

To the Graduate Council:

I am submitting herewith a dissertation written by James Jeffrey Morris entitled “The ‘Helper’ Phenotype: A Symbiotic Interaction Between *Prochlorococcus* and Hydrogen Peroxide Scavenging Microorganisms.” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Erik R. Zinser, Major Professor

We have read this dissertation
and recommend its acceptance:

Steven W. Wilhelm

Alison Buchan

Gladys Alexandre

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

The ‘Helper’ phenotype:

**A Symbiotic Interaction Between *Prochlorococcus* and
Hydrogen Peroxide Scavenging Microorganisms**

**A dissertation presented for the doctor of philosophy degree
The University of Tennessee, Knoxville**

James Jeffrey Morris

May 2011

To Milton C. Morris, who taught me that it's okay for smart people to play in the dirt.



*July, 1977
Fayetteville, Tennessee*



ACKNOWLEDGEMENTS

I would like to acknowledge a number of “helpers” who have contributed a great deal of assistance in the conduct of the scientific work contained herein. First, of course, my advisor, Erik Zinser, and my committee members Alison Buchan, Elizabeth Mann, Gladys Alexandre, and especially Steve Wilhelm, without whom I would not have done my graduate studies at the University of Tennessee. The chair of our department, Jeff Becker, deserves special recognition for the advice and support he has given me over the years, including helping me out with extra work opportunities during some financially difficult moments. I would also like to thank the co-authors of each of the chapters, as well as the crews of the research vessels *R/V Kilo Moana*, *R/V New Horizon*, *R/V Cape Hatteras*, *R/V Hugh Sharp*, and *CCGS Limnos* for keeping me alive while doing field work. I am exceedingly grateful for the early help and support of Bill Cooper at UC Irvine, who not only patiently helped me learn how to measure hydrogen peroxide but also arranged for me to get a generous aliquot of hard-to-synthesize acridinium ester reagent – in my first semester of graduate school. The Phycological Society of America deserves recognition for the resources they have given me, both for my research and for travel to their annual meetings where I have made many friends and contacts. Last, I would like to acknowledge the technicians whose hard work is often overlooked in writings such as these: Steve Allman at Oak Ridge National Laboratory, John Dunlap at the UT Microscopy Center, Robin Kirkegaard who helped me in my first years in the Zinser lab, and Joe May at the UT sequencing facility.

Of course, it should go without saying that my family – both my wife and children and my extended family – have been instrumental in making my doctorate possible. My parents, in particular, have had the utmost patience with my peculiar interests over the years, and without their material and emotional support, gods only know where I would be today. To all of you, my love, and my heartfelt thanks.

ABSTRACT

The unicellular cyanobacterium *Prochlorococcus* is the numerically dominant photosynthetic organism throughout the temperate and tropical open oceans, but it is difficult to grow in pure cultures. We developed a system for rendering spontaneous streptomycin-resistant mutants of *Prochlorococcus* axenic by diluting them to extinction in the presence of “helper” heterotrophic bacteria, allowing them to grow to high cell concentrations, and then killing the helpers with streptomycin. Using axenic strains obtained in this fashion, we demonstrated that *Prochlorococcus* experiences a number of growth defects in dilute axenic culture, including reduced growth rate, inability to form colonies on solid media, and higher incidence of mortality (i.e., catastrophic failure of liquid cultures). These defects were eliminated when *Prochlorococcus* was grown in co-culture with a phylogenetically diverse array of helper bacteria. The primary mechanism of helping was enzymatic removal of hydrogen peroxide (HOOH) from the culture medium. Axenic *Prochlorococcus* cultures were profoundly sensitive to HOOH additions in comparison with reported tolerance levels for all other wild-type aerobic bacteria, but in co-culture their resistance was similar to that of the helpers. Neither is dependence on helpers limited to the laboratory. Sterile-filtered seawater exposed to sunlight accumulated enough HOOH in 24h to kill ecologically relevant cell concentrations of *Prochlorococcus*. We also refined a method for delivering HOOH at a defined, steady rate using the buffer HEPES to more accurately simulate the steady accumulation of HOOH in natural waters. Even at the lowest production rates that could sustain the in situ HOOH concentration in the ocean, HEPES-generated HOOH was lethal to *Prochlorococcus*; again, co-culture with helpers prevented this effect. We speculate on the ecological consequences of *Prochlorococcus*’ dependency on other organisms for survival, as well as the evolutionary forces that have led to this lack of self-sufficiency.

TABLE OF CONTENTS

DEDICATION	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT.....	iv
CHAPTER 1: Hydrogen Peroxide in the Marine Environment	1
I. Introduction.....	3
II. Reactive oxygen (bio)chemistry: from O ₂ to H ₂ O.....	4
III. Sources of environmental HOOH.....	8
A) Biological sources	8
B) Abiotic sources	12
IV. Sinks of environmental HOOH.....	13
V. Effects of HOOH on the aquatic chemical environment	15
VI. Effects of HOOH on aquatic organisms	16
A) Targets.....	16
B) Defenses	19
VII. Evidence of HOOH-induced stress in aquatic ecosystems.....	20
VIII. HOOH and the evolution of oxygenic photosynthesis	23
IX. Conclusion: <i>Prochlorococcus</i> and the Catch-22 of phototrophy.....	25
CHAPTER 2: Facilitation of Robust Growth of <i>Prochlorococcus</i> Colonies and Dilute Liquid Cultures by “Helper” Heterotrophic Bacteria	27
I. Introduction.....	30
II. The “helper” phenotype of heterotrophic bacteria	30
III. Elimination of the “helpers” to obtain axenic <i>Prochlorococcus</i>	31
IV. The helper phenotype in liquid media	33
V. The helping phenomenon is common in marine heterotrophs	35
VI. The helping phenomenon is linked to oxidative stress	37
VII. Conclusion.....	39
CHAPTER 3: Light-driven Hydrogen Peroxide Production by Biological Buffers in Culture Media.....	40
I. Introduction.....	43
II. Measurement of HOOH	44
III. Kinetics of HOOH production in sterile media.....	47
A) Methods	47
B) Results.....	48
IV. Effects of buffers on growth of <i>Prochlorococcus</i>	51
A) Methods	51
B) Results.....	54
V. HEPES as a steady-state HOOH generator	56
VI. Discussion	59

CHAPTER 4: Dependence of the Cyanobacterium <i>Prochlorococcus</i> on Hydrogen Peroxide Scavenging Microbes for Growth at the Ocean's Surface	62
I. Introduction.....	65
II. Results	67
A) HOOH removal is necessary and sufficient for the helping phenotype	67
B) Ecologically relevant levels of HOOH inhibit axenic <i>Prochlorococcus</i>	77
C) HOOH stress results in catastrophic loss of cell integrity and photosynthetic capacity	83
D) Phylogenetic distribution of helper dependency	87
III. Discussion	90
A) HOOH removal is the primary mechanism responsible for the helper phenotype.....	90
B) Impact of helpers on <i>Prochlorococcus</i> ecology	91
C) Evolutionary impact of the helper- <i>Prochlorococcus</i> interaction	93
IV. Methods	95
A) Field sites and methods.....	95
B) Cultures and culture conditions.....	97
C) Quantification of <i>Prochlorococcus</i> and helpers	98
D) Transposon mutagenesis.....	99
E) HOOH exposure experiments.....	100
F) Chemical and enzymatic assays	100
G) Effects of HOOH on <i>Prochlorococcus</i> photophysiology and morphology	101
 CHAPTER 5: The Ecological and Evolutionary Implications of the Helper Phenotype ..	103
I. Introduction.....	106
II. Expression of oxidative stress-related genes in <i>Prochlorococcus</i>	106
III. Field acclimation experiments	113
IV. The catalase-deficient biosphere	116
V. Mutualists, commensals, or parasites?	123
VI. The Black Queen Hypothesis	128
VII. Final thoughts	131
 BIBLIOGRAPHY	133
 APPENDIX: Testing the Paradigm of Natural Hydrogen Peroxide Dynamics.....	161
I. Introduction.....	163
II. Methods.....	164
III. In situ HOOH dynamics	167
IV. Empirical tests of a mathematical model of the HOOH paradigm.....	176
V. Potential sources of deviation from the model.....	180
VI. Conclusions.....	183
 VITA.....	185

LIST OF TABLES

Table 1.1.	Equations describing the formation and degradation of ROS	5
Table 2.1.	Streak plate assay results.....	36
Table 3.1.	Buffers studied in this work.....	45
Table 4.1.	Strains used in this study	68
Table 4.2.	Results of helper assays in liquid Pro99	71
Table 4.3.	Field sites and HOOH methods	80
Table 4.4.	Estimated effects of <i>katG</i> loss from a hypothetical catalase-positive ancestor of <i>Prochlorococcus</i> MED4 on cell quotas for selected nutrients.....	96
Table 5.1.	Genes implicated in the HOOH response of <i>Prochlorococcus</i> MED4.....	108
Table 5.2.	Nature of the helper:beneficiary symbiosis for various trophic pairings	127
Table A.1.	Locations and dates of sampling.....	165
Table A.2.	Comparison of model predictions with in situ rate data	179

LIST OF FIGURES

Figure 1.1.	The reactive oxygen cycle of aquatic environments.....	9
Figure 1.2.	The sources and sinks of HOOH in and above the open ocean	10
Figure 1.3.	Propagation of lipid peroxides.....	18
Figure 1.4.	A scenario for HOOH involvement in the evolution of oxygenic photosynthesis	24
Figure 2.1.	Helper heterotrophs facilitate <i>Prochlorococcus</i> growth on solid media.....	32
Figure 2.2.	Growth of MIT 9215 in liquid Pro99 medium with or without helper heterotrophs	34
Figure 3.1.	Formation of HOOH in media containing various Good's buffers.....	50
Figure 3.2.	Effects on k_{HOOH} from the alteration of three parameters	52
Figure 3.3.	Kinetics and yield of buffer-driven HOOH formation	53
Figure 3.4.	Effects of buffers on the growth rate of <i>Prochlorococcus</i> MIT9215 _{AX}	55
Figure 3.5.	HEPES as a steady-state HOOH generator	58
Figure 4.1.	HOOH removal by <i>Prochlorococcus</i> spp. and <i>Alteromonas</i> sp. EZ55.....	69
Figure 4.2.	Influence of <i>Prochlorococcus</i> UH18301 and <i>Alteromonas</i> EZ55 on Pro99 medium chemistry.....	72
Figure 4.3.	Photoinactivation of purified catalase.....	73
Figure 4.4.	Removal of HOOH from media facilitates <i>Prochlorococcus</i> growth.....	75
Figure 4.5.	Alkaline phosphatase activity in EZ55-treated media.....	76
Figure 4.6.	Effects of chronic HOOH exposure on <i>Prochlorococcus</i> UH18301.....	78
Figure 4.7.	Removal of HOOH is necessary for <i>Prochlorococcus</i> ' survival at the ocean's surface	79
Figure 4.8.	Effects of HOOH exposure on <i>Prochlorococcus</i> ecotypes.....	82
Figure 4.9.	<i>Prochlorococcus</i> cell viability during HOOH exposure in unamended sterile seawater under low light.....	84
Figure 4.10.	Cell envelope and photophysiological effects of HOOH on <i>Prochlorococcus</i> grown under low light.....	85
Figure 4.11.	Photophysiological response of <i>Prochlorococcus</i> ecotypes following exposure to SMC (0.8 μM) HOOH exposure.....	86
Figure 4.12.	Growth at 0.2 μM HOOH as a function of helpers and light intensity for cultures with very dilute (~ 100 cells mL^{-1}) initial cell concentrations.....	89

Figure 5.1.	Diel expression of some oxidative stress-related genes for <i>Prochlorococcus</i> MED4	111
Figure 5.2.	Influences on HOOH degradation rates in the ocean	114
Figure 5.3.	SSU-rDNA phylogeny of microbes with the helper phenotype	117
Figure 5.4.	Catalase is much more common in cultured organisms compared to the open ocean	120
Figure 5.5.	Growth kinetics of helpers in <i>Prochlorococcus</i> seawater media.....	124
Figure A.1.	In situ HOOH concentrations along the WP2 transect	168
Figure A.2.	In situ HOOH concentrations along the DCM08 transect.....	169
Figure A.3.	In situ HOOH concentrations along the BC09 transect	170
Figure A.4.	In situ HOOH concentrations in Chesapeake Bay.....	171
Figure A.5.	In situ surface HOOH concentrations in Lake Erie	172
Figure A.6.	Diel fluctuations for in situ HOOH concentrations and dark HOOH dynamics.....	174
Figure A.7.	Mathematical model of the HOOH paradigm	178
Figure A.8.	Potential causes of conflict with the steady-state model	182

LIST OF ATTACHMENTS

File 1. JCVI-CMR catalase sequence database.....	Supplemental File 1.fasta
File 2. JCVI-CMR <i>rpsL</i> sequence database.....	Supplemental File 2.fasta
File 3. Taxonomic assignments of GOS BLAST hits.....	Supplemental File 3.csv
File 4. GOS catalase BLAST hits.....	Supplemental File 4.csv
File 5. GOS <i>rpsL</i> BLAST hits.....	Supplemental File 5.csv

CHAPTER 1
Hydrogen Peroxide in the Marine Environment

ABSTRACT

Hydrogen peroxide (HOOH) is generated in nature through a number of primarily photochemical processes. Although HOOH is a known toxin, and ubiquitous in natural waters, it has largely been overlooked by biologists due to its generally low in situ concentrations and the relatively high level of resistance exhibited by most cultured microbes. However, there is ample reason to suspect that HOOH may be of ecological importance. By altering the availability of important resources such as dissolved organic matter and trace metals, HOOH may provide a benefit to microbes, but it may also create stresses due to its ability to alter biological molecules. This chapter reviews what is known about the origins and fates of HOOH in the environment, as well as the conditions under which this common molecule may be beneficial or harmful to natural populations.

I. Introduction

The vast gyres of the open oceans cover an enormous fraction of Earth's surface area. Despite absorbing a sizable fraction of the total solar energy available to the planet, photosynthetic primary productivity in this biome is low per unit area [1]. For this reason, the open oceans have been described as “watery deserts.” Phytoplankton productivity in the open ocean is strongly nutrient-limited, mostly by N and/or P [2,3], although in some areas Fe may be more important [4,5,6]. In many areas, strong, year-round thermohaline stratification prevents vertical mixing of surface and deep waters, isolating sunlit regions from nutrient-rich sediments. Coriolis-driven movement of bulk waters and freshwater inflow lead to strong density gradients separating gyre waters from terrestrially-fertilized coastal waters. Thus, the surface, light-exposed stratum (the euphotic zone) of the gyres is, for the most part, permanently oligotrophic [7].

The combination of near-constant temperature regimes and predictably low nutrient concentrations has led to a pervasive conception of the open ocean as a “stable” environment. Supporting this description is the observation that the microbial communities inhabiting the open ocean are clearly dominated by a small number of taxa such as *Prochlorococcus* and *Pelagibacter* [8,9,10]. The remarkable stability of the gyres is often cited as a major driving force in the evolution of open ocean organisms, explaining, for instance, the reductive evolution apparent in both of these genera [11,12].

However, closer inspection reveals that the gyres are not as immutable as they first appear. Nutrient levels can change rapidly, both locally (e.g., through atmospheric deposition of terrestrial dust [13,14]) and regionally (e.g., by deep mixing from storms [15]). The photochemistry and photobiology of the ocean are also quite complex and clearly will vary dramatically due to changes in cloud cover and season. The impact of heterogeneously distributed, relatively rare organisms – “keystone” species – such as nitrogen fixing algae [16,17] and baleen whales [18] also cannot be ignored. In the wake of these stochastic perturbing forces, it is clear that the apparent stability of the open oceans is a holistic illusion, and local conditions are actually quite malleable. To a large degree, the chemical stability of the open ocean is a product of biological activity rather than abiotic stasis [19]. Thus, discovering the physiological

and evolutionary engines preserving marine homeostasis is an important ongoing task for oceanographers.

In this review chapter we will discuss one mutable element of seawater chemistry that is not widely acknowledged in aquatic biology, namely the formation and degradation of hydrogen peroxide (HOOH) in light-exposed natural water. First, we will discuss the basics of reactive oxygen chemistry, particularly as they apply to this system. We will then review research on the sources and sinks of HOOH in marine water, with a lesser focus on other aquatic environments. Finally, we will discuss what is known about the chemical and biological effects of HOOH in natural waters, with a focus on the impacts HOOH has on aquatic microorganisms. In conclusion, and to segue into the remainder of this dissertation, we will discuss ideas about the influence HOOH has had on the evolution of photosynthesis.

II. Reactive oxygen (bio)chemistry: from O₂ to H₂O

Molecular dioxygen (O₂) comprises approximately 20% of the modern atmosphere. Its solubility in water follows Henry's law, and is a function of its partial pressure at sea level and water temperature. O₂ solubility increases as temperature decreases, usually leading to higher availability in polar and deep ocean waters [7]. Because of its strongly positive reduction potential, O₂ is the most energetically attractive terminal electron acceptor for organisms inhabiting aerobic environments [20]. O₂ is also a primary product of algal and plant photosynthesis, which uses electrons obtained from splitting water molecules (via the Mn-containing oxygen evolving complex (OEC) enzyme) to regenerate the photosystem II reaction center (RCII) following the light-driven reduction of the primary photosynthetic electron carrier plastoquinone (PQ) (eq. 1 and 2, Table 1.1).

Ultimately, almost all O₂ derives from photosynthetic water-splitting. Earth's atmosphere was essentially free of O₂ until the evolution of oxygenic photosynthesis in cyanobacteria [21,22] more than 3 billion years ago [23]. As much of the biosphere experienced increasing levels of O₂ from this point onwards, organisms not only gained the energetic advantages of using O₂ metabolically, but also had to face the tendency of O₂ to enter into various "activated" states capable of nonspecifically oxidizing many chemical species, including all of the macromolecules responsible for cellular structure and function (e.g., nucleic acids,

Table 1.1. Equations describing the formation and degradation of ROS.

Type	Equation	Equation Number
B ¹	$\text{RCII} + 2h\nu + 2\text{H}^+ + \text{PQ} \longrightarrow \text{RCII}^{2+} + \text{PQH}_2$	(1)
B	$\text{H}_2\text{O} + \text{RCII}^{2+} \xrightarrow{\text{OEC}} \frac{1}{2}\text{O}_2 + 2\text{H}^+ + \text{RCII}$	(2)
A ²	$\cdot\text{O}_2\cdot + \text{D} \xrightarrow{h\nu} \cdot\text{O}_2^- + \text{D}^+$	(3) ³
A	$\cdot\text{O}_2^- + \text{H}^+ \longrightarrow \text{HO}_2\cdot$	(4)
A/B ⁴	$2\cdot\text{O}_2^- + 2\text{H}^+ \xrightarrow{\text{spont, SOD}} \text{HOOH}$	(5)
A/B	$2\text{HOOH} \xrightarrow{\text{spont, catalase}} \text{H}_2\text{O} + \cdot\text{O}_2\cdot$	(6)
B	$\text{HOOH} + \text{DH}_2 \xrightarrow{\text{peroxidase}} 2\text{H}_2\text{O} + \text{D}$	(7)
A	$\text{HOOH} + \text{Fe}^{2+} \longrightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$	(8)
A	$\text{Fe}^{3+} + \text{DOM} \xrightarrow{h\nu} \text{Fe}^{2+} + \text{DOM}^+$	(9)
A/B	$\cdot\text{OH} + \text{DH} \longrightarrow \text{H}_2\text{O} + \cdot\text{D}^+$	(10)
A/B	$\cdot\text{O}_2\cdot \xrightarrow{+e^-} \cdot\text{O}_2^- + 2\text{H}^+ \xrightarrow{+e^-} \text{HOOH} \xrightarrow{+e^-} \text{OH}^- + \cdot\text{OH} \xrightarrow{+e^-} \text{H}_2\text{O}$	(11)
A	$\text{H}_2\text{O} \xrightarrow{h\nu} \cdot\text{OH} + \text{H}$	(12)
A	$2\cdot\text{OH} \xrightarrow{\text{spont}} \text{HOOH}$	(13)

¹ B, Enzymatic (biological) reaction

² A, Abiotic reaction

³ D, electron donor

⁴ A/B, Abiotic reaction that may be enzymatically catalyzed

proteins, and lipids) [24]. This section will cover the chemistry of O₂ activation, including both strictly abiotic processes and enzymatic reactions.

O₂ is unusual in that its most stable state is a diradical, with 2 degenerate (i.e., of equal energies) molecular orbitals containing unpaired electrons [25]. This configuration, called a “triplet” state as the spins of the electron pair are uncoupled and can therefore exist in three possible combinations, is unusual. Most molecules are only stable in the “singlet” state, with all molecular orbitals filled and, therefore, having only a single possible combination of spins. There is a substantial energy barrier preventing reaction of triplet systems with singlets, so the O₂ diradical is very stable in comparison to the huge majority of free radicals. However, O₂ can become “activated” by one of two pathways to form a variety of “reactive oxygen species” (ROS). In the first, O₂ can absorb energy from electromagnetic radiation, often indirectly through light-absorbing compounds such as pigments, leading to the collapse of its unfilled degenerate orbitals and formation of a highly reactive singlet state (i.e. singlet oxygen, ¹O₂) [26]. ¹O₂ is a very important toxic by-product of photosynthetic light harvesting, and many of the accessory pigments found in plants and algae are effective scavengers of this compound [27]. Importantly, ¹O₂ is a *physically* excited state of O₂ rather than a *chemically* unique species. Because its half-life is too short to affect structures distant from its point of origin, and because it bears no causal relationship to HOOH, we will not consider it further in this review.

In the second pathway, O₂ may be *chemically* reduced by another molecule that has been excited and entered a triplet state. Like the formation of ¹O₂, this process can begin with the absorption of light energy by a compound [28], although it can also be facilitated in the active sites of enzymes such as xanthine oxidase [29]. Typically, this pathway begins with the transfer of a single electron from the excited compound to O₂ resulting in the formation of the superoxide anion radical O₂⁻ (eq. 3, Table 1.1), which may also exist in an uncharged protonated state as HO₂ (eq. 4, Table 1.1).

A subsequent single-electron transfer to O₂⁻ (or HO₂) produces HOOH. Perhaps the most common abiotic route to HOOH formation is the disproportionation of two O₂⁻ molecules (eq. 5, Table 1.1), resulting in the oxidation of one to O₂ and the reduction of the other to HOOH [25]. This reaction proceeds spontaneously, but in biological systems it is often accelerated by the enzyme superoxide dismutase (SOD).

Since it lacks the radical character of O_2 and O_2^- , HO_2H is quite stable on its own. The disproportionation of two HO_2H molecules to H_2O and O_2 (eq. 6, Table 1.1) occurs spontaneously at a slow rate, but in biological systems is commonly catalyzed by hydroperoxidase enzymes called catalases, many of which have the shortest turnover times known in biochemistry [30]. “Peroxidase” enzymes accomplish a similar reaction, but replace one of the HO_2H molecules with a secondary electron donor (e.g., ascorbate, glutathione, or thioredoxin, eq. 7, Table 1.1) [31]. Overall, electron flow from O_2 through catalase is cyclical (O_2 is regenerated by the enzyme), whereas peroxidase activity results in net O_2 consumption.

In the absence of biological hydroperoxidases, the most common route of HO_2H loss is the Fenton reaction [32], in which HO_2H is heterolytically cleaved by ferrous iron to form ferric iron, hydroxide ion, and hydroxyl radical ($\cdot OH$) (eq. 8, Table 1.1). The rate of the Fenton reaction in natural systems is likely limited by the availability of free $Fe(II)$, which is typically quite dilute (often $<10^{-9}$ M in seawater [33]) due to its rapid oxidation at higher pH values [34], the low solubility of $Fe(III)$ in the presence of O_2 [35], and the presence of strong chelators in natural waters [36]. However, the reduction of $Fe(III)$ by photochemical reactions involving dissolved organic matter (DOM) [37] maintains higher $Fe(II)$ concentrations than predicted by redox equilibria (eq. 9, Table 1.1). Thus, the rate of the Fenton reaction is controlled by this photochemical step, and most discussions of Fenton chemistry from an environmental perspective speak of the “photo-Fenton reaction” [38]. It has been suggested that direct reduction of $Fe(II)$ by O_2^- -- the Haber-Weiss cycle -- is important in perpetuating Fenton chemistry in natural systems, but the very existence of this reaction, much less its role in natural waters, is controversial [39,40].

