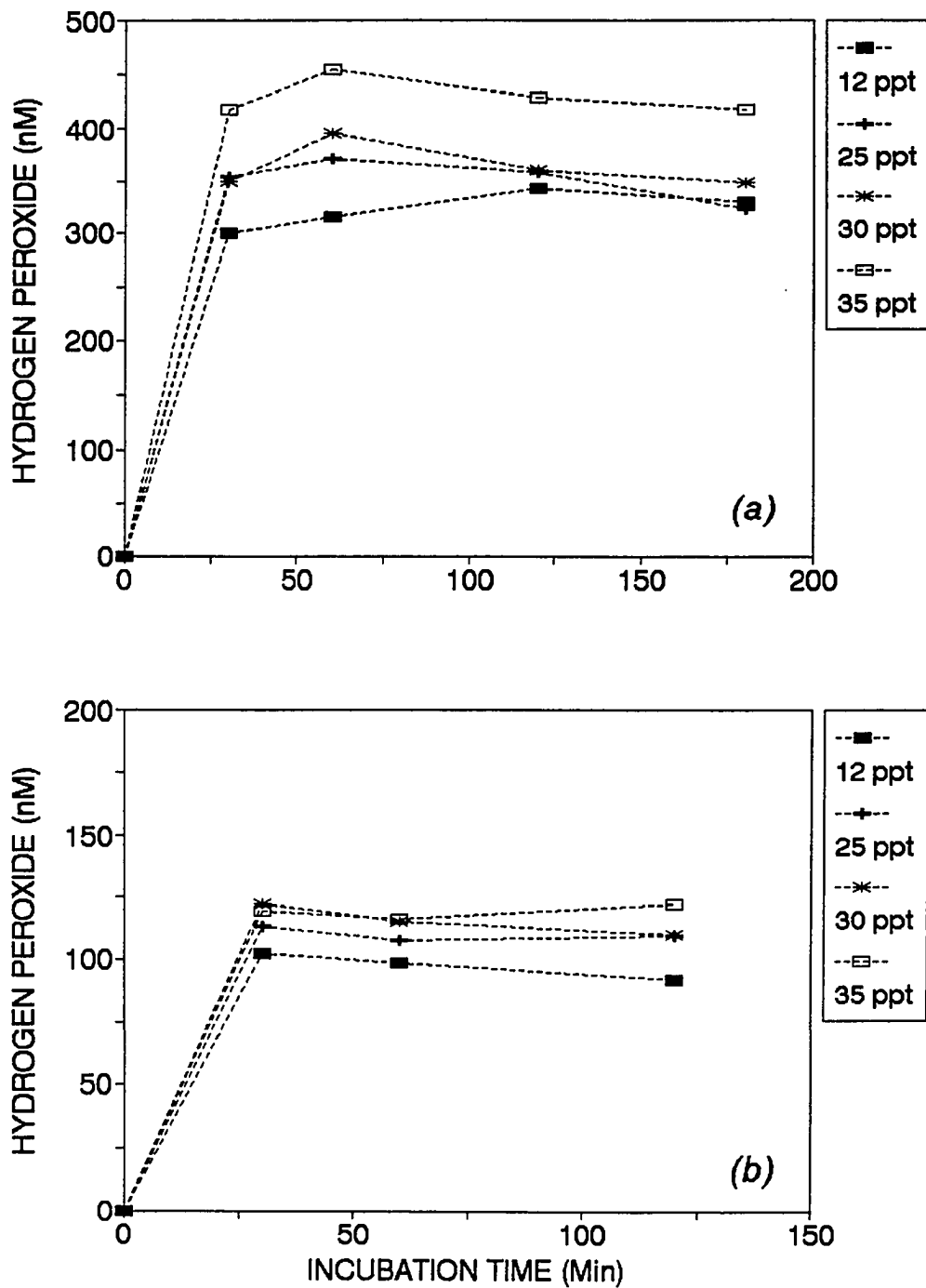


Fig. 2.6. Effect of varying salinity on the production of H₂O₂ by (a) *Pleurochrysis carterae* and (b) *Dunaliella tertiolecta*.

representative values of total dissolved free amino acids (DFAA) found in various oceans (Clark et al., 1972; Riley and Segar, 1970).

The two amino acids increased dark production of H_2O_2 profoundly (Fig. 2.7). This is in agreement with Palenik (1989) that amino acids are one of the major substrates for the production of H_2O_2 by marine phytoplankton. Addition of more amino acids resulted in higher P_{max} values. H_2O_2 production rates ($nmol \cdot \mu g \text{ chl-a}^{-1} \cdot hr^{-1}$) at $2 \mu M$ L-leucine and $2 \mu M$ L-tyrosine were 15.7 and 8.3, respectively. Assuming $1 - 10 \mu g \text{ chl-a} \cdot L^{-1}$ in open ocean water, these rates can result in 15.7 to 157 nM of H_2O_2 in an hour with the utilization of $2 \mu M$ L-leucine. Addition of L-tyrosine could result in 8.3 to 83 nM of H_2O_2 under the same conditions. According to Palenik (1989), many kinds of L-amino acids can be actively used by phytoplankton. The effect of the amino acids on the H_2O_2 production by phytoplankton appears to be remarkable.

Fig. 2.8 shows that the initial production rates increased as the substrate increased, following the Michaelis-Menten curve. Phytoplankton effectively use amino acids of a level lower than 0.5 to $2 \mu M$. The intercept on the y-axis indicates that amino acids were not the only substrates for H_2O_2 production in the medium. A preliminary experiment showed that the nitrate-grown cells without addition of ammonia and amino acids produced H_2O_2 in $f_{1/2}$ and $f_{1/20}$ media, implying that there are some unknown substrates or mechanisms responsible for the production of H_2O_2 . The ammonium-rich condition did

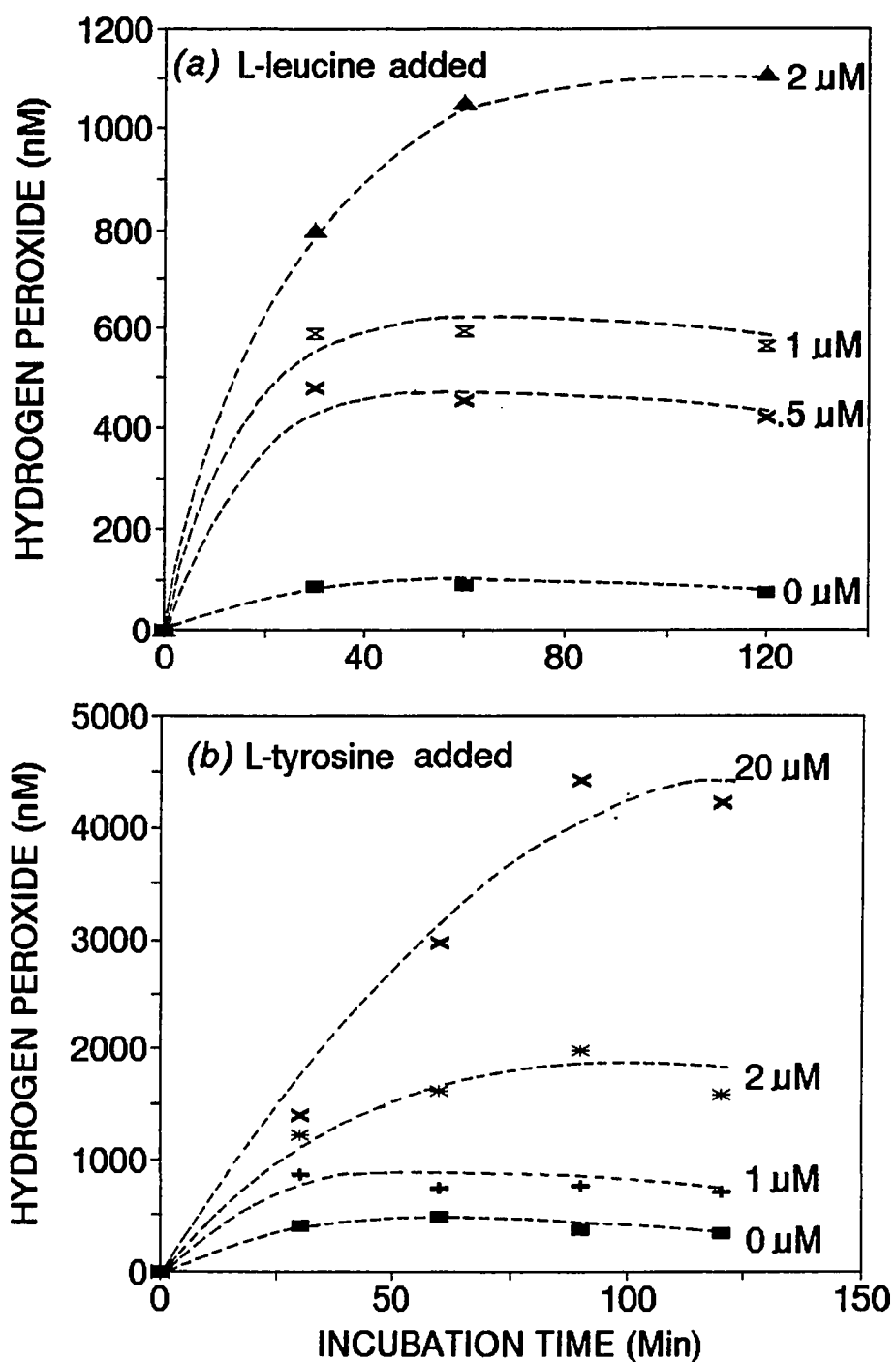


Fig. 2.7. Effect of amino acids on the production of H_2O_2 by *Pleurochrysis carterae*. (a) L-leucine addition (0 - 2 μM) (b) L-tyrosine addition (0 - 20 μM).

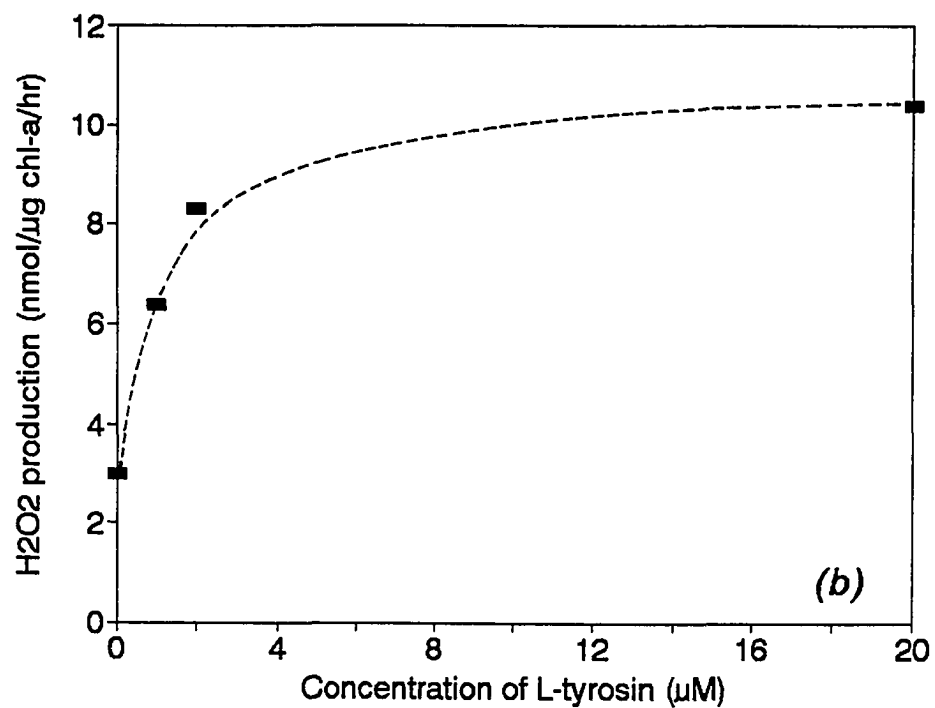
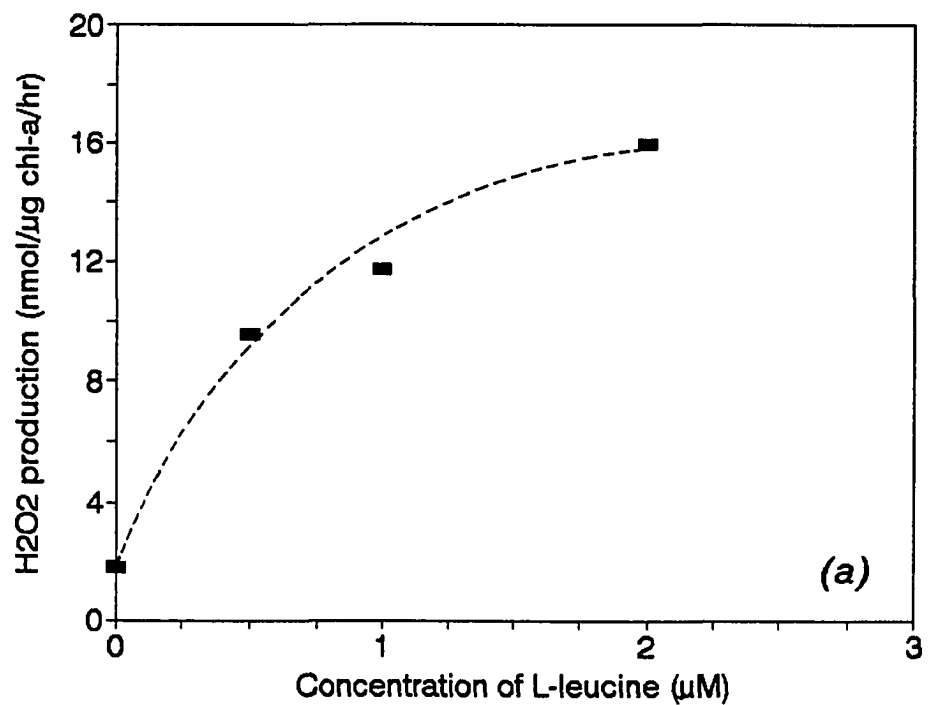
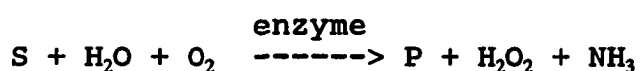


Fig. 2.8. Kinetics of H₂O₂ production as a function of amino acid concentrations. (a) L-leucine (b) L-tyrosine.

not produce H_2O_2 by phytoplankton (Palenik, 1989). Therefore, environments where the inorganic nitrogen, such as nitrate, nitrite and ammonia, is limited can stimulate phytoplankton to utilize amino acids as a nitrogen source and produce H_2O_2 as a by-product.

According to Palenik et al. (1991),



in which S is substrates such as L-amino acids or amines, P is products such as α -keto acids or aldehyde, and the enzymes are L-amino acid oxidases or amine oxidases. Additional candidates for the substrates are glucose, uric acid, galactose, etc (Mottola, 1988). H_2O_2 may come from either H_2O or O_2 . It was tested whether the depletion of O_2 could stop the production of H_2O_2 . Removal of O_2 from the incubation medium by He gas reduced H_2O_2 production from ca. 1100 nM to ca. 200 nM, which is more than 80 % decrease. H_2O_2 production was not completely stopped probably due to the incomplete purging of O_2 in the medium. Patterson found no sign of H_2O_2 production by green algae in the light when O_2 was expelled using N_2 gas (O_2 conc. = 1.1 ml $O_2 \cdot L^{-1}$). Moffett and Zafiriou (1990) also measured H_2O_2 production from dissolved O_2 using $^{18}O_2$. Therefore, H_2O_2 production by algae in an anoxic environment can be negligible. In addition, because H_2O_2 production by phytoplankton is light-independent (Moffett and Zafiriou, 1990), the above production mechanism (non-

photosynthetic) is potentially important both day and night in the nitrogen-limited but amino acid-rich water.

Effect of nutrient concentration : To examine the effect of nutrient concentration on the production of H_2O_2 , *Pleurochrysis carterae* was grown in different nutrient strengths, $f_{/2}$ (with and without the addition of trace metals), $f_{/10}$, $f_{/60}$ and $f_{/100}$. The concentration of added nitrate to each medium was 883 μM , 176.6 μM , 29.4 μM and 17.7 μM .

Fig. 2.9 shows the production rates of H_2O_2 normalized to chlorophyll-a amounts. The cells in the $f_{/2}$ medium showed lower P_{max} values, which indicates that the cells grown in the high nutrient concentrations cannot produce H_2O_2 at a higher rate per unit biomass. The depletion of trace metals neither reduced the production rates nor affected the production mechanisms of H_2O_2 . However, the lower nutrient strengths enhanced H_2O_2 production. The cells grown in very low levels of nutrients ($f_{/60}$ and $f_{/100}$), where probably they were most affected by nitrate limitation (no addition of ammonium), exhibited initial lag-phases for about 0.5 hour, probably resulting from adaptation processes or nutrient shocks after being transferred to the new incubation media. The same pattern was also observed in *Isochrysis galbana*. Once they adapted, they could take up more substrates for H_2O_2 production. P_{max} values, which are a function of environmental conditions and physiological status, can be important parameters in the diagnosis of the nutritional status of

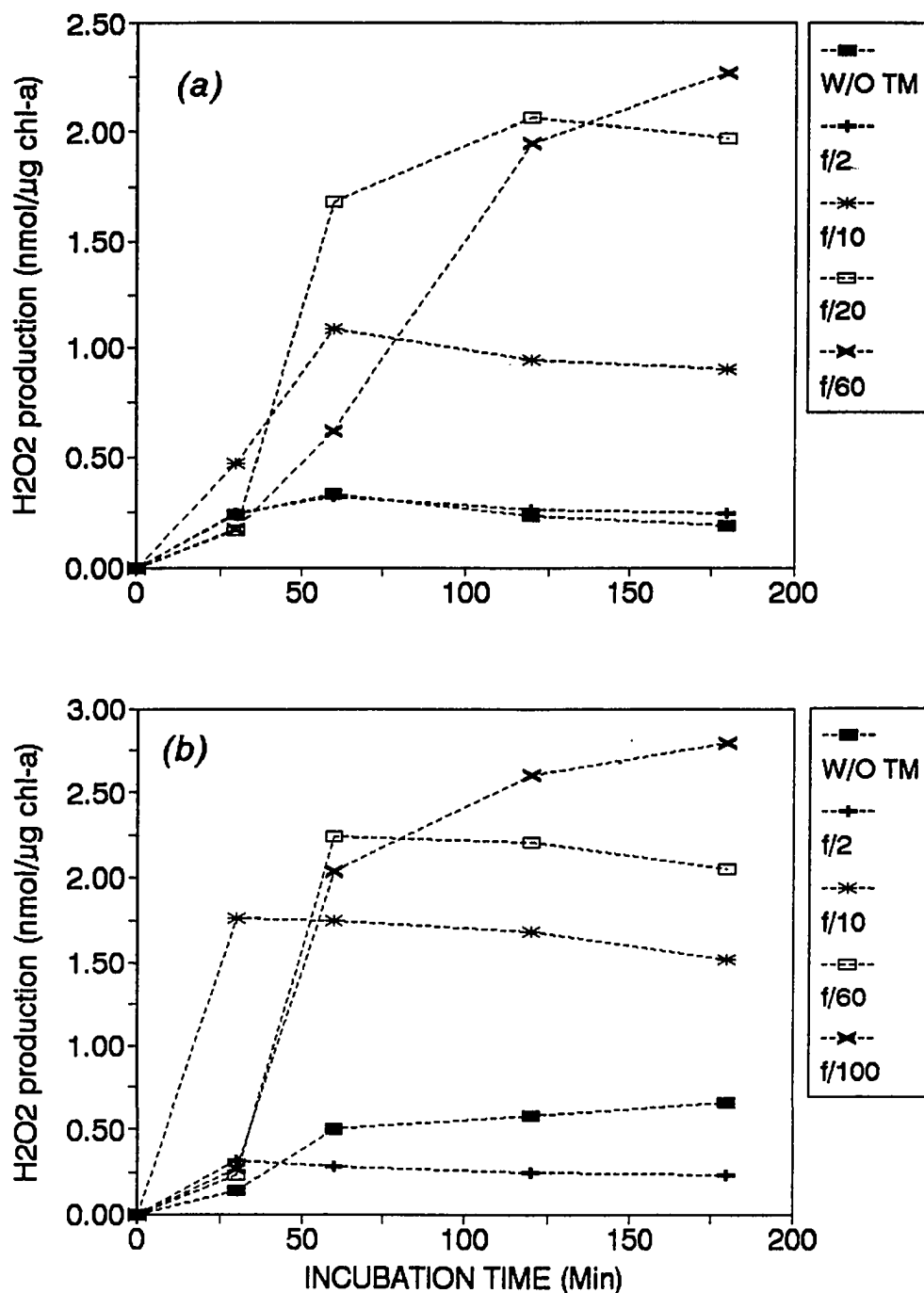


Fig. 2.9. Effect of nutrient concentration and trace metals on the production of H₂O₂ by (a) *Pleurochrysis carterae* (b) *Isochrysis galbana*. W/O TM represents no addition of trace metals to f_{1/2} medium.

cells. Fig. 2.10 clearly shows that P_{\max} tends to be increased as the nutrients become limited. The high P_{\max} values measured in the cells grown in the lower nutrient concentrations were observed probably because of the active uptake of nutrients as well as substrates of H_2O_2 production. However, it is unknown what is a responsible substrate for H_2O_2 production by phytoplankton in the medium with no addition of amino acids. Cells living in inorganic nitrogen-limited oligotrophic waters could show high levels of H_2O_2 production if amino acids are accessible to them. On the other hand, cells in inorganic nitrogen-rich coastal waters would show less H_2O_2 production.

Effect of growth phase : To examine whether cells from different growth phases can show different production rates of H_2O_2 , *Pleurochrysis carterae* was harvested at lag, mid-log, late-log and senescence phases. Fig. 2.10 shows that P_{\max} and the initial rates of H_2O_2 production have increased as the growth stage approached the senescence phase where nutrients become depleted. This result was in agreement with the previous experiment. That is, as the cells became nutrient-starved they produced more H_2O_2 per unit biomass. Therefore, cells living in nutrient-poor water, when amino acids or nutrients are available, could be potentially important in producing H_2O_2 . The nutrient experiment and the growth phase experiment have important implications on the biological production of H_2O_2 associated with low nutrient water such as that found in the Sargasso Sea.

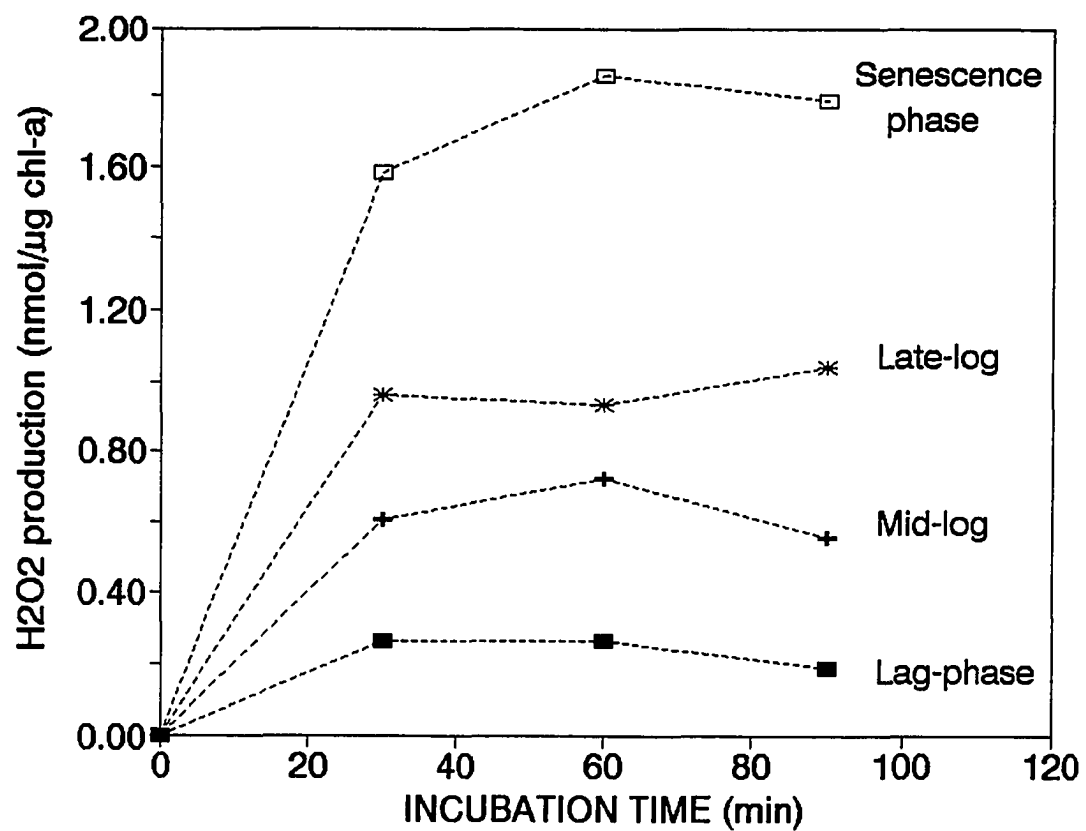


Fig. 2.10. Effect of varying growth phase on the production of H₂O₂ by *Pleurochrysis catarae*.