$\cdot OH$ is among the most reactive species known, oxidizing electron-rich biomolecules at near diffusion rates (eq. 10, Table 1.1) [26]. Hence, it is also one of the most toxic species known. Photo-Fenton chemistry is exploited in antimicrobial systems such as wastewater treatment [41], and $\cdot OH$ is implicated in a number of chronic afflictions of humans and other multicellular organisms [26]. Organisms deal with $\cdot OH$ by producing low molecular weight antioxidants, often containing conjugated double bonds capable of forming relatively stable cation radicals after reducing $\cdot OH$ [26]. While not specific scavengers of $\cdot OH$ in the sense that SOD and catalase are specific for their targets, these molecules are thermodynamically better

targets for $\cdot\text{OH}$ than the biomolecules they are meant to protect. They are also expendable, unlike the cell's DNA, membranes, and metabolic proteins. Since most antioxidants have conjugated bonding systems, they also tend to absorb visible light. Hence, many are colored, including well-known plant/algal antioxidants such as β -carotene and lycopene.

Thus, chemical ROS production proceeds by four sequential single-electron reductions of O_2 , ultimately resulting in the complete reduction of O_2 to H_2O (eq. 11, Table 1.1, and Fig. 1.1). Overall, this is a chemical reversal of the photosynthetic production of O_2 . Plants and eukaryotic algae exploit this fact to draw down O_2 generated by photosynthesis using the “water-water cycle [42,43]). First, electrons are transferred from the photosynthetic electron transport chain to O_2 near the OEC (the Mehler reaction). Then, nearby SOD and ascorbate peroxidase enzymes, sometimes actually affixed to the photosynthetic membranes, rapidly convert the reduced O_2 to H_2O . This results in a broad cycle, where electrons from the splitting of H_2O are ultimately returned to H_2O , protecting the organism in the process. Cyanobacteria undergo a similar process, only using flavoenzymes to reduce O_2 directly rather than proceeding through a ROS intermediary [44].

III. Sources of environmental HOOH

Naturally occurring HOOH in the ocean was first reported in the 1960's [45], although its significance was not appreciated until much later. In situ concentrations of HOOH rarely exceed $0.2\ \mu\text{M}$ in seawater (see Appendix I), well below the detection limit of traditional titrimetric methods used for HOOH quantification. As will be discussed in more detail in sections IV and V, this has led to the perception of HOOH as a trace component of seawater, and has led many researchers to discount its importance except in very specific chemical reactions (e.g., redox cycling of Fe). Over the decades, two models for the ultimate source of HOOH in natural waters have been developed, one biological, the other photochemical (Fig. 1.2). We will first consider the evidence for each model, then discuss the role each likely plays in controlling the actual HOOH flux in nature.

A) Biological sources. It has long been recognized that the formation of ROS is an unavoidable consequence of aerobic metabolism. Many enzymes capable of single-electron transfer to a substrate are also capable of reducing O_2 , and the ubiquity of O_2 in aerobic

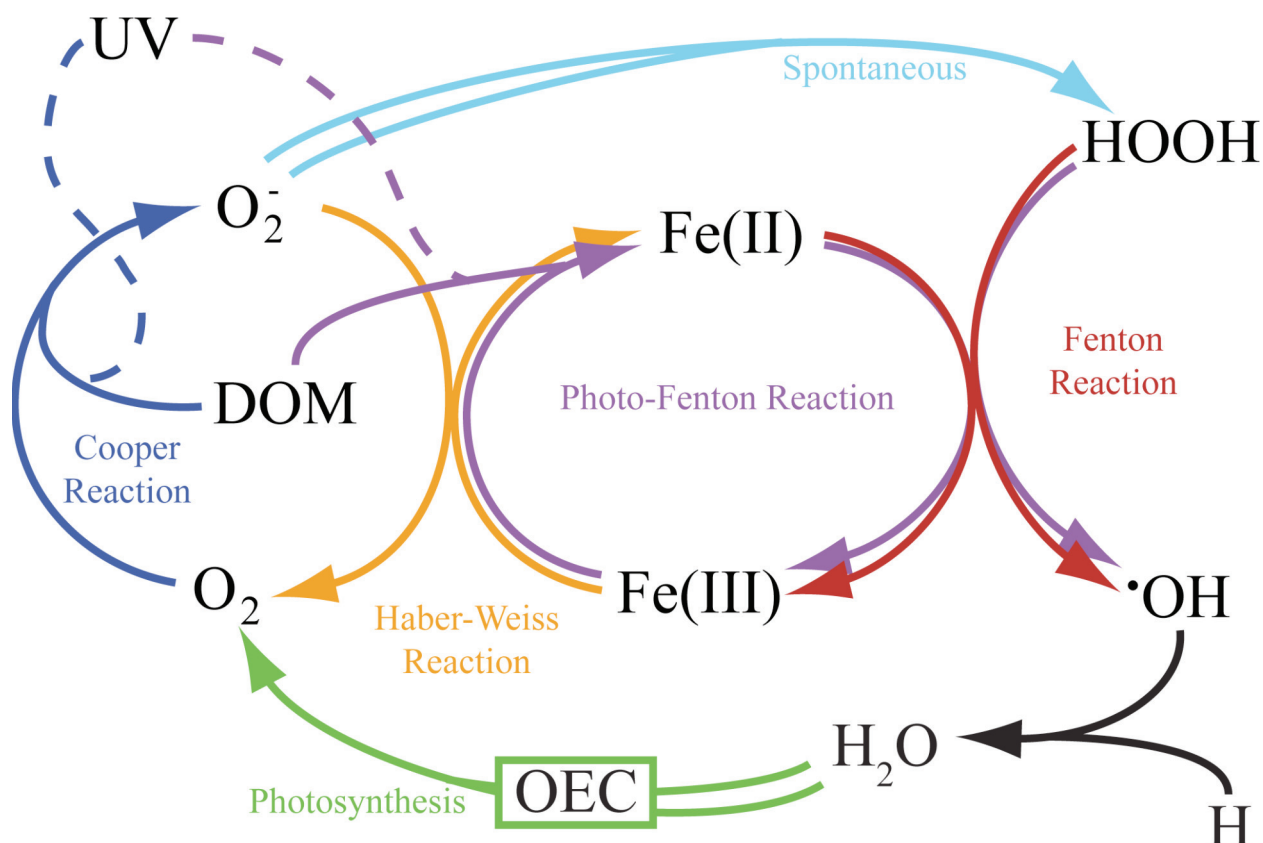


Figure 1.1. The reactive oxygen cycle of aquatic environments. O_2 arises as a consequence of photosynthesis, through the water-splitting activity of the oxygen evolving complex (OEC). In the Cooper Reaction [46], sunlight, particularly UV radiation, excites dissolved organic matter (DOM) into a triplet state that can reduce O_2 to superoxide (O_2^-), two molecules of which may spontaneously unite to form hydrogen peroxide (HOOH). In the Fenton reaction [32], HOOH oxidizes Fe(II) to Fe(III) , producing hydroxyl radical ($\cdot\text{OH}$), which may oxidize virtually any other compound by abstracting a hydrogen atom, producing H_2O and a radical species. Fe(II) can be regenerated to Fe(III) using electrons from an obscure source – probably UV-excited DOM – allowing the Fenton reaction to proceed without saturating the Fe(II) pool. Since this reaction involves solar irradiation, it is called the photo-Fenton reaction [38]. Some researchers also mention the Haber-Weiss reaction, in which O_2^- can reduce Fe(III) in a light-independent fashion; the existence of this reaction is, however, controversial [39,40].

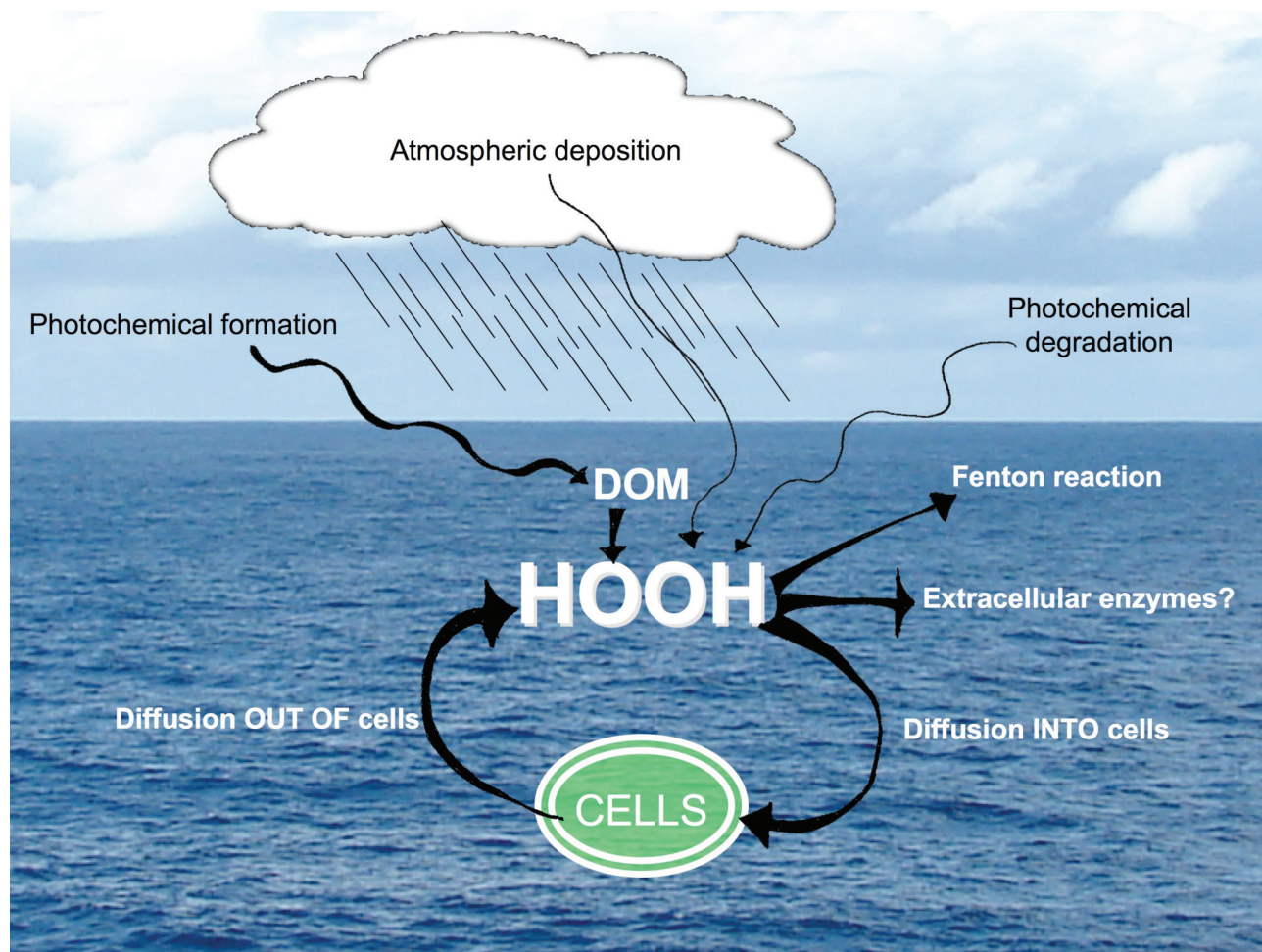


Figure 1.2. The sources and sinks of HOOH in and above the open ocean. Solar radiation produces HOOH in atmospheric water, which may be deposited in the ocean via rainfall. At the same time, sunlight excites dissolved organic matter (DOM), producing excited molecules that can reduce O_2 , leading ultimately to the formation of HOOH. HOOH may also arise as a by-product of cellular metabolism, which may readily escape cells since biological membranes are approximately as permeable to HOOH as to water. Once in seawater, HOOH can react with Fe(II) to produce hydroxyl radicals (the Fenton reaction), it may be photochemically degraded (perhaps also by the Fenton reaction), or it may be enzymatically degraded either intra- or extracellularly. Artwork courtesy of L. A. Morris.

environments (and even more so in photosynthetic organisms) ensures that some ROS will escape. Leakage to O_2 is dependent on the relative concentrations of O_2 and the preferred substrate of the enzyme. Excess O_2 near photosynthetic membranes can lead to ROS production by the Mehler reaction as described in the previous section. Similar reactions can occur with other electron transport enzymes such as NADH dehydrogenase [24] when O_2 is in excess. Another source of HOOH important when O_2 is in excess comes from the photorespiration pathway [47]. The first enzyme in the Calvin cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), can catalyze either its primary, CO_2 -fixing reaction or a competing, deleterious O_2 -fixation reaction (photorespiration). The byproduct of this latter reaction is glycolate, and must be processed by a series of enzymes to regenerate ribulose 1,5-bisphosphate. One enzyme in this cycle, glycolate oxidase, produces HOOH. In eukaryotic autotrophs this reaction occurs in the peroxisome, in the presence of high concentrations of catalase that rapidly eliminate HOOH. While the photorespiratory glycolate cycle is essential in cyanobacteria [48], they replace glycolate oxidase with a dehydrogenase that does not appear to produce HOOH [49].

O_2 reduction can also occur when a primary electron transport carrier is not available in sufficient quantities due either to excess electron flow, nutrient deficiency, or other problems. In Fe-starved cyanobacteria, oxidative stress occurs likely due to a lack of ferredoxin, the Fe-containing primary cytoplasmic electron acceptor for photosystem I [50]. When more electrons flow through the photosynthetic electron transport chain than can be accepted by ferredoxin, O_2 reduction becomes practically inevitable. ROS buildup from unbalanced metabolism could also explain the toxicity of high-nutrient media to organisms adapted to oligotrophic lifestyles [51]. Lastly, ROS accumulation is often observed as a general consequence of cellular stresses, such as temperature, osmotic, and dehydration stress [52,53,54,55,56,57], probably as a result of changes in protein and membrane structure. As the physical machines responsible for electron transfer become distorted by environmental perturbation, their capacity to discriminate between “proper” electron acceptors and O_2 is likely diminished.

HOOH release is not inevitably a result of cellular “accidents” caused by stress, however. Animals use HOOH at low concentrations as a signaling compound [58] and in high concentrations as an antimicrobial defense [59,60]. In plants, HOOH released by wounded

tissues can warn nearby cells to activate anti-herbivore defenses [61], and HOOH in the apoplastic space is used by peroxidases to catalyze the cross-linking of low molecular weight compounds into the chaotic, refractive net of lignin [62]. HOOH release is also a hallmark of programmed cell death (PCD) in the hypersensitive “immune” response of land plants [63]. Similar reactions occur in aquatic microorganisms. Green algae have been shown to produce HOOH as part of a defense mechanism to plug wounds [64]. Some dinoflagellates apparently produce HOOH as an allelopathic compound, producing very high local concentrations capable of seriously damaging many microbial species and even killing fish [65,66,67]. A relation between HOOH and PCD has also been observed for various algal taxa [68,69,70,71], and HOOH has been shown to accumulate in algal cultures during viral infection [72]. In both of these cases, however, it remains unclear whether HOOH formation is triggered as part of PCD, or rather is a consequence of general failure of cellular metabolism during necrotic death.

Cells have a number of HOOH scavenging systems (see section II), but it is possible that the generation of ROS will exceed these capacities and HOOH will accumulate. While intracellular ROS accumulation can result in direct damage to the cell, ROS can also diffuse out of the cell into the extracellular medium. Due to its charge, O_2^- is not membrane permeable, but HOOH is essentially as permeable as H_2O . Therefore there is no impediment to its diffusion out of the cell [26], and internal HOOH rapidly equilibrates with external HOOH. For this reason, it is possible that organisms under various kinds of stress could contribute significant amounts of HOOH to their environments.

B) Abiotic sources. Many studies in a wide variety of water types have supported the hypothesis that most HOOH in pelagic waters arises by the photooxidation of DOM [46,73,74,75]. As suggested by eq. 3 (Table 1.1), colored DOM can absorb photons and enter excited triplet states capable of reducing O_2 . The rate of HOOH accumulation is generally correlated with DOM concentration [76], although analysis of this rate is complicated in very oligotrophic waters where the concentration of DOM is near the limit of detection. Measurement of the rates of HOOH formation are also problematic in eutrophic waters where abundant Fe, often chelated by elements of the DOM pool, degrades HOOH by the Fenton reaction. Under such circumstances, rates of $\cdot OH$ formation are more commonly measured [37,77,78].

HOOH production is stimulated by light in the visual range, but is much faster under UV irradiation. While only a small fraction of the irradiance impacting natural seawater, photons in the UV-B range cause the majority of HOOH accumulation [79]. HOOH can also form in the atmosphere by the photolysis of water into hydrogen atoms and $\cdot\text{OH}$ (eq. 12, Table 1.1) [80]. These radicals can then join to form HOOH (eq. 13, Table 1.1). Models suggest that this was a primary route of HOOH formation and accumulation in primordial oceans, but in the modern atmosphere it is largely limited to the stratosphere and higher [81]. Still, photolysis of water can be an important source of HOOH in rainwater, as HOOH formed in the gas phase is recruited into rain droplets [82]. Rainwater typically contains 100 times the HOOH concentration found in surface seawater [82], and has been shown to dramatically impact the in situ HOOH of seawater following rainfall events [82,83,84,85,86,87,88].

While few reports have attempted to directly compare the two, the consensus seems to be that the abiotic route of HOOH formation is dominant in most natural waters. However, this could be a biased estimate, as biologists have largely ignored natural HOOH, probably because they perceive its concentration to be too low to impact biological processes. HOOH accumulation has been documented in the extracellular environment for a number of algal species, however, including both macro- and microscopic forms [52,56,65,66,67,89,90,91,92]. One recent study measured significant rates of HOOH production in dark-incubated samples of natural seawater [93] and suggested that a steady state between biological production and decomposition of HOOH was sufficient to explain in situ HOOH concentrations, at least in the Gulf of Alaska. More work is needed to separate these two sources in order to determine their relative influence on natural HOOH.

IV. Sinks of environmental HOOH

As early as the 1920's researchers were aware that HOOH added to seawater was not stable, but disappeared with enzyme-like kinetics [94]. Like HOOH production, we now know that HOOH removal in natural waters has both an abiotic and a biotic component (Fig. 1.2). The abiotic route – Fenton chemistry – has already been discussed in previous sections. This reaction is unquestionably an important route of HOOH elimination in acidic waters rich with humic substances [37,78] due to the greater solubility of Fe at low pH. Photochemical

degradation of HOOH has also been observed in the coastal ocean [95], and simultaneous decreases in HOOH and DOM in the presence of UV light have been measured in the open ocean [76]. Production rates for $\cdot\text{OH}$ were correlated with DOM concentrations across a wide variety of natural waters, including several open ocean stations [77]. All of these observations are consistent with photo-Fenton degradation of DOM in the ocean, despite the extremely low Fe concentrations in these waters. Laboratory experiments suggested that, in humic-rich, low pH freshwater, $\cdot\text{OH}$ production via photo-Fenton consumption of HOOH exceeded other mechanisms by orders of magnitude [37]. As Fenton rates increase with increasing pH in seawater [96] it is reasonable to conclude that the photo-Fenton reaction is also an important sink for HOOH in the ocean.

Even a low rate of abiotic HOOH consumption is important due to the toxicity of $\cdot\text{OH}$. For the same reasons, it is unsurprising that microbiota appear to be the primary agents responsible for HOOH removal in pelagic ecosystems. Cooper and Zepp [97] found that HOOH decomposition in freshwater was arrested in chemically sterilized samples. Similarly, Petasne and Zika [98] found that 0.2 μm filtration of samples greatly reduced HOOH decay rates, and that most of the HOOH degradation ability was contained within the 0.2-1 μm size fraction, implicating bacteria as the predominant players in HOOH removal. Both of these studies found that re-seeding sterilized samples with cultured organisms restored HOOH degradation. In a study of HOOH decomposition using ^{18}O -labelled HOOH, Moffett and Zafiriou [99] determined that the majority of HOOH was disproportionated to O_2 . As this reaction proceeds slowly without catalase, these investigators concluded that most of the HOOH was broken down by microorganisms. Many (perhaps most) aerobic organisms are able to eliminate HOOH from their environment for the reasons described in the previous sections. Since HOOH is freely membrane-permeable, any HOOH generated extracellularly should rapidly be available to intracellular enzymes for degradation. Hence, the bulk HOOH concentration of the medium is drawn down by the collective action of microorganisms detoxifying their immediate environment.

While 0.2 μm filtration removed most dark HOOH degradation capacity from seawater [98], some remained. It is possible that this residual capacity was Fenton-related, but it is also possible that it is a consequence of extracellular ROS scavengers. Extracellular SODs are well

characterized [26], and some bacteria and fungi [100,101,102] are known to produce extracellular catalases. Extracellular catalase activity was reported in polluted waters of the Mediterranean [103]. It is possible that extracellular enzymatic HOOH degradation is caused by enzymes that are actively secreted by organisms, but it is also possible that those enzymes are released as a product of cellular lysis. Enzymes responsible for other processes have been reported to retain activity for several days after cell death from viral lysis, apoptosis, or sloppy grazer feeding [51]. More work needs to be done to determine whether or not a biotic, extracellular sink for HOOH plays any role in the HOOH dynamics of natural waters.

V. Effects of HOOH on the aquatic chemical environment

The large majority of work to date on HOOH in aquatic ecosystems has focused on its biogeochemical role. Specifically, researchers have studied the relationships between HOOH, DOM, and redox-active metals such as Fe and Cu, considered in detail in previous sections (Fig. 1.1). In particular, much attention has been given to the importance of HOOH in controlling Fe speciation, and degrading DOM.

As previously mentioned, Fe is very scarce in the open ocean and potentially limits productivity in some regions. While Fe(II) is much more soluble than Fe(III) at the pH of seawater, it is rapidly oxidized to Fe(III) in the presence of O₂ [104]. In a dark, oxygenated ocean, all free Fe would probably exist as Fe(III). Due to its greater solubility, any Fe(II) produced by photochemistry (i.e., the photo-Fenton reaction) increases the pool of soluble Fe, with potentially profound implications for primary productivity [104]. The role of DOM in maintaining Fe(II) concentrations is pleiotropic: it provides electrons for the photoreduction of Fe(III) on the one hand, and O₂ on the other (leading to HOOH production and Fenton chemistry). DOM is therefore both a source and a sink for Fe(II). Elucidating the rates of these opposing stages in the photo-Fenton reaction is important for modeling the availability of Fe in the ocean.

HOOH may also be important in transformations of DOM. The DOM pool is highly heterogeneous, including species that are highly labile due to their predictable structure (e.g., nucleic acids and proteins) or their relative simplicity (e.g. straight-chain polysaccharides, low molecular weight acids), whereas other compounds are extremely resistant to degradation due to

their complex and/or energetically stable structures (e.g., aromatic compounds, lignin) [105]. This latter DOM fraction can have residence times in seawater of 10^3 yr or more, longer than the mixing time of the ocean as a whole [51]. However, as described above, the interaction of DOM with solar radiation can generate reactive oxygen species including HOOH and $\cdot\text{OH}$. As the strongest oxidant in the ocean, $\cdot\text{OH}$ is theoretically capable of breaking the more refractive components of the DOM pool into smaller, easily accessible compounds, potentially making them more bioavailable [106]. It is not clear to what extent this affects DOM bioavailability, however. Some investigators have reported an unambiguous increase in DOM lability following treatment of natural DOM-rich freshwater with UV or $\cdot\text{OH}$ generated by ionizing radiation [107,108]. Other results are less clear, reporting that $\cdot\text{OH}$ fluxes in nature are not sufficient to be an important factor in degrading natural DOM [109] or that UV/ $\cdot\text{OH}$ treatment actually makes some DOM *less* bioavailable [110,111]. In the latter case, it was suggested that radicalization of DOM species by $\cdot\text{OH}$ may encourage linkage of otherwise labile, low molecular weight compounds in a manner analogous to the HOOH-catalyzed formation of lignin, decreasing their bioavailability. The fact that assessments of lability are dependant on measurements of microbial growth offers another explanation of these results. It is possible that treatments designed to produce $\cdot\text{OH}$ could also lead to undetected accumulation of HOOH in the samples. If important elements of the microbial community were susceptible to HOOH damage, this could create an impediment to bacterial production that could counteract any benefit gained from $\cdot\text{OH}$ -mediated DOM degradation [110]. A more thorough appreciation of the toxicity, or lack thereof, of HOOH to various microbial communities will help in the interpretation of experiments such as these.

VI. Effects of HOOH on aquatic organisms

Compared to the chemistry of HOOH in natural waters, much less research has been done on its direct biological effects, despite the fact that ROS are well known to biologists as potent toxins. In this section, we will review the cellular targets of ROS attack as well as biological defenses against ROS. This review is not intended to be comprehensive, and interested readers are directed to the outstanding textbook of Halliwell and Gutteridge [26] for a more complete

treatment. In the next section, we will consider the current evidence that exogenous HOOH is a source of stress for aquatic organisms.

A) Targets. ROS, especially $\cdot\text{OH}$, can attack all classes of biological macromolecules, potentially causing extensive modifications with predictably deleterious results. DNA is vulnerable to several modifications caused by $\cdot\text{OH}$. First, $\cdot\text{OH}$ may attack the backbone deoxyribose sugars, fragmenting them in a variety of ways leading to single- or double-strand breaks [26,112]. Also, the nucleotide bases themselves may be modified, either before or after incorporation into a DNA molecule. 8-hydroxyguanine, one result of $\cdot\text{OH}$ attack on guanine, base pairs with thymine rather than cytosine, so if it is not repaired, it leads to GC-AT mutations [26]. RNA, including relatively long-lived rRNA and tRNA molecules, is even more vulnerable to oxidation than DNA [113], and oxidative mRNA damage predictably correlates with translational errors [114].

Proteins, too, are targeted by ROS, although their structural heterogeneity greatly amplifies the range of lesions that may be caused. $\cdot\text{OH}$ is capable of many non-specific reactions on proteins, oxidizing both side chains and polypeptide backbones. The typical result of such oxidation is the formation of carbonyl groups, and the detection of this “carbonylation” using antibodies is the primary method for measuring oxidative protein damage in vitro. Compared to $\cdot\text{OH}$, other ROS have more well-defined protein targets. O_2^- degrades proteins that bind Fe, such as ferritin, catalase, and Fe-S cluster enzymes [26,115,116], causing the release of free Fe into the cytoplasm, allowing destructive Fenton chemistry. HOOH itself, while more stable than either O_2^- or $\cdot\text{OH}$, attacks certain proteins even in the absence of free Fe, including important, constitutively expressed proteins such as the β -subunit of ATPase and translation elongation factor G [117,118].

Lipids are also prone to attack by $\cdot\text{OH}$, and oxidized lipids are capable of dramatically magnifying the effects of ROS attack via radical propagation (Fig. 1.3). In a typical scenario, $\cdot\text{OH}$ attacks a double bond in an unsaturated lipid and abstracts a hydrogen atom, leaving a carbon radical and water (Fig. 1.3A). This new lipid radical may react with any other radical with no energy barrier. One possible reaction is with a second lipid radical, quenching the radical character of the system and forming a stable, cross-linked species that terminates radical propagation (Fig. 1.3C). This reaction will be rare in aerobic systems, however, compared to

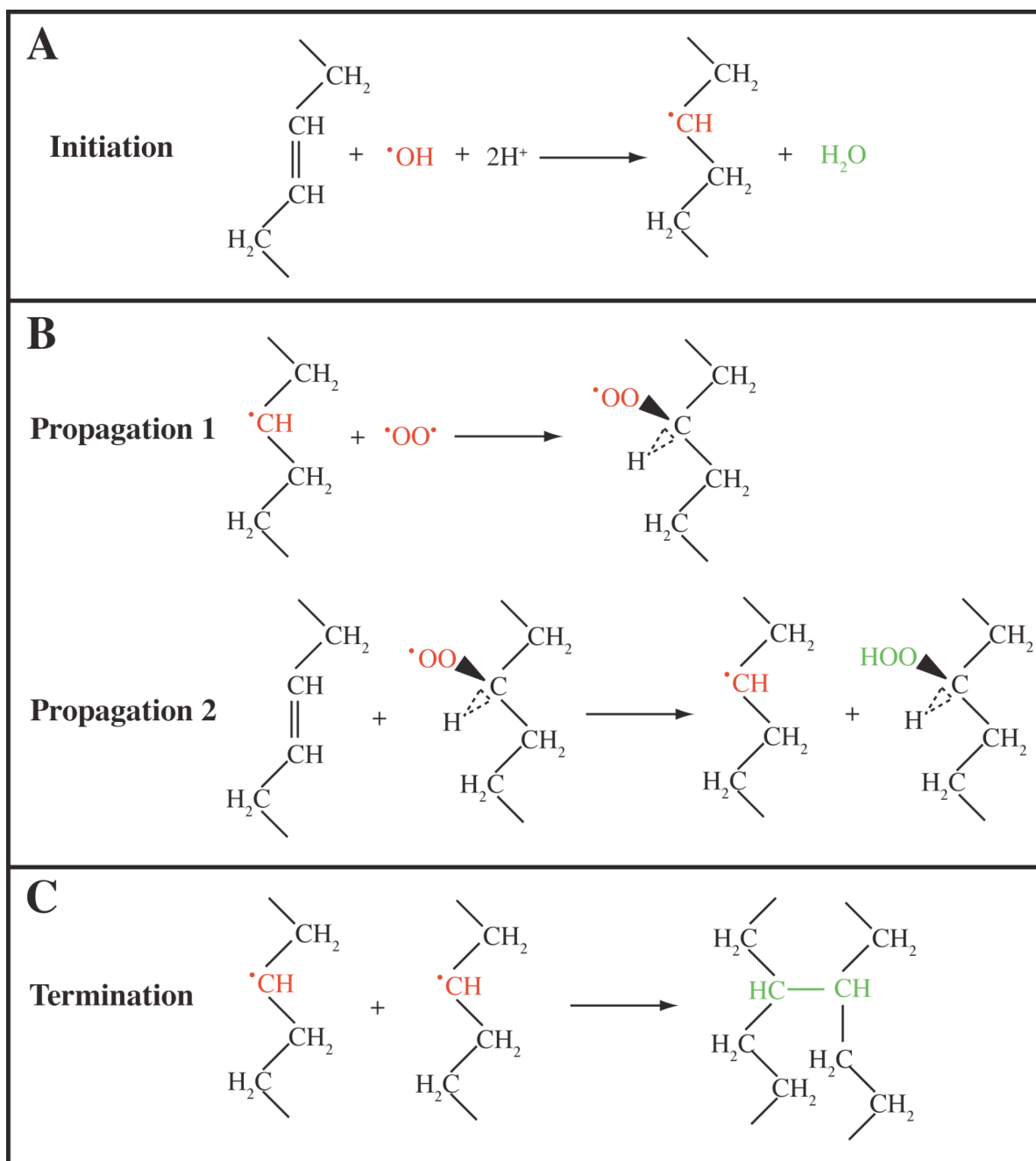


Figure 1.3. Propagation of lipid peroxides. A) A common initial step, where $\text{}^{\bullet}\text{OH}$ abstracts a hydrogen atom from an unsaturated fatty acid, leaving water and a more stable lipid radical. B) The most common reaction of a lipid radical in an aerobic atmosphere is with $\text{}^{\bullet}\text{OO}^{\bullet}$ (i.e., O_2), forming a lipid peroxy radical, which is then capable of abstracting another hydrogen atom from a nearby fatty acid, producing a lipid peroxide and a new lipid radical. Thus, step B may repeat itself many times as long as O_2 is available. C) The cycle terminates when two radicals encounter each other. In this case, two lipid radicals quench each other, resulting in a cross-linked species. Red moieties, free radicals; green moieties, “quenched” radicals.

reaction with a much more abundant radical – O_2 – to form a lipid hydroperoxide and a new lipid radical (Fig. 1.3B). This propagation step may be carried out hundreds of times before the original lipid radical is quenched by a termination reaction. Lipid hydroperoxides are capable of attacking membrane proteins as well as facilitating the transition of 3O_2 to 1O_2 in the cytoplasm [119]. Because of this propensity for magnification, lipid peroxidation is perhaps the most problematic biological effect perpetrated by ROS.

B) Defenses. The most effective strategy for a cell to protect itself from ROS is simply to avoid O_2 . While this is clearly not feasible for most organisms in aerobic environments, O_2 exposure can be minimized. For instance, filamentous cyanobacteria such as *Anabaena* sp. sequester their ROS-sensitive nitrogen-fixing proteins in special cells called heterocysts that a) retard diffusion of O_2 from the extracellular environment with 3 extra layers of extracellular matrix and b) consume O_2 faster than vegetative cells by accelerating aerobic metabolism [120]. In terrestrial and benthic environments bacteria are protected from ROS by being buried in low- O_2 biofilm matrices (e.g., [121]). At first glance, this seems to be an unlikely means for planktonic organisms to avoid O_2 , but there is increasing evidence that microscale associations between bacteria and inanimate particles provide biofilm-like habitats in pelagic ecosystems [122,123].

When O_2 cannot be avoided, a variety of systems exist to deal with the inevitable formation of ROS. The most conspicuous defenses are ROS-scavenging enzymes, including catalase and SOD (section II). Enzymes exhibiting these functions are phylogenetically diverse (i.e., not all catalases are homologous), but they share in common the use of redox-active metal cofactors, including Fe, Mn, Cu, and Ni, to facilitate the reduction of ROS. Canonical catalase disproportionates HOOH directly into water and O_2 , but other catalases as well as a number of metal-cofactor peroxidases reduce HOOH using electrons from other metabolites such as ascorbate. Still other peroxidases, including some that are common in marine bacteria – the so-called “peroxiredoxins” – do not have metal cofactors at all. Rather, they use redox-competent cysteine residues to reduce peroxides (e.g., HOOH as well as lipid and other organic peroxides) using electrons from small molecules (e.g., glutathione and thioredoxin) that are common intermediaries in cellular redox processes [124]. While some peroxiredoxins are clearly front-

line defenses against HOOH (e.g., AhpC in *Escherichia coli* [125]), the function of others appears to be directed more toward scavenging of lipid or other organic peroxides [126].

Enzymatic scavengers offer effective protection against O_2^- and HOOH, but the extremely rapid reaction rate of $\cdot OH$ presents a more difficult challenge. Cells typically counter $\cdot OH$ by one of two strategies. On the one hand, formation of $\cdot OH$ may be inhibited by limiting the availability of Fenton-reactive metals using, for instance, Fe-sequestration proteins such as Dps [127]. Alternatively, cells can accumulate high concentrations of “traps,” or molecules that can react with ROS and leave stable products. Examples of such molecules include vitamin E, which scavenges lipid peroxides [128], phenolic compounds derived from the shikimic acid pathway in plants and algae [47], and carotenoid pigments, which are also effective in preventing the formation of 1O_2 during photosynthesis [26,129].

VII. Evidence of HOOH-induced stress in aquatic ecosystems

Very few studies have been performed to examine the effects of exogenous HOOH on aquatic ecosystems. This is probably because in situ HOOH concentrations are much lower than concentrations reported to be toxic to bacteria in older studies. For instance, *E. coli* was able to tolerate acute exposure to 15 mM HOOH with only a moderate loss of viable counts and a short lag period [130]; this value is almost 5 orders of magnitude higher than any in situ concentration measured in the surface ocean. However, an *E. coli* population in natural river water exposed to light experienced ~99% mortality after 3 days unless catalase or sodium pyruvate (a $\cdot OH$ trap) was added to the water prior to illumination [131]. Protection from oxidative stress has also been linked to survival in stationary phase in *E. coli* and other bacteria [59,101,132,133,134], and it has been argued that the “viable but nonculturable” (VBNC) state of microbes is in fact indistinguishable from HOOH-induced cell damage [54,135]. Both stationary phase and VBNC states are commonly reported in planktonic microorganisms [51,136], consistent with oxidative stress being a common problem in natural waters. Regardless of whether the oxidative stress arises from external ROS or from metabolic imbalances, the membrane permeability of HOOH assures that stressed cells will leak oxidants into their environment, potentially amplifying the problem.

Cyanobacteria in particular appear to be vulnerable to lower doses of HOOH than most other studied organisms. Both unicellular cyanobacteria and filamentous forms experienced high mortality when exposed to $\sim 10\ \mu\text{M}$ HOOH, a value that was consistent across numerous experiments in different laboratories [137,138,139,140]. On the other hand, eukaryotic microalgae (green algae, diatoms) survived doses as high as 1 mM [138,139]. It is striking that even the “resistant” eukaryotes in these studies were much less resistant than cultured *E. coli*. As the toxicity of HOOH for both bacterial and eukaryotic algae increased with irradiance, it seems reasonable to conclude that photosynthesis is somehow responsible for the reduced resistance of algae relative to heterotrophic microbes.

While reports of HOOH tolerances for cultured aquatic organisms are rare, field studies are virtually non-existent. Xenopoulos and Bird [141] reported $\sim 50\%$ reduction of freshwater bacterioplankton productivity following exposure to $10\ \mu\text{M}$ HOOH. Algal primary productivity was more variable in these experiments, with different study sites exhibiting a wide range of tolerances, although 1 mM HOOH essentially eliminated primary production in all experiments. A large body of work from Russia and the former Soviet Union, summarized in English by Skurlatov and Ernestova [142], suggested that environmental HOOH has the positive effect of reducing the frequency and severity of toxic algal blooms. Consistent with laboratory results, these researchers found that cyanobacteria were more vulnerable to the toxic effects of ROS than eukaryotic algae. As a consequence, toxin-producing freshwater cyanobacteria were rare in waters where the concentration of HOOH exceeded $0.3\ \mu\text{M}$. When waters became loaded with ROS “traps” from wastewater effluents, however, toxic blooms frequently formed. In addition to killing cyanobacteria, joint addition of HOOH and Fe also destroys cyanotoxins via photo-Fenton chemistry [143,144]. As HOOH is generally toxic at lower concentrations to microorganisms than macroorganisms, it is an attractive treatment against nuisance plants and algae, and its effectiveness has been demonstrated in a number of cases [142,145,146,147,148,149]. Skurlatov and Ernestova [142] report the successful use of HOOH to control toxic algae in an industrial aquaculture facility, but to our knowledge no large-scale implementations of such plans have been attempted outside of Russia. These studies indicate that large-scale HOOH addition should be reconsidered as a cost-effective, non-toxic, and useful treatment for harmful cyanobacterial blooms. They also support the hypothesis that natural

populations of cyanobacteria are particularly vulnerable to HOOH compared to most cultured microbes.

Other studies provide indirect evidence of HOOH toxicity to microbial populations in the field. The toxic effects of UV radiation on aquatic communities are well documented [150,151,152,153,154], and anthropogenic increases in UV are a major future concern for the health of the oceans [155,156]. While UV mediates certain specific types of damage on its own (e.g., thymine dimers in DNA), it also is intimately linked to the production and cycling of HOOH in natural waters (Fig. 1.1). There have been many reports of UV-related inhibition of aquatic organisms, including macroalgae [157,158] and microalgae and cyanobacteria [154,159,160,161,162,163,164,165,166,167], caused both by the direct (DNA modification) and indirect (ROS generation) toxic effects of UV. Importantly for the work described in this dissertation, field samples of *Prochlorococcus*, but not their sister group *Synechococcus*, were extremely vulnerable to UV radiation, experiencing ~100% mortality after less than 24 hours of exposure to unfiltered sunlight [154,167]. While these experiments do not directly test the possibility, given the known susceptibility of cyanobacteria to HOOH it is likely that some of this damage is due to the generation of extracellular HOOH and the photo-Fenton reaction.

Similarly, there is ample reason to suspect that some of the toxicity of heavy metals to algae is mediated by ROS [168]. Redox-cycling metals, such as Fe and Cu, have a clear relationship to ROS through Fenton chemistry, and their toxicity probably involves the generation of $\cdot\text{OH}$ from HOOH. However, redox-stable metals, like Pb and Cd, are also associated with oxidative stress. In these cases, ROS probably arise from metal-stimulated metabolic impairment, leading to intracellular O_2 reduction. A number of studies have reported increases in phytoplankton productivity following Fe addition to seawater (e.g., [104]). However, Willey et al. [85,88] demonstrated that, in the presence of elevated HOOH such as that found in rainwater, the growth benefit of Fe addition was eliminated. Willey et al. [85] interpreted these results as indicating that HOOH reduced the bioavailability of Fe by changing its redox state; however, an explanation invoking $\cdot\text{OH}$ toxicity is equally attractive. These results should give pause to policymakers who would consider large-scale Fe additions to the oligotrophic oceans as a palliative for global CO_2 pollution. Cyanobacteria are numerically dominant throughout these waters [9], and their established HOOH vulnerability suggest that

they could be selectively impaired by such a scheme, with unpredictable consequences for the long-term health of the oceans.

In conclusion it is quite possible that the importance of naturally-occurring, low concentrations of HOOH as a toxin has been underestimated. HOOH is also probably involved in enhancing other environmental toxins, including UV and metal stress. As such, future work is needed to elucidate its exact role in structuring marine microbial communities.

VIII. HOOH and the evolution of oxygenic photosynthesis

It is intuitively clear that the oxygenation of the Earth's atmosphere by photosynthetic cyanobacteria had a profound impact on the evolution of the biosphere. However, the evolution of oxygenic photosynthesis has always been difficult to explain. First, what was the selective pressure to evolve a complex water-splitting mechanism, when, in an anaerobic world, reduced inorganic compounds (e.g. H₂S) were abundantly available as electron donors for anoxygenic phototrophs? Second, given the toxicity of ROS, how could the earliest water-splitters survive the by-products of their new ability? Clearly, they must have already had ROS detoxification capabilities – but why, if they lived in an anoxic environment?

Figure 1.4 shows a scenario wherein HOOH is instrumental in the evolution of oxygenic photosynthesis. Models suggest that in the ozone-free Archean atmosphere UV photolysis of water vapor could have led to an accumulation of HOOH at sea level even in the absence of an appreciable source of O₂ [80,81]. HOOH has been detected on the Martian surface [169], which is similar in terms of its irradiation profile to early Earth [170]. Thus, HOOH has probably always existed in the surface layers of the ocean, even before the presence of abundant DOM and gaseous O₂. It is possible that the earliest phototrophs were forced to develop an antioxidant capacity in order to exploit the sunlit surface waters, leading to “pre-adaptation” to the presence of O₂. The presence of HOOH would also have necessitated strong Fe scavenging systems, as Fe(II) would have been abundant in the absence of O₂. This could explain the extreme antiquity and phylogenetic ubiquity of iron-binding proteins such as ferritin [171].

Another interesting possibility is that O₂ production by catalase could have pre-dated oxygenic photosynthesis and actually could have created the selection pressure for the evolution

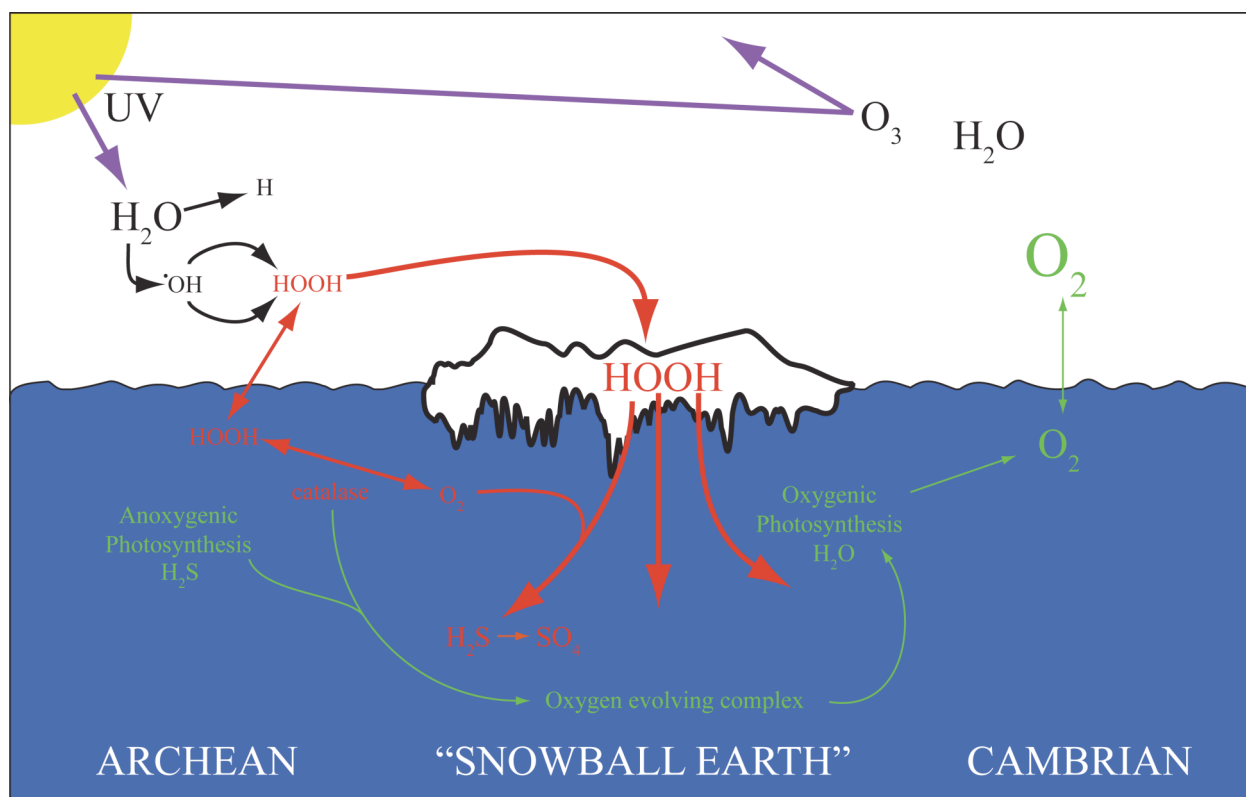


Figure 1.4. A scenario for HOOH involvement in the evolution of oxygenic photosynthesis. In the ozone-free Archean atmosphere, UV radiation cleaves water, allowing formation of gaseous HOOH from $\cdot\text{OH}$ radicals. In order to harvest sunlight, anoxygenic phototrophs must evolve anti-HOOH defenses such as catalase to survive at the surface. Unfortunately, O_2 generated from the disproportionation of HOOH oxidizes photosynthetic electron donors such as H_2S . This problem is exacerbated when HOOH concentrated in glacial ice is released, oxidizing most of the euphotic zone. In the absence of reduced compounds, phototrophs evolve the ability to split water into O_2 , harnessing electrons for photosynthesis in the process. It is possible that the Mn-containing oxygen evolving complex evolved from ancient Mn catalases. Oxygenic photosynthesis radically changed Earth's atmosphere; one effect of this was the generation of an ozone (O_3) layer that greatly restricts HOOH formation in the modern troposphere.

of water-splitting [172]. While most modern organisms use catalases that have a heme co-factor containing Fe, a less common version uses a Mn cofactor [173]. The active sites of Mn catalases share structural similarity to the active sites of the OEC of photosystem II, and these are among the very few enzymes capable of producing O₂. Thus, it is reasonable to conjecture that Mn catalases were evolutionary intermediates in the development of the OEC [174]. Moreover, the activity of catalases at the ocean surface by anoxygenic phototrophs could have led to an accumulation of O₂ that, while insufficient to radically alter planetary geochemistry, could have oxidized the local environment to the point that electron sources for anoxygenic photosynthesis could have become scarce. This phenomenon would have been exacerbated during melt-out from Snowball Earth events, as ice (like rain) can concentrate atmospheric HOOH [81,175]. Thus, protection from H₂O-derived HOOH could have been critical in oxidizing the euphotic ocean, providing strong selective pressure for evolution of a new form of photosynthesis deriving electrons from an even more abundant source – water.

IX. Conclusion: *Prochlorococcus* and the Catch-22 of phototrophy

In the above sections we laid out the framework for the studies described in the forthcoming chapters. HOOH is an important and understudied player in the oceans, particularly in terms of its biological effects. Evidence is strong that it may restrict the range of organisms that can survive in the photon-rich surface layer of the ocean. Light follows a “Subsidy/Stress” model [176], where both low and high abundances of a resource are problematic, with productivity peaking somewhere in the middle. Biological processes are only partly capable of controlling the pathways through which solar energy flows in their systems. The greater the energy flux, the greater the possibility of energy “escape” causing unwanted side reactions. Organisms that subsist on low levels of energy – e.g., deep-water chemoautotrophs, abyssal decomposers, etc. – have low risk of damage from radicals, at the cost of very slow metabolic rates. However, a great bounty awaits organisms that develop the capability of survival – however tenuous – in higher-energy environments. From the earliest days of life, the surface oceans have been rich with solar and chemical energy but also fraught with danger for systems requiring complicated, ROS-vulnerable macromolecules to function. Clearly, lineages have

conquered this environment several times, with huge consequences for Earth's biogeochemical history.

Prochlorococcus, the most abundant photosynthetic genus in the modern oceans, is one of these lineages. The earliest-branching *Prochlorococcus* species are obligately limited to the bottom of the euphotic zone where they are strongly light-limited [177]. At some point, this lineage developed a number of adaptations that granted them the ability to “return to the surface,” where they are able to exploit the greater solar energy resources and grow much faster. These organisms have enjoyed almost unparalleled success, outnumbering their sister taxon *Synechococcus* by at least an order of magnitude throughout the temperate and tropical open ocean gyres. As for all organisms that live in surface waters, the cost of abundant solar energy and the productivity it allows is that they must contend with HOOH and other ROS. As we will see, *Prochlorococcus*' solution is at the same time unexpected and fascinatingly simple, with profound implications for our understanding of evolutionary forces shaping microbial assemblages in the ocean.

CHAPTER 2

Facilitation of Robust Growth of *Prochlorococcus* Colonies and Dilute Liquid Cultures by “Helper” Heterotrophic Bacteria

Publication Note

This chapter was published in 2008 in a slightly different form [178].

Contributions

This chapter was prepared in collaboration with Robin Kirkegaard, Martin J. Szul, Zackary I. Johnson, and Erik R. Zinser. I performed the streak plate assays. RK performed the liquid culturing experiments. MJS did cloning and sequencing of helpers. ZIJ performed flow cytometry. ERZ isolated helpers and axenic MIT 9215. ERZ, ZIJ and I planned and designed the experiments and wrote the paper.