2.4. SUMMARY

1. The production of H_2O_2 in the dark was species-dependent. Among the 11 pure species of marine phytoplankton tested, five species produced H_2O_2 of various levels. Based on P_{max} normalized to biomass (chl-a), the relative significance of these species was *Pleurochrysis carterae* > *Isochrysis galbana* > *Dunaliella tertiolecta* > *Tetraselmis levis* > *Emiliana huxleyi*. Most of the producers were Prymnesiophyceae and most of the non-producers were Bacillariophyceae. Taxonomically, the effective producers of H_2O_2 were associated with the non-effective decomposers of H_2O_2 (chapter 3). H_2O_2 production will be seasonal and regional in nature and highly dependent on the numerical abundance of key species.

2. The rate of production of H_2O_2 could be described by pseudo-first order kinetics. Production rate constants (k) ranged from 0.0017 to 0.0072 ($\mu\text{g chl-a}\cdot\text{L}^{-1}$) $^{-1}\cdot\text{hr}^{-1}$, which were smaller than the decomposition rate constants (chapter 3). P_{max} values were less than 1 $\text{nmol}\cdot(\mu\text{g chl-a})^{-1}$, indicating that production of H_2O_2 by marine phytoplankton in nature is not important in the budget of H_2O_2 in surface oceans. P_{max} and k values were linearly related to biomass.

3. Production of H_2O_2 was more important in dimly-lit conditions and was partly light-dependent. Therefore, H_2O_2 would be produced more at night or in the subsurface water

column. The addition of amino acids stimulated the dark production of H_2O_2 by marine phytoplankton. Both of dissolved oxygen and amino acids were required for the H_2O_2 production. Temperature effects on the production of H_2O_2 were not extensive. Salinity had some effect on the production at higher salinity levels. Inorganic nitrogen-limited conditions stimulated phytoplankton to take up more amino acids with more production of H_2O_2 . Nutrient-limited conditions induced phytoplankton to produce more H_2O_2 per unit biomass. The production of H_2O_2 may have great relationships with nitrogen cycling by phytoplankton in the oligotrophic oceans.

Chapter 3

DECOMPOSITION OF H₂O₂ BY MARINE PHYTOPLANKTON

3.1. INTRODUCTION

Hydrogen peroxide (H_2O_2) may influence an ecosystem's biogeochemistry. For example, redox chemistry in marine environments can be profoundly affected by H_2O_2 in the water. H_2O_2 is a strong oxidant or reductant that affects the speciation of trace metals even at a very low concentration. H_2O_2 may also interfere with biological processes, because H_2O_2 is toxic to numerous organisms, causing growth inhibition, membrane damage, chlorophyll bleaching, etc. These influences may be very extensive, because H_2O_2 exists in almost all aquatic systems including freshwater (Cooper and Lean, 1989; Cooper *et al.*, 1989), seawater (Van Baalen and Marler, 1966; Zika *et al.*, 1985a, b; Johnson *et al.*, 1989), estuarine water (Szymczak and Waite, 1988), ground water (Holm *et al.*, 1987), clouds and rain water (Kok, 1980; Zika *et al.*, 1982).

H_2O_2 is in dynamic balance in surface lakes and oceans, showing a diurnal change. The maximum concentration of H_2O_2 occurs in the mid to late afternoon and the minimum concentration in the early morning. Photochemical production seems to be a major source of H_2O_2 in the day time (Zika, 1980). On a sunny day, H_2O_2 can accumulate up to levels of micromolar.

The accumulation rates and the photochemistry of H_2O_2 have been amply studied. However, the studies on the biological removal of H_2O_2 from water systems were not given much attention until it was found that H_2O_2 in filtered seawater

does not decay within 24 hours (Cooper and Zepp, 1990; Moffett and Zafiriou, 1990). Biological removal of H_2O_2 by cultures of freshwater algae (Zepp et al., 1987b), and by two cultures of bacteria (Cooper et al., 1992) have been reported. However, cultures of marine phytoplankton have not been studied for their decomposition of H_2O_2 .

The role of marine phytoplankton in the biogeochemical cycle of H_2O_2 in marine environments was studied by examining 11 pure cultures of marine phytoplankton (Table 2.1), and several environmental factors that may affect the capability of these organisms to decompose H_2O_2 . *Skeletonema costatum*, a common species in coastal oceans, and *Synechococcus* sp., a common species in open oceans, decomposed H_2O_2 most actively among the surveyed marine phytoplankton. The result of this study implies that marine phytoplankton play an important role in the decomposition of H_2O_2 in the surface ocean.

3.2. MATERIALS AND METHODS

Decomposition of H_2O_2 was studied in cultures of species obtained from the Provasoli-Guillard Center for Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, McKown Point, West Boothbay Harbor, Maine 04575, U.S.A.). Batch cultures of algae were maintained at 23 °C, 12:12 light:dark cycle, and $75 \mu E \cdot m^{-2} \cdot s^{-1}$. Culture medium, f_{12} , was prepared from Sargasso seawater according to Guillard and Ryther (1962).

In vivo fluorescence was monitored so that cells could be harvested at the late-log growth phase. After being conditioned in the dark for 2 - 3 hours, cells were transferred to sterile centrifuge tubes and then concentrated by centrifugation at 1000 rpm for 5 minutes. The supernatant was carefully decanted and the remaining concentrated cells were washed with fresh medium, f_{20} (no addition of trace metals) and then recentrifuged. The concentrated cells were quantitatively transferred into incubation bottles with f_{20} medium. Dark bottles (high density polyethylene) were used for the incubation to prevent photochemical reactions. The medium for the incubation was prepared from Sargasso seawater that had been filtered (GFF), aged, and sterile-filtered ($0.2 \mu m$) in the dark. The concentration of H_2O_2 in the medium prepared in this way was close to its detection limit.

The cell concentrations in the incubation bottles were determined either by chlorophyll-a measurement or by direct cell counting. The H_2O_2 solution (900 nM) was spiked into

each bottle at time 0 and samples were withdrawn at time intervals of 30 min to 60 min until the H_2O_2 concentration decreased by at least 75 %. Before and during the experiment, cells were examined under the microscope for any damage by the added H_2O_2 . Toxicity of H_2O_2 was tested also by monitoring growth curves after the addition of H_2O_2 .

Incubation bottles were incubated in the dark at 23 ± 1 °C. A subsample of 3 mL was taken for the determination of the concentration of H_2O_2 . Prior to the measurement of H_2O_2 , the subsample was filtered through a GFF filter to remove phytoplankton cells. The measurements were carried out in a dimly-lit room. The data presented here are the mean values of duplicate measurements. The pooled standard deviations of the duplicate data sets were less than ± 5 %.

H_2O_2 determination : H_2O_2 analysis was performed using the scopoletin-horseradish peroxidase (HRP) method (Andreae, 1955; Holm *et al.*, 1987). The method measures the fluorescence decay of scopoletin by the peroxidase mediated decomposition of H_2O_2 . A Perkin Elmer 650-10S fluorescence spectrophotometer was used. HRP and H_2O_2 working standard solutions were prepared fresh each day. For each medium or culture studied, separate standard curves were determined. The detection limit of the method was about 5 nM.

Preparation of controls : Control samples were prepared by filtering cell suspensions in the incubation bottles

through a GFF filter and then a 0.45 μm Millipore filter with low pressure (< 10 mm Hg) to avoid breaking cells. The medium ($f_{/20}$ without addition of trace metals) was tested as a control. Killed cells were tested for decomposition of H_2O_2 on the non-active cell surfaces. To prepare the killed cells, cells were either heated at 60 °C for 30 minutes or treated with sodium azide (10 mM) and formaldehyde (25 mM), buffered to ca. pH 7.5. H_2O_2 was also added to these control bottles and then monitored.

Chlorophyll-a determination : Chlorophyll-a was used as an index of biomass. Cell suspensions were filtered onto glass fiber filters. The retained cells on the filters were ground with a homogenizer and the pigments were extracted with 90 % acetone before fluorometric determination. The concentration of chlorophyll-a and phaeopigment-a were calculated using the method of Strickland and Parsons (1972). Often cell counts were made. For cell counting, samples were preserved with Lugol's iodine fixative and then counted on a 0.1 mm Neubauer hemocytometer.

Data treatment : To calculate rate constants (k) of the decomposition of H_2O_2 that appeared to follow pseudo-first order reaction (Cooper and Zepp, 1990), the following equation was used;

$$-d[\text{H}_2\text{O}_2] \cdot dt^{-1} = k \cdot \text{Ca} \cdot [\text{H}_2\text{O}_2] = k' \cdot [\text{H}_2\text{O}_2],$$

where $k' = k \cdot C_a$

by integrating,

$$\ln [H_2O_2]_t = -k' \cdot t + \ln [H_2O_2]_0,$$

$$t_{1/2} = 0.693 \cdot k'^{-1}.$$

where $[H_2O_2]_0$ is the concentration of H_2O_2 at time 0 and $[H_2O_2]_t$ at time t . C_a is a concentration of chlorophyll-a. The second-order rate constant (k) was obtained from the pseudo-first order rate constant (k') which were calculated by regression analysis using data points representing the first 2 - 3 half-lives, i.e. $[H_2O_2]_t/[H_2O_2]_0 = 0.125$. k' values were finally normalized to the amount of chlorophyll-a to get k in order to compare k values for each species.

3.3. RESULTS AND DISCUSSION

3.3.1. Biological decomposition of H_2O_2 and its kinetics

The decomposition studies were conducted using 11 pure cultures of marine phytoplankton by the the HRP-scopoletin method. The decomposition of H_2O_2 in phytoplankton samples and in control bottles without phytoplankton were compared with each other to prove that the decomposition was due to biological activities. The reaction rate constants for 11 species were obtained from the kinetic studies of the decomposition of H_2O_2 . $T_{1/2}$ of H_2O_2 estimated by the decomposition rate constants were applied to coastal ocean and open ocean water.

Decomposition of H_2O_2 in the controls : Medium, filtrates and killed cells were tested for the decomposition of H_2O_2 . No observable changes or decomposition of H_2O_2 occurred in these control samples during incubation periods (see Appendix C). The results from the medium indicated that chemical decomposition of H_2O_2 seems negligible or slow within the incubation periods. According to Cooper *et al.* (1992), chemical decomposition of H_2O_2 appears to play a minor role in the overall decomposition processes. However, the effect of H_2O_2 on the trace-metal speciation may be significant even if the loss of H_2O_2 due to the reaction is insignificant. No changes in H_2O_2 added to the filtrates ($0.45 \mu\text{m}$, $1 \mu\text{m}$) of the

cell suspension of *Skeletonema costatum* indicated that dissolved organic materials, including any enzyme, released into the medium by the cells have minor effects on the decomposition of H_2O_2 . This result also implies that the enzymes mediating H_2O_2 decomposition are tightly bound to the cells and are not water soluble and thus may not be easily released to the medium. Effects of free-living bacteria on the decomposition of H_2O_2 were tested with 1- μm filtrate of the algal suspension, assuming that a large portion of free-living bacteria can pass through a 1- μm filter (see Appendix C). Negligible effects were observed. Numbers of attached bacteria on algal cells accounted for ca. 5 % of total bacteria, thus no further experiment with attached bacteria was made. The results from the two control experiments are coincident with the previous conclusion by others that the decomposition of H_2O_2 is mainly caused by organisms living in water (Zepp et al., 1987b; Cooper and Zepp, 1990; Moffett and Zafiriou, 1990).

To investigate the decomposition of H_2O_2 on the surfaces of non-living particles, phytoplankton cells were killed and then H_2O_2 was added. Fig. 3.1 shows that dead cells could not decompose H_2O_2 within the incubation time, indicating that only living cells are responsible for the decomposition of H_2O_2 . Cooper and Zepp (1990) also showed that the decomposition of H_2O_2 in soil suspensions was because of the living organisms in the suspensions, not because of the soils. All the control experiments suggested the conclusion that H_2O_2 decomposition

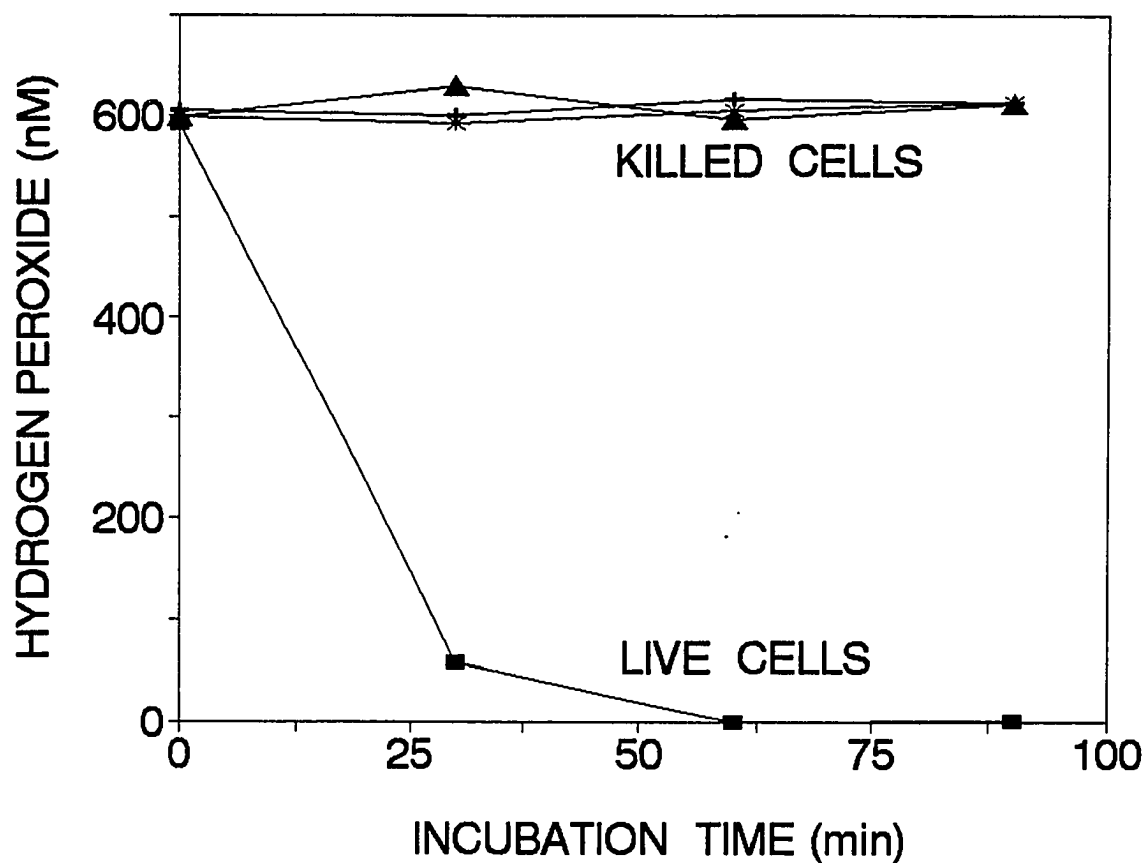


Fig. 3.1. Control experiment for H_2O_2 dark decomposition with the cell suspension of *Skeletonema costatum*. Killed cells (+: Heat-killed, *: NaN_3 -treated, \blacktriangle : Formaldehyde-treated) and live cells were prepared.

observed in the incubation bottles was the result of activity of living phytoplankton cells.

Inhibition of biological activities or deterioration of cells due to toxic effects of added H_2O_2 , may be possible. Thus, *in vivo* fluorescence and cell numbers were monitored for several species. However, no detectable changes occurred in both *in vivo* fluorescence and cell numbers during the incubation period (data not shown). As a separate experiment, H_2O_2 was spiked in cultures of *Skeletonema costatum* and *Synechococcus* sp. to monitor any effects on the growth curves due to toxicity of H_2O_2 (Fig. 3.2). *Skeletonema costatum* was unaffected up to $5 \cdot 10^{-2}$ mM of H_2O_2 . However, *Synechococcus* sp. was affected at concentrations above $2 \cdot 10^{-3}$ mM of H_2O_2 . *Synechococcus* sp. seemed to be more sensitive to H_2O_2 . The addition of $9 \cdot 10^{-4}$ mM of H_2O_2 for the decomposition study should not have any significant effect on either species.

Decomposition of H_2O_2 by marine phytoplankton: Most of the samples of marine phytoplankton demonstrated exponential decomposition of H_2O_2 with time (Fig. 3.3a), which could be described by pseudo-first order rate expressions. The decomposition rates were accelerated as the cell numbers of each species increased, showing a linear relationship to biomass (Fig. 3.3c). The second-order rate constants (k) were in the range of 10^{-4} to 10^{-2} $\mu\text{g chl-a} \cdot \text{L}^{-1} \cdot \text{hr}^{-1}$ (Table 3.1). The phytoplankton examined for the H_2O_2 decomposition may be subdivided into three groups based on their decomposition rate

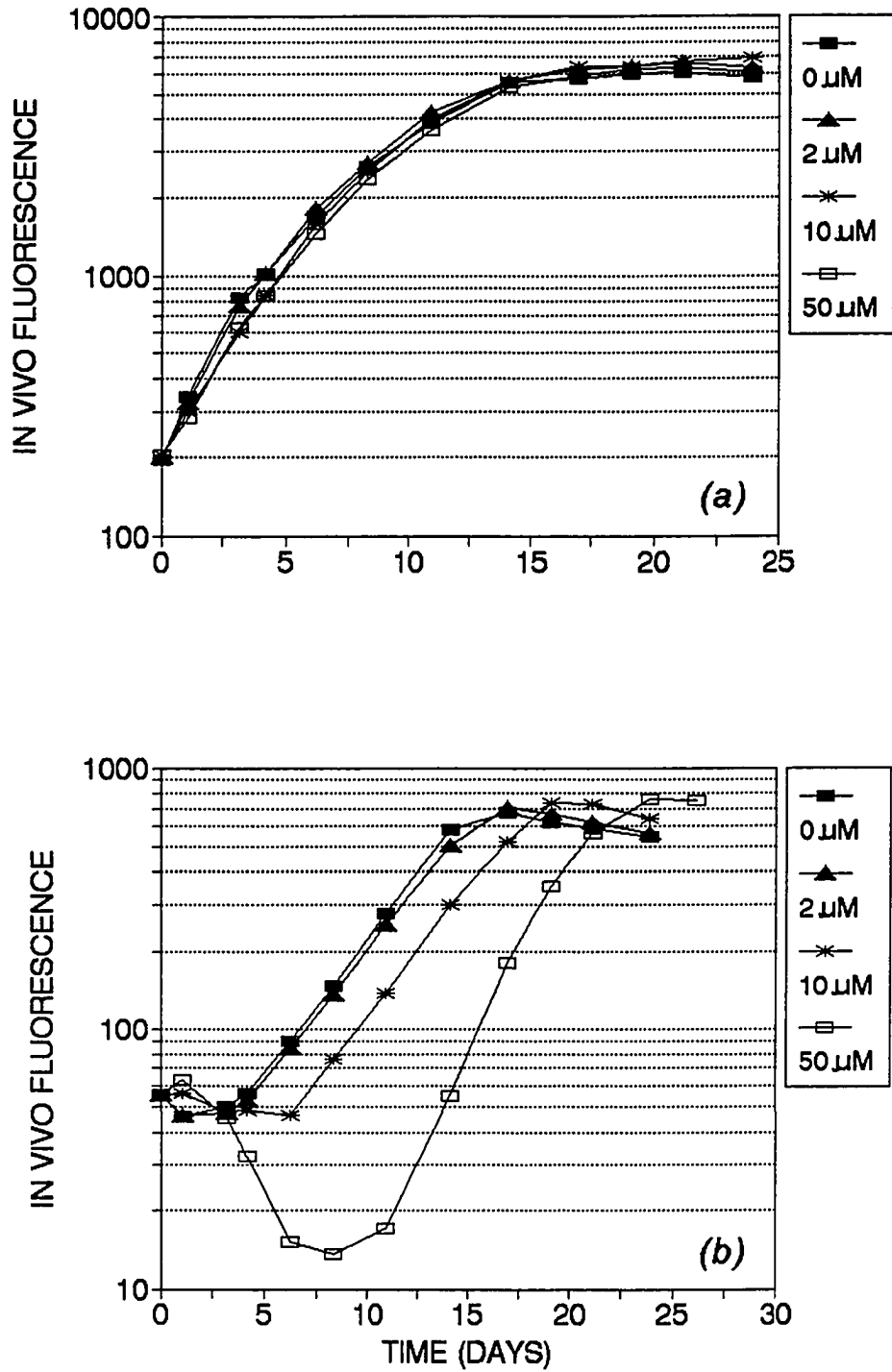


Fig. 3.2. Toxicity test of H_2O_2 on the growth curves of (a) *Skeletonema costatum* and (b) *Synechococcus* sp.

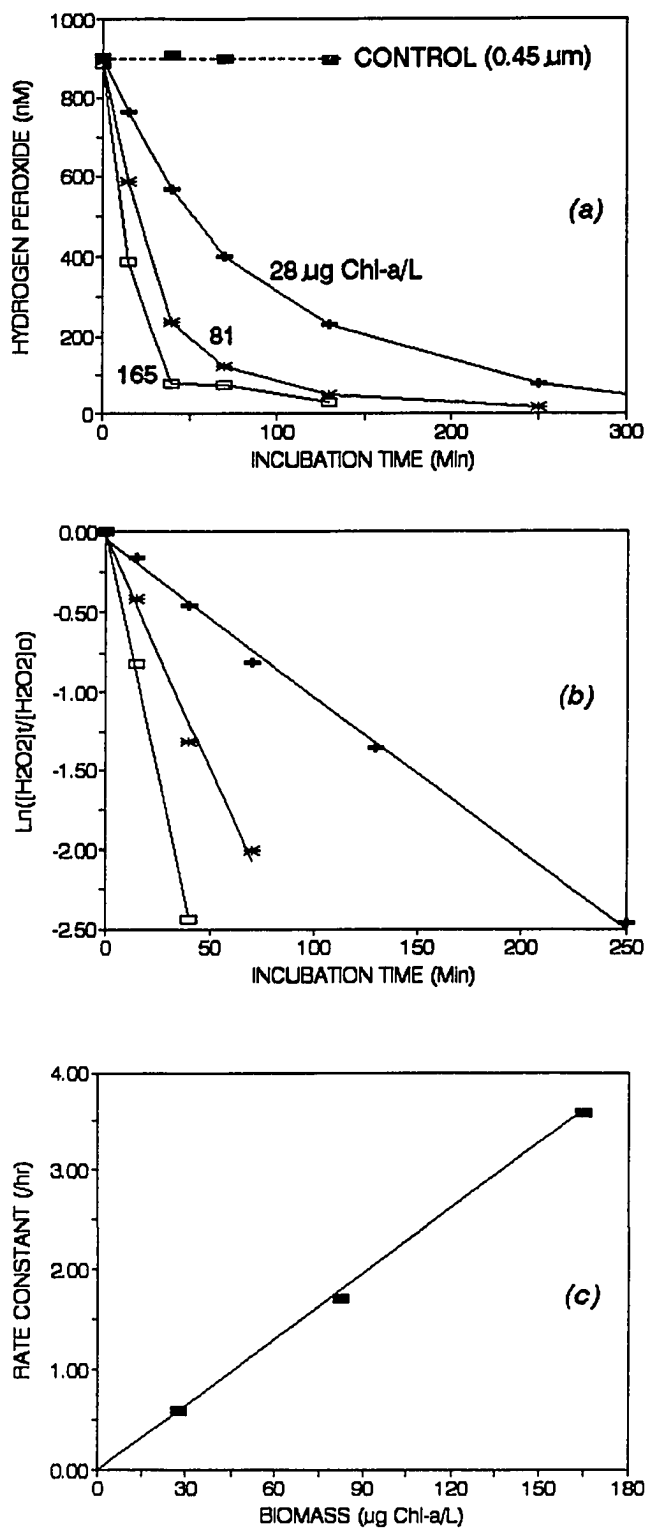


Fig. 3.3. Dark decomposition of H_2O_2 by *Skeletonema costatum*. (a) Kinetic curves. (b) Order of reaction. (c) Rate constants vs. chlorophyll-a concentration.

constants. The rate constants for group 1 were on the order of $10^{-2} \mu\text{g chl-a}\cdot\text{L}^{-1}\cdot\text{hr}^{-1}$, for group 2 on the order of $10^{-3} \mu\text{g chl-a}\cdot\text{L}^{-1}\cdot\text{hr}^{-1}$, and for group 3 on the order of $10^{-4} \mu\text{g chl-a}\cdot\text{L}^{-1}\cdot\text{hr}^{-1}$.

Group 1 : *Skeletonema costatum*, *Synechococcus* sp.