Acknowledgments

We thank A. Buchan, S. Chisholm, P. Fidopiastis, and K. Visick for bacterial strains. We also thank B. Fagg, R. Slightom and P. Jemes for technical assistance, and A. Buchan, E. Stabb, J. Waterbury and E. Webb for advice on culturing techniques. This project was supported by grants from the National Science Foundation (OCE-0526072, MCB-0527944) and the University of Tennessee.

ABSTRACT

Axenic (pure) cultures of the marine unicellular cyanobacterium *Prochlorococcus* grow efficiently only if the inoculation concentration is large; colonies form on semisolid media at low efficiency. In this work we describe a novel method for growing *Prochlorococcus* colonies on semisolid agar that improves recovery to approximately 100%. *Prochlorococcus* grows robustly at low cell concentration, in liquid or solid media, when co-cultured with marine heterotrophic bacteria. Once the *Prochlorococcus* cell concentration surpasses a critical threshold, the “helper” heterotrophs can be eliminated with antibiotics to produce axenic cultures. Our preliminary evidence suggests that one mechanism by which the heterotrophs help *Prochlorococcus* is to reduce oxidative stress.

I. Introduction

Prochlorococcus is the most abundant marine photosynthetic organism, and as such is a major contributor to photosynthesis in the ocean [9]. Over 30 strains of *Prochlorococcus* have been brought into culture, isolated from many locations within the 40° N to 40° S band, including the North Atlantic, North and South Pacific Oceans, the Mediterranean Sea and the Arabian Sea [9]. Despite this success, very few pure cultures of *Prochlorococcus* have been obtained (e.g. PCC 9511 and MIT 9313 [179,180]). The vast majority of cultures contain heterotrophic microbes as contaminants; these heterotrophs were co-cultured from the marine environment during the isolation procedure, which has relied thus far exclusively on liquid cultivation. While plating for contiguous lawns of *Prochlorococcus* has proven productive [181], attempts at colony formation (by pour plating or surface streak plating) have thus far met with significantly less success. Recovery efficiencies of 0.1 – 10 % have been reported for some strains with the pour plating technique [181,182], but have yet to produce pure cultures [181]. The inability to readily obtain clonal, pure cultures of *Prochlorococcus* has severely limited progress in the genetic and physiological analysis of this ecologically-important lineage.

II. The “helper” phenotype of heterotrophic bacteria

Standard dilution streaking of contaminated *Prochlorococcus* cultures onto semisolid media failed to produce axenic colonies. Colonies only formed within a visible mass of the contaminant heterotrophic bacteria; this mass was typically in the earliest, heaviest dilution streak(s) (data not shown). One interpretation of these results was that *Prochlorococcus* was only able to grow in the presence of the contaminating bacteria, perhaps because the bacteria provide a growth factor and/or remove an inhibitory factor. Co-culturing with heterotrophic bacteria is required for growth of some bacterial isolates [183], and is known to improve the growth of other cyanobacteria as well as dinoflagellates [184,185], suggesting a similar interaction may help *Prochlorococcus*. To test this hypothesis, a heterotrophic contaminant (EZ55) of a culture of the *Prochlorococcus* strain MIT 9215 was isolated on ProAC medium [75% Sargasso Sea water (pre-filtered with a 0.2 µm polycarbonate filter), supplemented with 17 g L⁻¹ of AC Difco Broth, 800 µM NH₄Cl, 50 µM NaH₂PO₄, 1X Va vitamin mix [186], and 15 g L⁻¹ granulated agar (unless noted, all chemicals are from Sigma)] and tested for its ability to help

Prochlorococcus grow on semisolid media. The 16S rRNA gene of EZ55 was PCR-amplified [187], cloned with the TOPO TA cloning kit (Invitrogen), and sequenced, identifying the strain as a member of the *Alteromonas* genus.

A “lawn” of 4.0×10^6 cells of EZ55 (pre-washed in un-supplemented Sargasso seawater) was spread evenly onto the 60 mm diameter Pro99-based agar plate [Pro99 nutrients [181] plus 1 mM sodium sulfite (all filter-sterilized separately), 0.42% Difco Agar Noble (Becton Dickinson) in a blend of 18 MΩ water and Sargasso seawater (75% v/v final concentration, autoclaved separately)], using acid-washed, autoclaved 2.5 mm glass beads (Bio Spec). As the Pro99 plates contained no organic carbon supplement, any growth of the EZ55 cells on the plates was undetectable, and no visible lawn formed. A dilution series of a late-log phase *Prochlorococcus* MIT 9215 culture was then titrated onto the plates, and was incubated under the standard incubation conditions for this study: 22 °C with continuous light at $30 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$, provided by cool white fluorescent bulbs. Plating efficiency was determined by comparing colony forming units (CFU) to counts determined by flow cytometry, which quantifies *Prochlorococcus* particles based on size and chlorophyll-based fluorescence [188,189]. The flow cytometry count for the culture was 3.3×10^8 cells mL^{-1} , while viable counts (Fig. 2.1A) averaged $2.7 \pm 1.6 \times 10^8$ CFU mL^{-1} . Thus, in the presence of EZ55, essentially every cell of MIT 9215 planted on the agar formed a colony. No colonies formed on control plates lacking EZ55 (data not shown), confirming that heterotrophs were necessary for the development of MIT 9215 colonies.

III. Elimination of the “helpers” to obtain axenic *Prochlorococcus*

To utilize this method to obtain axenic cultures of MIT 9215, a genetic selection was developed. We first obtained a streptomycin-resistant (Sm^r) mutant of MIT 9215 by inoculating approximately 10^{10} cells into 1L of Pro99 medium containing $100 \mu\text{g mL}^{-1}$ streptomycin (Sm_{100}). The Sm^r culture that grew out of this selection was not pure, as Sm^r heterotrophs could be isolated if grown on ProAC rich medium plates (data not shown). Axenic MIT 9215 Sm^r was then obtained by spread-plating a dilution of the culture (approximately 100 MIT 9215 Sm^r cells per 60 mm agar plate) with $\sim 5 \times 10^5$ cells of wild-type, streptomycin-sensitive (Sm^s) EZ55. The wild-type EZ55 facilitated the growth of MIT 9215, which was spatially separated from the Sm^r

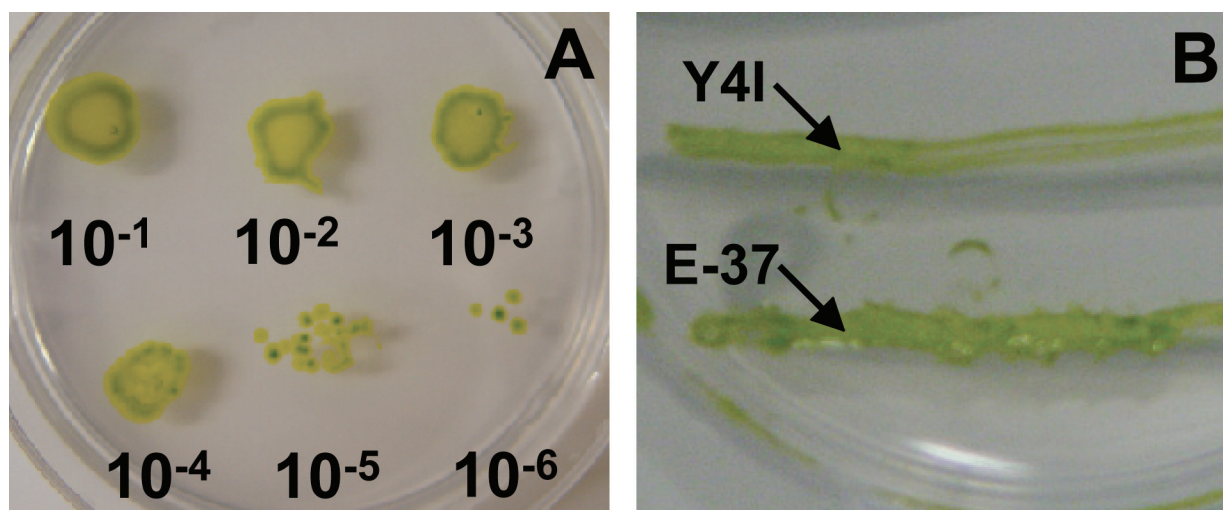


Figure 2.1. Helper heterotrophs facilitate *Prochlorococcus* growth on solid media. (A) Dilution series of *Prochlorococcus* MIT 9215 on a dilute lawn of approximately 1,000,000 cells of EZ55 on Pro99 agar media lacking organic carbon. Numbers above the dilution spots indicate the fold dilution of the 3.5×10^8 cells mL⁻¹ MIT 9215 source culture.; 10 µL volumes were spotted for each dilution. (B) Approximately 10⁶ *Prochlorococcus* MIT 9215 cells were spread evenly on the agar. Cells of heterotrophic Roseobacters *Phaeobacter* sp. Y4I and *Sagittula stellata* E-37 were then applied over the MIT 9215 cells as horizontal streaks (indicated by arrows). (Green) MIT 9215 cells grew only where the heterotrophs were streaked.

contaminants on the plates. Green colonies were transferred with a sterile wooden toothpick or plastic pipette tip into 5 mL of liquid Pro99 medium. At the first visible sign of growth, the liquid cultures were diluted 64-fold into Pro99 Sm₁₀₀ medium to eliminate the EZ55 cells, and thus establish a pure culture of MIT 9215. Due to the (low rate of) spontaneous mutation to streptomycin-resistance in EZ55, it was important to transfer as small a volume of cells as possible from Pro99 into Pro99 Sm₁₀₀, to ensure that all contaminants were killed upon exposure to streptomycin. Purity was confirmed by sub-culturing stationary-phase cultures into two types of purity test broth: a rich medium (1/10 ProAC: ProAC with only 1.7g L⁻¹ AC Difco broth) and a minimal medium (PLAG: 75% Sargasso seawater supplemented with 0.05% (w/v or v/v) each of sodium pyruvate, sodium lactate, sodium acetate, and glycerol; 800 µM NH₄Cl; 50 µM NaH₂PO₄; 1X Pro99 trace metal mix; and 1X Va vitamin mix). Cultures with no growth in the 1/10 ProAC or PLAG test media after four weeks of incubation were considered axenic.

IV. The helper phenotype in liquid media

The “helping” phenomenon was found to occur in liquid as well as on semisolid media. A dilution series of axenic MIT 9215 Sm^r was inoculated into fresh Pro99 medium, and chlorophyll-based fluorescence, quantified with a TD700 fluorometer (Turner Designs), was used to measure relative *Prochlorococcus* cell concentration during the incubation [177]. At the highest cell inoculum tested, 3.5 * 10⁶ cells mL⁻¹ (determined by flow cytometry), axenic MIT 9215 Sm^r grew well as pure cultures (Fig. 2.2A), confirming that the “helping” phenomenon is not necessary for concentrated cultures of axenic *Prochlorococcus*. However, growth of cultures with lower inocula was severely limited. Cultures at an initial concentration of 3.5 * 10⁵ cells mL⁻¹ grew, but exhibited a variable and overall lower final yield of chlorophyll-based fluorescence than the cultures initiated with ten times as many cells. Cultures inoculated with 35 - 35,000 cells mL⁻¹ failed to grow to detectable levels of fluorescence. Hence, the ability of pure cultures of MIT 9215 Sm^r cultures to grow was strongly dependent upon the initial cell density.

In contrast, there was effectively no density dependence for growth of MIT 9215 Sm^r when co-inoculated with approximately 10⁶ cells mL⁻¹ of EZ55. From 35 to 3,500,000 cells mL⁻¹ inoculum, growth was robust and highly reproducible: triplicate cultures exhibited the same

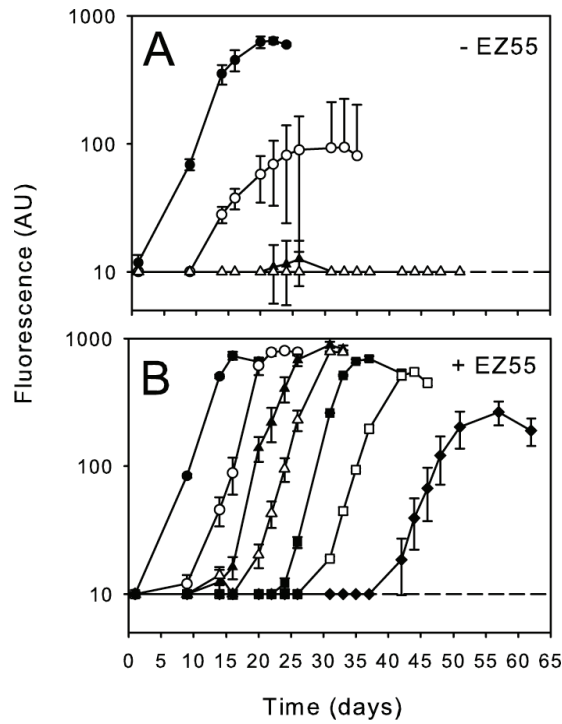


Figure 2.2. Growth of MIT 9215 in liquid Pro99 medium with or without helper heterotrophs. Dilutions of MIT 9215 were grown in the absence (A) or presence (B) of $\sim 5 \times 10^5$ CFU mL⁻¹ EZ55. Initial concentrations of MIT 9215 were 3.5×10^0 (◆), 10^1 (□), 10^2 (■), 10^3 (△), 10^4 (▲), 10^5 (○), or 10^6 (●) cells mL⁻¹. Error bars indicate one standard deviation of the mean for three replicate cultures. The dotted horizontal line is the limit of detection for chlorophyll-based fluorescence [10 arbitrary units (AU)]; values below this limit were reported as 10 AU.

growth rates and yields (Fig. 2.2B). The lowest initial cell dilution assayed, $3.5 \text{ cells mL}^{-1}$, was the one exception, exhibiting a slower growth rate and maximum fluorescence yield. Nevertheless, all three cultures started at this very low inoculum grew to detectable levels of fluorescence by day 40 post-inoculation. Thus, as with semisolid media, the ability of initially low concentration cultures of MIT 9215 Sm^r to grow was dependent on the presence of heterotrophic helper bacteria.

V. The helping phenomenon is common in marine heterotrophs

To address the specificity of the “helping” interaction, one member of each ecotype (ecologically-distinct lineage) of *Prochlorococcus* [190] was assayed with (i) marine heterotrophs isolated as for EZ55 above, from seven cultures of *Prochlorococcus* representing all six ecotypes, (ii) representatives of the Roseobacter clade, and (iii) bioluminescent species of the genus *Vibrio*. Several Roseobacters have been shown to improve the growth of phototrophic dinoflagellates and algae [184,185], suggesting that they may be able to perform a similar role for *Prochlorococcus*. A rapid screening assay was developed to facilitate the high throughput *Prochlorococcus*-by-heterotroph interaction survey. In contrast to the prior plating method, the *Prochlorococcus* strain, rather than the heterotroph, was spread on the plate as the dilute lawn (approximately 10^6 cells of exponentially-growing cultures per 60 mm diameter plate). Heterotrophs were then overlayed on top of the *Prochlorococcus* by streaking a patch of cells from a colony using a wooden toothpick. Accumulations of green biomass indicating *Prochlorococcus* growth occurred only where “helpers” were streaked (Fig. 2.1B). While the *Prochlorococcus* cultures tested in this assay were not axenic, the contaminants did not affect the assay, since *Prochlorococcus* grew only where the much denser test heterotrophs were streaked on top. For the low-light ecotype strains NATL2A, SS120, MIT 9211, and MIT 9313 [191,192], plates contained only 0.29% agar, as this provided more robust growth than the 0.42% plates (data not shown).

The majority of strains tested helped strains MIT 9215, MED4, NATL2A, and MIT 9313 grow, although the amount of time required for visible cyanobacterial growth to appear varied extensively both between heterotrophic and *Prochlorococcus* strains (Table 2.1). In contrast, far fewer heterotrophic strains facilitated the growth of *Prochlorococcus* strains SS120 and MIT

Table 2.1. Streak plate assay results. Numbers are the number of weeks required for *Prochlorococcus* growth to become visible on at least two of three replicate streaks of the indicated heterotrophic bacteria. '--' indicates that no growth occurred in the allotted time (16 weeks).

			Prochlorococcus Strain						
Organism			Catalase ^j	MIT 9215	MED4	NATL2A	MIT 9211	SS120	MIT 9313
Co-isolates with <i>Prochlorococcus</i> strains	<i>Alteromonas</i> sp. EZ32 ^a	+/-	1	3	--	--	--	--	
	Rhodospirillaceae str. EZ35 ^a	+/-	2	3	3	--	--	--	
	<i>Alteromonas</i> sp. EZ33 ^b	+	1	2	5	--	--	--	
	<i>Halomonas</i> sp. EZ34 ^b	++	2	3	7	--	--	--	
	Rhizobiaceae sp. EZ36 ^b	++	2	3	5	--	--	--	
	Flavobacteriaceae str. EZ40 ^c	+/-	1	3	5	--	--	9	
	<i>Sphingomonas</i> sp. EZ41 ^c	+	1	3	3	--	--	9	
	<i>Alteromonas</i> sp. EZ42 ^c	+/-	2	3	5	--	--	9	
	<i>Marinobacter</i> sp. EZ43 ^d	+	2	3	5	--	--	9	
	<i>Marinobacter</i> sp. EZ44 ^d	+/-	5	3	3	--	--	--	
	<i>Alteromonas</i> sp. EZ45 ^e	+/-	5	3	3	--	--	--	
	<i>Alcanivorax</i> sp. EZ46 ^e	+	--	3	3	--	--	--	
	<i>Pseudoalteromonas</i> sp. EZ48 ^f	+/-	5	3	--	--	--	--	
	Rhodospirillaceae str. EZ49 ^f	+/-	2	3	1	--	9	7	
<i>Alteromonas</i> sp. EZ55 ^g	+/-	2	2	1	--	--	7		
Rhodospirillaceae str. EZ54 ^g	+/-	2	2	3	--	--	7		
<i>Vibrio</i> spp. ^h	<i>Vibrio fischeri</i> ES114	+	3	2	4	10	--	9	
	<i>V. fischeri</i> 7744	+	3	3	5	10	--	9	
	<i>V. harveyi</i> B392	+/-	2	2	3	9	--	--	
	<i>V. logei</i> 15382	+	2	3	3	14	--	9	
Roseobacters ⁱ	<i>Phaeobacter</i> sp. Y4I	+	3	3	3	14	--	--	
	<i>Phaeobacter</i> sp. Y3F	+	3	3	--	--	--	7	
	<i>Ruegeria lacuscaerulensis</i> ITI-1157	+/-	3	2	5	14	9	5	
	<i>Ruegeria</i> sp. TM1040	+/-	3	--	--	--	--	5	
	<i>Ruegeria pomeroyi</i> DSS-3	+	2	3	3	--	--	5	
	<i>Sagittula stellata</i> E-37	+/-	2	5	5	--	9	7	
	<i>Sulfitobacter</i> sp. NAS-14.1	+	3	--	--	--	--	5	
	<i>Sulfitobacter</i> sp. EE36	++	2	4	3	--	--	5	
	<i>Sulfitobacter pontiacus</i> ChLG10	+	3	4	5	--	9	5	
	Rhodobacteraceae str. SE62	+/-	2	5	5	--	--	5	
	Rhodobacteraceae str. PSPC-2	++	2	--	4	--	--	5	
	<i>Citricella</i> sp. SE45	+	1	3	3	--	--	5	
	<i>Roseovarius nubinhibens</i> ISM	+	2	2	6	--	--	5	

^a Isolated from *Prochlorococcus* MED4 culture (S. Bertilsson, unpub.)

^b Isolated from *Prochlorococcus* MIT 9313 culture (S. Bertilsson, unpub.)

^c Isolated from *Prochlorococcus* MIT 9312 culture (this study)

^d Isolated from *Prochlorococcus* MIT 9211 culture (this study)

^e Isolated from *Prochlorococcus* NATL2A culture (this study)

^f Isolated from *Prochlorococcus* SS120 culture (this study)

^g Isolated from *Prochlorococcus* MIT 9215 culture (this study)

^h source: P. Fidopiastis

ⁱ source: A. Buchan

^j Relative vigor of bubble formation upon exposure to HOOH

9211. Additionally, the amount of time before growth was evident was typically greater for these strains. Only one heterotroph, *Ruegeria lacuscaerulensis*, universally helped all 6 strains of *Prochlorococcus* grow, although 7 others (including all 4 *Vibrio* spp. tested) helped 5 strains. Heterotrophs isolated from the *Prochlorococcus* cultures exhibited no preferential “helping” of their cognate *Prochlorococcus* strains over the other *Prochlorococcus* strains tested, and not all isolates from the *Prochlorococcus* cultures were able to help their cognate strains. Hence, these initial studies indicate that the helping phenomenon appears to be common, though not universal for all marine heterotrophs. Despite the relative crudity of this assay, it is nevertheless a significant observation that all strains of *Prochlorococcus* tested in this study - representing each of the six known ecotypes - were helped by at least 4 of the heterotrophic species surveyed.

VI. The helping phenomenon is linked to oxidative stress

Heterotrophs appeared to play an active role in the facilitation of *Prochlorococcus* growth. EZ55 Sm^s cells helped MIT 9215 Sm^r form colonies on standard Pro99 plates (see above), but not on plates containing Sm₁₀₀ (data not shown). Thus, streptomycin-killed cells were unable to facilitate MIT 9215 growth. One heterotroph activity we hypothesized to be important in the helping phenomenon is the scavenging of reactive oxygen species (ROS) from the medium. There is growing evidence that standard agar media contain levels of ROS that restrict the growth of many bacteria: the overall plating efficiency of bacteria from marine, soil, and atmospheric environments can be dramatically improved by the introduction of hydrogen peroxide (HOOH) scavenging agents such as catalase or pyruvate to the medium [54,193,194,195,196,197,198]. Of note, cells of the marine heterotroph *Vibrio vulnificus* that appear to have entered a “viable but non-culturable” (VBNC) state during exposure to low temperature have lost their catalase activity (9), and can in fact be cultured if the medium contains catalase [198]. In light of these earlier studies, we found it intriguing that the genomes of *Prochlorococcus* lacked homologs to all known catalases and heme-containing peroxidases [199]. We therefore hypothesized that the removal of ROS from the medium by ROS-scavenging heterotrophic bacteria might be an important component of the “helping” phenomenon.

Our preliminary evidence supports this oxidative stress reduction hypothesis. Unlike its wild-type parent (ESR1, a derivative of ES114) (Table 2.1), a *katA* mutant (KV433) of *Vibrio fischeri*, which lacks the periplasmic catalase [59], was unable to help MIT 9215 grow on plates (data not shown). This indicates that catalase activity is necessary for the helping phenomenon in this strain. The *katA* mutant has no growth defects (rate or yield) in rich medium; indeed *katA* expression is low during logarithmic growth but is induced by HOOH additions or entry into stationary phase [59]. These results thus suggest that elimination of extracellular HOOH by *V. fischeri*'s periplasmic catalase is a necessary component of this organism's helping phenotype.

Direct tests for the sufficiency of purified catalase to help *Prochlorococcus* grow were complicated by the fact that catalase is vulnerable to photoinactivation [200,201,202]. This loss of activity is likely very significant given the long incubation periods in the light that are required for growth on solid media (1-2 months for colonies). Nevertheless, purified catalase showed a significant positive effect on the growth of dilute lawns of *Prochlorococcus*. Without catalase, an even spread of 10^6 cells of MIT 9215 was unable to form a lawn on Pro99 semisolid media. However, on plates containing 50, 100, and 200 U mL⁻¹ catalase, the same amount of cells grew robustly into lawns (data not shown), demonstrating it can significantly enhance the growth of *Prochlorococcus* on plates.

Clearly, hydrogen peroxide scavenging is implicated as a mechanism of helping by *V. fischeri*, and may play a similar role for the other helpers. All of the heterotrophs assayed for ability to help *Prochlorococcus* grow also demonstrated visible signs of catalase activity (as judged by "bubbling" of colonies upon exposure to 3 or 30% HOOH, at 22°C) (Table 2.1). However, there was no clear correlation between the intensity of catalase activity and the ability of an organism to help; indeed, *Ruegeria lacuscaerulensis*, the only organism capable of helping all strains of *Prochlorococcus* tested, also had the lowest catalase activity. Whether peroxidases (which do not form gas bubbles) substitute for catalase in the low-catalase-activity helpers or not is currently unknown, but we suspect that ROS scavenging may not be the only mechanism of helping. The apparent specificity of some helpers for certain strains of *Prochlorococcus* (e.g. EZ46, Table 2.1), and the identification of only one "universal helper" out of 33 tested suggests that multiple factors are responsible for the helping phenomenon.

VII. Conclusion

In this work we have presented a two-step method whereby strains of *Prochlorococcus* may be made clonal and eventually pure. By plating with heterotrophic helper bacteria, isolated clones can be obtained as colonies, with plating efficiencies approaching 100%. Subsequently, the clones can be grown in liquid culture and made axenic by the addition of streptomycin to eliminate the helpers once the *Prochlorococcus* is dense enough to grow as a pure culture. This advance in our ability to grow *Prochlorococcus* colonies should dramatically enhance our ability to isolate new, clonal strains from the oceans and to improve the genetic manipulation of this organism, which has thus far been limited by the inability to isolate individual mutants [182]. In addition, our preliminary evidence indicates that a wide range of marine heterotrophs can facilitate the growth of the different ecotypes of *Prochlorococcus*, with reduction of oxidative stress as an important component of this “helping” phenomenon. Clearly, much work remains to understand the mechanism(s) of this helping phenomenon, and to determine what connection, if any, this interaction between heterotroph and phototroph plays in the oceans.

CHAPTER 3
Light-driven Hydrogen Peroxide Production
by Biological Buffers in Culture Media

Publication Note

This chapter has been prepared for submission to the journal *Phycologia*.

Acknowledgements

We would like to thank the crews of the R/Vs *Kilo Moana* and *Hugh R. Sharp* for assistance in seawater collection, S. Wilhelm and A. Buchan for use of equipment, G. Sayler and S. Chen for TOC analysis, and T.F. Williams for chemistry advice. Many thanks to W. Cooper and S. Rusak for early advice on AE chemiluminescence, and M. Gonsior for the gift of our acridinium ester. This work was supported by NSF grant OCE-0526072 to ERZ and by a Phycological Society of America Grant-In-Aid to JJM.

ABSTRACT

Researchers in mammalian cell culture have previously observed the generation of toxic levels of hydrogen peroxide (HOOH) in light-exposed culture media containing the pH buffer HEPES. Seeking to bring this work to the attention of environmental microbiologists, we investigated the production of HOOH in seawater media containing a variety of zwitterionic “Good’s buffers.” Production rates varied extensively amongst buffers, with HEPES highest and TAPS lowest. Further, rates for a given buffer increased linearly with buffer concentration, natural seawater concentration, and light level. Concentrations of HEPES commonly used in culture media (1-10 mM) generated enough HOOH to kill the axenic *Prochlorococcus* strain MIT9215 during growth at low, ecologically-relevant cell concentrations and/or under high light exposure. Evidence supports a model of HOOH formation where an unknown organic contaminant in the basal medium photosensitizes the buffer, leading to O₂ reduction. HOOH accumulation is asymptotic, with a peak level that correlates well with the rate of formation, suggesting a steady-state process where HOOH production competes with an HOOH decomposing reaction such as the Fe(II)-catalyzed Fenton reaction that converts HOOH into highly reactive hydroxyl radicals. We offer several recommendations (e.g., replacing HEPES with TAPS) for modifying culturing practices to avoid buffer-mediated oxidative stress. Finally, we demonstrate that HEPES can be used experimentally to study the biological effects of chronic exposure to sublethal levels of HOOH such as may be experienced by organisms in the euphotic zone of natural aquatic ecosystems.

I. Introduction

Natural waters are buffered to a greater or lesser degree by the equilibrium between dissolved CO₂ and carbonate sediments [203]. In most aquatic systems, both freshwater and marine, this results in a pH between 7.5 and 8.5. This strong chemical buffering system largely offsets the effects of microbial metabolism on proton concentrations, resulting in a very stable pH that is predominantly controlled by abiotic forces. On the other hand, organisms typically attain much greater cell densities in culture than in the field, and pH often becomes dominated by biological effects, particularly in the later stages of batch culture. Bacterial fermentation pathways, for instance, may generate organic acids as by-products, leading to lower pH when these products accumulate. Conversely, many aquatic algae use a carbon concentrating mechanism (CCM) that actively transports bicarbonate (HCO₃⁻) into the cell where it is enzymatically converted to CO₂ [204,205]. By depleting the medium of inorganic carbon more rapidly than it can be replenished from the atmosphere, the algae shift equilibrium toward carbonate and pH rises [206]. This may become problematic when CO₂ resupply is restricted, i.e., due to large culture volume and/or insufficient aeration. Beyond a certain point, changes in external pH result in the loss of a cell's ability to maintain pH homeostasis, leading to the collapse of the transmembrane proton gradient, cessation of growth, and eventual loss of viability [207,208].

To avoid this pH shift, it is helpful to artificially buffer culture media. Inorganic carbonates and phosphates, commonly used as buffers in a wide range of solutions, are macronutrients and may affect the growth of cultured organisms. This problem is particularly acute for photoautotrophs, which are typically growth-limited by these nutrients. Tris buffer is used in some media, but it is known to permeabilize biological membranes [209] and thus can be toxic to many microbes. Good's buffers [210] such as HEPES, MOPS, and TAPS, on the other hand, appear to be optimal choices for buffering media. They each have a pKa in the range of 7.0-8.5, they are chemically stable over time, and their ring structures make them biologically inert. Additionally, these buffers were characterized as chemically unreactive under physiological conditions and largely optically transparent in the UV-VIS range [210]. These last properties were emphasized because the buffers were intended to be essentially invisible in

experimental conditions, interacting neither with the chemical matrix of the medium nor with the detection methods, such as spectrophotometry, used to follow the progress of the experiment.

Contrary to these initial observations, however, studies have shown that HEPES is not chemically inert, and is actually quite photoreactive, forming hydrogen peroxide (HOOH) in light-exposed media [211]. Lepe-Zuniga et al. [212] reported that a 30 min exposure to approximately $90 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light was sufficient to kill 99% of thymocytes cultured in HEPES-containing media, while unbuffered cells were unaffected. While HOOH production in HEPES-containing thymocyte cultures can be circumvented by incubation in the dark [212], such a remedy is not possible for cultured photoautotrophs or other experiments requiring light exposure. Notably, several recent studies demonstrate that many unicellular photosynthetic microbes are susceptible to damage by low concentrations of HOOH [69,138,178], and some ubiquitous marine heterotrophic bacteria such as *Pelagibacter ubique* [12] are conspicuously lacking in antioxidant enzymes such as catalase. Therefore, we sought to confirm the original findings of Zigler et al. in the context of seawater growth media, as well as to explore alternatives to HEPES for buffering.

In this work we quantified the photochemical production of HOOH in HEPES-buffered seawater media as a function of buffer concentration and light intensity. We also examined the effects of HEPES on the unicellular marine cyanobacterium *Prochlorococcus*, known to have high HOOH sensitivity (Chapters 2 and 4). We assayed HOOH production from several other buffers with similar pKa values (Table 3.1) in an attempt to identify a suitable replacement for HEPES. Finally, we show how HEPES may be exploited as a reliable, steady-state generator of HOOH in culture media.

II. Measurement of HOOH

HOOH concentration was measured using an acridinium ester chemiluminescence method [213] modified for use in an Orion-L microplate luminometer (Berthold Detection Systems, Pforzheim, Germany). 10-methyl-9-(*p*-formylphenyl)acridinium carboxylate trifluoromethanesulfonate (acridinium ester, AE) was a generous gift of M. Gonsior (University of Otago, NZ). Replicate 200 μL aliquots of sample were loaded onto white, flat-bottomed 96-well plates (Costar #3912, Corning Life Sciences, Lowell, MA) along with HOOH standards

Table 3.1. Buffers studied in this work.

Buffer	pKa
Bicine	8.3
DIPSO	7.6
Glycylglycine	8.2
HEPES	7.5
MOPS	7.2
TAPS	8.4
Tricine	8.1
Tris	8.1

(2-fold dilutions ranging from 1000 nM to 15.625 nM) prepared using a seawater blank collected from 900 m depth in the oligotrophic South Pacific (32° 25' S, 159° 5' E). The HOOH stock solution was prepared by diluting a 30% solution to approximately 25 mM, and then standardized using its absorbance at 240 nm on a DU-800 spectrophotometer (Beckman Coulter) using the molar absorbance coefficient $38.1 \text{ L mol}^{-1} \text{ cm}^{-1}$ [214]. Where necessary, samples were diluted in blank water to bring them into the useful range for the AE response (0-1000 nM). Additionally, samples of low salinity were diluted into blank water to compensate for the pronounced difference in clarity of seawater and deionized water after alkalization. After loading, plates were incubated for 10 minutes in the same room as the luminometer to allow temperatures to equilibrate. The assay consisted of sequential injections of 50 μL 2 M Na_2CO_3 (pH 11.3) followed by 50 μL of 2.2 mg L^{-1} AE (prepared in 1 mM phosphate buffer, pH 3). Immediately after addition of AE, light output was measured for 1 s. HOOH levels were determined by comparison of experimental aliquots to a regression line prepared from the HOOH standards run on the same plate; standard deviations were calculated from the means of two mechanical replicates and the standard error of the slope and intercept of the standard curve.

To test the precision of this method, twelve different standard curves were run in pairs on six different days, using different batches of reagents and HOOH standards. The AE responses were linear with HOOH concentration, with an average $r^2 = 0.9988 \pm 0.0006$ for individual curves. The limit of detection, defined as 3 times the standard error of the blank, was 2.5 ± 2.0 nM. This value is significantly higher than the 0.35 nM limit reported by King et al. [213] for similar seawater samples using their AE method, which employed a FeLume flow injection analysis system. However, our resolution was sufficient for our purposes and the time saved by the higher throughput microplate format afforded us much more flexibility in our experimental design. On average, we could process 40 samples in 30 minutes or less, counting standard preparation and plate loading and reading. In comparison, the same number of samples and standards took us more than 2 h to process using the FeLume. Reagent usage was also much lower using the luminometer: 40 samples required less than 5 mL of AE reagent, compared to > 200 mL using the FeLume.

Initial measurements with the luminometer and the FeLume suggested that the chemiluminescence response might vary over the time required to read all the samples. We

therefore ran the standard curves in pairs, one at the beginning of the reading of a plate and again at the end of the plate. While measurements for the same standards were significantly higher in the second set of readings than in the first (paired one-tailed t-test, $p < 0.001$), the slopes of the lines were not different (paired t-test, $p > 0.5$). When standard curves were made from paired measurements, they were still strongly significant (average $r^2 = 0.9986 \pm 0.0005$). The limit of detection increased to 7.7 ± 4.4 nM, still within an acceptable range for our experiments. Therefore, we used these paired standard curves to calculate HOOH values in all our experiments.

To confirm the specificity of the AE reaction for HOOH, at the end of one experiment all tubes were treated with 5 U mL^{-1} bovine liver catalase (see below) overnight and then assayed again. In all cases, the AE response was lowered to a level that was at or slightly below the level of the seawater blank (data not shown). We also tested the possibility that buffers in the samples could skew estimates of HOOH by perturbing the pH of the AE reaction. Standard curves prepared either in blank water or blank water supplemented with 10 mM TAPS (pH 8.0) did not have significantly different slopes or intercepts (t-tests, $P > 0.05$).

III. Kinetics of HOOH production in sterile media

A) Methods. We followed the generation of HOOH in test tubes containing seawater amended with a variety of buffers (Table 3.1). All buffers were from Sigma (St. Louis, MO), except tricine, which was from Fisher (Waltham, MA). All buffers were of $\geq 99.5\%$ purity. Buffers were prepared as 0.5 M stocks in milli-Q water, titrated to pH 8.0, sterile filtered using $0.22 \mu\text{m}$ PVDF Steriflip units (Millipore, Billerica, MA), and stored at 4°C .

Early experiments indicated that HOOH production was notably slower in Pro99 natural seawater medium than in unamended sterile seawater. This removal of HOOH was likely a consequence of the Fenton reaction [26] caused by the addition of Fe(III):EDTA as a nutrient. Briefly, HOOH reacts with photoproducted Fe(II) to produce hydroxide anion (HO^-) and hydroxyl radical ($\text{HO}^\bullet$), the latter of which is one of the most reactive species known, oxidizing a wide variety of molecules at near-diffusion rates [24,78]. As we wished to focus on rates of HOOH production, we therefore minimized the Fenton reaction by using Chelex-treated, trace-metal free seawater [181] without nutrient amendments. Experiments were conducted either in

0.22 μm filtered seawater harvested from the continental shelf near the mouth of Chesapeake Bay (37° 6' N, 75° 42' W, 32 ppt salinity) or in artificial seawater (Turk's Island Sea Salts, Moore et al. 2007, 35 ppt salinity). In both cases, water was passed through a Chelex-100 column (Bio-Rad, Hercules, CA) into an acid-washed polycarbonate bottle in a trace metal clean laminar flow hood [181]. Water was subsequently sterilized by boiling in a microwave oven three times on three consecutive days. Each time, the water was allowed to cool in the trace metal clean hood. Dissolved organic carbon (DOC) levels were assayed on triplicate samples using a TOC-VCP analyzer (Shimadzu, Kyoto, Japan) after the above treatments. Absorption spectra of buffer solutions and seawater pre-filtered through 0.22 μm Millex-GV syringe filters (Millipore) were obtained using a DU-800 spectrophotometer (Beckman Coulter, Fullerton, CA) with milli-Q water as a blank.

All experiments were conducted on 8 mL of sterile sample in acid-washed borosilicate test tubes incubated at 22° C under cool fluorescent illumination. Aliquots were removed aseptically periodically over the course of several weeks for HOOH determination. The zero-order rate constant of HOOH formation, k_{HOOH} , was calculated as the slope of HOOH vs. time based on the first 7 d of the experiment, except in cases of very slow production when longer periods were used to obtain statistically significant constants. In one experiment, we lowered the dissolved oxygen content of the seawater by incubating the reaction vessels in an airtight anaerobe chamber containing a GasPak Plus O₂ removal system (BD). Samples were equilibrated with the anoxic atmosphere in the dark for 1 week, then removed for buffer addition (either 10 mM TAPS or HEPES, or unbuffered control). At this time, HOOH was measured as described and the samples were re-sealed in the anaerobe chamber with a fresh GasPak, then incubated at 22° C, 65 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for 2 weeks. At the end of this incubation period, HOOH levels were measured again.

B) Results. We tested a number of buffers with pKa values in the relevant range for seawater media (Table 3.1) for the ability to produce HOOH in natural or artificial seawater when exposed to light. Control tubes lacking light, buffer, or the natural/artificial seawater solutes (i.e., buffer prepared in milli-Q water) did not generate detectable levels of HOOH (data not shown). Tris and glycylglycine, the two non-Good's buffers examined, did not generate HOOH under any conditions tested. All of the Good's buffers produced HOOH when exposed

to light in a seawater matrix, and the accumulation of HOOH was linear with time. For the Good's buffers, the zero-order rate constant of HOOH accumulation (k_{HOOH}) varied dramatically based on the specific buffer used and whether natural or artificial seawater was used as a basal medium (Fig. 3.1). In natural seawater, HEPES and DIPSO were comparable, and produced almost twice as much HOOH per day as MOPS, four times as much as tricine, and almost an order of magnitude more than bicine or TAPS. The general pattern was similar in artificial seawater, but with significantly lower k_{HOOH} values in each case. In contrast to natural seawater, however, there was no difference in k_{HOOH} between HEPES, DIPSO and MOPS in artificial media. Of note, HOOH generation was not detectable in TAPS-buffered artificial seawater. Depletion of O_2 in the medium drastically limited the rate of HOOH formation for HEPES (Fig. 3.1) and TAPS (data not shown), confirming that O_2 is the ultimate source of HOOH in these systems.

The linearity of HOOH formation over the first week of observation in the above experiments suggests that the reaction rate is not sensitive to the concentration of any reactant, at least over this time frame. One explanation for these kinetics is that the rate is limited by the photochemical step, and the light level we used was insufficient to saturate the chromophore in the medium involved in the photochemical production of HOOH. However, none of the Good's buffers had measurable absorbance in the UV-VIS range (250 nm to 800 nm), even in highly concentrated stock solutions up to 2 M (data not shown), consistent with the original observations [210]. Likewise, neither the natural nor artificial seawater background used for media absorbed in the visible range. Since the rate of HOOH formation was higher in natural seawater (110 μM DOC) than in artificial seawater (17 μM DOC), we speculate that photochemistry proceeds through unidentified organic chromophores that are too dilute to be detected by spectrophotometry, analogous to the formation of HOOH from DOC in the ocean [46].

The production of HOOH thus requires four factors: the buffer, the unidentified light-absorbing seawater component, light, and O_2 . To establish the relative contribution of the first three factors, we manipulated each one separately from a "reference condition" of 1 mM buffer, 65 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and 100% natural seawater. Regardless of the light level or seawater concentration, HOOH did not accumulate to detectable levels in buffer-free controls or media

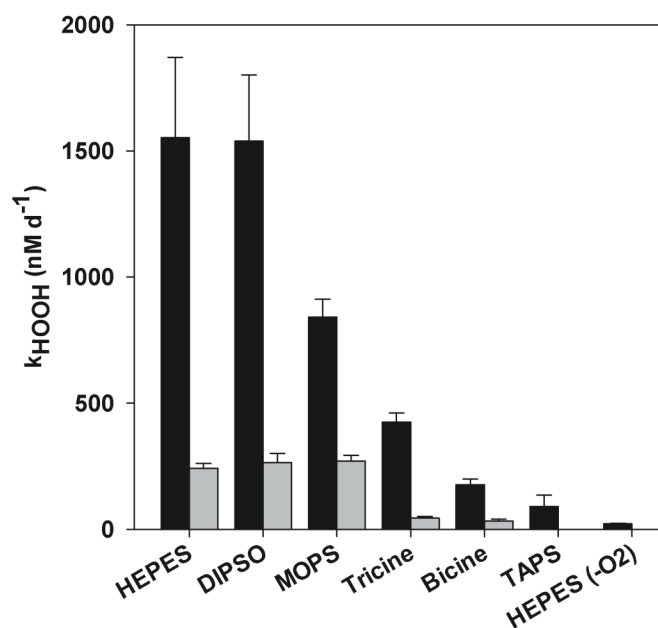


Figure 3.1. Formation of HOOH in media containing various Good's buffers. The zero-order rate constants for HOOH formation (k_{HOOH}) in sterile, iron free natural seawater (black bars) or Turk's Island artificial seawater mix (gray bars) under $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for six Good's buffers at 10 mM concentration. Error bars are the standard deviation of 3 averaged replicates.

containing less than 10 mM TAPS (data not shown). Conversely, in HEPES-containing solutions k_{HOOH} increased linearly with increases in buffer concentration, percent seawater, or light (Fig. 3.2). This suggests that all three factors co-limit the reaction rate and argues for a mechanism in which each of these three parameters is tightly linked. One hypothesis is that the buffer acts as a photosensitizer, facilitating the transfer of electrons from the unknown chromophore to O_2 . The primary source of HOOH in natural waters is the photooxidation of dissolved organic matter by sunlight [46,215], primarily by ultraviolet radiation [79]. We did not observe HOOH generation in buffer-free seawater (see above), but this may be a consequence of using light sources that do not generate significant amounts of UV, as well as light levels significantly less intense than natural sunlight. We suspect that the presence of the buffer may lower the energy requirement for reduction of O_2 by light-excited trace carbon compounds in our media, facilitating the generation of HOOH by a similar mechanism to that existing in the field.

In long-term exposures to light (weeks to months) HOOH concentrations in buffered media leveled off asymptotically (Fig. 3.3A). Maximum HOOH concentration was strongly correlated with maximum k_{HOOH} across all experiments conducted in the same seawater background, regardless of buffer type or concentration (Spearman Rank Order Correlation, $R = 0.87$, $P < .001$, Fig. 3.3B). If the ultimate HOOH yield was the result of a limiting reagent being exhausted, we would expect the asymptote concentration and rate to be independent of each other; samples with higher k_{HOOH} values would simply achieve the asymptote more quickly. If on the other hand the asymptote was the result of a steady-state balance with an invariable competing reaction that consumes HOOH, we would expect it to vary based on the rate constants of these two reactions. Our observations (Fig. 3.3) support the latter explanation, and suggest that the competing rate – HOOH consumption – is not affected by any of the parameters we manipulated, i.e. buffer concentration, seawater concentration, and light level.

IV. Effects of buffers on growth of *Prochlorococcus*

A) Methods. Non-axenic cultures of *Prochlorococcus* were able to grow in media treated with 10 mM of each of the Good's buffers we tested, but not in Tris or glycylglycine (data not shown). However, heterotrophic contaminants present in these wild-type cultures can confer protection against oxidative stress on *Prochlorococcus* (Chapters 2, 4). Therefore, to test

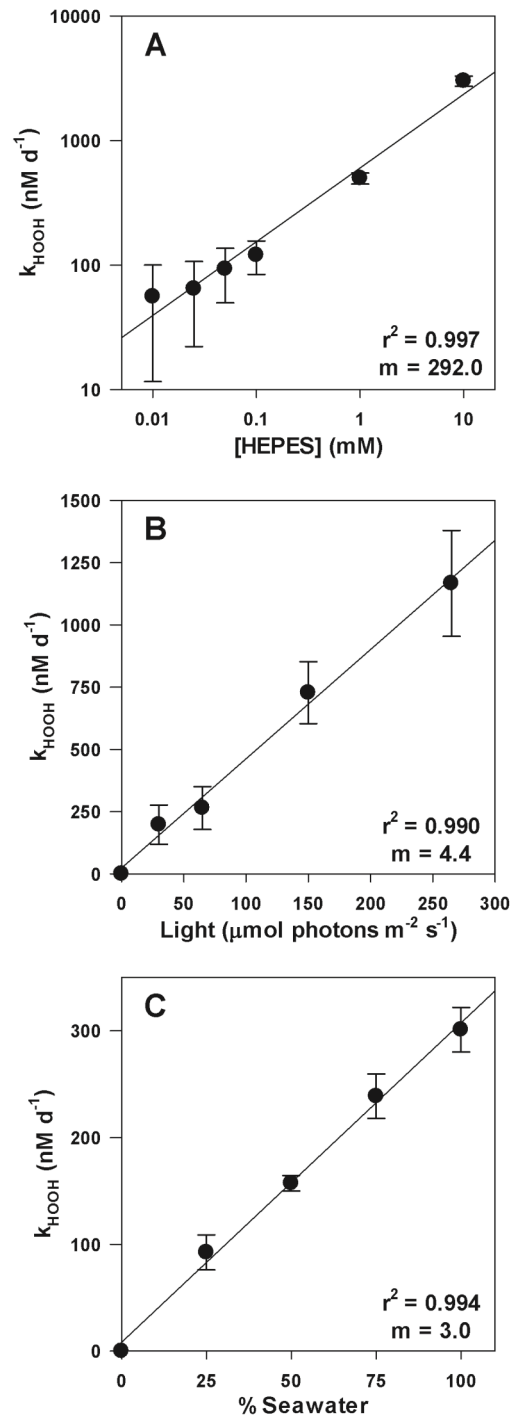


Figure 3.2. Effects on k_{HOOH} from the alteration of three parameters. A) buffer concentration; B) light intensity; C) % natural seawater. All alterations were made from the standard conditions of 1 mM HEPES, 65 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and 100% natural seawater. Error bars are the standard deviation of 3 averaged replicates.