Among the tested species, *Skeletonema costatum* and *Synechococcus* sp. decomposed H_2O_2 most rapidly. The rate constant of *Skeletonema costatum* was $2.2\cdot 10^{-2} \mu\text{g chl-a}\cdot\text{L}^{-1}\cdot\text{hr}^{-1}$ and that of *Synechococcus* sp. was $2.4\cdot 10^{-2} \mu\text{g chl-a}\cdot\text{L}^{-1}\cdot\text{hr}^{-1}$. It took only 1.5 hours for *Synechococcus* sp. ($98 \mu\text{g chl-a}\cdot\text{L}^{-1}$) to reach a steady state from 900 nM H_2O_2 . The half-life of H_2O_2 decomposition was only about 30 minutes. H_2O_2 at the steady state remained at a very low concentration close to the detection limit. *Skeletonema costatum* ($165 \mu\text{g chl-a}\cdot\text{L}^{-1}$) required 1 - 1.5 hours to reach a steady state from 900 nM. The half-life of H_2O_2 decomposition was less than 30 minutes. The H_2O_2 concentration at the steady state also remained at a very low concentration with this species.

Group 2 : *Tetraselmis levis*, *Chaetoceros flexosus*, *Chaetoceros simplex*, *Isochrysis galbana*, *Thalassiosira oceanica*

Almost all of the species in this group retained some different characteristics from group 1, such as longer half-lives, higher levels of steady state H_2O_2 concentrations, and one order of magnitude smaller k values than those of group 1 (Table 3.1). The values of k ranged from $4.4\cdot 10^{-3}$ to $1.3\cdot 10^{-3}$

$\mu\text{g chl-a}\cdot\text{L}^{-1}\cdot\text{hr}^{-1}$. The effectiveness of these phytoplankton species in mediating the decomposition of H_2O_2 decreased in the order of *Tetraselmis levis* > *Chaetoceros flexosus* > *Chaetoceros simplex* > *Isochrysis galbana* > *Thalassiosira oceanica*.

Group 3 : *Amphidinium carterae*, *Pleurochrysis carterae*, *Dunaliella tertiolecta*.

The members in this group had some different characteristics from the previous groups. Examples of differences include much longer half-lives than any other group, high levels of steady state, and initial lag-phases. The initial lag-phase implies that the decomposition processes by the species in this group were slow and less effective. It is possible that phytoplankton species in this group have higher tolerance against toxicity of H_2O_2 . The decomposition rate constants were two orders of magnitude smaller than those in group 1. The sequence in the order of decreasing ability of decomposition of H_2O_2 is *Amphidinium carterae* > *Pleurochrysis carterae* > *Emiliana Huxleyi* > *Dunaliella tertiolecta*. *Dunaliella tertiolecta* was least able to decompose H_2O_2 among the examined species.

Different initial concentrations of H_2O_2 : These experiments were conducted at a constant cell concentration as a function of H_2O_2 concentration. H_2O_2 concentrations in the range of 900 nM to 200 nM were used as the initial

Table 3.1. Summary of the second order rate constants (k) for the H₂O₂ decomposition by 11 marine phytoplankton.

Species	k ($\mu\text{g chl-a}\cdot\text{L}^{-1}$) ⁻¹ ·hr ⁻¹
<i>Synechococcus</i> sp.	0.0242
<i>Skeletonema costatum</i>	0.0224
<i>Tetraselmis levis</i>	0.0044
<i>Chaetoceros flexosus</i>	0.0032
<i>Chaetoceros simplex</i>	0.0031
<i>Isochrysis galbana</i>	0.0017
<i>Thalassiosira oceanica</i>	0.0013
<i>Amphidinium carterae</i>	0.0009
<i>Pleurochrysis carterae</i>	0.0003
<i>Emiliana huxleyi</i>	0.0003
<i>Dunaliella tertiolecta</i>	0.0002

concentration. *Skeletonema costatum* and *Synechococcus* sp. were tested. Both species decomposed all levels of H_2O_2 rapidly (Fig. 3.4). A very low steady state concentration near the detection limit was observed within two hours. As the initial concentration was reduced, the initial slope of decomposition also decreased. However, regardless of H_2O_2 concentration, the decomposition rate constants were almost the same. Therefore, the half-lives were almost the same about 30 minutes, and were independent of the initial H_2O_2 concentration. Because the kinetics of the H_2O_2 decomposition by marine phytoplankton were independent of the initial concentration of H_2O_2 , the decomposition rate constants obtained in the lab could be applied to the various natural levels of H_2O_2 .

Kinetics of the H_2O_2 decomposition : The decomposition of H_2O_2 by marine phytoplankton appeared to follow first-order kinetics with respect to H_2O_2 concentration. In addition, the relationship between the rate constant and algal biomass proved to be linear. Therefore, the overall kinetic expression can be written as following :

$$-d[\text{H}_2\text{O}_2] \cdot dt^{-1} = k' \cdot [\text{H}_2\text{O}_2] = k \cdot [\text{biomass}] \cdot [\text{H}_2\text{O}_2],$$

where k' is the pseudo-first order rate constant and k is the second-order rate constant normalized to unit biomass ($\mu\text{g chl-a} \cdot \text{L}^{-1}$). k values for each species and half-lives of H_2O_2 are

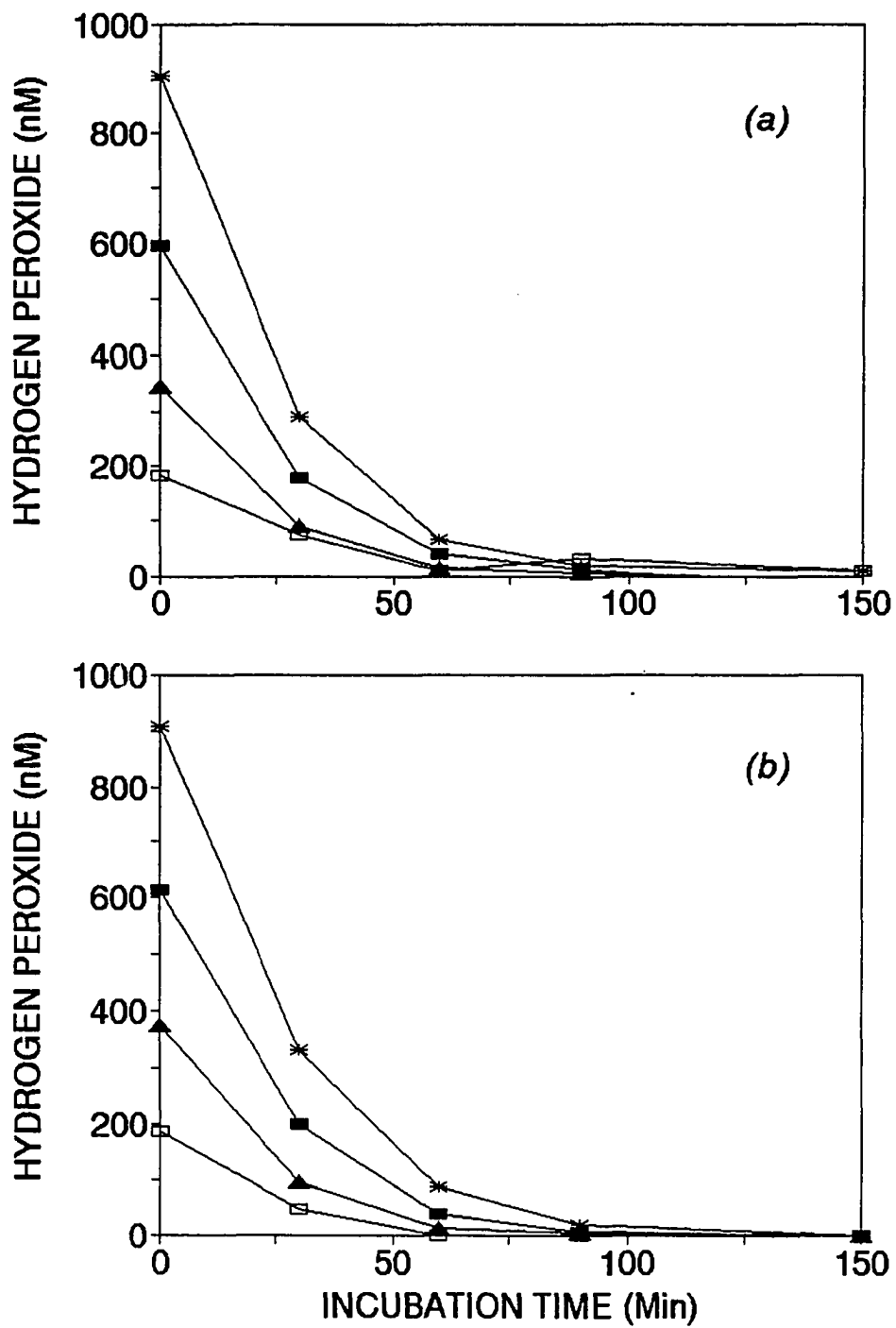


Fig. 3.4. Decomposition of H₂O₂ as a function of H₂O₂ concentration. (a) *Skeletonema costatum* (b) *Synechococcus sp.*

summarized in Table 3.1. k values for marine phytoplankton are compared with k values for 11 freshwater phytoplankton and two bacteria (Table 3.2). k values reported by Zepp *et al.*, (1987b) ranged from 10^{-3} to 10^{-4} order of magnitude, which are 2 - 3 times smaller than our k values. Marine phytoplankton can play as much of a role in seawater as freshwater phytoplankton play in freshwater systems. There are two available k values for bacteria, one a brackish species and the other a marine species (Cooper *et al.*, 1992). The decomposition rate constants for *Skeletonema costatum* in this study was about two-orders higher than bacterial species per cell. In this comparison, size differences have not been counted.

The half-lives of H_2O_2 in coastal and open ocean waters were estimated using a typical phytoplankton biomass in each water environment (Table 3.3). Using an average k value of 0.01, the estimated times required for the decomposition of H_2O_2 by marine phytoplankton in the coastal ocean and in open ocean water are in the range of a few hours to 18 hours over the representative range of chlorophyll-a concentrations (Table 3.3). The diel variations of H_2O_2 could then be explained by the activities of marine phytoplankton; They could decompose more than 50 % of the maximum H_2O_2 in the coastal ocean and about 20 % in the open ocean. These results indicate that marine phytoplankton play an important role in regulating the diel change and budget of H_2O_2 in the surface oceans by mediating its rate of decomposition.

Table 3.2. Comparison of k values of marine phytoplankton with k values from other studies.

		k VALUES
11 FRESHWATER PHYTOPLANKTON		$(\mu\text{g chl-a}\cdot\text{L}^{-1})^{-1}\cdot\text{hr}^{-1}$
	<i>Nostoc muscorum</i>	8.8×10^{-3}
	<i>Anabaena variabilis</i>	7.7×10^{-3}
	<i>Chlamydomonas reinhardtii</i>	1.8×10^{-4}
	<i>Chlamydomonas</i> sp.	1.0×10^{-4}
		(Zepp et al., 1987b)
1 FRESH, 1 MARINE BACTERIA		$\text{mL}\cdot\text{cells}^{-1}\cdot\text{min}^{-1}$
	<i>Enterobacter cloacae</i>	5.1×10^{-9}
	<i>Vibrio alginolyticus</i>	2.3×10^{-9}
		(Cooper et al., 1992)
11 MARINE PHYTOPLANKTON		$(\mu\text{g chl-a}\cdot\text{L}^{-1})^{-1}\cdot\text{hr}^{-1}$
	<i>Synechococcus</i> sp.	2.4×10^{-2}
	<i>Skeletonema costatum</i>	2.2×10^{-2}
	<i>Pluerochrysis carterae</i>	3.0×10^{-4}
	<i>Dunaliella tertiolecta</i>	2.0×10^{-4}
	<i>Skeletonema costatum</i>	$1.1 \times 10^{-7} \text{ mL}\cdot\text{cells}^{-1}\cdot\text{min}^{-1}$
		(This Study)

Table 3.3. Estimated time required for the decomposition of H_2O_2 by marine phytoplankton in coastal and open ocean with the specified ranges of chlorophyll-a concentrations ($\mu g \text{ chl-a} \cdot L^{-1}$).

	k=0.02		k=0.01		k=0.005	
	Coastal	Open	Coastal	Open	Coastal	Open
	$T_{(0.5)}$	$T_{(0.2)}$	$T_{(0.5)}$	$T_{(0.2)}$	$T_{(0.5)}$	$T_{(0.2)}$
Time	0.6- 3	2 - 9	1.2- 6	4 - 18	2.4-12	8 -36
chl-a	50 -10	5 - 1	50 -10	5 - 1	50 -10	5 - 1

(Time unit in hours)

3.3.2. Factors affecting the decomposition rates of H₂O₂

Instead of the previous experimental conditions (temperature: 23 °C, salinity: 30 ppt, dark incubation, growth phase: late-log phase, medium: f₁₂ grown), several different conditions were tested because the reaction kinetics can be affected by changes in the experimental conditions.

Effect of light intensity : *Skeletonema costatum* and *Dunaliella tertiolecta* were tested for the decomposition of H₂O₂ both in the dark and in the light. The light intensity was in the range of 0 to 289 μE·m²·s⁻¹. *Skeletonema costatum* and *Dunaliella tertiolecta* were selected because the former has one of the highest k values and the latter has the lowest k value. *Skeletonema costatum* (63 μg chl-a·L⁻¹) exposed to several light intensities resulted in no apparent changes in the decomposition rates (Fig. 3.5). Regardless of the light intensity, *Skeletonema costatum* decomposed H₂O₂ at the similar rate. The rate of H₂O₂ decomposition by *Dunaliella tertiolecta* slightly increased with increasing light intensity. However, it was not clear whether the increase was caused by the cells or some other factors such as photolysis. Thus, the decomposition of H₂O₂ by marine phytoplankton seemed to be light-independent. Marine phytoplankton may decompose H₂O₂ continuously during day and night, keeping down the level of H₂O₂ in seawater.

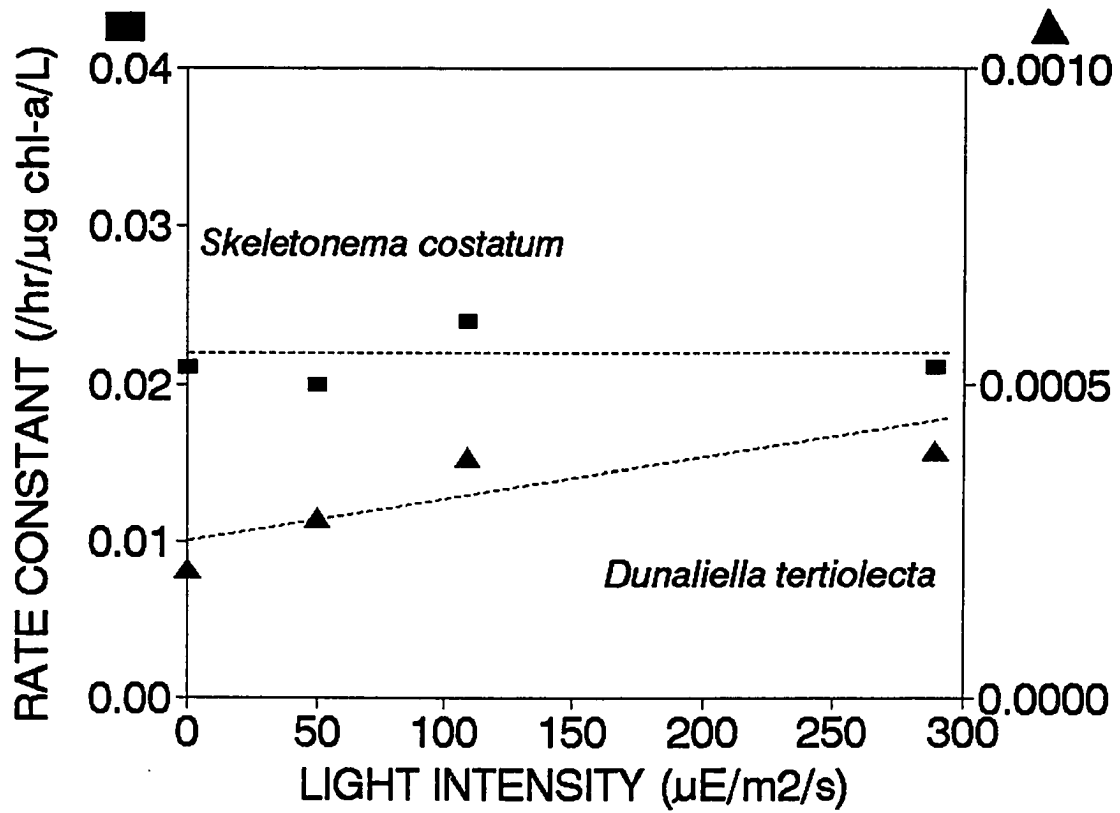


Fig. 3.5. Effect of varying light intensity on the rate constants of H_2O_2 decomposition by *Skeletonema costatum* and *Dunaliella tertiolecta*.

Effect of temperature : *Skeletonema costatum* and *Synechococcus* sp. were incubated at several water temperatures ranging from 5 °C to 33 °C to study temperature effects on the decomposition rates of H₂O₂. Temperature did not have much effect on the decomposition rates of H₂O₂ by *Skeletonema costatum* (Fig. 3.6). At 5 °C, the decomposition rate slightly decreased. However, between 13 °C and 33 °C, there was little change in decomposition rate constants. This implies that the decomposition process by *Skeletonema costatum* may not be temperature-dependent and may occur rapidly both in summer and winter. Thus, regardless of the seasons, the decomposition rate of H₂O₂ by *Skeletonema costatum* can be estimated by biomass data. *Synechococcus* sp. displayed a different pattern from *Skeletonema costatum*. The decomposition rates were significantly affected by temperature. As temperature decreased from 33 °C to 5 °C the rate constants decreased considerably. *Synechococcus* sp. has a narrow optimum range of temperature for the growth (20 - 25 °C) whereas *Skeletonema costatum* has a broader range of temperature (12 - 30 °C). *Synechococcus* sp. placed in cold water, such as upwelling water, may not function in the decomposition of H₂O₂. The decomposition rate constants of *Synechococcus* sp. may be seasonally variable.

Effect of Salinity : *Skeletonema costatum* was examined for the effect of salinity on its capability of decomposing H₂O₂. Cells were grown at 12, 25, 30 and 35 ppt because

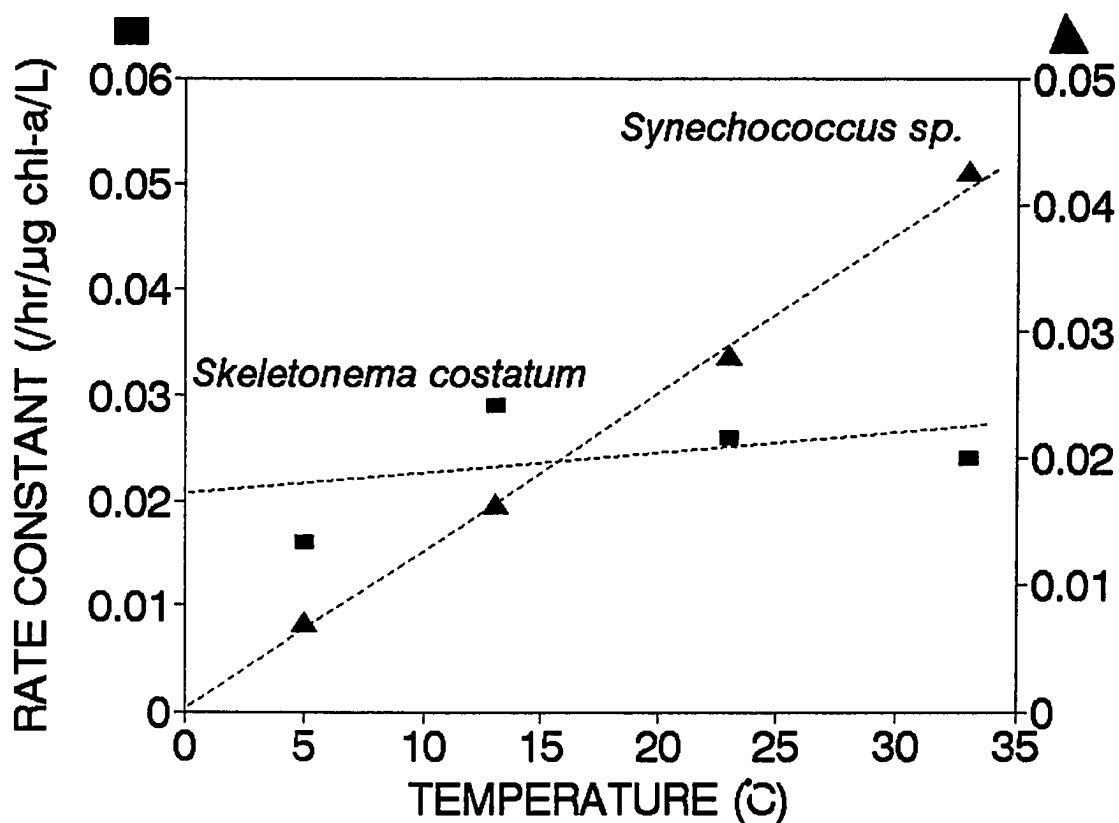


Fig. 3.6. Effect of varying temperature on the rate constants of H_2O_2 decomposition by *Skeletonema costatum* and *Synechococcus sp.*

Skeletonema costatum has the optimum salinity range for growth between 12 and 32 ppt. Fig. 3.7 shows that salinity between 25 and 35 ppt did not affect significantly the decomposition of H_2O_2 . However, a considerably higher rate constant was observed at the salinity of 12 ppt. Probably saltwater species placed in low salinity environments, such as high runoff areas or during heavy rains, may decompose H_2O_2 more effectively. Because rainwater contains a high level of H_2O_2 (Kok, 1980; Cooper and Lean, 1989), *Skeletonema costatum* may have to decompose more H_2O_2 during a rainy season. *Skeletonema costatum* in estuaries could potentially decompose H_2O_2 faster than in open oceans of high salinities. It may be that phytoplankton cells in low salinity have more dilated cell walls and thus be more vulnerable to the permeability of H_2O_2 .

Effect of nutrient concentration : To study the effect of nutrients on the decomposition of H_2O_2 *Skeletonema costatum* and *Synechococcus* sp. cultures were grown in different nutrient strengths; $f_{/2}$, $f_{/20}$ and $f_{/100}$. The initial nitrate concentration in each medium was 883 μM , 88.3 μM and 17.7 μM , and phosphate was 36.3 μM , 3.63 μM and 0.73 μM , respectively.

Nutrient levels had a dramatic effect on the decomposition rate constant for *Skeletonema costatum* (Fig. 3.8); the cells grown in a lower nutrient concentration decomposed H_2O_2 much faster and showed a higher k value. *Skeletonema costatum* confined in a nutrient-limited layer above a thermocline can play an important role in removing H_2O_2

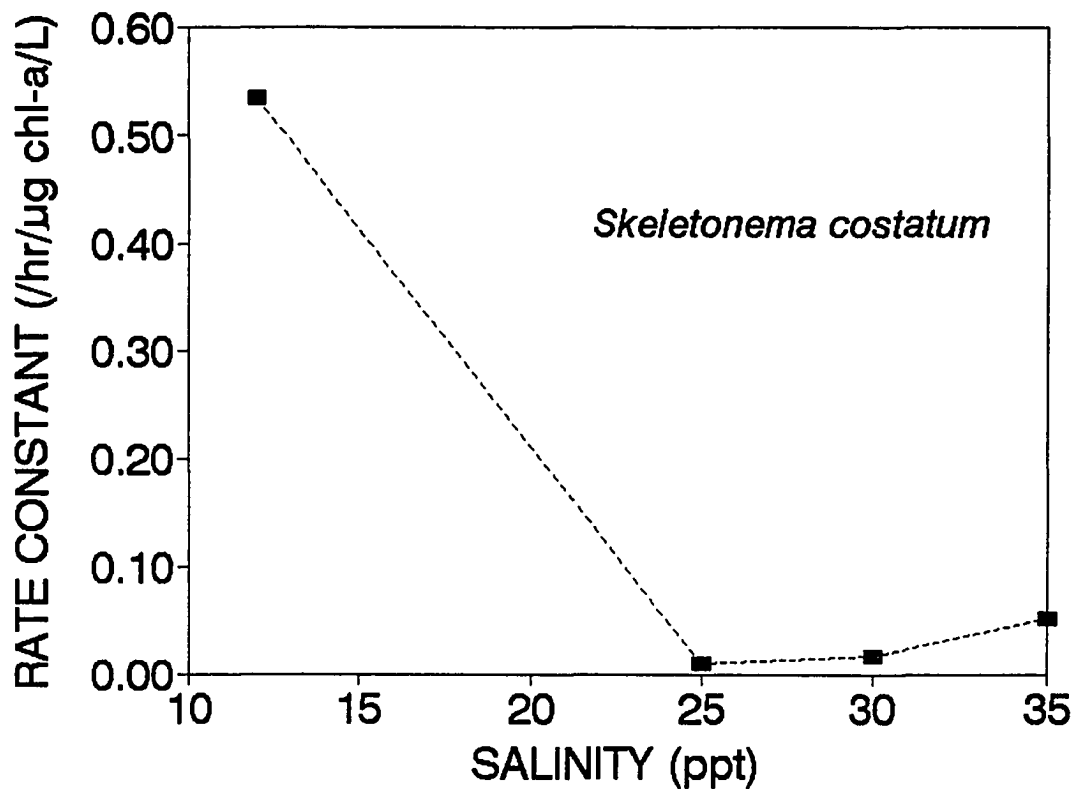


Fig. 3.7. Effect of varying salinity on the rate constants of H_2O_2 decomposition by *Skeletonema costatum*.