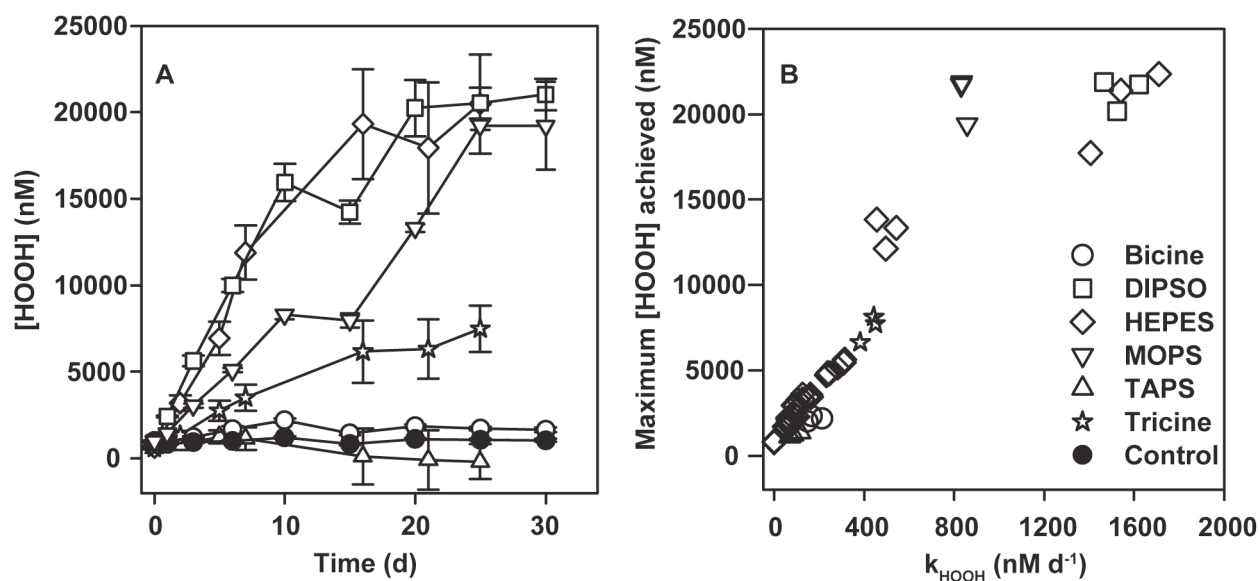


Figure 3.3. Kinetics and yield of buffer-driven HOOH formation. A) Kinetics of HOOH formation for six different buffers at 10 mM concentration in natural seawater under $65 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Error bars are the standard deviation of three replicate samples. B) Relationship between the rate of HOOH accumulation and the ultimate HOOH yield for 6 different Good's buffers in natural seawater.

for toxicity of buffer-generated HOOH on laboratory cultures, we used an axenic strain of *Prochlorococcus*, MIT9215_{AX} (Chapter 2). MIT9215_{AX} was grown in Pro99 natural seawater medium [181] supplemented either with HEPES or TAPS under a variety of conditions. Importantly, Pro99 contains 1 μM Fe(III):EDTA as a nutritional supplement, which may enhance the toxicity of HOOH by accelerating the Fenton reaction. MIT9215_{AX} was acclimated to growth conditions (other than presence/absence of buffer) over at least two passages into fresh media prior to the start of experiments. Mid-exponential phase cultures ($\approx 10^7$ - 10^8 cells mL^{-1}) were diluted 200-fold into Pro99 medium either with or without HEPES or TAPS as a buffer and growth was followed by chlorophyll fluorescence using a TD700 fluorometer (Turner Designs, Sunnyvale, CA, USA) equipped with an *in vivo* chl *a* filter set (excitation 340–500 nm; emission ≥ 665 nm). Buffers were added either at 1 mM, the level used in several artificial seawater media recipes for *Prochlorococcus* [180,181], or 10 mM, a higher level used for large-volume batch cultures of *Prochlorococcus* in natural seawater [181] such as those used for a recent diel microarray experiment [216]. If no growth was detectable after 60 d, the culture was assumed to be dead (growth rate = 0). At the end of each experiment, samples were tested for purity by the addition of 1/10 volume of ProAC medium (Chapter 2) to the culture tubes; if no turbidity developed within 60 d, the culture was assumed to be axenic.

Crystallized bovine liver catalase (1,340 units $\cdot$ mg protein⁻¹) was resuspended in 1 mM sodium phosphate buffer (pH 7.0) pre-heated to 37° C at a concentration of 1000+ units mL^{-1} . The resulting solution was sterilized using a 0.22 μm Millex-GV syringe filter (Millipore) prior to dilution into culture media.

B) Results. With an inoculum of 10^6 cells mL^{-1} of MIT9215_{AX}, the deleterious effects of buffer additions were most pronounced under constant illumination (Fig. 3.4A, first set of bars). Under a modest light level of 65 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, equivalent to approximately 3% of the irradiance at the ocean surface, 1 mM HEPES was sufficient to prevent growth of *Prochlorococcus*. Conversely, TAPS-treated cultures and controls were not significantly different, consistent with the lower k_{HOOH} of TAPS relative to HEPES. Addition of 1 U mL^{-1} catalase to HEPES cultures completely restored growth, confirming that there is no HEPES-based toxicity independent of HOOH formation.

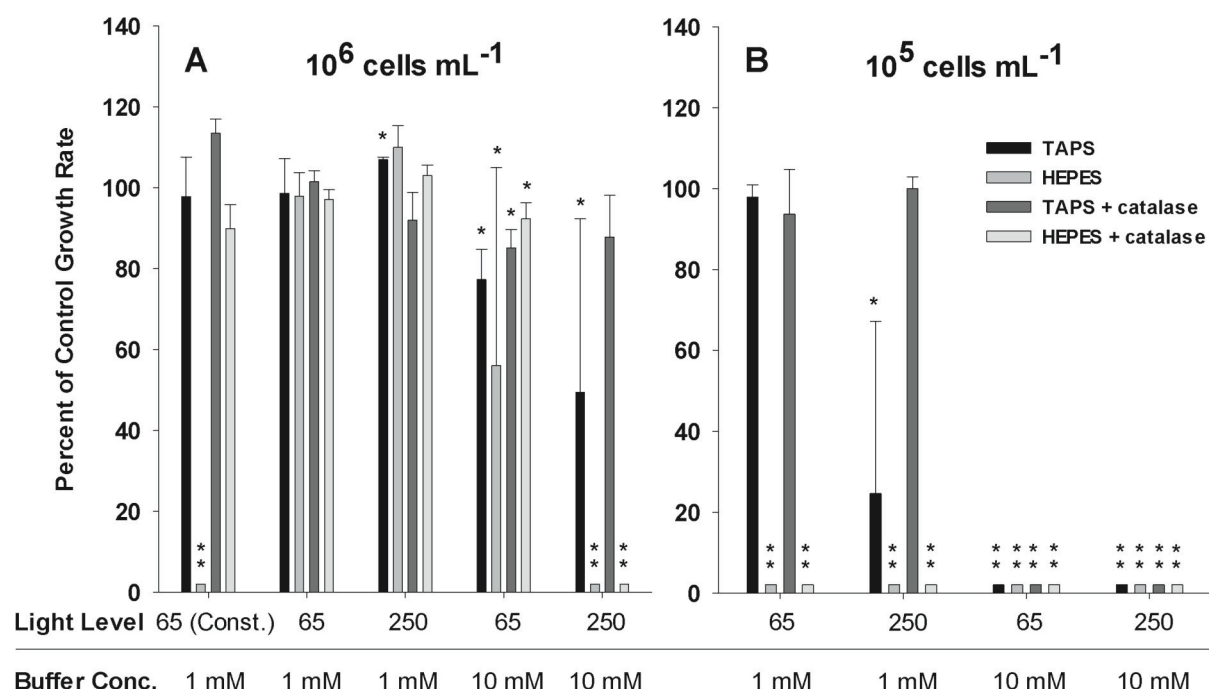


Figure 3.4. Effects of buffers on the growth rate of *Prochlorococcus* MIT9215_{AX}. Cultures were grown under either moderate or high light intensities (indicated values are in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Most cultures were grown under a 12/12 light/dark photoperiod; the first set of bars in graph A were grown under a constant light regime. Cultures were initially inoculated with 10^6 (A) or 10^5 (B) cells mL⁻¹ of MIT9215_{AX}. All growth rates are expressed as a percentage of the growth rate of a simultaneously inoculated control (without buffer) culture. Single asterisks indicate the samples are significantly different from the relevant control either based on unpaired t-tests ($P < 0.05$) or because 1 or more test cultures failed to grow. Double asterisks indicate absence of detectable growth after 60 days in all 3 replicate cultures. Error bars are the standard deviation of three replicate cultures.

Under the more natural light/dark cycle, 1 mM HEPES or TAPS did not affect growth at moderate ($65 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or high ($250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) light levels (Fig. 3.4A). When the buffer concentration was raised to 10 mM, however, pronounced growth rate decreases were observed for all cultures. Both TAPS and HEPES lowered growth rates, suggesting that even the lower rate of HOOH production by TAPS become deleterious at this buffer concentration. Moreover, one HEPES replicate failed to grow entirely. At high light ($250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), no HEPES cultures survived, whereas 2 out of 3 TAPS cultures survived. Catalase exerted a clear protective effect. At both light levels, catalase-treated 10 mM TAPS cultures had higher growth rates than untreated cultures. Catalase also prevented mortality in HEPES at low light and TAPS at high light; it was not, however, capable of allowing growth in the presence of 10 mM HEPES at high light.

When the initial inoculum of *Prochlorococcus* was lowered down to the ecologically-relevant level of $10^5 \text{ cells mL}^{-1}$ the effects of the buffers on growth became even more pronounced (Fig. 3.4B). Under these conditions no HEPES-containing cultures grew, regardless of light level, buffer concentration, or the presence of catalase. On the other hand, 1 mM TAPS cultures continued to grow normally at moderate light, although only 1 of 3 replicates grew at high light. The addition of catalase restored the control growth rate to the 1 mM TAPS cultures grown at high light. Strikingly, no cultures with an ecologically relevant inoculum survived in the presence of 10 mM buffer at either light level, even with catalase.

As a final observation, we noted that storage of media bottles on the bench top, exposed to light, had a significant effect on the survival of cultures inoculated into that media. Buffered media in bottles stored under ambient lab lighting (approximately $30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 12 hours a day) for 1 week were universally lethal to cultures regardless of incubation conditions (data not shown). This effect was observed for both HEPES and TAPS buffered media, but was eliminated completely by the addition of catalase prior to inoculation.

V. HEPES as a steady-state HOOH generator

We used multiple linear regression of our empirical HOOH production data in natural seawater to provide a framework for predicting k_{HOOH} under varying light levels, buffer concentrations, and seawater concentrations. This regression was strongly significant ($r^2 =$

0.958, $P < 0.001$ for all three experimentally manipulated parameters), suggesting HEPES concentration (or, for that matter, light or seawater concentration) could be manipulated to generate a variable rate of HOOH accumulation over time. Furthermore, introduction of a light-dark photoperiod, and the addition of catalase (to simulate biological HOOH decomposition) to this system, might create a diel oscillation in HOOH concentration similar to those observed in illuminated natural waters [217,218,219]: in both systems, HOOH consumption is constant, while production only occurs in the light. To test this, we used 100% natural seawater, $65 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and 10 mM HEPES, calculated to produce approximately 70 nM h^{-1} HOOH. We then supplemented this medium with a range of catalase concentrations to vary the competing HOOH removal rate and sampled the system for HOOH concentration periodically over two photoperiods of incubation (16:16 hours of light:darkness during the first photoperiod, and 12:12 during the second).

In the catalase-free buffered samples, HOOH accumulated at a consistent rate during both diel light periods as expected, although the rate was almost twice as fast as predicted (Fig. 3.5A). HOOH accumulation ceased at “night” and resumed immediately upon illumination in the “morning.” An examination of dk_{HOOH}/dt (Fig. 3.5B) shows that, at the beginning of each light period, HOOH accumulation was initially rapid but tapered off over the course of the day before finally ceasing in the dark. Since the regression equation was produced using continuously illuminated samples, this diel variation in k_{HOOH} could explain the discrepancy between our predictive model and our experimental results.

In catalase-treated samples, net HOOH accumulation was attenuated as a function of catalase concentration, with net HOOH accumulation in the light and net HOOH removal over the dark period (Fig. 3.5B). However, the degree of attenuation and night-time consumption decreased on day 2 relative to day 1, suggesting a loss of catalase activity over the course of the experiment. This is consistent with previous reports indicating that catalase is itself photosensitive [202] and is also susceptible to damage from ROS [26]. Nevertheless, it appears that 0.1 to 1 U mL^{-1} of catalase may be used in cooperation with HEPES to simulate the HOOH periodicity observed in the ocean, at least for the first 1 or 2 d.

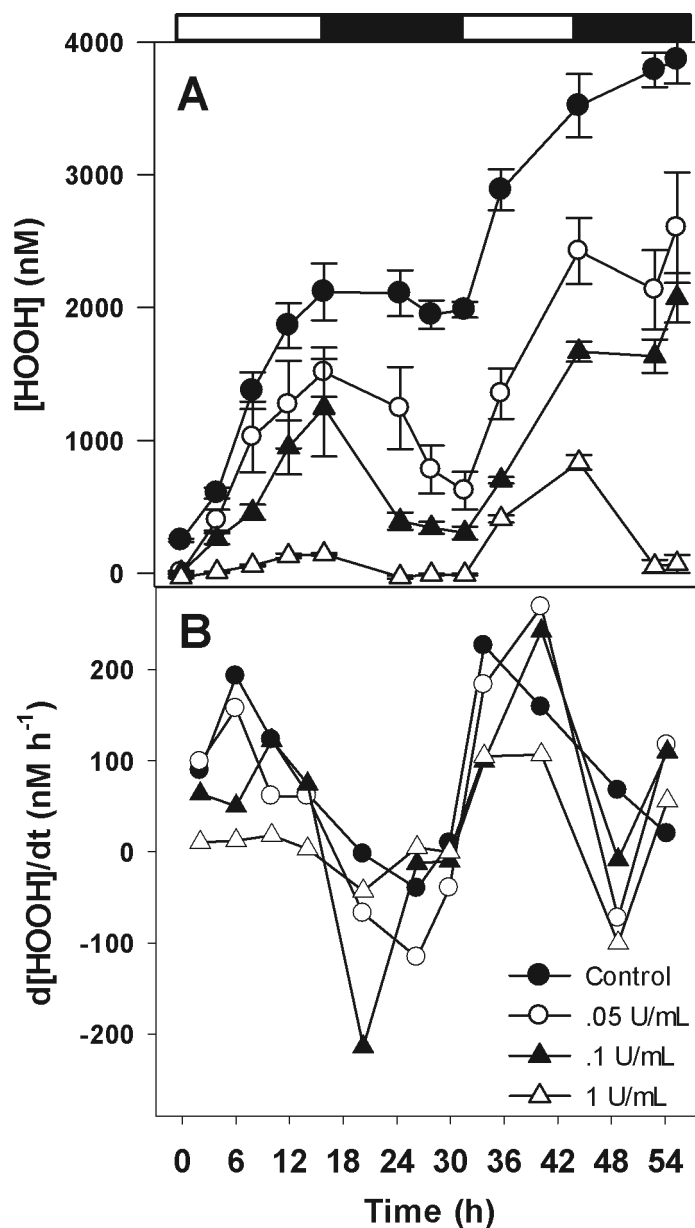


Figure 3.5. HEPES as a steady-state HOOH generator. A) Time series of HOOH accumulation in natural seawater with 10 mM HEPES either with or without catalase (see legend in panel B). Samples were incubated under a light/dark regime (16/16 on day 1, 12/12 on day 2, indicated by white and black bars at the top of the figure). Error bars are the standard deviations of three replicate treatments. B) First derivatives of the data in 5A. Each data point is the average change in [HOOH] between the two flanking time points in 5A.

VI. Discussion

In this work we confirmed the photochemical production of HOOH in HEPES-buffered media and demonstrated its toxic effects on an axenic strain of *Prochlorococcus*. We utilized this relatively vulnerable organism to emphasize the toxicity of HEPES-generated HOOH, but HEPES toxicity has also been demonstrated for *Skeletonema costatum* [220] despite the higher HOOH resistance of diatoms relative to cyanobacteria [138,221]. While we readily acknowledge that both *Prochlorococcus* [180,181] and diverse eukaryotic algae [222] have been successfully cultured in HEPES-containing media, we suspect that special handling of these cultures (e.g., high transfer volumes, lower light levels) facilitated their survival. Even in these cases, however, it is necessary to keep in mind that the presence of Good's buffers in culture media produces a constant flux of ROS whenever the media are exposed to light. Though this flux may not be sufficient to result in cell death or growth arrest, it may still alter gene expression, metabolism, cell composition, etc. [223,224,225,226,227], potentially leading to erroneous interpretations of experimental results. As an example, *Escherichia coli* exhibits an oxidative stress response when first exposed to rich media agar plates [228], which are known to contain HOOH [198]. While these *E. coli* cells do not demonstrate an obvious stress phenotype, i.e. they remain viable and grow following a lag phase, exposure to solid media induces a cryptic response that has far-reaching consequences on the organism's physiology.

Because of its inherent buffering capacity, it is often unnecessary to add exogenous buffers to natural seawater media. However, buffers are necessary for some applications (e.g. artificial seawater, large volume cultures) and our work suggests a number of procedural improvements to avoid their deleterious side effects:

1. To minimize HOOH production, buffers that are poor producers of HOOH should be chosen. By this metric, Tris and glycylglycine, already in use for several algal media recipes [229], may be ideal choices as they produce no HOOH. However, as both were toxic to *Prochlorococcus* for reasons apparently unrelated to oxidative stress, the use of such buffers should be determined on a case-by-case basis. If HOOH-producing buffers must be used, the lowest concentration capable of maintaining an acceptable pH should be empirically determined and used for all media recipes. For culturing

Prochlorococcus, we recommend AMP-J, a modified form of AMP-1. This medium replaces 1 mM HEPES with 1 mM TAPS. Also, it is sterilized by passage through a 0.2 μ m PES membrane, as autoclaving is another major source of HOOH in culture media (data not shown).

2. Media should be stored in the dark until inoculation.
3. When cultures are grown under high light and/or concentrated buffer conditions, starting inocula should be kept high to counter the elevated HOOH concentrations that may occur.
4. Catalase may be added in small amounts to ameliorate buffer toxicity in cases where dilute cultures are required. However, our results indicate that exogenous catalase degrades fairly quickly, so its protective power will probably not last more than a day or two. Further, we have observed in other studies that inactivated catalase is itself capable of catalyzing the photoproduction of HOOH, probably by means of its heme cofactor (data not shown). If culture purity is not required, the best solution may be to intentionally contaminate the algal culture with a heterotrophic catalase-positive “helper” microbe to maintain a low HOOH environment (Chapter 2).

Our work has focused on the use of buffers in culture media. However, we feel that these findings are of significance to *in vitro* systems as well. A number of common molecular biology procedures such as protein purification call for HEPES or another Good’s buffer. Since ROS are capable of extensively modifying all classes of biological macromolecules [26], researchers using these recipes are encouraged to protect their solutions from light as much as possible.

While in some situations in nature microorganisms are exposed to acute, lethal levels of ROS (e.g., oxidative bursts inside macrophages), a more common scenario is chronic exposure to lower, sub-lethal levels (e.g., in corals and their symbionts [230]). Even though the toxic and chemically reactive properties of HEPES make it undesirable as a general buffer for light-exposed cultures, it may be useful in studies of such long-term exposures of cells to HOOH. This is not only true for microorganisms but also for the tissues of animals and plants. Several steady-state systems exist for the generation of different types of ROS: rose Bengal for singlet oxygen, the xanthine/xanthine oxidase (XXO) couple for superoxide anion (O_2^-), and the Fenton

reaction for hydroxyl radical [26]. While the XXO couple ultimately generates HOOH via the spontaneous disproportionation of O_2^- , this process is relatively slow and limited by the concentration of xanthine in the system and the lifespan of the xanthine oxidase enzyme. The HEPES system, in contrast, appears to produce HOOH as its primary product. HEPES does not appear to generate stable O_2^- since HOOH formation does not continue into the dark period; otherwise the light-independent process of disproportionation of O_2^- into HOOH would have been observed once the light was removed. Further, the net accumulation of HOOH is continuous over at least several days and apparently much longer in our system. The level of HOOH production may be fine-tuned by adjusting any of the three parameters measured here: seawater (or perhaps DOC) concentration, buffer concentration, or light level. By using an equimolar amount of total buffer amongst treatments, but varying the ratio of TAPS:HEPES, one may separate the effect of increased HOOH production from any effect obtained from changes in the buffering capacity of the culture media. We feel that the HEPES system is suited for a number of experimental applications, providing a valuable new method for exploring the long-term effects of ROS production in living cells, with the caveat that each investigator should calibrate the rates of HOOH production in their own experimental milieu. In Chapter 4 we use this system to simulate the diel pulse in HOOH concentrations observed in natural waters; however, the system would be equally useful for measuring the effects of, for instance, HOOH production on the metabolism of mammalian mitochondria in cell culture.

In conclusion, we have demonstrated that Good's buffers, in the presence of light, O_2 , and an unidentified component of seawater such as DOC, cause the rapid accumulation of HOOH in culture media. We have provided a number of suggestions for the improvement of culture techniques in light of this information, including the replacement of HEPES with TAPS. Further, we hope that this work can inform future studies of chronic ROS exposure and allow for the use of HEPES as a long-term HOOH generator rather than strictly as a pH buffer.

CHAPTER 4

Dependence of the Cyanobacterium *Prochlorococcus* on Hydrogen Peroxide Scavenging Microbes for Growth at the Ocean's Surface

Publication Note

This chapter has been submitted in a slightly altered form to the journal *PLoS One*.

Contributions

This chapter was prepared in collaboration with Martin J. Szul, Zackary I. Johnson, Martin Keller, and Erik R. Zinser. I performed the experiments. MJS created catalase mutants of helpers. ZIJ assisted with photophysiology. MK assisted with flow cytometry. ERZ and I designed the experiments and wrote the paper.

Acknowledgements

The authors would like to thank J. Chandler, E. Mann, K. Barbeau, B. Binder, L. Poorvin, A. Anderson, W. Cooper, and G. O'Toole for advice and technical support; the captains and crews of *R/V Kilo Moana*, *R/V New Horizon*, and *R/V Cape Hatteras*; S. Allman for assistance with flow cytometry; J. Dunlap for assistance with scanning electron microscopy; and S. Wilhelm and G. Sayler for access to equipment and facilities.

ABSTRACT

The phytoplankton community in the oligotrophic open ocean is numerically dominated by the cyanobacterium *Prochlorococcus*, accounting for approximately half of all photosynthesis. In the illuminated euphotic zone where *Prochlorococcus* grows, reactive oxygen species are continuously generated via photochemical reactions with dissolved organic matter. However, *Prochlorococcus* genomes lack catalase and additional protective mechanisms common in other aerobes, and this genus is highly susceptible to oxidative damage from hydrogen peroxide (H₂O₂). In this study we showed that the extant microbial community plays a vital, previously unrecognized role in cross-protecting *Prochlorococcus* from oxidative damage in the surface mixed layer of the oligotrophic ocean. Microbes are the primary H₂O₂ sink in marine systems, and in the absence of the microbial community, surface waters in the Atlantic and Pacific Ocean accumulated H₂O₂ to concentrations that were lethal for *Prochlorococcus* cultures. In laboratory experiments with the marine heterotroph *Alteromonas* sp., serving as a proxy for the natural community of H₂O₂-degrading microbes, bacterial depletion of H₂O₂ from the extracellular milieu prevented oxidative damage to the cell envelope and photosystems of co-cultured *Prochlorococcus*, and facilitated the growth of *Prochlorococcus* at ecologically-relevant cell concentrations. Curiously, the more recently evolved lineages of *Prochlorococcus* that exploit the surface mixed layer niche were also the most sensitive to H₂O₂. The genomic streamlining of these evolved lineages during adaptation to the high-light exposed upper euphotic zone thus appears to be coincident with an acquired dependency on the extant H₂O₂-consuming community. These results underscore the importance of (indirect) biotic interactions in establishing niche boundaries, and highlight the impacts that community-level responses to stress may have in the ecological and evolutionary outcomes for co-existing species.

I. Introduction

The open ocean is the largest biome on the surface of the earth, but due to its distance from coastal and deep ocean sediments, is also one of the most oligotrophic. Nutrient scarcity is especially prevalent in the surface mixed layers of highly stratified systems, with inputs of nutrients often restricted to new production (e.g. nitrogen fixation) or atmospheric (dust) deposition [13]. Microbes dominate biomass in this “wet desert” [51] and the most abundant phytoplankter in the tropics and subtropics ($\sim 40^\circ$ N to 40° S latitude) is the unicellular cyanobacterium, *Prochlorococcus* [9]. This oligotrophic specialist has the smallest cell size (0.4 to $1.2\ \mu\text{m}$ in diameter) and genome ($1.7\text{--}2.5 \times 10^6$ bp) [11,231] of any known photoautotroph. The small cell size results in a superior surface to volume ratio that is believed to provide a key advantage in nutrient scavenging versus larger competitors [232]. The small genome size, together with a reliance on sulfo- rather than phospholipids [233], greatly diminishes its cellular P quota, providing an additional advantage over larger competitors. As a result of these and other adaptations, *Prochlorococcus* populations span the entire euphotic zone, often exceeding 10^5 cells mL^{-1} , and due to this numerical dominance have been credited for roughly half of all photosynthesis in the oceans [234,235,236,237,238].

Genetically distinct ecotypes of *Prochlorococcus* partition the oligotrophic euphotic zone niche with respect to depth and latitude, in response to gradients of light and temperature, respectively. The upper euphotic zone is dominated by two closely-related ecotypes, eMED4 (“e” for ecotype, “MED4” for the type strain of the lineage) and eMIT9312, which are high-light adapted (HL), while the lower euphotic zone is dominated by low-light adapted (LL) ecotypes that include eNATL2A, eMIT9313, and eSS120 [177,190,191,239,240]. In seasonally-stratified regions of the subtropics, deep mixing events facilitate an invasion of the surface mixed layer by the LL ecotype eNATL2A [177,241], which in certain instances may lead to numerical dominance of the LL ecotypes in the mixed layer [242]. The high irradiances found near the ocean surface restrict the growth of eNATL2A [177] as well as the other LL ecotypes [191] but eNATL2A appears unique amongst the LL ecotypes in its ability to survive temporary exposures to high light, such as would be experienced during deep vertical mixing events [241]. The two HL ecotypes further partition the upper euphotic zone by latitude: the eMIT9312 ecotype dominates the middle band from 30° N to 30° S latitude, while eMED4 dominates the higher

latitudes at the extremes of *Prochlorococcus*' distribution [243]. This latitudinal niche partitioning is driven primarily by ocean temperature [177,241,242,243,244], and is consistent with the growth properties of cultured ecotype representatives [243].

The pattern of diversification within the *Prochlorococcus* lineage is consistent with an evolutionary progression from LL ancestors restricted to the deep euphotic zone, towards HL strains able to exploit the high light niche in the surface mixed layer. The LL eMIT9313 ecotype is the earliest branching lineage from the last common ancestor of *Prochlorococcus* and *Synechococcus*, and like all *Prochlorococcus* lacks the phycobilisome light harvesting complexes characteristic of many cyanobacteria including *Synechococcus* [231,245]. Instead, *Prochlorococcus* utilizes transmembrane light harvesting complexes containing divinyl chlorophyll a and b, which optimize the cells for utilization of the blue light wavelengths that penetrate deepest into the euphotic zone [246]. The LL lineages are polyphyletic, with the eNATL2A ecotype the most recently derived. Concurrent with the emergence of the eNATL2A lineage was the ability of *Prochlorococcus* to invade the surface mixed layer, albeit only in instances of deep vertical mixing that minimize exposure to high light [177]. Acquisition of a DNA photolyase and an elevated number of high light inducible proteins may have been responsible for this adaptation for high light tolerance [247]. With the emergence of the eMED4 and eMIT9312 “true” high light ecotypes – the most derived lineages – *Prochlorococcus* gained a sustained presence in the mixed layer, irrespective of mixed layer depth. The HL ecotypes have a common “core” of ~100 genes not found in the LL ecotypes, and many of these may be responsible for the exploitation of high light near the surface [247].

In addition to the high light and low nutrient stresses associated with the oligotrophic surface mixed layer, the invasion of the mixed layer by *Prochlorococcus* may have also involved elevated oxidative stress. While hydrogen peroxide (HOOH) is ubiquitous in the ocean, the photooxidation of dissolved organic carbon (DOC) by sunlight [46], particularly by light in the UV range [79], results in near-surface HOOH maxima. Along with iron, HOOH is enriched in rainwater [83,84,86], and the combination of the two may cause periodic oxidative stress in marine organisms via the generation of highly reactive hydroxyl radicals ($\cdot\text{OH}$) by the Fenton reaction [96,248]. HOOH has been shown to inhibit the growth of diverse marine algae, including cyanobacteria, macro- and microscopic chlorophytes, diatoms, and coral-associated

zooxanthellae [56,85,137,138,141,225], albeit at levels higher than those typically measured in pelagic waters.

In prior work, we demonstrated that *Prochlorococcus* was dependent on “helper” heterotrophic bacteria to thrive in dilute laboratory cultures (Chapter 2). Many heterotroph strains were capable of helping *Prochlorococcus*, including members of the α - and γ -Proteobacteria and the Bacteroidetes cluster, suggesting the mechanism(s) are common bacterial activities. In these experiments, the helping phenomenon occurred when *Prochlorococcus* and heterotrophic bacteria were inoculated at ecologically relevant concentrations, suggesting that the helping mechanism might also play an important role in natural communities. Preliminary evidence suggested the helper phenotype was associated with protection from HOOH, compensating for *Prochlorococcus*’ conspicuously diminished suite of antioxidant genes (e.g. catalase) compared to other aerobes [173,231]. In this study, we were driven by the following questions regarding the heterotroph/*Prochlorococcus* interactions: is the removal of HOOH from the medium necessary and sufficient for the helping phenotype? Does this mechanism of protection have relevance to natural communities in the open ocean? How universal is the dependency on helpers for the growth of the *Prochlorococcus* lineages? Finally, what does the apparent loss of endogenous HOOH-protection mechanisms imply regarding the genomic streamlining of the *Prochlorococcus* genus?

II. Results

A) HOOH removal is necessary and sufficient for the helping phenotype. From our prior work, we hypothesized that heterotrophs help *Prochlorococcus* by the removal of HOOH from the medium (Chapter 2). To test this hypothesis we began by analyzing the impacts of *Prochlorococcus* and heterotrophic growth on the medium chemistry. Standard Pro99 medium prepared with filtered, autoclaved seawater was found to contain approximately $0.2 \pm 0.02 \mu\text{M}$ HOOH. Axenic cultures of *Prochlorococcus* UH18301 (a novel isolate of the eMIT9312 ecotype, Table 4.1) depleted medium HOOH either very slowly or not at all, depending on the initial inoculum (Fig. 4.1). Compared to these axenic controls, however, co-cultures with the “helper” strain *Alteromonas* sp. EZ55 rapidly depleted this HOOH to below detection ($<0.01 \mu\text{M}$) (Figure 4.1). In contrast, pH, dissolved oxygen, bicarbonate, and dissolved organic carbon

Table 4.1. Strains used in this study.

Strain	Parent Strain	Genotype	Ecotype/Species	Reference ¹	Genome Accession Number
VOL1	MIT9215	Sm ^r ²	<i>Prochlorococcus</i> eMIT9312	[247] and Chapter 2	NC_009840 ⁴
MIT9313	MIT9313	WT ³	<i>Prochlorococcus</i> eMIT9313	[245]	NC_005071
VOL3	NATL2A	Sm ^r	<i>Prochlorococcus</i> eNATL2A	[247] and this study	NC_007335 ⁴
VOL4	MIT9312	Sm ^r	<i>Prochlorococcus</i> eMIT9312	[247] and this study	NC_007577 ⁴
VOL5	AS9601	Sm ^r	<i>Prochlorococcus</i> eMIT9312	[247] and this study	NC_008816 ⁴
UH18301	UH18301	Sm ^r	<i>Prochlorococcus</i> eMIT9312	[247] and this study	MMI ⁵
VOL7	MED4	Sm ^r	<i>Prochlorococcus</i> eMED4	[245] and this study	NC_005072 ⁴
VOL8	MIT9515	Sm ^r	<i>Prochlorococcus</i> eMED4	[247] and this study	NC_008817 ⁴
EZ55	WT	WT	<i>Alteromonas</i> sp.	Chapter 2	MMI ⁵
EZ111	EZ55	<i>KatA::Tn5</i>	<i>Alteromonas</i> sp.	This study	MMI
ESR1	<i>V. fischeri</i> ES114	Rf ^r ⁶	<i>Vibrio fischeri</i>	[249]	NC_006840, NC_006841 ⁴
KV433	ESR1	Rf ^r <i>KatA::Erm</i>	<i>Vibrio fischeri</i>	[59]	NC_006840, NC_006841 ⁴
EZ121	WT	WT	<i>Ruegeria lacuscaerulensis</i>	[250]	NZ_ACNX000000000
EZ145	EZ121	<i>KatG::Tn5</i>	<i>Ruegeria lacuscaerulensis</i>	This study	NZ_ACNX000000000 ⁴
WH7803	WT	WT	<i>Synechococcus</i> sp.	[251]	NC_009481

¹ Reference describing strain and/or genome

² Sm^r, spontaneous streptomycin-resistant mutant

³ WT, wild type

⁴ The published genome is from this strain's parent.

⁵ Sequencing in progress. Data available from the Gordon and Betty Moore Foundation Marine Microbiology Initiative (<https://moore.jcvi.org/moore/>).

⁶ Rf^r, spontaneous rifampicin-resistant mutant

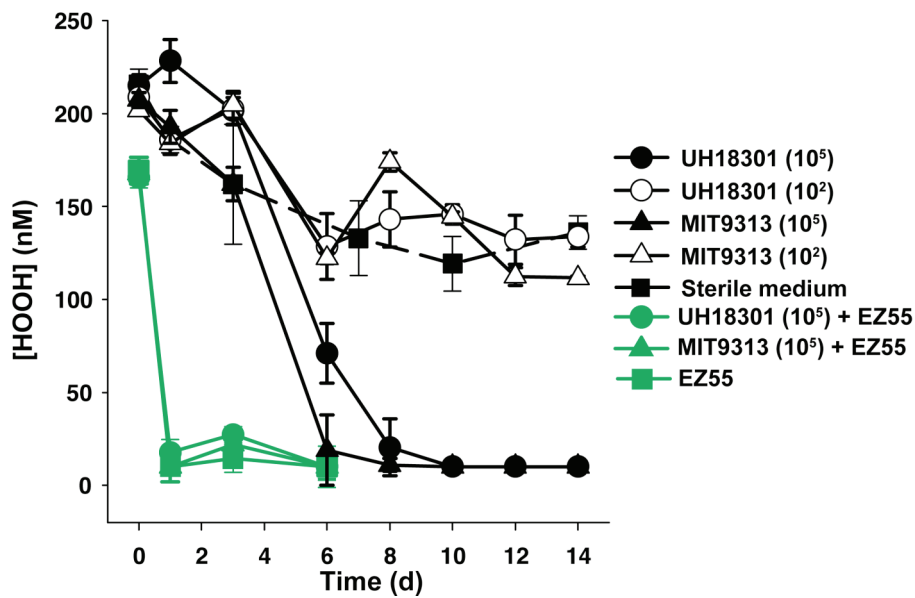


Figure 4.1. HOOH removal by *Prochlorococcus* spp. and *Alteromonas* sp. EZ55. *Prochlorococcus* was added to autoclaved Pro99 media at the indicated initial inoculum (cells mL^{-1}) either with or without 10^6 cells mL^{-1} EZ55. Changes in [HOOH] were followed using acridinium ester chemiluminescence (Chapter 3).

were not significantly different in axenic versus mixed cultures of *Prochlorococcus* during the first week of culture (t-tests for individual data points, $df=4$, $p>0.05$, Figure 4.2). While CO_2 concentration was different in mixed versus axenic *Prochlorococcus* cultures, this potential carbon source for *Prochlorococcus* was *less* abundant in co-cultures as a consequence of slightly elevated pH (Fig. 4.2, C and E). While EZ55 on its own had the predicted effect of lowering O_2 concentrations, and raising inorganic carbon concentrations, this change was relatively minor, short-lived, and not reflected in a difference between axenic UH18301 and co-cultures with EZ55 (Fig. 4.2). Thus, of all the potential means by which helpers may benefit *Prochlorococcus* through their modification of the bulk medium, whether by adding a nutrient or removing a growth inhibitor, the removal of HOOH was the only one consistent with a beneficial effect for *Prochlorococcus*.

All confirmed helpers are catalase-positive (Chapter 2), but the helping phenotype could not be unambiguously assigned to the catalase activity of these bacteria. While insertional inactivation of catalase in *Alteromonas* sp. EZ55, *Vibrio fischeri* ES114, and *Ruegeria lacuscaerulensis* ITI-1157 resulted in the loss of ability to help *Prochlorococcus* growth on agar media (Chapter 2 and data not shown), the results in liquid media were less clear. Catalase mutants of EZ55 and ES114 were still capable of helping dilute liquid cultures of *Prochlorococcus*, but they also showed negligible loss of HOOH scavenging ability in liquid (Table 4.2). Redundant HOOH scavenging pathways are common in aerobic heterotrophs [252,253], and these are likely to maintain the heterotrophs in a help-competent state, at least for experiments in liquid (semisolid agar media may have higher HOOH levels that do require active catalase). In contrast, the *R. lacuscaerulensis* catalase mutant was unable to help *Prochlorococcus* in liquid, but by an unknown mechanism actually raised HOOH levels in culture media relative to sterile controls (Table 4.2). Studies with purified bovine liver catalase likewise provided ambiguous results. While catalase provided some benefit to UH18301 in comparison to untreated, axenic controls, it was clearly inferior in comparison to co-culture with bacterial helpers (Table 4.2). Catalase is photo-inactivated ([202] and Fig. 4.3), and heat-killed catalase resulted in higher *Prochlorococcus* mortality than unamended controls (Table 4.2). Thus, light-driven degradation of catalase to toxic end products may account for these ambiguous results.

Table 4.2. Results of helper assays in liquid Pro99. *Prochlorococcus* was inoculated at 100 cells mL⁻¹ in media with the indicated treatment.

	Treatment	MIT9313	UH18301
Helper-naïve media	Axenic	186.1 ± 20.6 ¹ (9/10) ²	189.9 ± 25.5 (1/7)
	Catalase ³	-0.9 ± 6.4 (6/6)	12.1 ± 13.6 (5/7)
	Boiled Catalase ⁴	159.2 ± 79.3 (1/3)	153.2 ± 43.8 (0/3)
Wild-type helpers	<i>Alteromonas</i> sp. EZ55	8.3 ± 14.4 (12/12)	7.4 ± 14.9 (9/9)
	<i>V. fischeri</i> ESR1	10.7 ± 19 (3/3)	73.1 ± 29.7 (5/5)
	<i>R. lacuscaerulensis</i>	5.6 ± 7.8 (3/3)	-1.6 ± 12.7 (5/5)
Catalase mutants	<i>Alteromonas</i> sp. EZ111	54.1 ± 34.5 (5/5)	28.9 ± 25.4 (6/6)
	<i>V. fischeri</i> KV433	7.3 ± 17.4 (3/3)	79.5 ± 35.4 (5/5)
	<i>R. lacuscaerulensis</i> EZ145	78.6 ± 100.8 (2/4)	207 ± 137.3 (1/7)

¹ Top values, integrated daily HOOH exposure ([HOOH] $\bar{x}$) for the first week of culture, plus or minus the standard deviation of all replicates.

² Bottom values in parentheses, number of cultures that survived to late exponential phase ($> 10^7$ cells mL⁻¹)/total number of replicates tested.

³ Catalase was prepared as a 1% stock of crystals (~1340 U mg⁻¹ solid) in 37° C milli-Q water buffered to pH 7.0 with 10 mM phosphate buffer, then sterilized by passage through a 0.2 μ m Millex GV syringe filter. Experiments were performed with a final concentration of 1 U mL⁻¹ catalase.

⁴ Catalase was inactivated by heating at 95° C for 5 min.

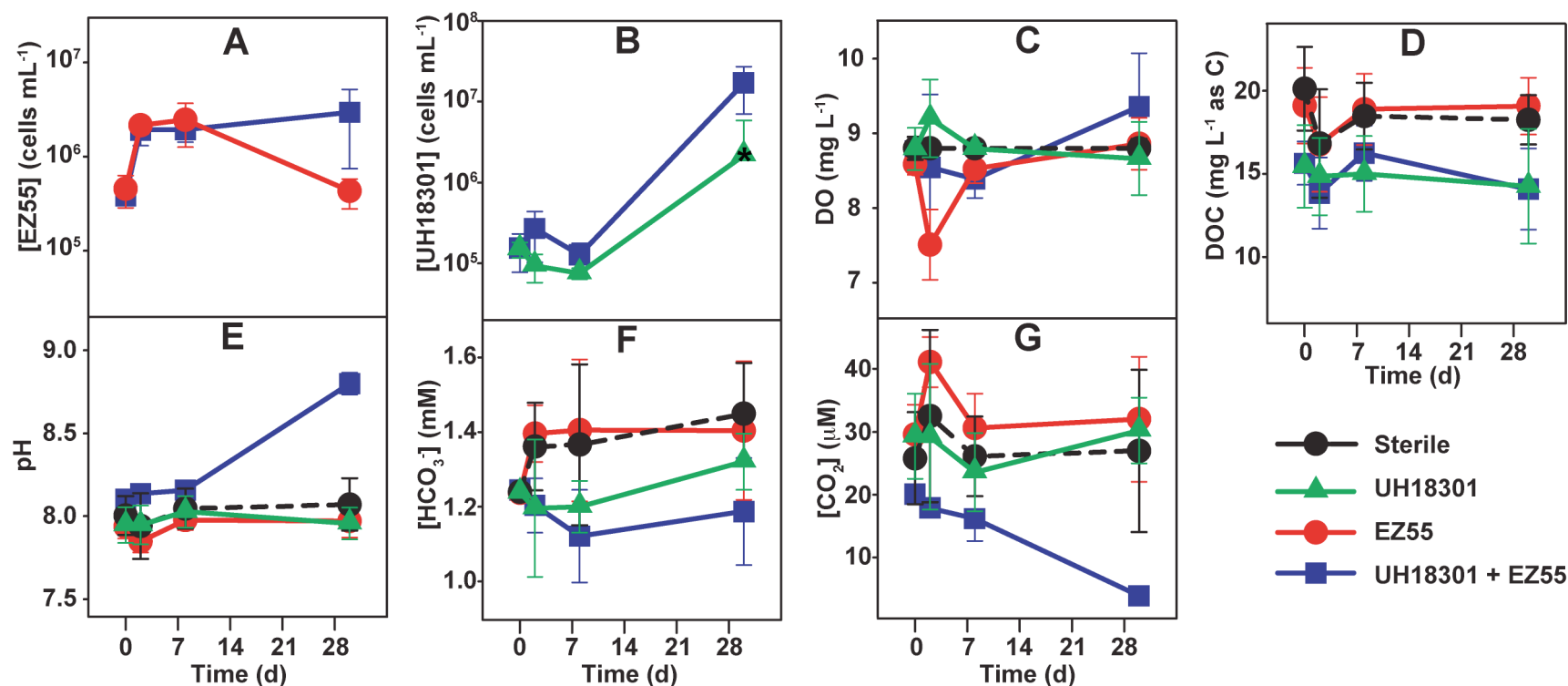


Figure 4.2. Influence of *Prochlorococcus* UH18301 and *Alteromonas* EZ55 on Pro99 medium chemistry. A) EZ55 cell concentration; B) *Prochlorococcus* UH18301 cell concentration; C) pH; D) HCO₃⁻ concentration; E) CO₂ concentration, representing the sum of dissolved CO₂ and H₂CO₃; F) dissolved O₂ concentration; G) Total organic carbon in 0.22 μm-filtered media. Error bars are the standard error of three biological replicates. Black circles, sterile media; green triangles, axenic UH18301; red circles, axenic EZ55; blue squares, co-cultured UH18301 and EZ55. The * in Panel B indicates that only 1 of 3 replicates survived to this point, leading to a very large standard deviation for cell counts.

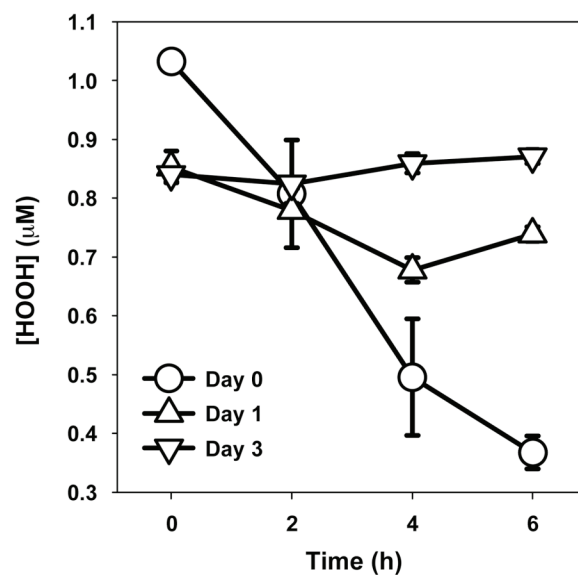


Figure 4.3. Photoinactivation of purified catalase. Catalase was added to sterile Pro99 media at 1 U/mL and incubated in a Sunbox incubator under low light conditions (see Methods). Aliquots were removed immediately after sample preparation (circles), after 1 d (up triangles), and after 3 d (down triangles). 0.8 μ M HOOH was added to these aliquots, and the change in HOOH was monitored for 6 h.

Because complications obscured the results of the catalase studies, we confirmed the role of HOOH scavenging in the helping phenotype by another line of experimentation, which was to manipulate the HOOH and heterotroph activities in media prior to inoculation with *Prochlorococcus*. Dilute (~ 100 cells mL⁻¹) axenic *Prochlorococcus* UH18301 was unable to lower the initial HOOH of the autoclaved medium, resulting in a mean daily exposure ($[\text{HOOH}]\bar{x}$) of $0.2 \mu\text{M}$, and consequently exhibited very poor growth over this period (Fig. 4.4A). In contrast, pre-inoculation of the medium with the EZ55 helper strain resulted in robust growth of UH18301. This significant growth improvement was associated with a much lower $[\text{HOOH}]\bar{x}$ ($< 0.02 \mu\text{M}$) as a result of the activity of EZ55. The activity of EZ55 was sufficient to pre-condition the medium for *Prochlorococcus* growth, as evidenced by the robust UH18301 growth in media pre-incubated with EZ55 for 24 hours, followed by removal of the EZ55 cells via filtration prior to inoculation of the *Prochlorococcus* cells. As for the co-culture treatment, this pre-conditioning treatment also resulted in a significantly lower $[\text{HOOH}]\bar{x}$ for *Prochlorococcus*. Filtration of the EZ55 did not appear to release intracellular components into the medium, as no detectable hydroperoxidase or alkaline phosphatase activity remained in the filtrates, in contrast to the unfiltered cultures themselves (Fig. 4.5). Finally, if HOOH was added back to the EZ55-conditioned medium, restoring the HOOH concentration to $0.2 \pm 0.02 \mu\text{M}$, the enhanced growth effect of the EZ55 preconditioning step on *Prochlorococcus* growth was lost (Fig. 4.4A). This indicates that any activity of EZ55 other than the removal of HOOH is not sufficient to explain the helping phenotype of these cells.

The ability of axenic *Prochlorococcus* to grow in concentrated but not dilute cultures (Chapter 2) suggests that *Prochlorococcus* can help itself in a density-dependent manner. To confirm this, we conducted co-culture experiments between two strains of *Prochlorococcus* distinguishable by quantitative PCR. Axenic VOL1 (a streptomycin-resistant mutant of MIT9215, Table 4.1) had a minimal influence on the medium $[\text{HOOH}]$ and was incapable of growth from 100 cells mL⁻¹ (Fig. 4.4B and Chapter 2). However, when co-cultured with 10^5 cells mL⁻¹ of VOL7 (a streptomycin-resistant mutant of MED4, Table 4.1), VOL1 survived and grew at approximately the same rate as VOL7 (Fig. 4.4B). VOL1 grew slower in co-culture with VOL7 than in co-culture with the heterotroph, EZ55, and consistently, the ability of VOL7 to remove HOOH from the medium was also substantially lower than EZ55 (Fig. 4.4B). These

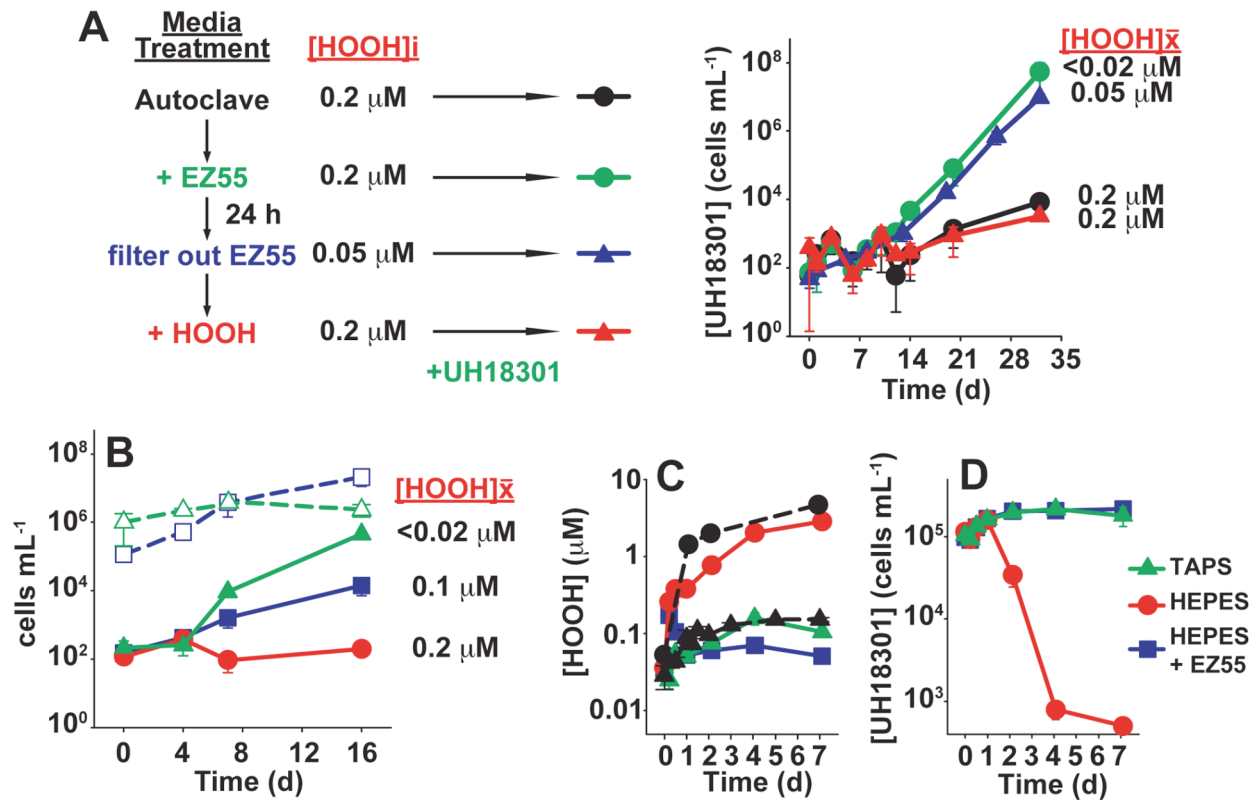


Figure 4.4. Removal of HOOH from media facilitates *Prochlorococcus* growth. A) Effects of pre-treatment of Pro99 medium on growth of dilute (initially ≈ 100 cells mL⁻¹) axenic *Prochlorococcus* UH18301 cultures grown under low light. Prior to inoculation of UH18301, medium was autoclaved (black circles), pre-inoculated with 10^6 cells mL⁻¹ EZ55 (green circles), pre-incubated for 24 hours followed by removal of EZ55 via filtration (blue triangles), and then supplemented with HOOH to restore its initial concentration (red triangles). [HOOH]_i, HOOH concentration at the time of inoculation; [HOOH]_̄, mean daily HOOH exposure, obtained by integrating [HOOH] over the first 7 d and dividing by the elapsed time. B) Growth kinetics of dilute *Prochlorococcus* VOL1 under high light in axenic cultures (red circles) and in co-culture with more concentrated *Prochlorococcus* VOL7 (solid blue squares) or EZ55 (solid green triangles). Also shown are the kinetics of the co-cultured VOL7 (open blue squares, dashed line) and EZ55 (open green triangles, dashed line). Error bars are the standard errors of 3 biological replicates. C) HOOH accumulation in high light-exposed UH18301-inoculated seawater containing 3.75 mM HEPES either with (blue squares) or without (red circles) EZ55, compared to an axenic control grown in media with 3.75 mM of the non-HOOH producing buffer TAPS (green triangles). Dashed lines, sterile media containing HEPES (black circles) or TAPS (black triangles). D) Cell concentrations over time in buffer-treated cultures. Symbols represent the same cultures as in 4.4C.