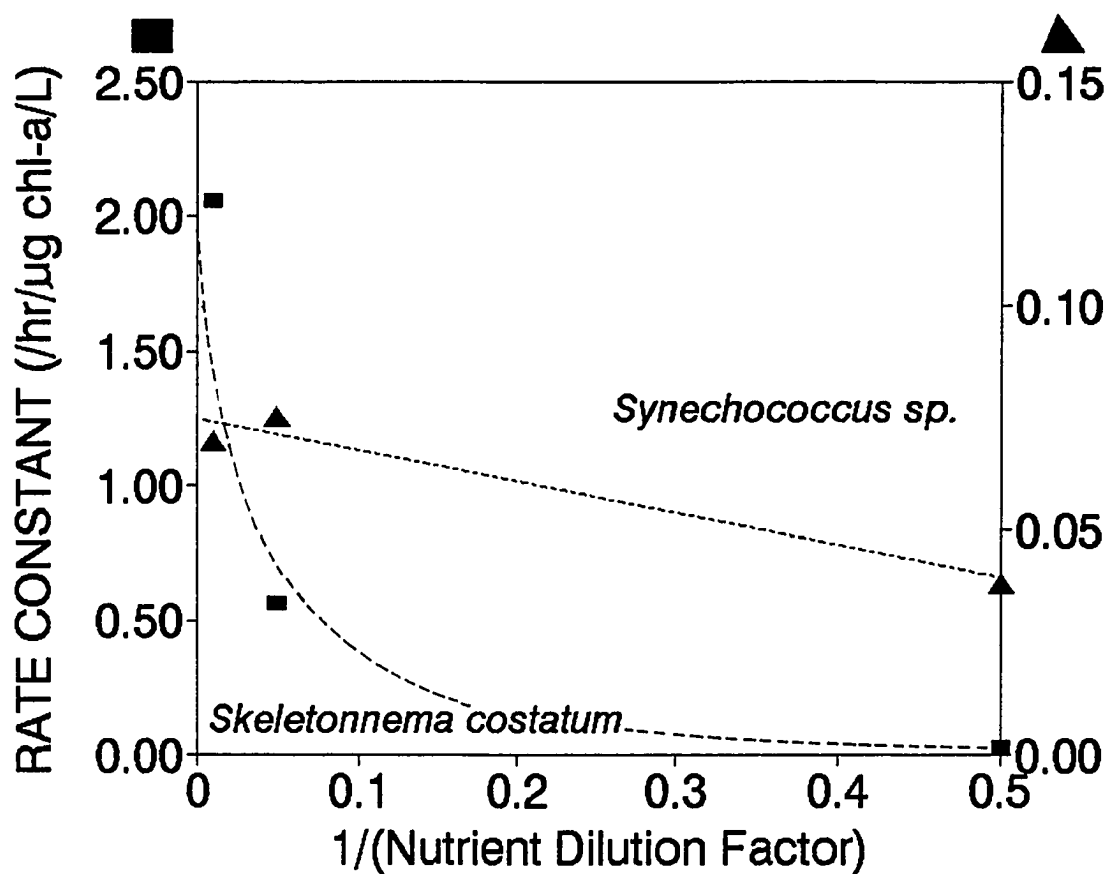


Fig. 3.8. Effect of nutrient concentration on the rate constants of H_2O_2 decomposition by *Skeletonema costatum* and *Synechococcus sp.*

from coastal or shelf waters. Strong sunlight during summer, which forms a thermocline, can also produce high concentration of H_2O_2 through photochemical reaction with dissolved organics, especially in coastal ocean waters (Zika et al., 1985a). The high concentration of H_2O_2 produced by strong sunlight above the thermocline can be effectively removed by the nutrient-starved *Skeletonema costatum*. Even if they are nutrient-starved, however, *Skeletonema costatum* in oligotrophic regions cannot play an important role because of the low abundance of the species.

Meanwhile, the decomposition of H_2O_2 by *Synechococcus* sp. was less affected by the nutrient limitation than *Skeletonema costatum*. The decomposition rate constants did not show much difference between samples. The cells grown under nutrient-depleted conditions decomposed slightly more H_2O_2 per biomass than ones in nutrient-rich conditions. *Synechococcus* sp., cyanobacteria, is an oceanic species thriving in a very low nutrient condition. They have developed some strategies, such as small cells, and low V_{max} and k_s to maximize nutrient uptake in low nutrient waters. Therefore, *Synechococcus* sp. may decompose H_2O_2 similarly even under the low nutrient conditions.

Effect of growth phase : *Skeletonema costatum* and *Synechococcus* sp. at different growth phases were tested to determine the effect of growth phase on the decomposition rates of H_2O_2 . Special attention was focused on the senescence

phase in which nutrients become limited. Cells were harvested at late log, early senescence, mid senescence and late senescence phases. The normalized decomposition rate constant for *Skeletonema costatum* showed a dramatic increase at the late senescence phase where nutrients are limited (Fig. 3.9a). In the late senescence phase the ratio of phaeopigment-a vs. chlorophyll-a increased. It is uncertain whether phaeopigment-a contributes to the decomposition of H_2O_2 . *Synechococcus* sp. showed a much different result (Fig. 3.9b). The decomposition rates did not vary much between growth phases. There was no significant trend in the change of the rate constants. Even in the nutrient depleted condition, *Synechococcus* sp. did not increase their decomposition rate constants. This is in agreement with the result of the experiment with the cells grown in the different nutrient strengths.

The results of the experiments on the effects of the nutrient conditions and the growth phase showed that the nutrient-depleted condition stimulated *Skeletonema costatum*, a common coastal species, to increase the decomposition rate, but did not stimulate *Synechococcus* sp. a common ocean species. This suggests that during summer *Skeletonema costatum* may be an important species in removing excess H_2O_2 under strong sunlight.

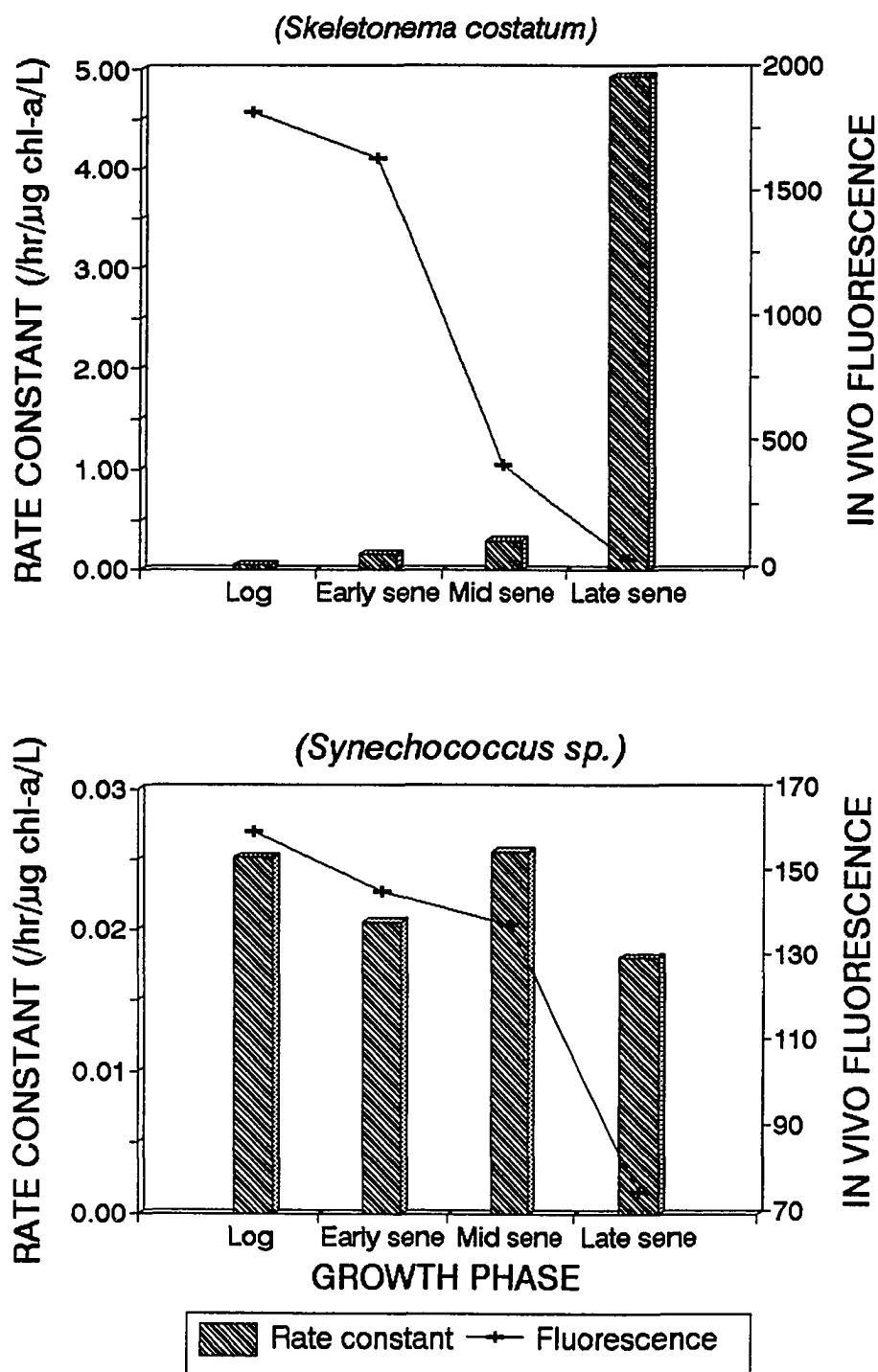


Fig. 3.9. Effect of growth phase on the rate constants of H_2O_2 decomposition by (a) *Skeletonema costatum* (b) *Synechococcus sp.*

3.4. SUMMARY

1. All of 11 marine phytoplankton in cultures decomposed H_2O_2 at various rates. *Skeletonema costatum*, a widely distributed coastal species and *Synechococcus* sp., a widely distributed oceanic species had the highest decomposition rates of H_2O_2 . These two species can play significant roles in removing H_2O_2 from a broad range of surface oceans.

2. The decomposition of H_2O_2 by marine phytoplankton could be described by pseudo-first order kinetics. The rate of H_2O_2 decomposition was proportional to biomass and concentration of H_2O_2 . The reaction rate constants ranged from 10^{-4} to 10^{-2} ($\mu\text{g chl-a}\cdot\text{L}^{-1}$) $\cdot\text{hr}^{-1}$. The estimation of half-lives of H_2O_2 in open oceans and in coastal oceans using the rate constants of marine phytoplankton could explain a diel variation of H_2O_2 in the surface oceans.

3. The decomposition of H_2O_2 by marine phytoplankton was light-independent. Even in the presence of light, the decomposition of H_2O_2 occurred at a similar rate. Temperature and salinity effects on the k values appeared to be related to the optimum ranges of temperature and salinity for the growth of each species. Nutrient effects on the k values were remarkable in coastal species but not in oligotrophic species. The decomposition of H_2O_2 by *Skeletonema costatum* was dependent on the changes of salinity and nutrients and independent of

temperature changes. The decomposition of H_2O_2 by *Synechococcus* sp. was dependent on temperature changes and independent of the changes in salinity and nutrients.

Chapter 4

PRODUCTION AND DECOMPOSITION OF H₂O₂ IN OPEN OCEAN AND COASTAL WATERS

4.1. INTRODUCTION

Hydrogen peroxide (H_2O_2) may have significant effects on the biogeochemical processes in the oceans. Chemically, it is well known that, under certain conditions, H_2O_2 can act as an oxidizing and/or a reducing agent (Moffett and Zafiriu, 1990). Recent studies also indicate that the oxidation-reduction reactions between H_2O_2 and some trace metals in the sea may have profound effects on the speciation of these metals. For example, a number of studies have suggested that the speciation of iron in seawater may be affected by the oxidation of Fe^{2+} by H_2O_2 (Waite and Morel, 1984; Moffett and Zika, 1987a; Millero and Sotolongo, 1989) while the speciation of copper may be affected by its reduction of Cu^{2+} to Cu^+ and oxidation of Cu^+ to Cu^{2+} (Moffett and Zika, 1983, 1987b; Sharma and Millero, 1989). The speciation of these metals have been shown to be important in determining their biogeochemistry and their availability and/or toxicity to marine organisms (Sunda, 1989). Biologically, above certain concentration, it has been shown that H_2O_2 can inhibit the growth of many marine organisms (Marler and Van Baalen, 1965) and damage cell membranes via lipid peroxidation (Borg *et al.*, 1981; Dixit *et al.*, 1982).

H_2O_2 exists rather ubiquitously in the surface waters of the hydrosphere (Zika *et al.*, 1982; Cooper and Zika, 1983; Draper and Crosby, 1983a; Zika *et al.*, 1985a; Cooper *et al.*, 1988; Johnson *et al.*, 1989). In the ocean, typical profiles show the maximum concentration of H_2O_2 at the surface. This

maximum concentration varies from 100 to 200 nM in open oceans (Moffett and Zika, 1983; Zika, 1985a). However, in coastal waters, the concentration tends to be higher and more variable. The concentration of H_2O_2 decreases quasi-exponentially with depth to the detection limit, usually of about 5 nM, at the base of the euphotic zone (Zika, 1984; Zika *et al.*, 1985b; Johnson *et al.*, 1989). Diurnal variation of H_2O_2 occurs in surface waters; the maximum concentration occurs in the late afternoon and the minimum concentration occurs in the early morning (Palenik and Morel, 1988; Szymczak and Waite, 1988).

In comparison with the well-known distribution of H_2O_2 , the biochemical cycling of H_2O_2 in the sea is not well understood and poorly quantified. Photo-oxidation of dissolved organic matter is widely accepted as the major source of H_2O_2 in the surface oceans (Zika, 1981; Cooper and Zika, 1983; Mopper and Zika, 1987). However, there is some evidence suggesting that there may be a contribution from the biologically-mediated production of H_2O_2 (Stevens *et al.*, 1973; Patterson and Myers, 1973; Zepp *et al.*, 1987b; Palenik *et al.*, 1987; Palenik and Morel, 1988). The biological production of H_2O_2 has been demonstrated in the field only at one station in the Sargasso Sea (Palenik and Morel, 1988). In addition, studies on the biological production of H_2O_2 may be complicated by the biological decomposition of H_2O_2 . The decomposition of H_2O_2 is believed to be controlled by the enzymes, peroxidase and catalase, which are commonly found in plants (Zepp *et al.*,

1987b; Moffett and Zafiriou, 1990). The rates of decomposition of H_2O_2 by several species of fresh-water algae have been determined (Zepp et al., 1987b). Little work has been done with specific species of marine phytoplankton. Moffett and Zafiriou (1990) reported the only measurements of the dark decomposition of H_2O_2 . Their data from Vineyard Sound coastal waters indicate a decomposition rate of about 6 nM/hr.

Therefore, more field studies on biological production and decomposition are necessary to understand biological roles in the H_2O_2 budget of the oceans. This study also discussed how environmental factors, such as inorganic nitrogen, and species composition of phytoplankton can be related to the biological roles in different sub-marine environments. We have measured hydrography, nutrients, the biomass and composition of phytoplankton, the rates of dark production and/or decomposition of H_2O_2 at three stations in a transect from the oligotrophic Sargasso Sea to the mesotrophic inner shelf of the South Atlantic Bight in Long Bay.

4.2. THE STUDY AREA AND SAMPLING

The shelf off the southeastern coast of the United States is commonly known as the South Atlantic Bight. It extends from Cape Hatteras, North Carolina, at about 35 °N to West Palm Beach, Florida, at 27 °N. The waters in the South Atlantic Bight may be subdivided into three zones: the inner shelf, the mid-shelf and the outer shelf. The turbid inner shelf is usually confined within the 20-m isobath. The Gulf Stream lies just seaward of the outer shelf and represents the eastern boundary of the Bight.

Twenty three stations were located in a transect from the Sargasso Sea to around the axis of the Gulf Stream and then from the Gulf Stream, normal to the coast, across the South Atlantic Bight to Long Bay, South Carolina between November 3 and 6, 1990 on board R/V Endeavor during Cruise EN-220 (Fig. 4.1). Station 1 was located in the Sargasso Sea close to the eastern edge of the Gulf Stream. Stations 2 and 3 were in the Gulf Stream. Stations 4 to 7 were located at the upper slope and the outer shelf. Stations 8 to 15 and 16 to 23 were in the mid-shelf and inner shelf respectively. Station 23 was within sight of land. Thus, a range of oceanographic conditions were covered in this transect from the clear and oligotrophic water of the Sargasso Sea to the turbid and mesotrophic water of the inner shelf. Discrete water samples were collected with depth at each station with 5-L Niskin bottles mounted on a rosette sampler for the determination of

salinity, phosphate, nitrate, silicate, nitrite and ammonia. Surface water samples from these stations were also analyzed for concentrations of H_2O_2 . The distributions of temperature, salinity and *in vivo* fluorescence were monitored with a Seabird CTD mounted on the rosette sampler and equipped with the proper sensors. The rates of dark biological production or decomposition of H_2O_2 , concentrations of chlorophyll-a and phaeopigment-a, and cell counts and species of phytoplankton were determined in discrete water samples collected at various depths at stations 1, 4 and 23. The variation of light intensity with depth at these stations were also monitored.

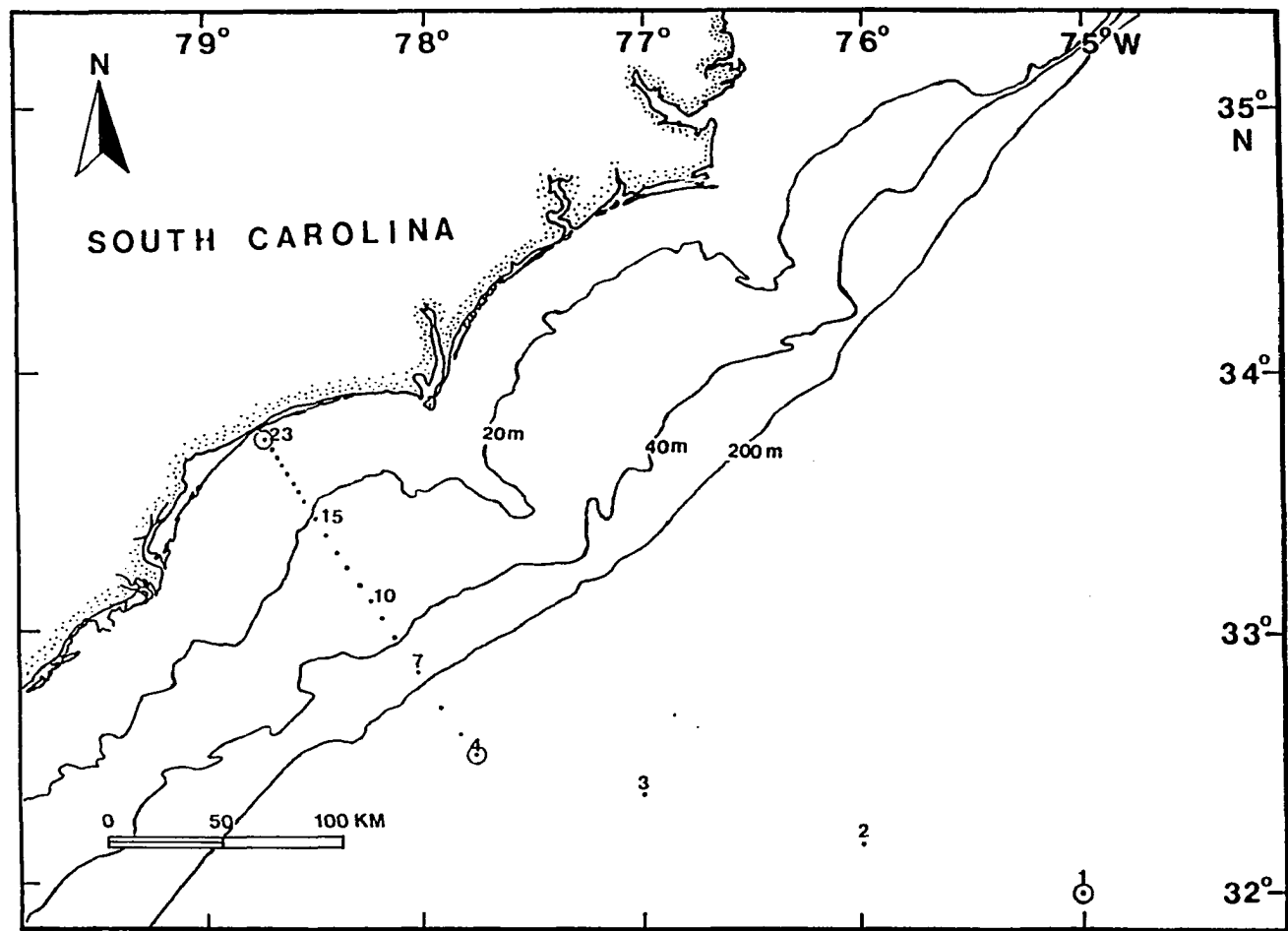


Fig. 4.1. The map showing the locations of 23 stations studied during EN-220 cruise, Nov. 3 - 6, 1990. Stations 1,4, and 23 were selected for dark incubation experiments.

4.3. METHODS AND MATERIALS

Sample preparation and incubation : The determination of the rate of dark production/decomposition of H_2O_2 was started with the measurement of the initial concentration of H_2O_2 in each sample immediately after the collection of water samples. Incubation was performed in the dark at 20 to 22°C using 250 mL HDPE (High Density Polyethylene) bottles. Water samples were incubated in the bottles to distinguish decay losses of H_2O_2 from diffusive(mixing) losses (Cooper *et al.*, 1989). Changes in the concentration of H_2O_2 in these bottles were monitored for up to 30 hours. During the incubation period, no nutrient additions into the samples were made. All glassware and the incubation bottles were cleaned beforehand by soaking overnight successively with distilled deionized water, 6-N hydrochloric acid and distilled deionized water to minimize any contamination of trace metals.

Determination of H_2O_2 : Before the determination of H_2O_2 , samples were filtered through a 0.45- μ m membrane filter. The concentration of H_2O_2 in a 4-mL filtrate was determined with a Perkin Elmer model 650-10S fluorescence spectrophotometer by using the scopoletin horseradish peroxidase method (Andreae, 1955; Perschke and Broda, 1961; Kieber and Helz, 1986; Holm *et al.*, 1987). A calibration curve was constructed for each sample by the internal standard addition method. The detection limit of the method was about 5 nM. Doubly

distilled deionized water was used for preparing all reagents and standard solutions. HRP (Horseradish Peroxidase) and H_2O_2 working standard solutions were prepared freshly each day. All the analyses were carried out on board ship.

Determination of pigments : Samples for the determination of chlorophyll-a and phaeophytin-a were filtered through a Nucleopore filter on board ship. The filters were kept frozen at $-5\text{ }^\circ\text{C}$ and returned to the laboratory for analyses. The pigments were extracted from the filters and determined fluorometrically with a Turner Designs Model 10-000R Fluorometer within a month of sample-collection. Concentrations of chlorophyll-a and phaeophytin-a were calculated using the method of Strickland and Parsons (1972).

Other determinations : To calculate light extinction coefficient, underwater light intensity was measured with a LI-COR LI-193SB underwater spherical quantum sensor at stations, 1 and 23, during the day time. Light measurements, for scheduled stations 3 and 4, were canceled due to a strong Gulf stream current. Salinity in discrete water samples was determined with a Minisal 2100 salinometer. Samples for the determination of phosphate, nitrate and silicate were stored frozen and returned to the laboratory for analyses. These nutrients were determined manually by the methods of Strickland and Parsons (1972). The analyses were completed within a week of the termination of the cruise.

Data treatment : To explain the production of H_2O_2 which appears to be described by pseudo-first order kinetics (chapter 2);

$$\text{Rate} = d[H_2O_2] \cdot dt^{-1} = k \cdot (P_{\max} - [H_2O_2])$$

The following hyperbolic equation was fitted to the observed time course of change of the concentration of H_2O_2 ;

$$P_t = (P_o - P_{\max}) \cdot e^{-kt} + P_{\max}$$

in which P_o is $[H_2O_2]$ at time 0, P_t is $[H_2O_2]$ at time t , P_{\max} is $[H_2O_2]$ at a steady state, t is time in hour, k is a pseudo-first order rate constant for production of H_2O_2 (' k_p ') in the unit of hr^{-1} . P_{\max} was obtained by averaging two to three data points at a steady state section of the curve. The above equation was rearranged to get k ;

$$k = t^{-1} \cdot \ln\{(P_{\max} - P_o) \cdot (P_{\max} - P_t)^{-1}\}$$

Decomposition of H_2O_2 can be described by pseudo-first order kinetics as well (Zepp *et al.*, 1987b; Cooper *et al.*, 1989; Cooper and Zepp, 1990; Moffett and Zafirou, 1990; Cooper *et al.*, 1992)

$$[H_2O_2]_t = [H_2O_2]_o \cdot e^{-kt}$$

$$k = -t^{-1} \cdot \ln([H_2O_2]_0 \cdot [H_2O_2]_t^{-1})$$

in which $[H_2O_2]_0$ is H_2O_2 concentration at time 0 and $[H_2O_2]_t$ is H_2O_2 concentration at time t . k is a pseudo-first order decomposition rate constant (' k_d ').

4.4. RESULTS AND DISCUSSION

4.4.1. Hydrography and H₂O₂ distributions

Hydrography : CTD data obtained from 23 stations on the cruise track are plotted to show surface distributions of salinity, temperature, density and *in vivo* fluorescence (Fig. 4.2a-d). Salinity decreased quickly from 36.75 psu to 34.30 psu in the inner shelf area towards the coast. Presumably, the low salinity was due to either freshwater discharge from nearby rivers (Atkinson *et al.*, 1983) or some intrusion of low salinity subsurface Gulf stream water on the shelf along the southeast U.S. coast (Stefansson *et al.*, 1971). From the edge of the outer shelf, temperature dropped rapidly toward the inner shelf showing the minimum (20 °C) at the coast. At the surface of the inner shelf relatively high densities between 25 and 25.5 kg·m⁻³ were observed. The highest density and the lowest temperature and salinity near the coast could be a result of the intrusion of cold subsurface Gulf stream water.

In vivo fluorescence, as an index of phytoplankton biomass, increased greatly at the coastal region, about 20 to 30 times greater at stations 22 and 23 than at stations 1 and 2. The cell numbers were 25 times higher at the coast than at station 1. Biomass increase on a shelf caused by the intrusion of nutrient-rich bottom water has been observed in a near coast water by Dunstan and Atkinson (1975).

Surface distributions of nutrients (Fig. 4.2e-g) also

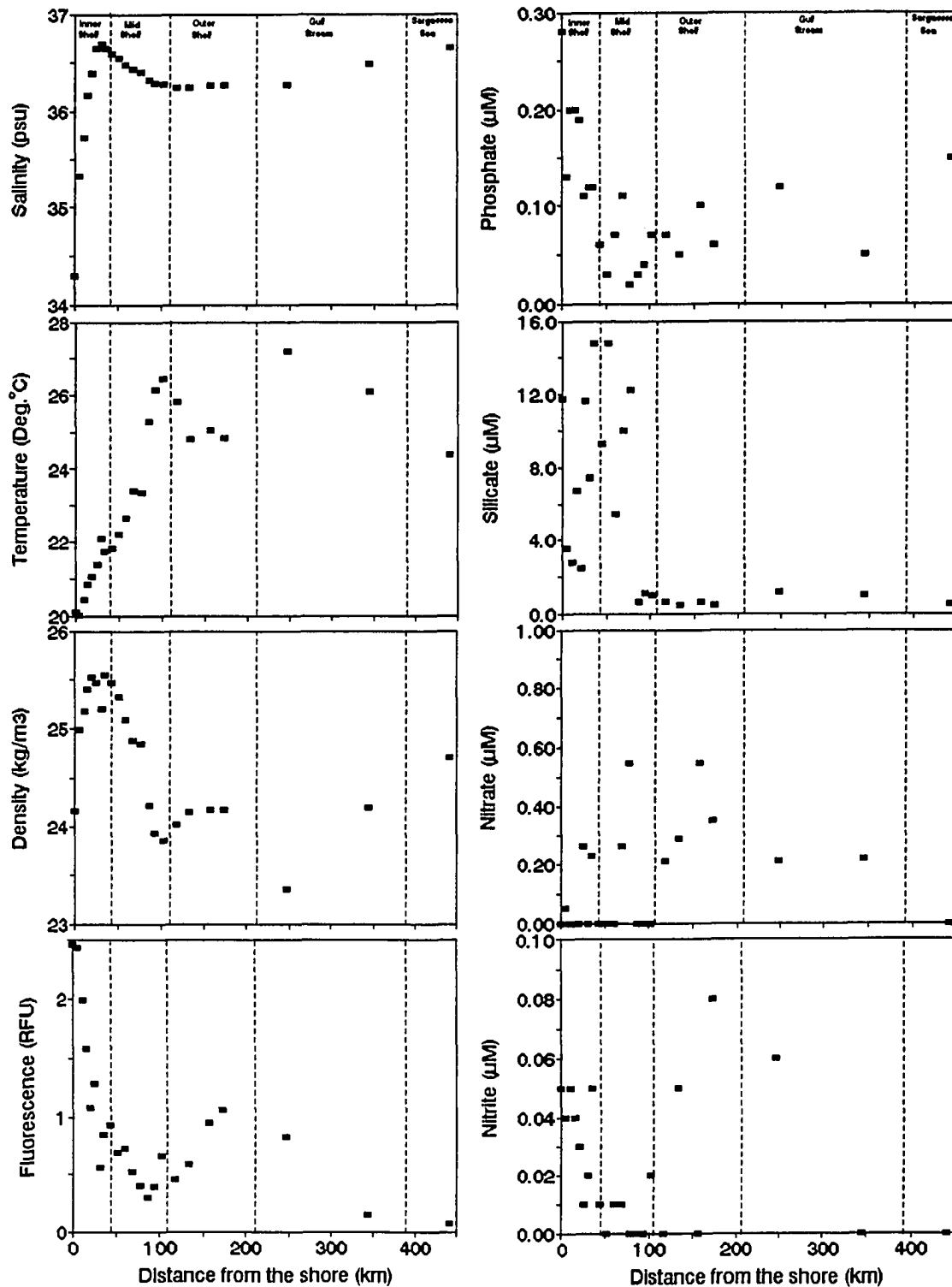


Fig. 4.2. Surface distributions of (a) salinity, (b) temperature, (c) density, (d) *in vivo* fluorescence, (e) phosphate, (f) silicate, (g) nitrate and (h) nitrite.

reflected a potential intrusion of nutrient-rich deep water. Phosphate, silicate and nitrite increased starting from the edge of the shelf break (100 km off-shore) toward the coast. Surface distribution of nitrate, however, was different; Elevated concentrations of nitrate in the outer shelf decreased towards the inner shelf, which is probably a result of the active consumption of the nitrate by phytoplankton in bloom at the shore. However, the low concentration of nitrate accompanied by low biomass found in the oligotrophic stations was an indication of nutrient depletion.

Vertical distributions of hydrographic factors of the selected three stations were plotted (Fig. 4.3). Hydrography of these stations were markedly different from each other. Station 1 had a seasonally stratified water column with the thermocline around 60 to 80 m depth. The density gradient at the pycnocline was rather strong compared to other stations (Table 4.1). The layer above the thermocline appeared to be well mixed. The fluorescence maximum was found with the thermocline and the chlorophyll maximum was about $0.28 \mu\text{g}\cdot\text{L}^{-1}$. Nutrients in the mixed layer hovered around their detection limits. Particularly, nitrate and nitrite were depleted severely compared to phosphate (N:P ratio was about 1) because the average N:P ratio of the sea is 15. The water of this station was clear with an extinction coefficient of 0.04 m^{-1} . Station 4, on the top of the nutrient-rich slope water, showed a shallow mixed layer and a thermocline between 25 m and 100 m. A remarkably high level of nutrients were reaching to the

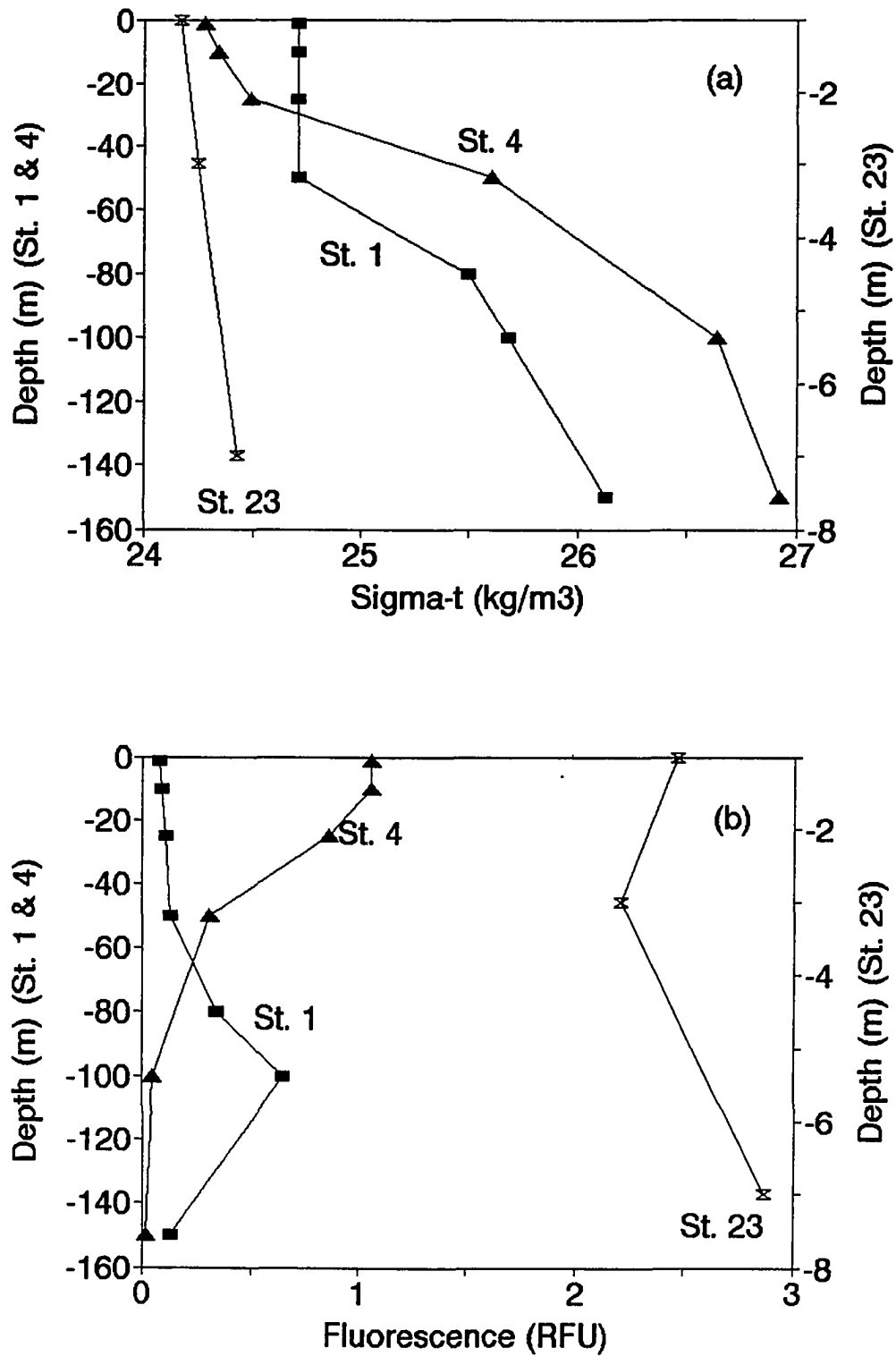


Fig. 4.3. Depth profiles of (a) density and (b) *in vivo* fluorescence at the three selected stations

Table 4.1. Summary of physical parameters at stations 1, 4 and 23.

	St.1	St.4	St.23
20 °C depth	140 m	50 m	0 - 7 m
Sigma-t*	25.40-24.80	25.40-25.18	24.25-24.38
Thermocline depth	70 m	40 m	.
Thermocline thickness	60 m	30-50 m	.
Maximum stability (m ⁻¹) ⁺	19.45	10.95	13.0
Extinction coeffi.(m ⁻¹) [§]	0.04		0.20

*: Density differences at the pycnocline.

+: Density gradient at the pycnocline was calculated by using $d\sigma_t \cdot dz^{-1} \cdot 100$.

§: Extinction coefficient of light was calculated by using $I=I_0 \cdot e^{-zk}$

near surface (20-30 m). The fluorescence maximum was observed above the thermocline. Station 23, the coastal station, had no thermocline. The water column was well mixed at this time. Phosphate and nitrate were low through the water column, but silicate was not. The water of this station was turbid with an extinction coefficient of 0.2 m^{-1} .

Distribution of H_2O_2 : Surface concentrations of H_2O_2 were measured at the 23 stations (Fig. 4.4). Station 1 showed 122 nM H_2O_2 at 6-7 p.m. on a calm day. At night the concentration of H_2O_2 decreased quickly to 40 nM by the time of arrival at station 2 in the early morning. As the sunlight irradiated the surface water, the concentration started to increase (stn. 3 and 4), which was the indication of a diurnal variation of H_2O_2 . However, some stations (5, 6 and 7) deviated from the diel pattern probably due to a geographical variation over the broad cruise track and local weather conditions. It was slightly rainy at station 3 and 4, and windy at station 5. Concentrations of H_2O_2 at stations, 6, 7 and 8, measured at night between 7 p.m. and 10 p.m. were high for unknown reasons. At night H_2O_2 concentrations in the surface waters of the outer and inner shelves decreased continually and never recorded more than 80 nM. An average concentration of H_2O_2 on this cruise track was about 80 nM, which is comparable with other reported values (Table 1.1). The surface concentration of H_2O_2 can be affected by several factors such as amounts of organics and trace metals in water mass, diurnal variation,

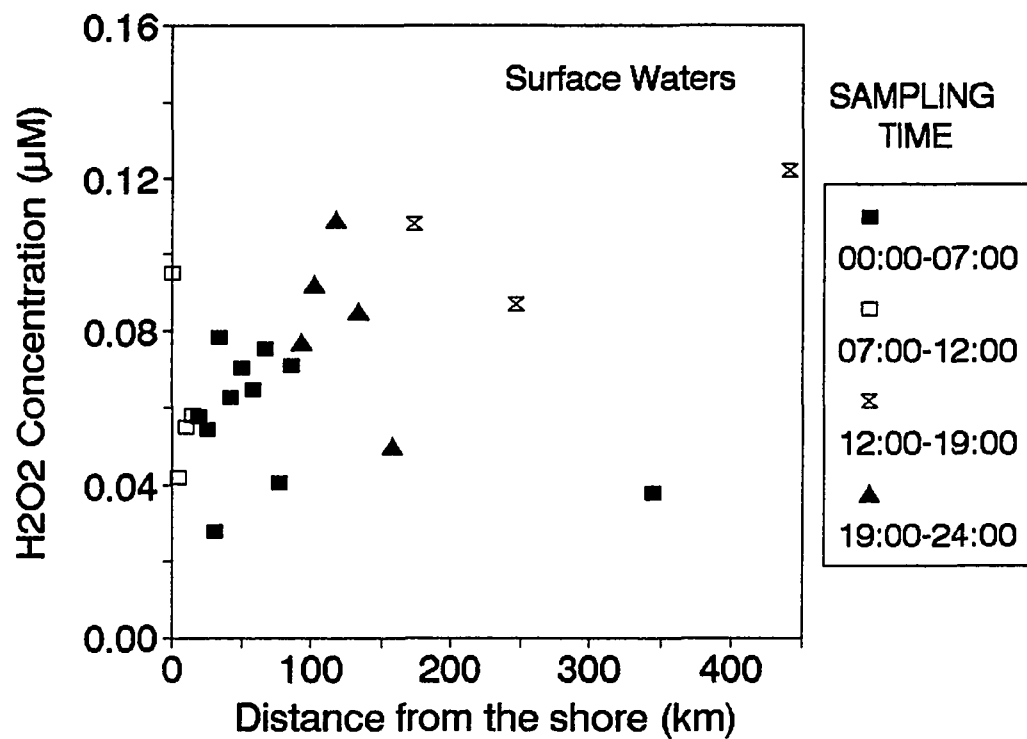


Fig. 4.4. Surface distributions of H₂O₂ of the 23 stations.

biological production and decomposition of H_2O_2 , and vertical mixing in mixed layers (Zika *et al.*, 1985a; Palenik and Morel, 1988; Cooper and Lean, 1989; Johnson *et al.*, 1989).

Vertical profiles of H_2O_2 were measured at 3 stations (Fig. 4.5). In agreement with other studies (Zika *et al.*, 1985a; Johnson *et al.*, 1989; Palenik and Morel, 1988), all profiles exhibited maximum concentrations at the surface and then exponential decreases with depth. These profiles were fitted into an equation, $[H_2O_2] = [H_2O_2]_0 \cdot e^{-kz}$, where z is a depth (m) and k (m^{-1}) is an attenuation coefficient of H_2O_2 .

$$\text{Station 1 ; } \ln[H_2O_2] \text{ (nM)} = 4.455 - 0.028 \cdot \text{depth(m)}, \quad r^2 = 0.86$$

$$\text{Station 4 ; } \ln[H_2O_2] \text{ (nM)} = 4.457 - 0.020 \cdot \text{depth(m)}, \quad r^2 = 0.95$$

$$\text{Station 23; } \ln[H_2O_2] \text{ (nM)} = 4.662 - 0.106 \cdot \text{depth(m)}, \quad r^2 = 0.99$$

Light extinction coefficients (Table 4.1) were greater than the attenuation coefficients of H_2O_2 at stations, 1 and 23, which implies that H_2O_2 concentration decreased with depth at a slower rate than the light did. This may be caused by some factors other than light, for example, *in situ* biological production of H_2O_2 in near-surface water columns (Palenik and Morel, 1988), and physical transport of H_2O_2 from the surface to a subsurface layer (Plane *et al.*, 1987; Johnson *et al.*, 1989).

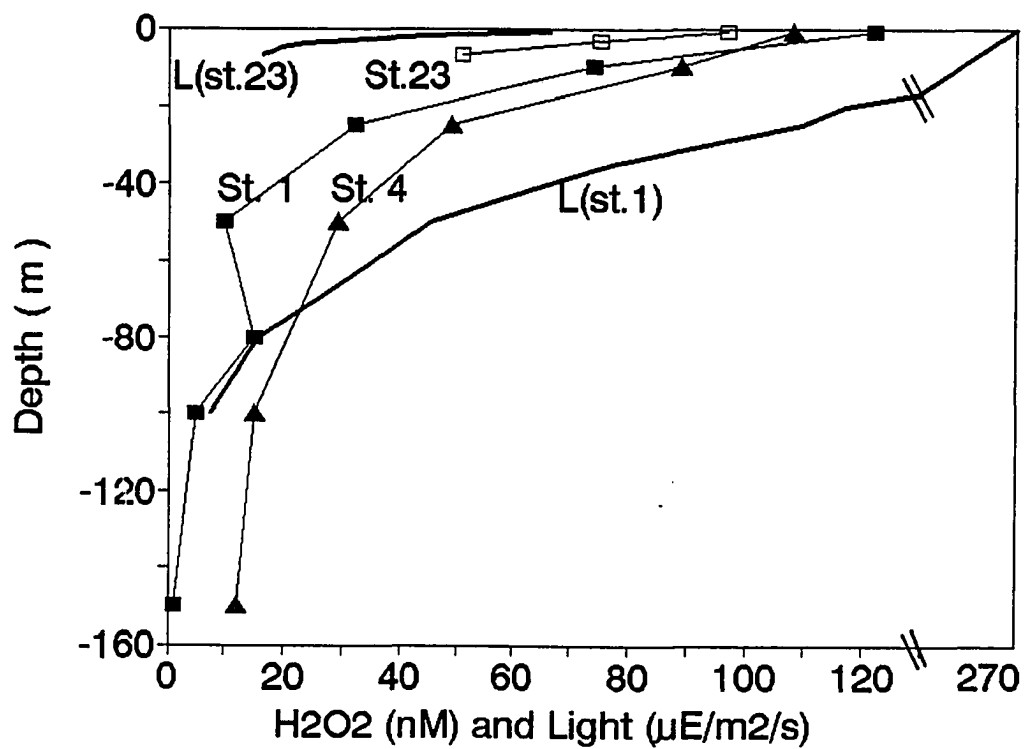


Fig. 4.5. Depth profiles of H₂O₂ at the three stations 1, 4 and 23. Light profiles (L) were measured at stations 1 and 23.

4.4.2. Dark incubation experiments

Kinetics and rate constants : H_2O_2 concentrations measured with time in each incubation bottle were plotted (Fig. 4.6-4.8). Rate constants were determined based on pseudo-first order kinetics. k values (k_p : production rate constant, k_d : decomposition rate constant) and P_{\max} values, which are the two most important parameters determining the observed kinetics, are summarized in Table 4.2. A total of 3 stations and 16 depths were investigated. The data presented here are "net results" of H_2O_2 production and decomposition in the dark.

Station 1 : H_2O_2 production was observed in most samples (Fig. 4.6). The sample from the 50 m depth produced a maximum amount of H_2O_2 , 155 nM over 30 hours ($5.3 \text{ nM}\cdot\text{hr}^{-1}$). k_p values ranged from 0.06 to 0.31 with an average of 0.24 (hr^{-1}) (Table 4.2). On average, P_{\max} was about 80 nM. P_{\max} values may be underestimated due to some decay processes after the steady state. A reason for the maximum production of H_2O_2 at the subsurface water column between 25 and 50 m as well as some relationship between P_{\max} and nutritional status of phytoplankton will be discussed later. The production rates and net increases of H_2O_2 indicate the significance of dark production of H_2O_2 in the oligotrophic ocean under certain condition. Maximum production of H_2O_2 (50 m) was about 176 nM after about 20 hrs. The surface sample (1 m) exhibited a

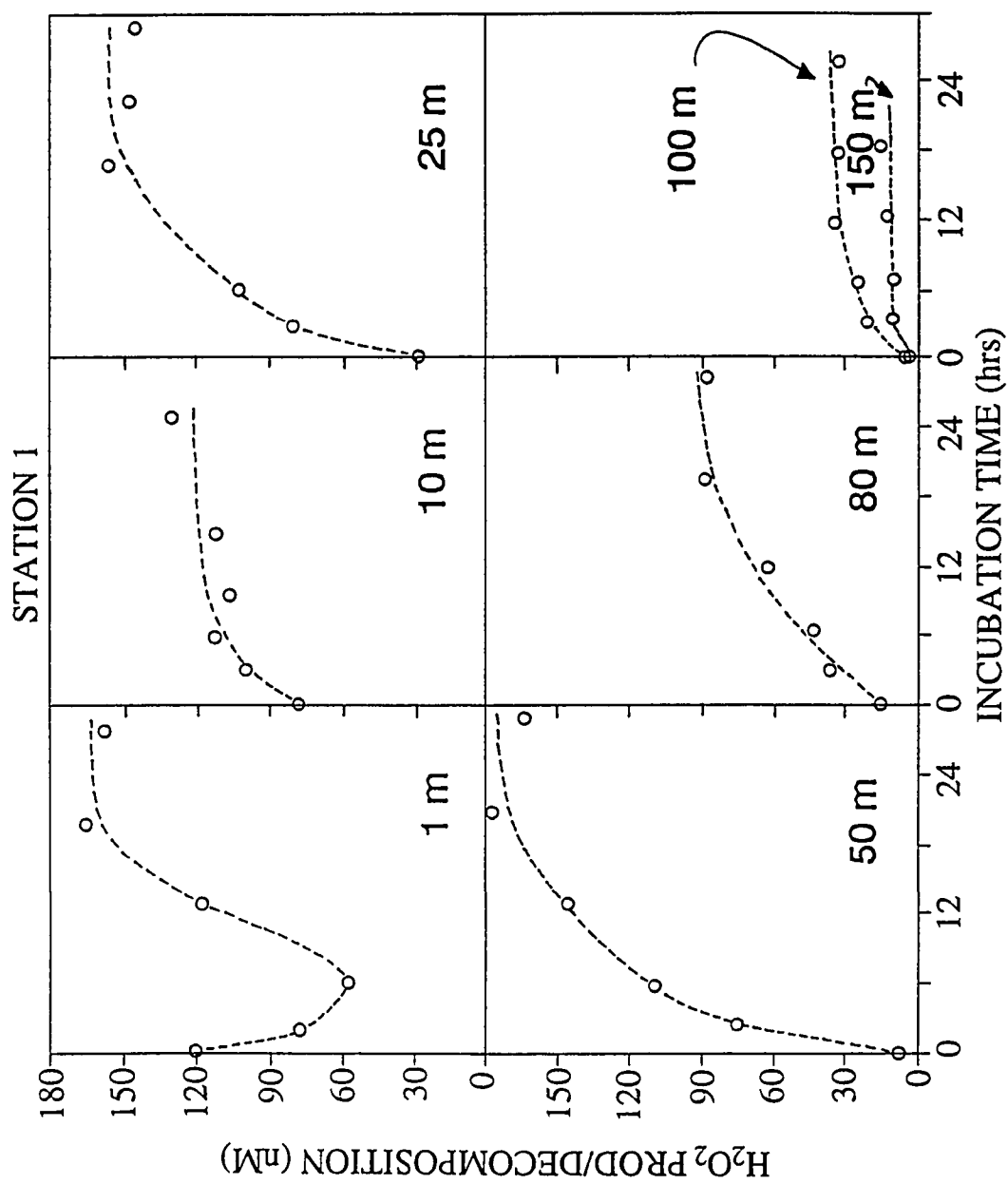


Fig. 4.6. Kinetic curves obtained from the dark incubation experiment at station 1.

different pattern; Decomposition ($k_d = 0.25$) occurred before the initiation of production ($k_p = 0.14$). Thus, the net increase over 28 hours was only 18 nM. The decomposition of H_2O_2 continued down to about 60 nM. The initial decomposition observed in 1 m sample was possibly due to the highest initial concentration of H_2O_2 , which could be toxic to some population of phytoplankton. H_2O_2 production in the deep samples (100 m and 150 m) was much smaller.

Station 4 : Production of H_2O_2 at station 4 showed substantially different results from station 1 (Fig. 4.7). P_{max} values were much lower than station 1, only 39 nM on average (Table 4.2). But rate constants ranged from 0.33 to 0.65 with an average value of 0.44 (hr^{-1}), which is higher than station 1. The higher k values indicate that H_2O_2 production reaches P_{max} levels quickly at this station. Unlike station 1, a major production of H_2O_2 occurred at the surface. All samples between 1 m and 50 m showed production of H_2O_2 . However, they could sustain their P_{max} level only for a short period of time (except for 25 m). And then, rapid losses of H_2O_2 proceeded during remaining incubation time. Thus, the net increases of H_2O_2 over 30 hours were small, maximally 35 nM in the 25 m sample (The maximum rate was only $1.23 \text{ nM}\cdot\text{hr}^{-1}$). H_2O_2 concentrations before the initiation of decomposition were different sample to sample. The deep-depth samples (100 m and 150 m) did not show any significant production or decomposition of H_2O_2 .

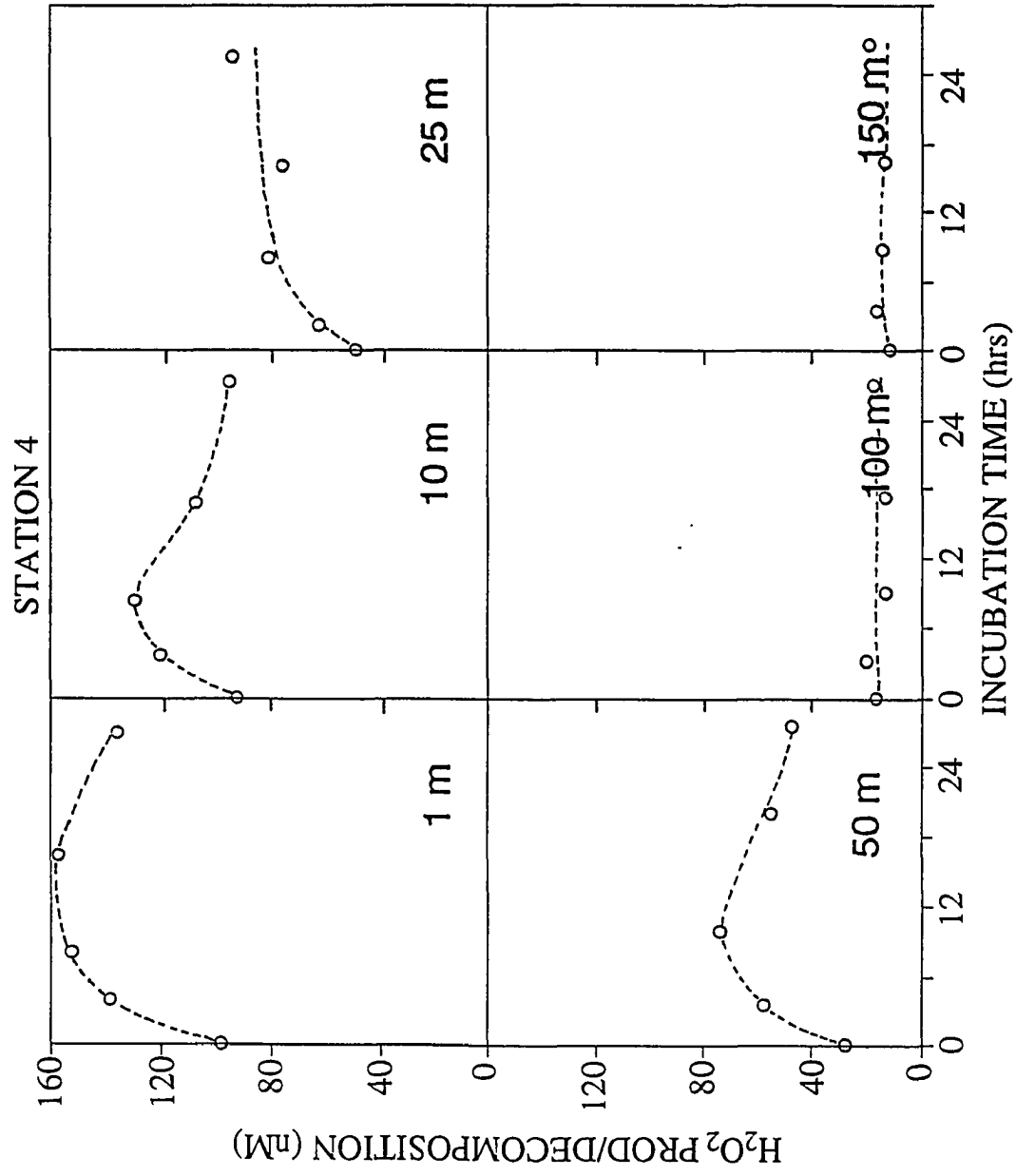


Fig. 4.7. Kinetic curves obtained from the dark incubation experiment at station 4.

Station 23 : Fig. 4.8 exhibits features remarkably different from the two previous stations. Decomposition of H_2O_2 occurred predominantly at this station. The decomposition rate constant, k_d , values ranged from 0.11 to 0.25 hr^{-1} with half-lives of 2.8 to 6.3 hrs. All samples showed decomposition of H_2O_2 with a consistent pattern except for a slight initial production in the 3 m sample. Net decreases of H_2O_2 ranged from 47 nM to 90 nM. The net decrease rates were 1.9 $nM \cdot hr^{-1}$ to 3.7 $nM \cdot hr^{-1}$. Even in the lowest initial concentration of H_2O_2 in the 7 m sample, the decomposition of H_2O_2 still occurred down to a very low concentration. Our results showed that the biological decomposition was very important in coastal areas compared to open oceans. However, the possibility of H_2O_2 decomposition by dissolved organic materials and trace metals cannot be ruled out, especially in extremely polluted areas.

Comparison with other data : Palenik and Morel (1988) reported on the dark production of H_2O_2 in the Sargasso Sea (location : 32 °N, 62 °W). They observed dark production in the subsurface water between 40 m and 60 m with an initial production rate of 11 $nM \cdot h^{-1}$, which is similar to our results in the magnitude.

Moffett and Zafirioiu (1990), at the Vineyard Sound coast, measured a dark decomposition rate of 6.14 $nM \cdot hr^{-1}$. Cooper et al., (1992) observed dark decomposition of H_2O_2 in several lake

STATION 23

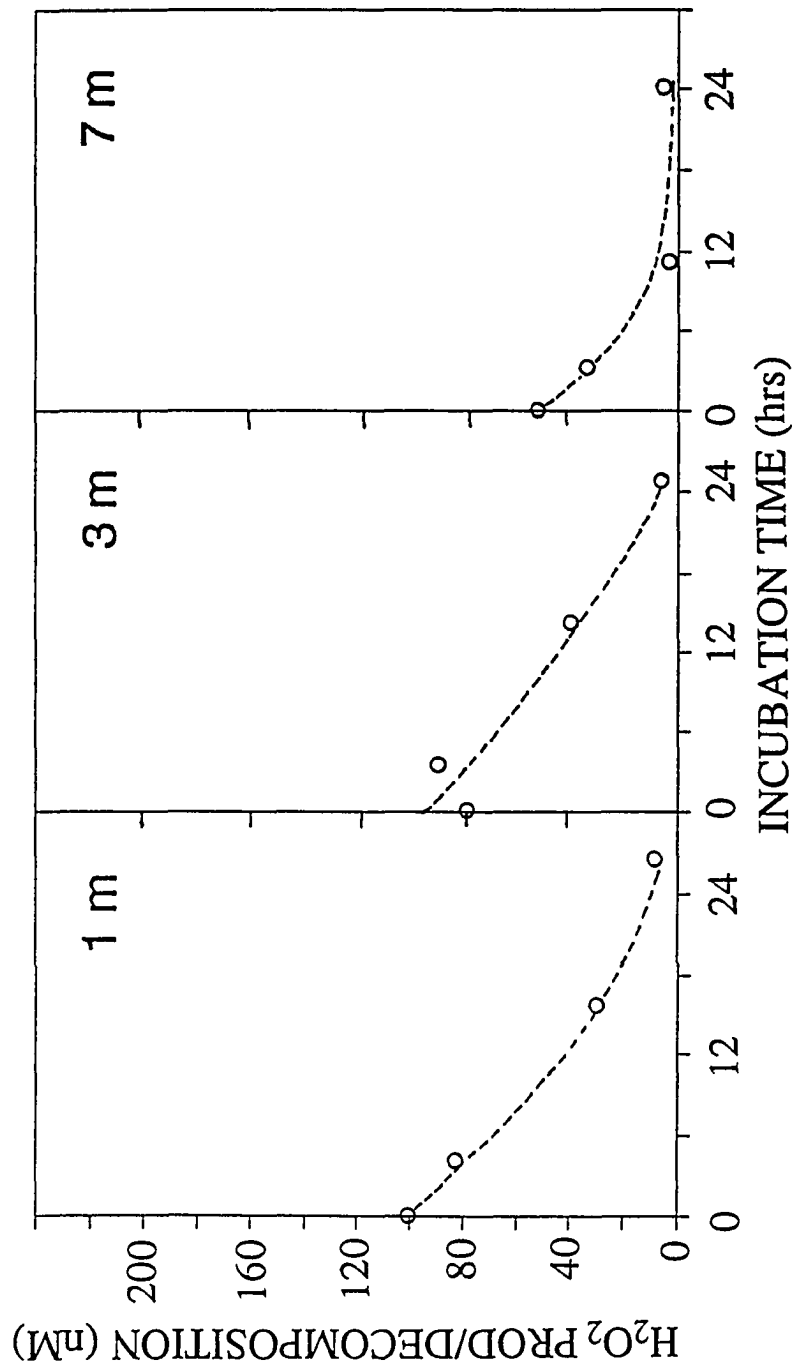


Fig. 4.8. Kinetic curves obtained from the dark incubation experiment at station 23.

Table 4.2. Summary of the kinetic parameters calculated from the kinetic curves of the dark production and decomposition of H₂O₂.

St. No.	Depth (m)	k _p (hr ⁻¹)	k _d (hr ⁻¹)	P _{max} * (nM)	Net change (nM)	Total time (hr)	Net rate (nM/hr)	Chl-a (µg/L)
1	1	0.14	0.25	81.0	18.0	28.2	0.64	0.119
1	10	0.22		31.5	46.0	27.6	1.67	0.073
1	25	0.14		115.0	109.0	28.9	3.77	0.104
1	50	0.15		161.5	155.0	29.5	5.25	0.060
1	80	0.06		70.5	68.0	29.5	2.31	0.281
1	100	0.31		21.2	16.0	29.0	0.55	0.077
1	150	NS		NS	17.0	30.0	0.57	0.051
avg		0.24		80.5	61.3	29.0	2.11	
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4	1	0.40		50.0	25.0	28.2	0.89	0.542
4	10	0.65		37.0	2.0	28.4	0.07	0.650
4	25	0.38		28.0	35.0	28.5	1.23	0.807
4	50	0.33		42.0	9.0	28.9	0.31	0.040
4	100	NS		NS	-2.0	29.1	-0.07	0.035
4	150	NS		NS	12.0	29.4	0.41	0.010
avg		0.44		39.3	13.5	28.8	0.47	
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23	1		0.11	90.0	-90.0	24.3	-3.70	1.80
23	3		0.15	84.0	-71.0	24.6	-2.89	2.23
23	7		0.25	49.0	-47.0	24.9	-1.89	3.30
avg			0.17	74.3	-69.3	24.6	-2.83	

NS : not significant

k_p : production rate constant, k_d : decomposition rate constant

* : P_{max} when P₀ is 0 nM

systems (half-life of H_2O_2 ; 4.4 - 6.3 hr) and in the Chesapeake Bay (half-life of H_2O_2 ; 2.2 - 12.2 hr) with little evidence of dark production. Cooper et al. (1989) also reported half-lives of 10 - 22 hr in lake waters. Cooper and Lean (1989) estimated a half-life of H_2O_2 of 7.8 hr in a lake. Pamatmat (1988) measured a biological decomposition of H_2O_2 in San Francisco Bay. Szymczak and Waite (1988) found a rapid decomposition of H_2O_2 at the inside of an estuary but no decomposition at the outside of the estuary.

All the samples from shores, bays and lakes appeared to dominantly decompose H_2O_2 in the dark, bringing down H_2O_2 produced by sunlight to maintain diel cycles of H_2O_2 in these water systems.

Control experiments : Control experiments were performed to test whether the observed production and decomposition of H_2O_2 at the three stations were due to phytoplankton activity. Assuming most bacteria pass through a 1- μm filter, 1- μm filtrates were incubated at stations 1, 4, and 23. In general, the filtration did not stop the production of H_2O_2 at station 1, but the production and the decomposition were reduced considerably after the filtration at station 4 and 23 (Table 4.3).

Station 1 : Phytoplankton less than 1- μm size (ultra- and pico-phytoplankton) accounted for 56 % of the total chlorophyll-a. Thus, these small phytoplankton may be responsible for the produced H_2O_2 in the 1- μm filtrate.

Palenik and Morel (1988), however, observed that H_2O_2 production completely stopped in the incubation of 1- μm filtered Sargasso seawater, thus they believed that the production of H_2O_2 was probably a result of the activity of eukaryotic algae. No report on the production of H_2O_2 by marine bacteria was published. H_2O_2 is generally known to be toxic to bacteria, thus bacteria may not produce H_2O_2 . Incubation of a 0.2- μm filtered Sargasso seawater did not show any meaningful production or decomposition in the dark (Table 4.3). Dark chemical reactions for the formation of H_2O_2 are not thought to be significant in most waters (Cooper *et al.*, 1992), such as a chemically complex coastal water (Moffett and Zafirion, 1990).

Stations 4 and 23 : Levels of H_2O_2 production and decomposition in 1- μm filtrates were reduced by more than 50 % (Table 4.3), probably due to the reduction of total chlorophyll-a in the filtrates by about 56 % and 20 %, respectively. We cannot rule out the role of non-living particles, such as suspended sediments from shallow bottoms (8 m) and dead cells, in the decomposition of H_2O_2 at station 23. However, Cooper and Zepp (1990) found that the decomposition of H_2O_2 in a soil suspension was due to the organisms living in the soils, not due to the soils.

Table 4.3. Summary of the control experiment data.

	Unfiltered	Filtered [@]	Filt/Unfilt	< 1- μ m [§] vs total
St.1 (50m)	+144 nM	+119 nM	0.82	56 %
St.4 (1 m)	+ 52 nM	+ 9 nM	0.17	56 %
St.23 (1 m)	- 70 nM	- 1 nM	0.01	19 %
(3 m)	- 42 nM	- 19 nM	0.45	19 %
(7 m)	- 49 nM	- 26 nM	0.53	23 %

Incubation of 0.2- μ m filtered Sargasso seawater*

Incubation Time	0	0.5	1	2	3	5 hrs
	Measured H ₂ O ₂ (nM)					
	0	5	2	3	7	7
	900 ^{&}	897	903	895	887	888

+ : increase of H₂O₂ during incubation

- : decrease of H₂O₂ during incubation

* : Non-parallel incubation with aged water in the dark

@ : Filtered through an 1- μ m Nucleopore filter

§ : Size fraction of <1- μ m chlorophyll-a vs. total chl-a

& : 900 nM of H₂O₂ was spiked at time 0.

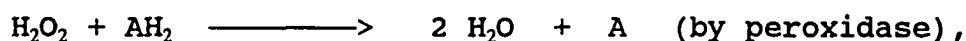
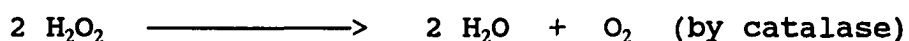
4.4.3. Biological production and decomposition of H_2O_2 in different sub-marine environments

The mechanisms of biological production and decomposition of H_2O_2 are different because the enzymes mediating these processes are different. Palenik and co-workers (1990a, b, 1991) demonstrated a biological mechanism for the dark production of H_2O_2 . According to them, nitrogen-limited phytoplankton convert L-amino acids to α -keto acids, H_2O_2 , and ammonia using cell-surface amino acid oxidases to take up ammonia as their nitrogen source.

L-aa oxidases



However, the decomposition of H_2O_2 is believed to be mediated by catalase and peroxidase, which prevent the accumulation of H_2O_2 from reaching a toxic level (Fridovich, 1975a; Zepp *et al.*, 1987b; Pamatmat, 1988; Moffett and Zafiriou, 1990).



where AH_2 is a hydrogen donor molecule.

To meet the H_2O_2 production model, nitrogen-starved phytoplankton should be supplied with L-amino acids. Nitrogen

limitation and the availability of amino acids in the open ocean may have relationships with the biological H_2O_2 production.

Nutrient limitation : Fig. 4.9 shows that the concentrations of inorganic nitrogen (nitrate, nitrite and ammonium) at station 1 above the thermocline were close to their detection limits. Assuming this thermocline was formed in May, the age of the water mass above the thermocline is about 5 months, which is sufficient time for phytoplankton community to consume all the new inorganic nitrogen. In oligotrophic waters, except for some episodic pulses of nutrient input, nutrients in the surface waters during summer and fall are extremely low (Glover *et al.*, 1986, 1988).

According to Wheeler *et al.* (1974) and Admiral *et al.* (1984), phytoplankton do not take up amino acids until nitrogen in culture media become limited. Amino acid uptake rates are accelerated when nitrogen is "depleted" (North and Stephens, 1971; Stephens and North, 1971; Lee and Bada, 1977; Wheeler *et al.*, 1974; McCarthy and Goldman, 1979; Flynn and Syrett, 1985).

Availability of amino acids : In general, amino acids show their maximum above thermocline in oligotrophic waters (Lee and Bada, 1977; Liebezeit *et al.*, 1980; Mopper and Lindroth, 1982). Meanwhile, dissolved free amino acids, which are needed for H_2O_2 production, show constant concentrations

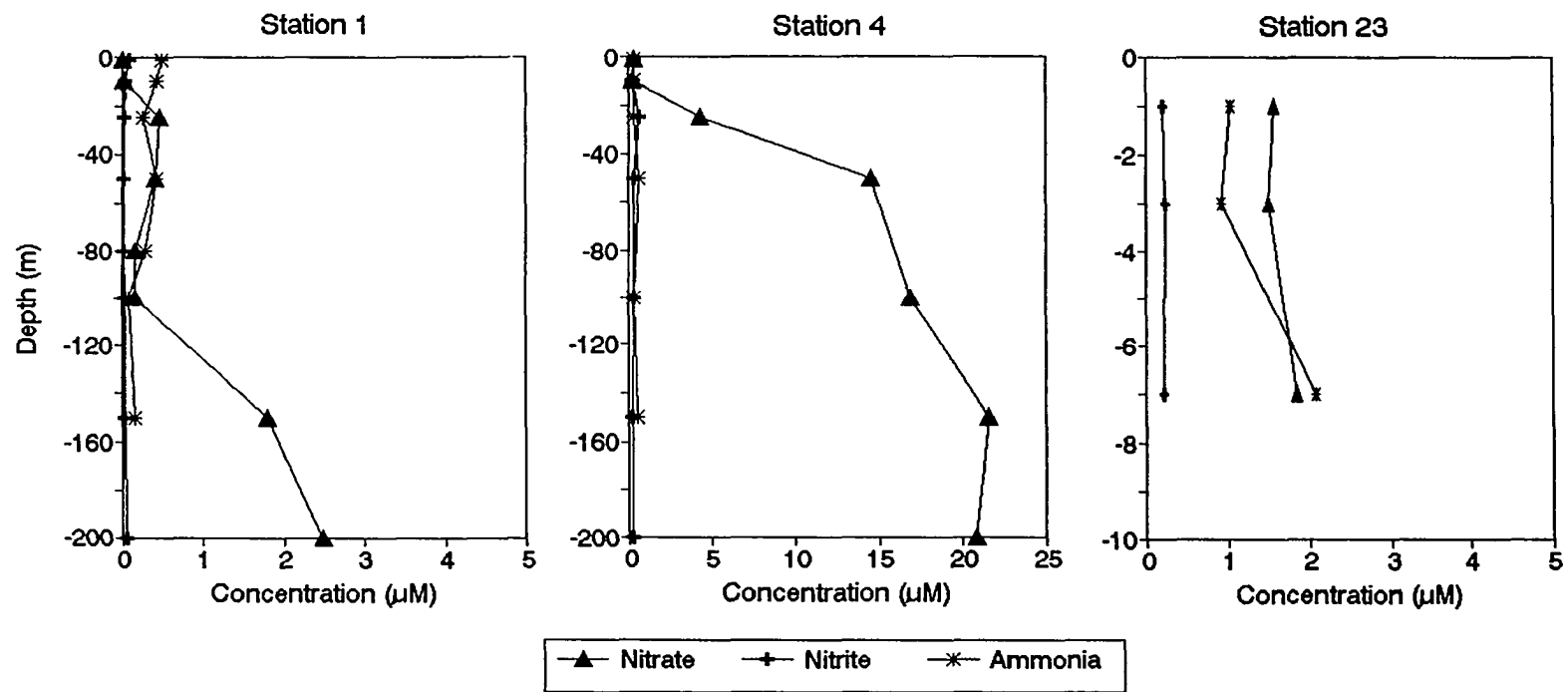


Fig. 4.9. Depth profiles of nitrate, nitrite, and ammonia at stations 1, 4 and 23.

from the coast to the Sargasso Sea throughout the year (Lee and Bada, 1977). Therefore, station 1 with the lowest biomass could have a greater amount of available amino acids per unit biomass compared to the coastal station 23. Nitrogen-starved phytoplankton in the subsurface water (25 - 50 m) could have even greater availability of amino acids, which may explain the maximum production of H_2O_2 . Phytoplankton biomass in deep-water samples (100 m and 150 m) were similar with the 50 m sample (Table 4.2). However, the production of H_2O_2 decreased significantly, probably because phytoplankton below the thermocline were not nitrogen-starved.

Moreover, the minimum ratio of phaeophytin-a/chlorophyll-a ('minimum number of senescent cells') was found at 25-50 m (Fig. 4.10), thus number of bacteria attached to phytoplankton cells could be minimum (Albright *et al.*, 1986). Therefore, phytoplankton could take up more amino acids per cell with less competition from bacteria at this water layer, thereby producing more H_2O_2 at their cell surfaces. Before 1980 it had been widely believed that bacteria outcompete algae in the uptake of dissolved free amino acids (DFAA). But, now, there is enough evidence that algae could 'out-compete' bacteria for DFAA (Wheeler *et al.*, 1974; Saks and Kahn, 1979; Amano *et al.*, 1982; Lu and Stephens, 1984; Ming and Stephens, 1985). Bacterial utilization of DFAA might have been overestimated because of the addition of unnatural concentrations of test amino acids in field experiments (Flynn and Butler, 1986). In addition, the size fraction collected through nets or filters,

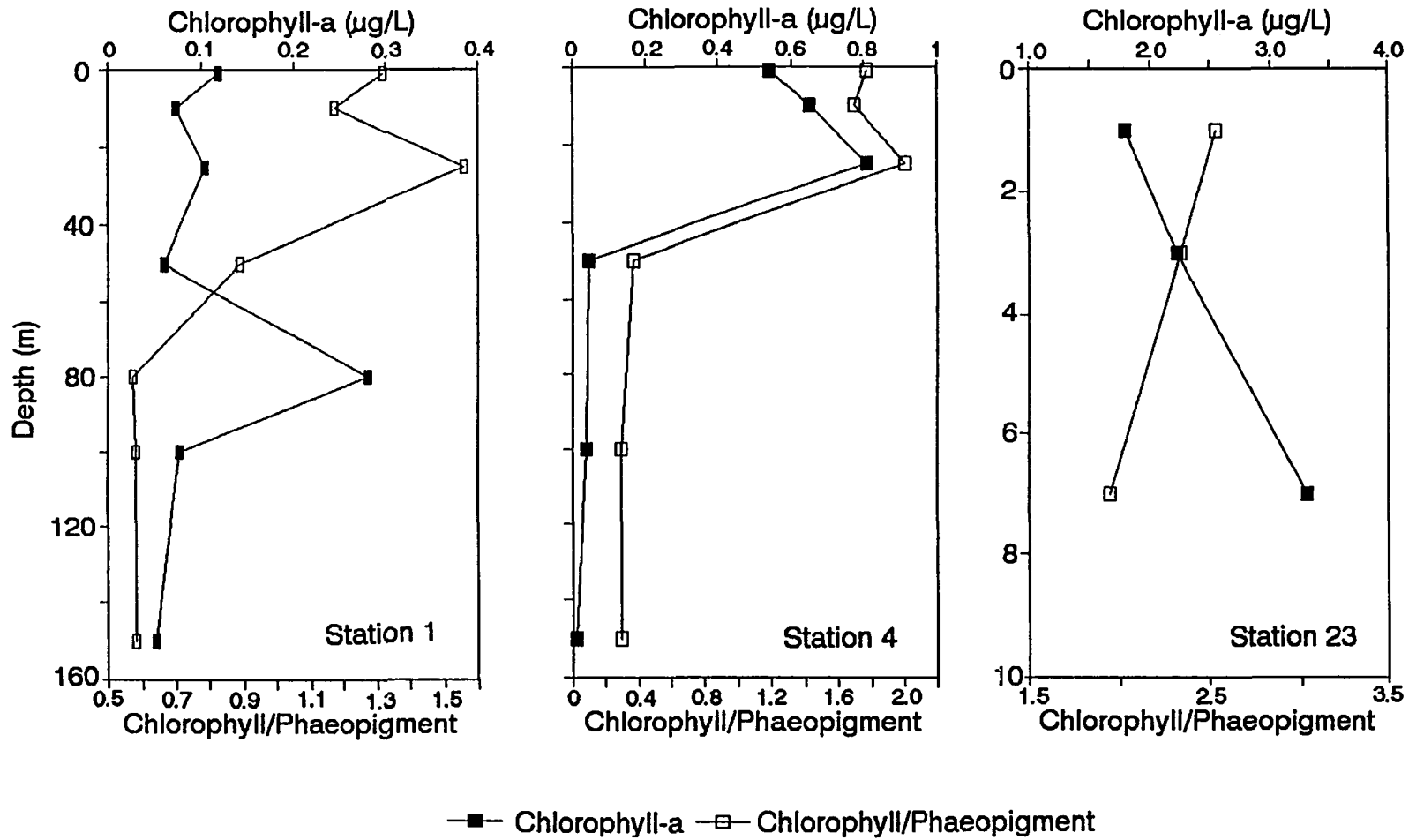


Fig. 4.10. Depth profiles of chlorophyll-a concentration ($\mu\text{g/L}$) and ratios of chlorophyll-a concentration vs. phaeopigment concentration.

which was thought to contain only bacteria, might have included a significant proportion of nano- and picophytoplankton. Thus, it is possible that a significant proportion of DFAA has been used by these small algae in addition to bacteria (Li and Dickie, 1985; Flynn and Butler, 1986).

The seasonal thermocline at station 1 was located at about 70 m. Nitrogen-limited phytoplankton in the mixed layer above the thermocline in the oligotrophic ocean probably take up amino acids actively as an alternative nitrogen source, while producing a considerable amount of H_2O_2 . This has been demonstrated by Palenik and co-workers (1989, 1990a, b) in the lab using nitrogen-limited phytoplankton. However, a problem occurs in the diel cycles of surface H_2O_2 observed by Palenik and Morel (1988) and Zika et al. (1985a) because of the lack of biological decomposition at this station. Although significant net decomposition occurring in the surface sample within 6 hours may partially explain the problem, the following net production remains as a question (Fig. 5.6).

Compared to station 1, station 4 had a much shallower nitracline, which brings nutrient-rich water up to 25 m (Fig. 4.9). Therefore, phytoplankton in this water column consume NO_3^- or NH_4^+ first before they use amino acids; thus H_2O_2 production would be reduced.

Meanwhile, station 23 exhibited no thermocline and represented a mesotrophic condition. Phytoplankton were in

bloom ($> 1.8 \times 10^6$ cells/l), probably due to the continual supply of nutrients by the intrusion of nutrient-rich slope water, by nitrogen fixation of blue green algae (Table 4.4), and by riverine inputs from adjacent areas. Therefore, phytoplankton would not take up amino acids until they are limited in nitrogen. Therefore, the production of H_2O_2 was either very small (3 m) or could not keep up with the decomposition of H_2O_2 showing the predominant net decomposition.

Cell sizes of phytoplankton may relate to the efficiency of production of H_2O_2 (Zepp et al., 1987b). In general, bigger cells are abundant in nutrient-rich coastal waters than in oligotrophic waters. Bigger cells have a smaller ratio of surface to volume. It is speculative whether bigger cells have more catalase or peroxidase than amino acid oxidases compared to smaller cells (commonly smaller by two orders of magnitude). Amino acid oxidases are known to be located at the cell surface membranes (Palenik, 1989), and catalase and peroxidases are located in peroxisomes. Thus, probably catalase and peroxidase could be more important in the cells living in coastal waters, where they play major roles in the decomposition of H_2O_2 .

Species composition of phytoplankton and H_2O_2 production and decomposition may have important relationships. At station 1, dinoflagellate and cryptophyta were abundant at the surface but they decreased dramatically at the chlorophyll maximum layer (80 m). Instead, spores showed up as one of the

Table 4.4. Phytoplankton species composition and chlorophyll-a amounts at stations 1, 4, and 23.

	St.1 (1 m)	St.1 (80 m)	St.4 (1 m)	St.4 (25 m)	St.23 (3 m)
Diatoms	4		50	34	128
Dinoflagellate	13,252 (18)	8	13,242 (5)	19,893 (4)	27,240 (2)
Microflagellate	52,888 (73)	33,055 (71)	198,355 (68)	323,941 (72)	1,632,927 (87)
Cryptophyta	6,611 (9)	2	79,338 (27)	105,787 (24)	218,176 (12)
Blue-green algae					376
Spores		13,226 (28)			6
Unknown			2		
Total (cells/L)	72,755	46,293	290,987	449,655	1,878,855
Total chl-a ($\mu\text{g/L}$)	0.12	0.28	0.54	0.81	2.23

Numbers in parenthesis indicate percentages over total numbers of the cells.

dominant populations (Table 4.4). At station 23, number of diatoms increased significantly compared to the two other stations. Many blue-green algae showed up at this coastal station. Our studies with pure cultures of phytoplankton showed that *Skeletonema costatum* decomposed H_2O_2 very effectively (chapter 3). Because this species are common in coastal waters, *Skeletonema costatum* could be an important species in the decomposition of H_2O_2 in coastal waters. The increased number of diatoms and net decomposition of H_2O_2 at station 23 agrees with the lab results that most diatoms could decompose H_2O_2 actively and they were not involved in H_2O_2 production. In general, the coastal waters are abundant in dissolved organic materials and are subject to accumulate H_2O_2 to maximum levels under the sunlight. Therefore, phytoplankton in coastal waters might have developed the decomposition ability as a defense mechanism against the potential toxicity of H_2O_2 .

4.5. SUMMARY

1. H_2O_2 distribution appeared to vary diurnally although there was inconsistency due to geographic variation. Profiles of H_2O_2 had maxima at the surface and decreased quasi-exponentially towards the euphotic depths. Light profiles were important in determining the depth profiles of H_2O_2 during daytime. The H_2O_2 profiles were not related to either biomass profiles or the rates of the H_2O_2 production and decomposition.

2. H_2O_2 production by phytoplankton in the dark from three different water environments resulted in remarkably different patterns. Most production of H_2O_2 in the dark occurred in oligotrophic waters where inorganic nitrogen sources are limited. Most decomposition of H_2O_2 occurred in mesotrophic coastal water where nitrogen sources are abundant. Production rates ranged from 0.1 to 5.3 $\text{nM}\cdot\text{hr}^{-1}$ and decomposition rates from 0.1 $\text{nM}\cdot\text{hr}^{-1}$ to 3.7 $\text{nM}\cdot\text{hr}^{-1}$ over the incubation period of about 30 hours. Both the production and decomposition of H_2O_2 could be described by pseudo-first order kinetics.

3. The dark production and decomposition of H_2O_2 seemed to be the results of biological activity. The mechanism of H_2O_2 production could be a result of amino acid uptake by inorganic nitrogen-limited phytoplankton affected by physical and chemical dynamics of the water column. Three selected stations were distinctively different in terms of nutrient

concentrations, physical parameters, and phytoplankton biomass and species composition.

4. Production of H_2O_2 in the dark in the oligotrophic waters could make a significant contribution to the budget of H_2O_2 in the surface water. Dark decomposition of H_2O_2 by phytoplankton could explain the prominent diurnal change of H_2O_2 in the coastal water. Further field studies are needed to confirm the mechanism of H_2O_2 production, i.e., amino acid uptake by N-limited phytoplankton, and biological roles in controlling the H_2O_2 budget in various marine environments.

Chapter 5

GENERAL SUMMARY AND CONCLUSIONS

General summary : The results of the production of H_2O_2 tested with 11 pure cultures of marine phytoplankton have shown that five species produced H_2O_2 . A representative species for the production of H_2O_2 was *Pleurochrysis carterae*. Most of the H_2O_2 producers were Prymnesiophyceae. Because of the somewhat evident taxonomical pattern of H_2O_2 producers, the distribution of phytoplankton biomass and species composition could influence the distribution of H_2O_2 in surface oceans. Low ratio of P_{max} vs biomass from the kinetics of H_2O_2 production indicated that the production of H_2O_2 by marine phytoplankton could not contribute significantly to the budget of H_2O_2 in the surface oceans. However, nitrogen-starved phytoplankton could produce a higher level of H_2O_2 by using amino acids. The production of H_2O_2 by marine phytoplankton may have great implications on the amino acid study in surface oceans, particularly in oligotrophic oceans.

Decomposition of H_2O_2 took place in all 11 species of marine phytoplankton. Among the 11 species, *Skeletonema costatum* and *Synechococcus* sp. decomposed H_2O_2 most efficiently. Because these two species are the most commonly found species in coastal and open oceans, respectively, their roles in removing H_2O_2 could be very important in surface oceans. The kinetics of the decomposition of H_2O_2 could be described by a pseudo-first order kinetics. Half-lives of H_2O_2 estimated from the rate constants can explain diel variations of H_2O_2 in coastal oceans. Nutrient effects on H_2O_2 decomposition were more profound in coastal species than in

oligotrophic species. Amino acids and trace metals did not have significant effects on the decomposition of H_2O_2 . This study indicates that a large number of marine phytoplankton play an important role in regulating the budget and chemistry of H_2O_2 in the surface oceans by mediating its rate of decomposition.

The results from the field study indicated that the production and the decomposition of H_2O_2 occurred at the three different marine environments can be explained by the hydrochemical structure of the water columns. Most of the production occurred in the nutrient-limited oligotrophic waters (in dimly-lit water column) and the most active decomposition occurred in the mesotrophic waters with coastal species. As indicated in the lab studies, kinetics of both production and decomposition of H_2O_2 could be explained by a pseudo-first order reactions. The result was not just a function of biomass (chl-a), even though it was a biological process. Inorganic nitrogen-limited phytoplankton cells produce H_2O_2 while they utilize amino acids as their nitrogen sources. Therefore, we should consider not only the phytoplankton species composition and their abundance but also amino acid concentrations and nutrient-limitation conditions to understand the H_2O_2 budget of the ocean in association with biological roles.

Conclusion : A large number of marine phytoplankton play an important role in the diurnal changes of H_2O_2 in surface oceans, especially in coastal oceans, by mediating its rate of decomposition. Their contribution of H_2O_2 was not important in nutrient-rich conditions. However, under nutrient-depleted conditions, H_2O_2 production by marine phytoplankton was significant. The effects of light, temperature and salinity were not as significant as nutrient effects. To understand the biological roles in the H_2O_2 budget, it is necessary to consider not only the species composition and abundance but also hydrography associated with nitrogen dynamics.

Future goals : Correlationships between biological H_2O_2 production and DFAA concentrations in oligotrophic oceans should be studied to find how they control the budget of H_2O_2 in open oceans with focuses on how they maintain the diel variation of H_2O_2 . Species composition of phytoplankton and bacterial abundance should be studied at the same time.

Benefits of production and decomposition of H_2O_2 have been barely studied. It should be answered whether cultured algal cells can develop stronger defense mechanisms to fight against toxic effects of H_2O_2 , and also whether the production of H_2O_2 by phytoplankton can be a strong mechanism to compete with bacteria for the uptake of nutrients. In addition, more species of bacteria should be tested for their capability of decomposition of H_2O_2 to understand better relationships between bacteria and phytoplankton in association with

biochemistry of H_2O_2 in the ocean. Production of extracellular H_2O_2 may control the adverse effects of other microorganisms, e.g., bacteria, on the health and growth of phytoplankton. Phytoplankton may control concentrations of H_2O_2 , which, in turn, affect the availability and/or toxicity of trace metals because H_2O_2 can act as a strong oxidant and/or reductant.

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APPENDIX A : THE DETAILS ON THE MATERIALS AND METHODS

A-1: Maintenance of Phytoplankton Stock Cultures

A-2: Preparation of Medium

A-3: Preparation of Samples

A-4: Kinetic Experiment Procedures

A-5: Determination of H₂O₂ in Natural Waters

A-1. MAINTENANCE OF STOCK CULTURES

A list of phytoplankton tested for the production and decomposition of H_2O_2 are shown in Table A-1. These organisms, which were obtained from Provasoli-Guillard Center for Marine Phytoplankton (Bigelo Laboratory for Ocean Sciences, McKown Point, West Boothbay Harbor, Maine 04575, U.S.A.), have been chosen because; 1) they cover some of the major types of marine phytoplankton; 2) some of them have been used traditionally in culture studies; 3) some are quantitatively important primary producers in the sea.

Stock cultures were maintained using batch culture modes routinely in f_{12} media. As the culture conditions, temperature of 23 °C, light intensity of $75 \mu E m^{-2} s^{-1}$, and 12:12 hr L:D cycle were used. Cells grown in this way were routinely used for experiments. Stock cultures were handled in the Labconco Tissue Culture Enclosure sterilized by ultraviolet radiation at least for an hour but the cultures may not be axenic. Some culture stocks were treated with antibiotics purchased from Guillard Center to prevent bacterial growth in the cultures. Stock cultures were grown in 125-ml Erlenmeyer glass flask cleaned in acid bath and autoclaved prior to use.

Table A-1. The details of the tested phytoplankton for the production and decomposition of H₂O₂

Class						
Species Name	Strain	L/W (μ m)	Medium	Sal (psu)	Temp (°C)	
Bacillariophyceae						
<i>Skeletonema costatum</i>	SKEL	6-8/	f/2	12-32	12-30	
<i>Chaetoceros simplex</i>	BBSM	3-6/	f/2		15-23	
<i>Chaetoceros flexosus</i>		20-40/8	f/2			
<i>Thalassiosira oceanica</i>	13-1	4-11/	f/2	26-36	14-25	
Dinophyceae						
<i>Amphidinium carterae</i>	AMPHI	12-18/9-13	f/2-Si		16-23	
Prymnesiophyceae						
<i>Emiliana huxleyi</i>	BT-6	4-8/	f/2	25-35	15-23	
<i>Coccolithus pelagicus</i>	COPEL		f/2		12-20	
<i>Pleurochrysis carterae</i>	COCCO II	10-14/	f/2	3-45	16-23	
<i>Isocrysis galbana</i>	ISO					
Prasinophyceae						
<i>Tetraselmis levis</i>	PLATY1	10-12/6-10	f/2		12-20	
Chlorophyceae						
<i>Dunaliella tertiolecta</i>	DUN	6-9/	f/2-Si	<36	14-28	
Cyanophyceae						
<i>Synechococcus sp.</i>	DC2	1.3-2.5/1.3-1.5	f/2-Si		20-25	

Notes

1. L/W indicates length and width of cell.

A-2. PREPARATION OF MEDIUM

Culture medium : Culture medium was made from Sargasso seawater filtered through Gelman type A/E glass fiber filter (nominal pore size, $0.7 \mu\text{m}$) and salinity was adjusted to 30 ppt with Milli-Q water using a refractometer. Nutrients were added according to Guillard and Ryther (1962). Medium was then sterilized by autoclaving (2 minutes, 250°F and 13 lb/in^2) and cooled to a room temperature slowly.

Incubation medium : To avoid a high blank of H_2O_2 caused by heat and pressure during autoclaving (Marler and Van Baalen, 1965), incubation medium (Sargasso seawater) for this study was sterilized by filtering with $0.2 \mu\text{m}$ Nucleopore filter in a dim-lit room instead of autoclaving. The above Sargasso seawater had been stored in a dark place for several months to decay any remaining H_2O_2 in the water. Nutrient enrichment, f_{120} , was made with axenic nutrients. To prevent interferences of trace metals with H_2O_2 , trace metals were not added to the medium. The medium prepared with this method showed a very low background of H_2O_2 concentration near detection limit.

A-3. PREPARATION OF SAMPLES

A brief experimental scheme is shown in Fig. A-1. Stock cultures from the late-logarithmic phase were typically sampled. Growth phase was determined from growth curves measured by *in vivo* fluorescence using a fluorometer (Turner Design 10). Stock cultures were placed in a dark place about 2 to 3 hours prior to use. The cell suspension in a culture flask was transferred to disposable sterile centrifuge tubes (50 ml) wrapped with aluminium foils. The samples were then centrifuged (1000 rpm and 5 min) to obtain a properly condensed cell suspension (The centrifugation of 2000 rpm and 10 minutes was used for some small cells). The supernatant was then discarded and the concentrated cells were washed with fresh medium of f_{20} (no addition of trace metals) and recentrifuged. A small volume of the concentrated cells collected in the bottom of the centrifuge tube was quantitatively added into incubation medium of choice for the dark incubation study.

The purpose of washing and concentrating cells was to minimize the introduction of dissolved organic materials, trace metals and H_2O_2 existing in the original culture flasks. The estimated dilution factor was about 10^4 , because only few mL of the concentrated cells was resuspended in the 50 ml of fresh medium and only a few hundred μL of reconcentrated cells was resuspended in 100 - 200 ml of the incubation medium.

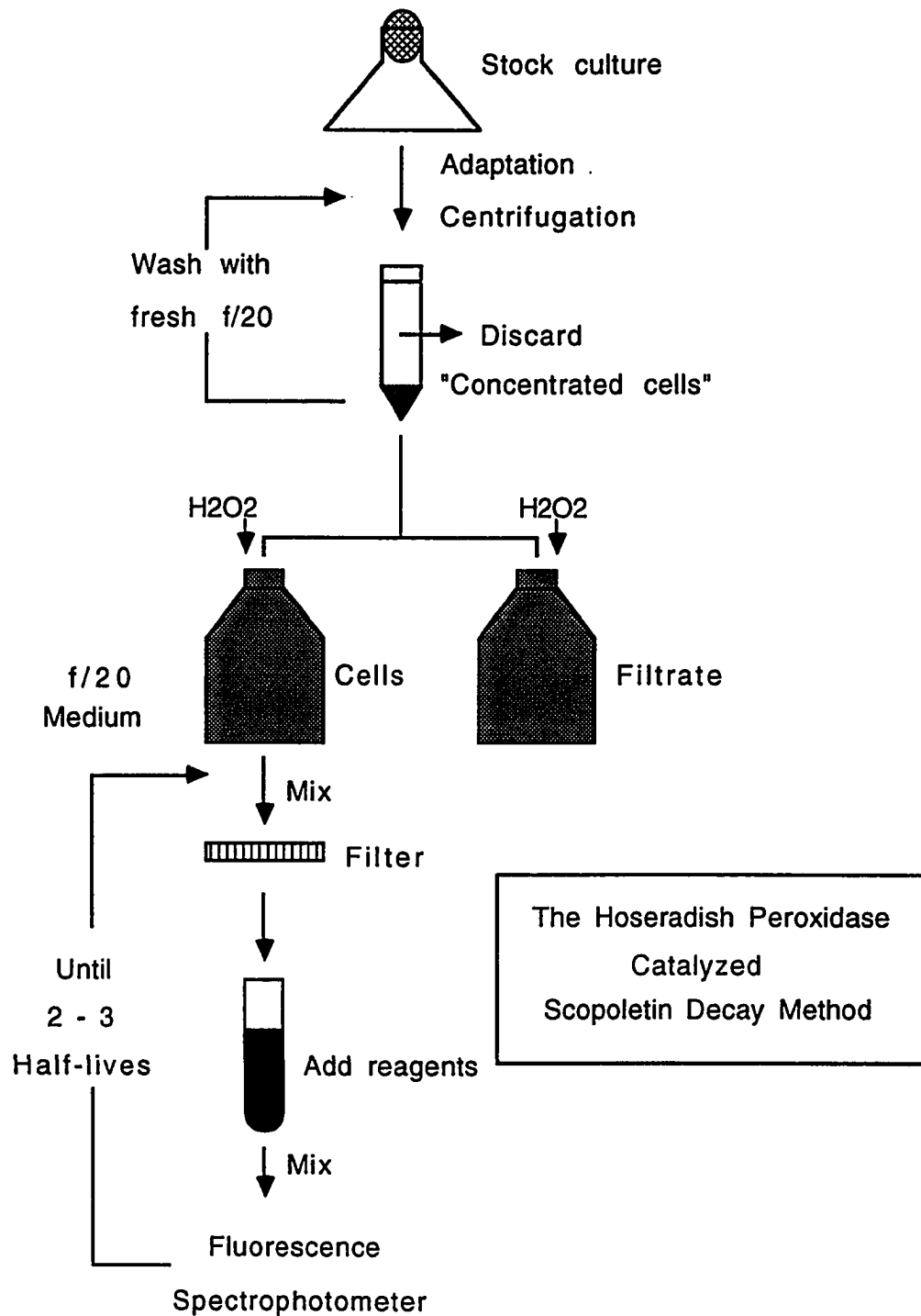


Fig. A-1. A brief experimental procedures for the kinetic studies of H_2O_2 production and decomposition by cultured marine phytoplankton.

Cell densities in incubation media were determined by either chlorophyll-a measurements or cell countings. Cell suspensions were filtered through GFF filters and then 0.45 μm Millipore filters with low pressure (< 10 mm Hg) to prepare control samples. Often, media (f_{20} without trace metals) were used as the control samples. At least replicate samples for each experiment were measured.

Incubation bottles (250-ml amber HDPE) were soaked in acid overnight and cleaned with distilled deionized water. Glassware for the sterile filtering was autoclaved before use. The experiments were conducted at the room temperature, 23 ± 1 °C in a dimly-lit room.

Because centrifugation is used to concentrate cell suspensions, centrifugal force may damage the cells. *Pleurochrysis carterae* (used often for H_2O_2 -production experiment) was tested with C^{14} radioisotope to measure C^{14} uptakes by centrifuged cells and non-centrifuged cells. Centrifugation up to 2500 rpm for 20 min did not affect C^{14} uptake by *Pleurochrysis carterae*. According to the t-test at the 95 % probability level ($t=2.776$), two means were not significantly different (calculated t, 1.252) (Table A-2). Any significant amounts of broken cells in the centrifuged samples were not observed under microscope as well.

C^{14} uptake experiment : Each BOD bottle was added with 1 mL of C^{14} (5 μCi) and then incubated for 2 hours. Volume of each sample was calibrated. After 2 hours, 10-ml aliquot of each sample was filtered onto 0.45 μm membrane filter. The

filter was washed with 1 ml of fresh medium at the last moment of filtration. Filters were acid-fumed with HCl for 30 min and dried in the desiccator for an hour and placed in a vial containing 15 ml of Aquasol. An activity of C¹⁴ from each filter was measured with a Liquid Scintillation Counter.

Table A-2. Activity (CPM) of C¹⁴ assimilated by phytoplankton

	Sample(ml)	CPM(A)	CPM(B)	CPM ⁺ (avg)	CPM [@] (avg)
Centrifuged					
C1	140	76728.5	77696.5	77212.5	216195.0
C2	142	77265.0	77160.5	77212.8	219284.2
C3	139	78869.5	78408.5	78639.0	218616.4
Intact					
I1	137	77975.0	78258.0	78116.5	214039.2
I2	127	83313.0	84597.5	83955.3	213246.3
I3	136	80672.0	80424.0	80548.0	219090.6
		CPM/ μ Ci	SD(n-1)	n	RSD (%)
C		218031.9	1625.5	3	0.75
I		215458.7	3170.0	3	1.47

⁺ Counts from 10 ml filtered sample

[@] Counts normalized to unit activity of C¹⁴ solution

A-4. Kinetic Experiment Procedures

Kinetic experiments for H_2O_2 production were started by taking subsamples from incubation bottles immediately after spiking prepared concentrated cells. Subsamples were drawn with syringes and filtered through filter holders containing glass fiber filters. Reagents were added to a 3-ml filtered sample for H_2O_2 analysis. This method, in terms of avoiding the light scattering by cell suspensions, is better than the methods used by others (Patterson and Myers, 1973; Stevens et al., 1973; Palenik, 1989). They incubated cells with reagents in a small quartz cuvet.

After an initial sampling at time 0, every 30 minutes for up to first 2 hours and then, every 1 hour for the next 3 - 4 hours. The total incubation period was confined to less than 6 - 7 hours. Measurements were performed in duplicate. Often, subsamples for *in vivo* fluorescence and cell-counting were drawn to monitor activity changes of the cells due to the added H_2O_2 or prolonged dark periods. Always control bottles were run in parallel. All procedures were done under a dim light condition. The dim light condition did not affect the analysis of H_2O_2 . The result was the same as under the complete darkness.

Kinetic experiments for H_2O_2 decomposition were started by adding the H_2O_2 of a known initial concentration, close to concentrations in natural waters. Before and during experiments, cells were often drawn to examine the possible breakage of the cells due to the added H_2O_2 . In addition, potential toxic effects of H_2O_2 on cells were tested by

monitoring growth curves after the addition of various amounts of H_2O_2 . Subsamples were drawn with the intervals as mentioned above until about 2 - 3 half-lives is complete. After the completion of the kinetic runs, 20 - 30 ml of the remaining algae suspensions were used for the determination of chlorophyll-a amounts.

A-5. DETERMINATION OF H₂O₂ IN NATURAL WATERS

The fluorometric scopoletin-horseradish peroxidase method for the determination of hydrogen peroxide in natural waters is based on the procedures published by Andrea (1955), Perschke and Broda (1961), and Holm et al. (1987). This method is known to be most sensitive and relatively easy for use. The determination of H₂O₂ is basically the measurement of the depletion of scopoletin fluorescence mediated by HRP. As HRP decomposes H₂O₂ the fluorescence of the reduced scopoletin decreases because the oxidation of scopoletin occurs.

1.0 Procedures

1.1 Preparation of labwares

Labwares or reagent bottles are cleaned with laboratory detergent, rinsed with de-ionized water, leached in a acid bath with 10 % hydrochloric acid for at least one day, and then rinsed with de-ionized water repeatedly.

1.2 Materials

A. Chemicals

1. Scopoletin (Sigma, S-2500, C₁₀H₈O₄, MW = 192.2) - reagent grade, no further purification
2. Horseradish peroxidase (Sigma, P-8250, 200 purpurogallin units/mg, Type IV) (HRP) - reagent grade, no further purification
3. Hydrogen peroxide solution (30 % w/w) - purified grade, no further purification
4. Tris (hydroxymethyl) aminomethane - purified grade, no further purification

B. Reagents and Preparation

1. 0.1 M Tris buffer solution (pH 8)

Transfer 12.114 g of Tris to 1-l volumetric flask and dilute to volume with doubly-distilled deionized water (DDDW). Add 29.2 ml of 0.1 M HCl to 50 ml of 0.1 M Tris and dilute to 100 ml with DDDW.

2. 10^{-4} M HRP solution
Transfer 20 mg of HRP into 5 ml of double-distilled water in a small vial. Prepare it on the day of use.
3. Scopoletin stock solution (e.g. 160 μ M)
Transfer 15.4 mg of scopoletin crystals to a 500 ml volumetric flask and add 400 ml of DDDW. Dissolve it in a ultrasonicator until complete dissolution. Dilute to 500 ml with DDDW.
4. H_2O_2 stock solution (e.g. ca. 10 mM)
Transfer 1 ml of 30 % H_2O_2 to 1 l of DDDW in a 1-l marked HDPE (High Density PolyEthylene) bottle. The concentration of H_2O_2 should be periodically determined by iodometric method or sodium thiosulfate titration method. Keep it in a refrigerator.
5. H_2O_2 standard solution (e.g. 1 - 10 μ M)
Dilute H_2O_2 stock solution with DDDW using volumetric flasks immediately before use.

1.3 Equipments

1. Perkin-Elmer 650-10S Fluorescence Spectrophotometer equipped with digital display and Zenon Lamp. The unit should be warmed for about 30 minutes. Set excitation and emission wavelength to 385 nm and 460 nm for best result.

1.4 Stoichiometry between Scopoletin and H_2O_2

Preliminary H_2O_2 determinations are necessary to optimize the amount of scopoletin added, the volume of the H_2O_2 spikes and the fluorometer sensitivity for the best range. The reaction stoichiometry between scopoletin and H_2O_2 is 1 mole : 1 mole assuming no interferences by other elements in the sample. However, the final concentration of scopoletin in the sample should be at least 120 % of the concentration of H_2O_2 in the sample for the complete reaction between scopoletin and H_2O_2 . In addition, scopoletin concentrations should be adjusted to bracket the concentration of H_2O_2 standard spikes. The fluorescence of the calculated amount of scopoletin

should be adjusted to 100 fluorescence unit using range and sensitivity knobs. The optimum volume of the H_2O_2 spikes should be calculated to quench approximately half of the scopoletin fluorescence for the best result.

1.5 Sample Collection

1. Mount a Gelman A/E 47 mm glass fiber filter (nominal pore size $0.7 \mu\text{m}$) on to a filter holder with syringe.
2. Filter the sample through the filter.
3. Discard first portion of the filtered sample and then collect with quick manner.

1.6 Determination of H_2O_2

1. Zero the fluorometer using zero adjustment knob after block the light path with the equipped shutter.
2. Transfer 3 ml of the filtered sample into the fluorometric cell with an eppendorf pipette.
3. Add 0.1 ml of 0.1 M tris buffer with an eppendorf pipette, mix for 5 seconds with a vortex mixer, and then measure the background fluorescence (F_b).
4. Add scopoletin solution (e.g., 10 - 20 μl of 160 μM or 80 μM) to the sample, mix for 5 seconds, and then measure the scopoletin fluorescence (F_s).
5. Add 5 μl of HRP, mix for 5 seconds, wait 3 minutes for the complete reaction between H_2O_2 , HRP and scopoletin. Measure the fluorescence (F_h).
5. At least two spikes of H_2O_2 standard solution should be made. Record the fluorescence after each addition (F_{st}).

1.7 Blank Determination

Treat blanks (DDDW or artificial seawater) identically to samples.

1.8 Calibration

A calibration curve is prepared for each sample by internal standard additions.

1.9 Treatment of Raw Data

Raw data :

Volume of sample (ml) - V_{sm}

Volume of scopoletin added (ml) - V_s

Volume of HRP added (ml) - V_h

Volume of standard H_2O_2 solution spiked (ml) - V_{st}

Unknown concentration of H_2O_2 in sample (μM) - C_{sm}

Concentration of scopoletin added (μM) - C_s

Concentration of standard H_2O_2 solution spiked (μM) - C_{st}

F_b - background fluorescence

F_s - fluorescence after the addition of scopoletin

F_h - fluorescence after the addition of HRP

F_i - fluorescence after the addition of H_2O_2 standard

$$F_s = k \cdot (V_s \cdot C_s) \cdot (V_s + V_{sm})^{-1} + F_b$$

$$F_h = k \cdot (V_s \cdot C_s - n \cdot V_{sm} \cdot C_{sm}) \cdot (V_s + V_{sm} + V_h)^{-1} + F_b$$

$$F_i = k \cdot (V_s \cdot C_s - n \cdot V_{sm} \cdot C_{sm} - i \cdot n \cdot C_{st} \cdot V_{st}) \cdot (V_s + V_{sm} + V_h + i \cdot V_{st})^{-1} + F_b$$

n = stoichiometry between scopoletin and H_2O_2

Calculation :

Arrange ($F_s - F_i$) as following

$$(F_s - F_i) \cdot (V_s + V_{sm} + V_h + i \cdot V_{st}) = k \cdot ((V_s \cdot C_s \cdot V_h) \cdot (V_s + V_{sm})^{-1} + n \cdot V_{sm} \cdot C_{sm}) \\ + k \cdot ((V_s \cdot C_s \cdot V_{st}) \cdot (V_s + V_{sm})^{-1} + n \cdot V_{st} \cdot C_{st}) \cdot i$$

i.e. $Y_i = A + B \cdot i,$

where $Y_i = (F_s - F_i) \cdot (V_s + V_{sm} + V_h + i \cdot V_{st})$

$$A = k \cdot ((V_s \cdot C_s \cdot V_h) \cdot (V_s + V_{sm})^{-1} + n \cdot V_{sm} \cdot C_{sm})$$

$$B = k \cdot ((V_s \cdot C_s \cdot V_{st}) \cdot (V_s + V_{sm})^{-1} + n \cdot V_{st} \cdot C_{st})$$

Determine A (intercept) and B (slope) through regression analysis.

Since $(F_s - F_b) = k \cdot (V_s \cdot C_s) \cdot (V_s + V_{sm})^{-1}$

$$A = (F_s - F_b) \cdot V_h + k \cdot n \cdot V_{sm} \cdot C_{sm}$$

$$B = (F_s - F_b) \cdot V_{st} + k \cdot n \cdot V_{st} \cdot C_{st}$$

Rearrange A and B,

$$A - (F_s - F_b) \cdot V_h = k' \cdot V_{sm} \cdot C_{sm} \quad \dots (1)$$

$$B - (F_s - F_b) \cdot V_{st} = k' \cdot V_{st} \cdot C_{st} \quad \dots (2)$$

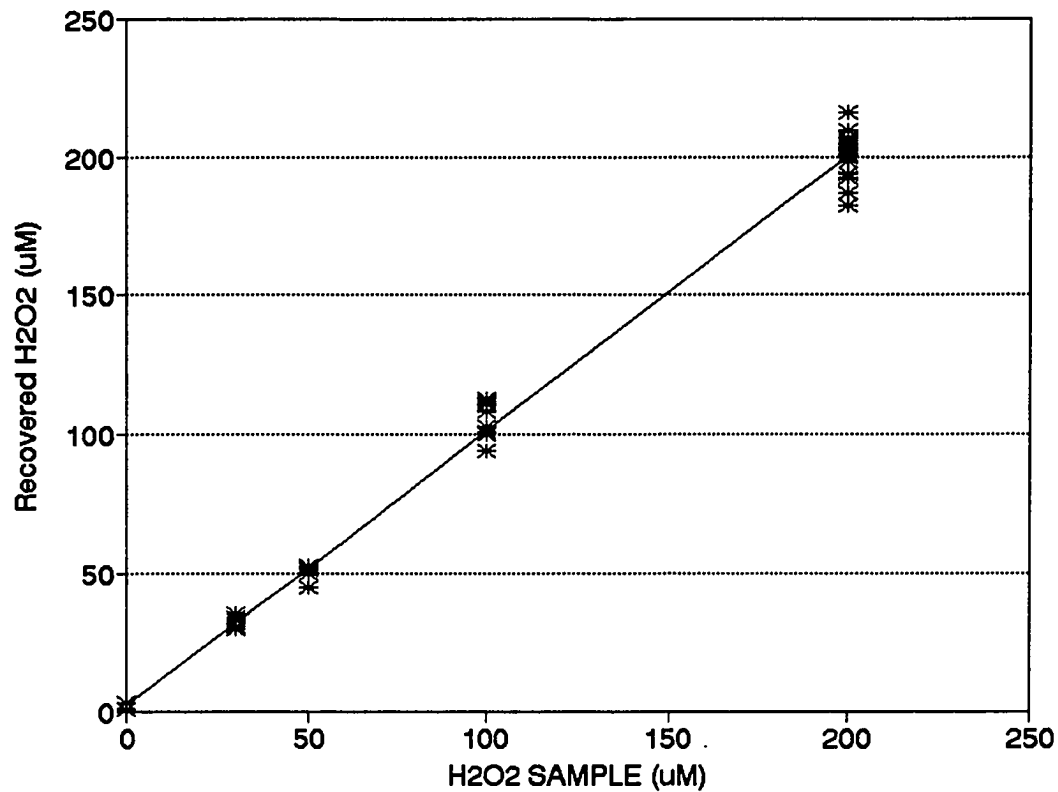
where $k' = k \cdot n$

Divide eqn 1 with eqn 2

Then,

$$C_{sm} = (A - (F_s - F_b) \cdot V_h) \cdot (B - (F_s - F_b) \cdot V_{st})^{-1} \cdot (V_{st} \cdot C_{st}) \cdot V_{sm}^{-1}$$

Notes : The accuracy of H₂O₂ analysis was tested by determining a recovery of the added H₂O₂. The recovery rate was more than 95 % (Fig. A-2).



Regression Output:

Constant	2.580334
Std Err of Y Est	7.220128
R Squared	0.991304
No. of Observations	41
Degrees of Freedom	39
X Coefficient(s)	0.989934
Std Err of Coef.	0.014846

Fig. A-2. A determination of H₂O₂ recovery, and the result of regression analysis.

**APPENDIX B : TIME COURSE DATA FOR H₂O₂ NON-PRODUCERS
AND CONTROL BOTTLES**

<i>Amphidinium carterae</i>			<i>Chaetoceros flexosus</i>			<i>Chaetoceros simplex</i>		
Time	CTR	CELL	Time	CTR	CELL	Time	CTR	CELL
0	0	0	0	0	0	0	0	0
30	3	5	30	5	6	30		4
60	7	8	60	8	10	60	<0	<0
120	5	4	120	16	5	120	<0	<0
180	7	11	180	<0	10	180		<0
240	10	14	240	6	9	240	<0	<0
300	11	8	300	10	<0	320		<0
360	7	6	360	4	<0			

<i>Thalassiosira oceanica</i>			<i>Skeletonema costatum</i>			<i>Synechococcus sp.</i>		
Time	CTR	CELL	Time	CTR	CELL	Time	CTR	CELL
0	0	0	0	0	0	0	0	0
30	5	11	60	9	<0	30	11	<0
60	2	14	90	10	<0	60	11	<0
90	2	14	150	8	<0	180	10	<0
150	4	14	210	15	<0			
			270	10	<0			

Notes

1. Time is in the unit of minutes.
2. CTR means the control bottle without cells, i.e., contains only 0.45 μm filtrate of cell suspension.
3. CELL means the bottle with cell suspension.
4. Numbers under CTR and CELL are in the unit of nanomolar.

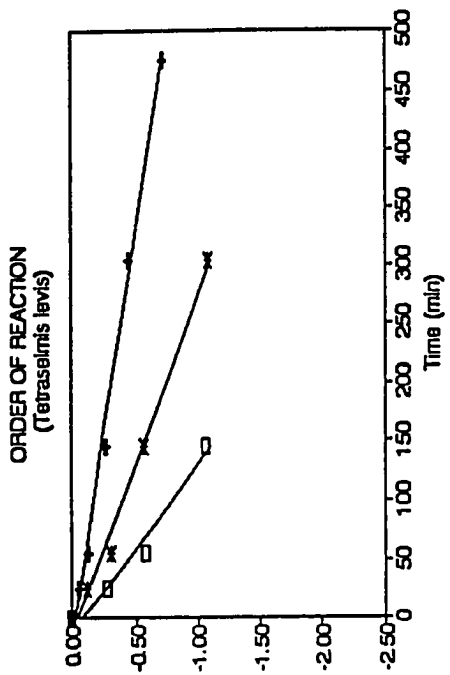
APPENDIX C : TIME COURSE DATA FOR H₂O₂ DECOMPOSITION STUDIES

C-1: Decomposition Studies with Phytoplankton

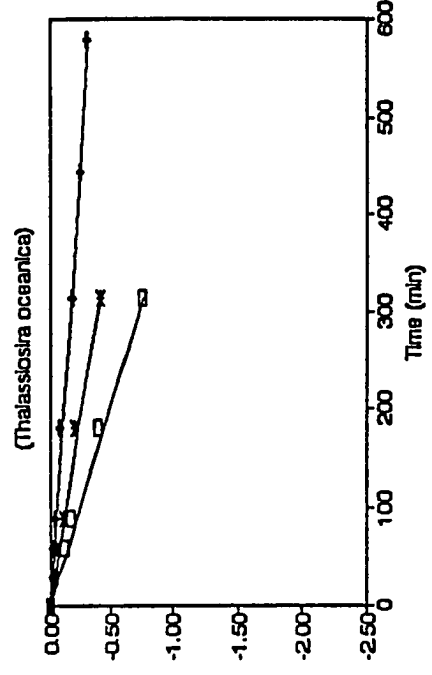
(Solid lines represent regression lines)

C-2: Decomposition Studies with Control Bottles

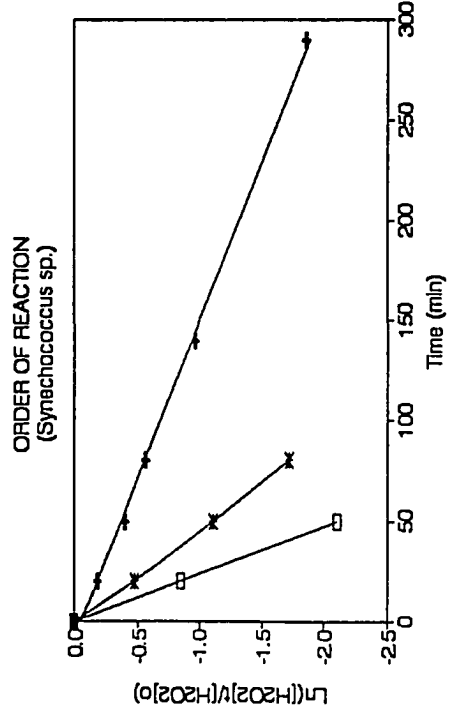
C-1



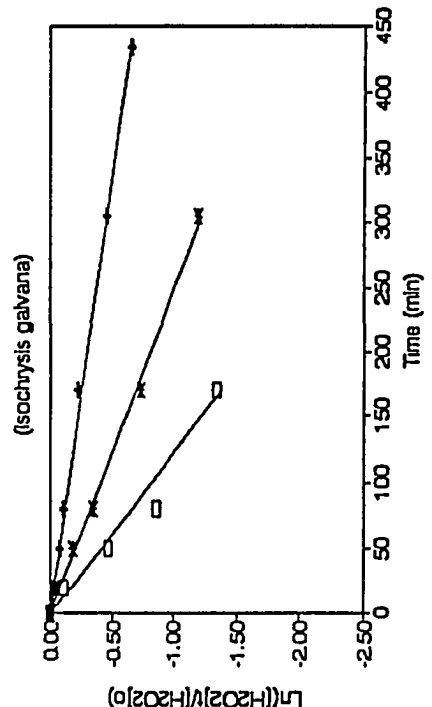
15 ug/L x 46 ug/L x 83 ug/L



22 ug/L x 58 ug/L x 114 ug/L

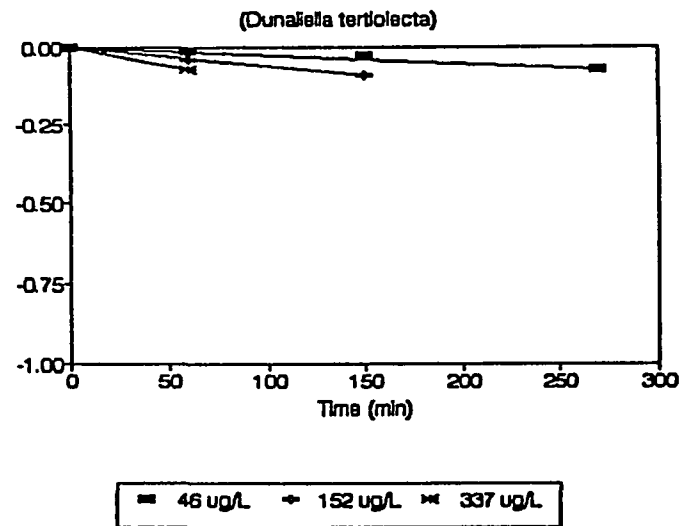
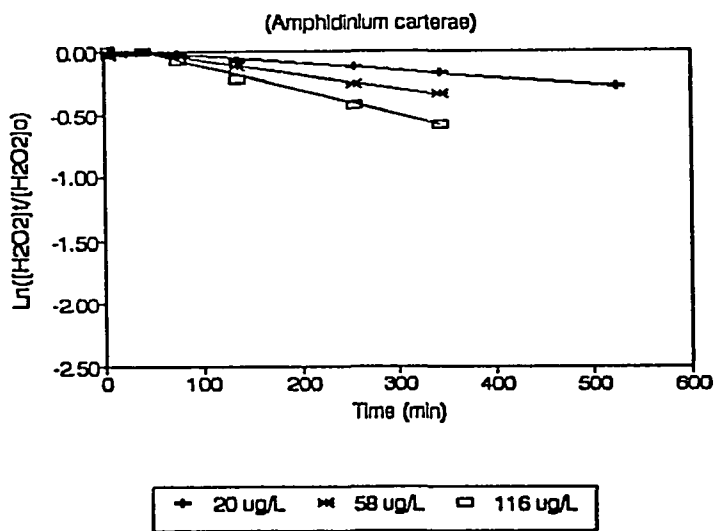
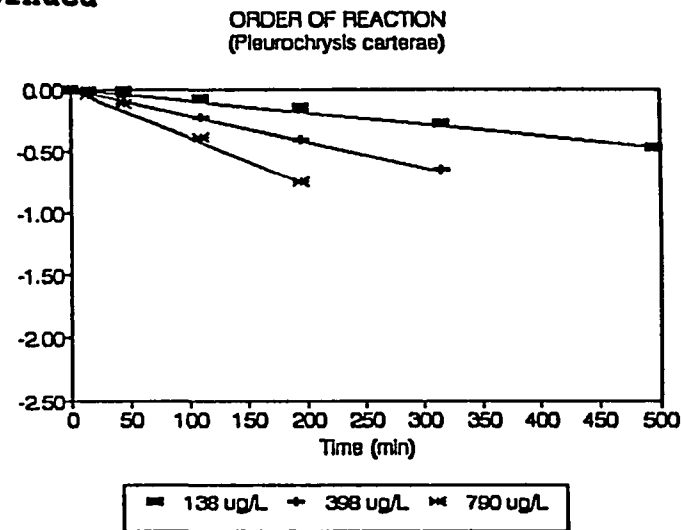
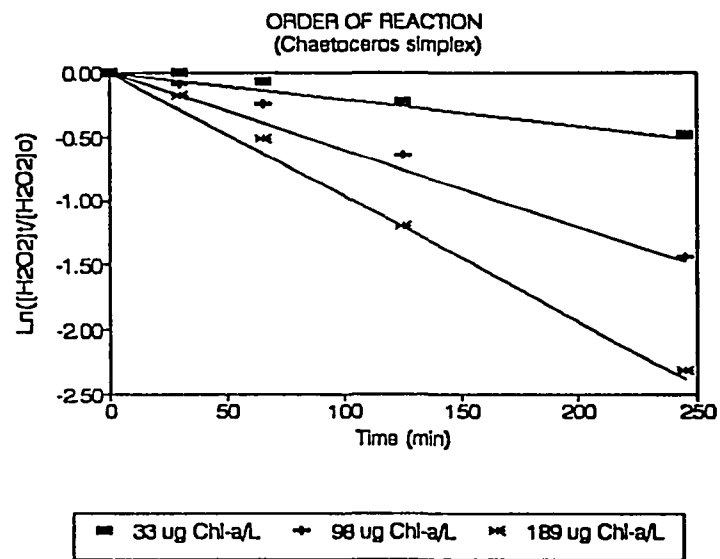


14 ug/L x 50 ug/L x 98 ug/L



50 ug/L x 142 ug/L x 288 ug/L

- Continued



C-2

 1) Media only (no cells)

Time (min)	0	30	90	180
f/20 (+TM)	600	605	599	603
f/20 (-TM)	608	608	599	602
f/2 (+TM)	615	613	596	592
f/2 (-TM)	617	602	595	602

 2) Filtrates of cell suspensions

Time (min)	0	30	60	140	240
a) H ₂ O ₂ (nM)	604	608	608	608	609

Time (min)	0	5	35	65	125
b) H ₂ O ₂ (nM)	903	908	900	895	891
c) H ₂ O ₂ (nM)	885	750	110	50	20

Notes

- +TM; Addition of trace metals according to the recipes of Guillard and Ryther (1962).
-TM; No addition of trace metals
- 0.2- μ m filtrate of *Synechococcus* sp.
 - 1- μ m filtrate of *Skeletonema costatum*
 - Unfiltered (*S. costatum*)
- Initial conc of H₂O₂ in b) and c) are 900 nM.

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