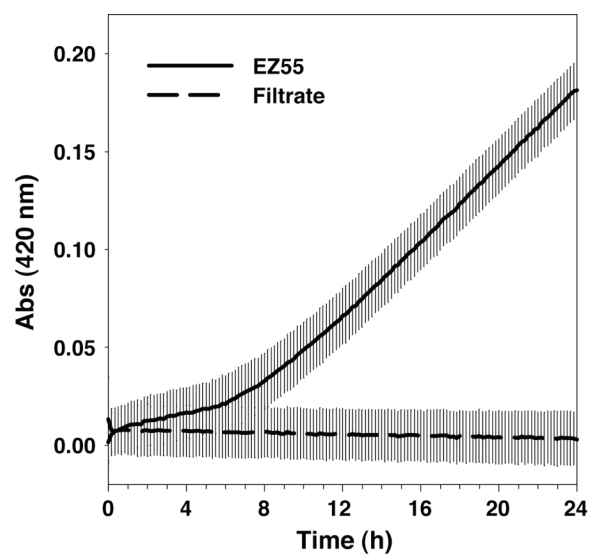


Figure 4.5. Alkaline phosphatase activity in EZ55-treated media. *Alteromonas* sp. EZ55 was added to Pro99 at 10^6 cells mL^{-1} and incubated in the dark for 24 h. Alkaline phosphatase activity was measured as described in the Methods either before (solid line) or after (dashed line) $0.2 \mu\text{m}$ filtration of this medium through low-protein-binding PVDF membranes. Readings were taken every 10 min for 24 h. Error bars are the standard deviation of three replicate well on a single 96-well plate.

observations support the hypothesis that *Prochlorococcus* is a poor scavenger of exogenous HOOH, but by mass-action at high densities can draw down HOOH to sub-lethal levels.

The experiments described above involved exposing dilute *Prochlorococcus* cultures to the elevated HOOH of autoclaved media. However, the poor HOOH scavenging capacity of *Prochlorococcus* and its dependency on help is also apparent when ecologically-relevant concentrations of cells ($\sim 10^5$ cells mL⁻¹) are challenged with HOOH that accumulates gradually in the medium. To avoid potential influences of the concentrated Fe, N, and P in growth media, we performed these experiments in sterile unamended seawater. In the presence of light, HEPES buffer generates HOOH ([211,212] and Chapter 3), and under high light (~ 250 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), the rate of HOOH generation in sterile seawater containing 3.75 mM HEPES was approximately 0.4 $\mu\text{M d}^{-1}$ (Fig. 4.4C). Axenic *Prochlorococcus* UH18301 was unable to prevent HOOH accumulation to lethal levels (Fig. 4.4C), resulting in a rapid decline in cell counts (Figure 4.4D). The same outcome was observed even when the HEPES concentration was lowered to reduce the rate of HOOH production by 75% (Fig. 4.6). However, if the *Prochlorococcus* cultures also contained EZ55, or if the HEPES buffer was substituted with the buffer TAPS, which generates much less HOOH (Fig 4.4C), HOOH concentrations were maintained at or below 0.1 μM , and subsequently *Prochlorococcus* cells survived, doubling about once before running out of nutrients in this unamended seawater medium (Fig. 4.4D).

B) Ecologically relevant levels of HOOH inhibit axenic *Prochlorococcus*. For the experiments described above, initial HOOH concentrations were between ~ 0 -0.2 μM . Importantly, this is well within the range observed *in situ* in the ocean euphotic zone [84,87,217,254,255,256,257], and as some of these experiments (e.g., the HEPES experiments, Fig. 4.4C-D) also involved ecologically-relevant concentrations of *Prochlorococcus* and helper, our results may reflect important interactions that occur in natural communities. The primary sources of HOOH in the ocean are rainfall events [83,84,85,86] and photochemical (especially UV) degradation of dissolved organic carbon [73,74,79]. Accordingly, the highest concentrations of HOOH in the oceans are in the upper euphotic zone, which we confirmed in a 2007 meridional transect in the Pacific Ocean (Fig 4.7A, Table 4.3). With a few exceptions, the concentration of HOOH in the surface mixed layer ranged from 0.075 - 0.15 μM , whereas the concentration rapidly dropped below the mixed layer and fell below the limit of detection (< 0.01

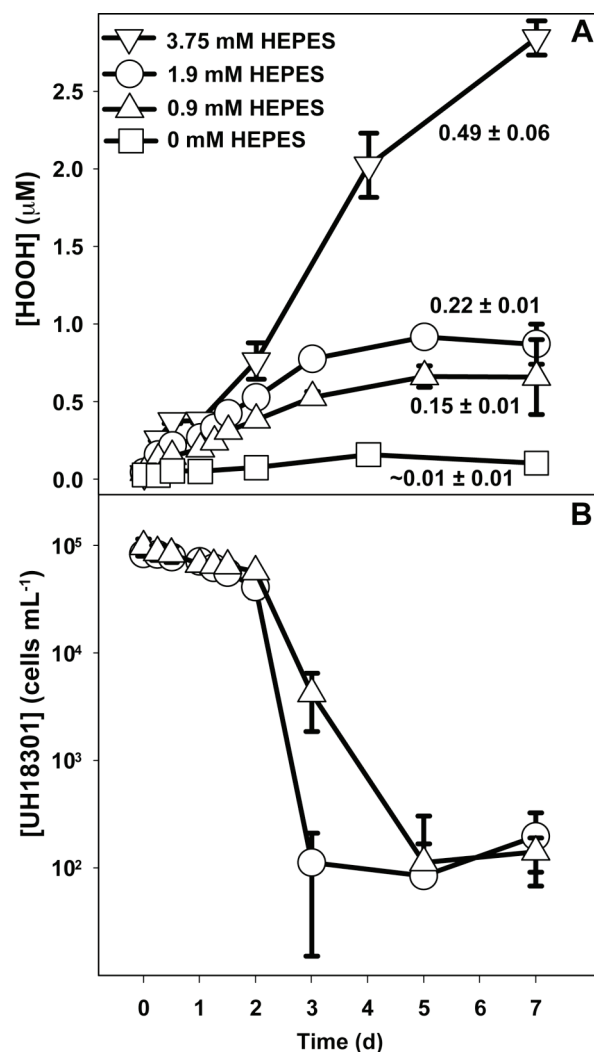


Figure 4.6. Effects of chronic HOOH exposure on *Prochlorococcus* UH18301. UH18301 was inoculated into sterile, unamended seawater containing the indicated concentration of HEPES at 10^5 cells mL^{-1} . All cultures had a constant 10 mM concentration of buffer; the difference was made up using TAPS. The control cultures for this experiment are plotted in Fig. 4.4C. A) HOOH accumulated in proportion to the concentration of HEPES in the medium. Values are the zero-order rate constants for HOOH formation, in $\mu\text{M d}^{-1}$, calculated over the first 3 d. Down triangles plot the same data shown by red circles in Fig. 4.4C for comparison. B) Changes in cell concentration were observed over time.

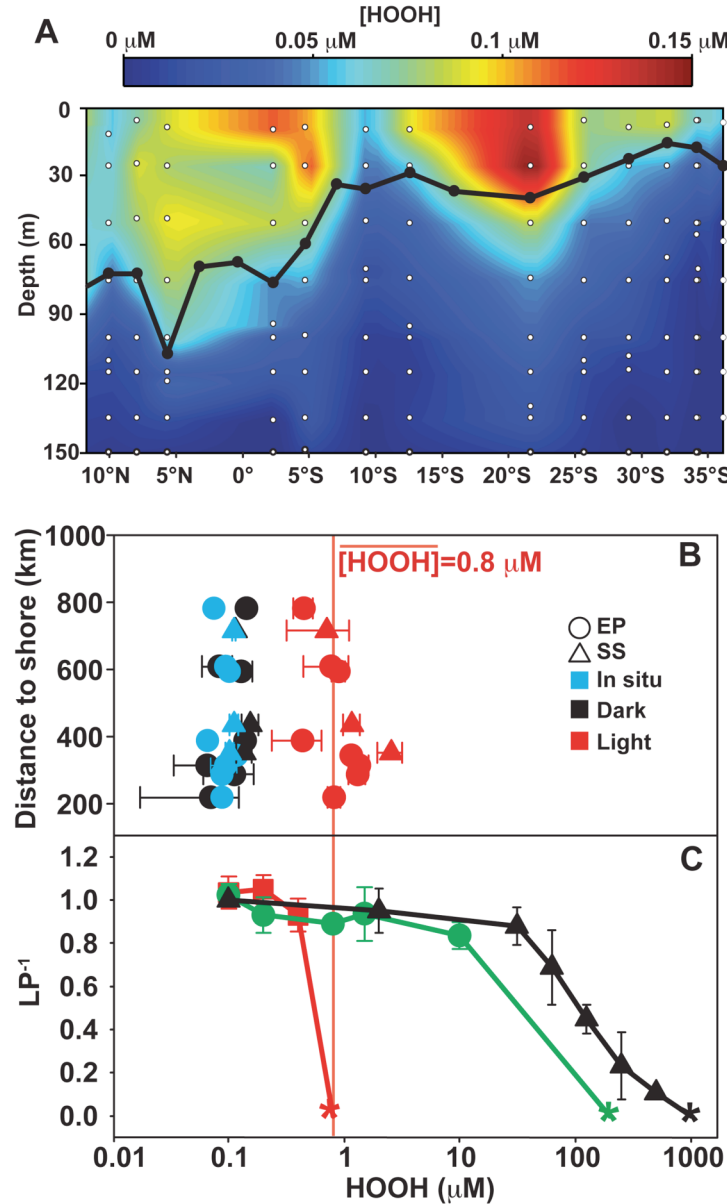


Figure 4.7. Removal of HOOH is necessary for *Prochlorococcus* survival at the ocean's surface. A) [HOOH] in the euphotic zone along a transect from Hawai'i to Australia, Jan-Feb 2007. Sampled depths (open circles) used in the linear interpolation of HOOH, and surface mixed layer depth (black line) are reported. B) Effect of solar irradiation on [HOOH] of seawater. "Light" and "Dark" indicate HOOH concentrations after 24h incubations as described in Methods. Vertical line represents the mean [HOOH] in light incubations, represented by the SMC value as described in the text. EP, Eastern Pacific stations; SS, Sargasso Sea stations. C) Effect of HOOH exposure on the long-term growth of *Prochlorococcus* UH18301 cultures. Lag phase duration, expressed as the inverse ratio of each sample to a no added HOOH control (LP^{-1}) as a function of HOOH dosage with (green circles) or without (red squares) EZ55. Black triangles, LP^{-1} of EZ55 in YT3 medium; *, all replicate cultures failed to show detectable growth after 60 d. Error bars are the standard error of three biological replicates. Cultures were grown under low light with an initial inoculum of $\sim 10^5$ cells mL^{-1} .

Table 4.3. Field sites and HOOH methods.

Cruise	Date	Latitude	Longitude	HOOH Method
WP2	1/7/07	12° 28.47' N	167° 41.46' W	FeLume ¹
WP2	1/8/07	10° 6.15' N	170° 7.29' W	FeLume
WP2	1/9/07	7° 56.33' N	172° 17' W	FeLume
WP2	1/10/07	5° 41.31' N	174° 27.93' W	FeLume
WP2	1/14/07	2° 18.04' S	177° 26.22' E	FeLume
WP2	1/15/07	4° 43.25' S	174° 44.07' E	FeLume
WP2	1/17/07	9° 14.58' S	169° 57.64' E	FeLume
WP2	1/20/07	12° 34.55' S	169° 51.54' E	FeLume
WP2	1/23/07	21° 37.42' S	169° 39.5' E	FeLume
WP2	1/26/07	25° 39.5' S	165° 27.23' E	FeLume
WP2	1/27/07	29° 2.43' S	164° 20.27' E	FeLume
WP2	1/28/07	31° 54.48' S	163° 20.82' E	FeLume
WP2	1/29/07	34° 9.41' S	162° 31.34' E	FeLume
WP2	1/30/07	36° 9.31' S	161° 46.59' E	FeLume
WP2	2/2/07	34° 14.09' S	160° 21.28' E	FeLume
DCM08	6/16/08	30° 7.28' N	118° 24.81' W	Orion ²
DCM08	6/17/08	28° 22.81' N	117° 16.27' W	Orion
DCM08	6/17/08	26° 4.22' N	115° 46.64' W	Orion
DCM08	6/18/08	24° 59.35' N	113° 24.86' W	Orion
DCM08	6/20/08	24° 59.93' N	117° 59.82' W	Orion
DCM08	6/23/08	19° 59.97' N	114° 0.05' W	Orion
DCM08	6/23/08	19° 4.15' N	111° 20.76' W	Orion
DCM08	6/24/08	17° 56.64' N	108° 5.99' W	Orion
DCM08	7/5/08	19° 21.85' N	105° 22.65' W	Orion
DCM08	7/6/08	21° 17.99' N	107° 48.08' W	Orion
BC	5/24/09	30° 10.98' N	72° 1.83' W	Orion
BC	5/31/09	31° 39.9' N	74° 26.4' W	Orion
BC	5/31/09	32° 13.2' N	75° 2.55' W	Orion

¹ [213]² Chapter 3

μM). From these measurements we conclude that the greatest threat of HOOH-mediated oxidative damage for *Prochlorococcus* is within the mixed layer.

HOOH concentrations in the ocean are instantaneous measurements that reflect competing rates of production and removal. While sunlight is the primary source of HOOH, the primary sink is the microflora [97,98]. To investigate the role that the extant microbial community plays in maintaining HOOH concentrations at the surface within the tolerable range for *Prochlorococcus*, we examined the effect that removal of this primary sink had on the local concentrations of HOOH. In total, 11 Pacific and Atlantic open ocean stations were chosen for analysis (Table 4.3), each with mixed layer HOOH concentrations of $\sim 0.1 \mu\text{M}$ (Figure 4.7B) and *Prochlorococcus* present in typical abundance (10^4 - $10^5 \text{ cells mL}^{-1}$, data not shown). Extant microbiota were removed from surface seawater via $0.2 \mu\text{M}$ filtration, and samples were assessed for gross HOOH abiotic production in the absence of the sink. After 24 hours incubation, dark controls were not significantly different than the in situ values (paired t-test, $p > 0.05$), whereas the experimental samples incubated at approximately 80% of surface intensity sunlight (visible + UV), simulating exposure at 5m depth, showed dramatic increases in HOOH (Figure 4.7B). This maximal concentration varied from station to station but had a mean of $0.8 \mu\text{M} \pm 0.6 \mu\text{M}$. In the experiments that follow we refer to this HOOH concentration as the surface monoculture (SMC) challenge, as it represents the hypothetical situation where *Prochlorococcus* is exposed to naturally generated HOOH in the surface mixed layer in the absence of HOOH-removing helpers.

In the absence of helpers, the SMC concentration of HOOH was lethal to all three of the mixed layer ecotypes. While concentrations of HOOH within the range of observed field values ($< 0.2 \mu\text{M}$) had minimal impact on axenic cultures at ecologically relevant cell concentrations of $10^5 \text{ cells mL}^{-1}$, exposure to the SMC concentration of $0.8 \mu\text{M}$ HOOH was lethal to representative strains of the eMIT9312 (UH18301 and VOL1), eMED4 (VOL7) and eNATL2A (VOL3) ecotypes (Fig 4.7C and 4.8). This lethality occurred for all these strains under both high ($250 \mu\text{M quanta m}^{-2} \text{ s}^{-1}$, data not shown) and low ($40 \mu\text{M quanta m}^{-2} \text{ s}^{-1}$, Fig. 4.7C and 4.8) light conditions. In contrast, when co-cultured with ecologically relevant concentrations of EZ55 ($10^6 \text{ cells mL}^{-1}$) these strains were able to tolerate HOOH exposures at least an order of magnitude higher than the SMC value (Fig. 4.7C and 4.8). Indeed, the resistance of these strains

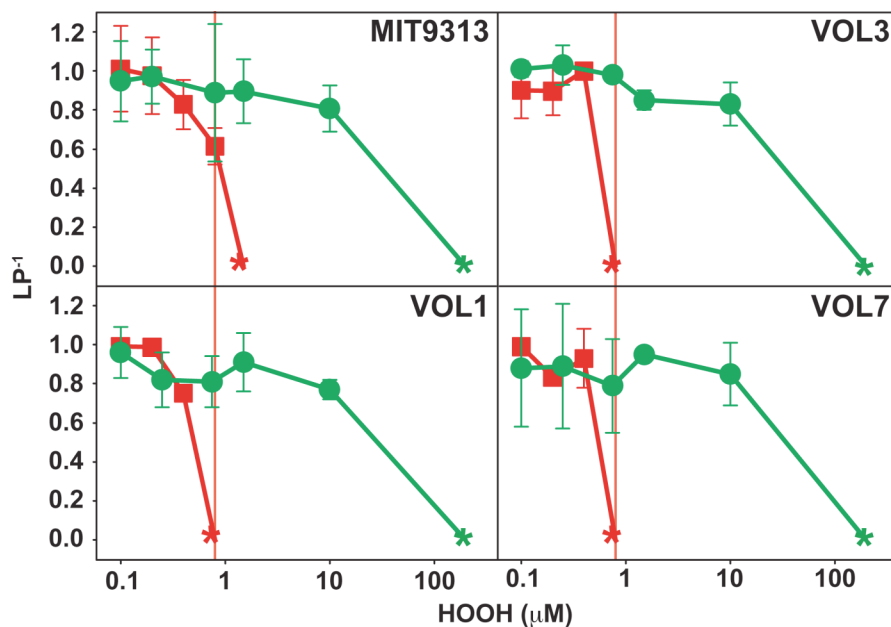


Figure 4.8. Effects of HOOH exposure on *Prochlorococcus* ecotypes. Cultures representing 2 LL (MIT9313 and VOL3) and 2 HL (VOL1 and VOL7) ecotypes with (green circles) or without (red squares) EZ55 were exposed to the indicated [HOOH]. Cultures were inoculated at ecologically relevant concentrations (10^5 *Prochlorococcus* and 10^6 EZ55 cells mL^{-1}) in Pro99 medium and grown under low light. Vertical red line represents the SMC HOOH concentration described in the text. LP^{-1} , inverse lag proportion as described in the text and in the legend for Fig. 4.7; *, no growth was detectable after 60 d in all 3 biological replicates.

in co-cultures approached that of the EZ55 helpers themselves (Fig. 4.7C and 4.8), well beyond any naturally occurring HOOH concentration observed in the open ocean.

C) HOOH stress results in catastrophic loss of cell integrity and photosynthetic capacity. The lethal effects of the SMC challenge were further characterized for axenic UH18301 in unamended seawater. Cell concentrations (detected by flow cytometry) remained constant or increased slightly during 48 h exposure to HOOH dosages up to 0.2 μM , but exposures of 0.4 μM and greater resulted in significant loss of cells (Fig. 4.9A). At the SMC concentration of 0.8 μM , > 99% of the initial UH18301 cell population was undetectable after 48 h. Moreover, as time progressed, an increasing proportion of the remaining cells stained positive for the membrane-impermeable vital stain Sytox Green (Fig. 4.9B), strongly suggesting that cell loss is due to death and subsequent lysis. Sytox Green uptake was greatly attenuated in co-cultures with EZ55 (Fig. 4.9B), suggesting that helpers protect *Prochlorococcus* against HOOH-mediated membrane permeabilization.

Consistent with the Sytox Green results, scanning electron microscopy revealed pronounced cell envelope damage in HOOH-stressed *Prochlorococcus* cells. Untreated (≤ 0.05 μM HOOH) axenic UH18301 cells had the typical spherical morphology of HL *Prochlorococcus* strains, and this strain appeared to produce an extracellular matrix (Fig. 4.10A). Damaged cells were rare, and showed signs of lysis due to osmotic stress during the fixation process. In contrast, after 24 h of exposure to HOOH, UH18301 exhibited profound envelope damage (Fig. 4.10B). While cell size and basic shape were unchanged, cell population density was greatly diminished in these samples (e.g. Fig 4.9A), and no matrix of any kind was visible. Many of the intact cells had holes in their membranes, and partial fragments of cell envelopes were also observed (Fig. 4.10B). These images strongly suggest that the decline in cell concentration upon HOOH exposure (Fig. 4.9A) is due to a catastrophic loss of cell integrity, such that the cells lose the light-scattering and chlorophyll-based fluorescence properties used in their detection.

Concurrent with the decline in cell concentration, *Prochlorococcus* cells exposed to the SMC concentration of HOOH also suffered in their capacity for photosynthesis. Fv/Fm, a measure of photosynthetic efficiency and health, declined steadily in UH18301 cultures during 24 h of HOOH exposure, but was rescued by the presence of EZ55 helpers (Fig. 4.10E and Fig. 4.11). Similar results were observed for another eMIT9312 strain (VOL1), as well as

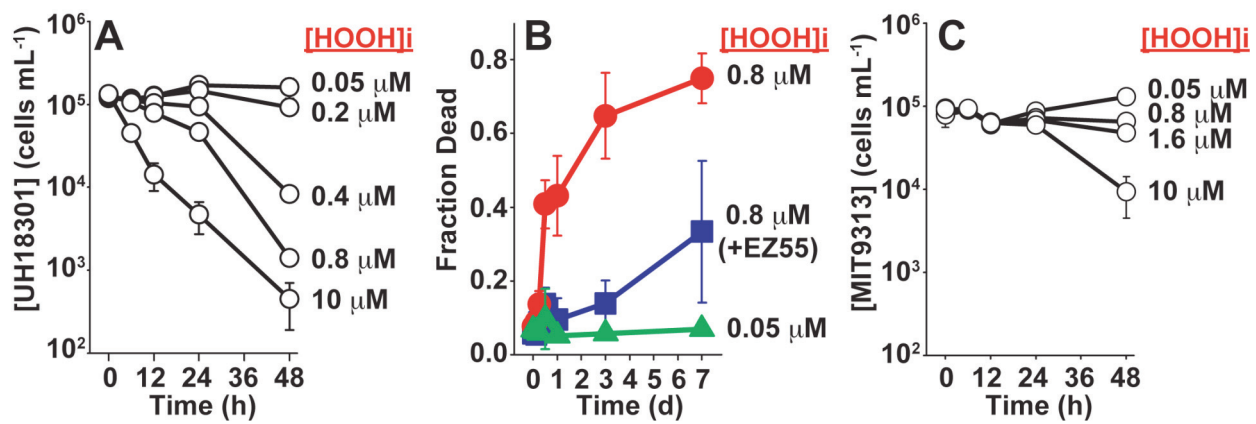


Figure 4.9. *Prochlorococcus* cell viability during HOOH exposure in unamended sterile seawater under low light. A) Change in cell concentration following exposure to the indicated [HOOH]_i for axenic UH18301 in low light. B) Proportion of Sytox Green-positive (i.e. dead) UH18301 cells following exposure to the indicated [HOOH]_i. Green triangles, axenic UH18301 with no added HOOH; red circles, axenic UH18301 with added HOOH; blue squares, co-culture of UH18301 with EZ55 and added HOOH. C) As in A, but for MIT9313.

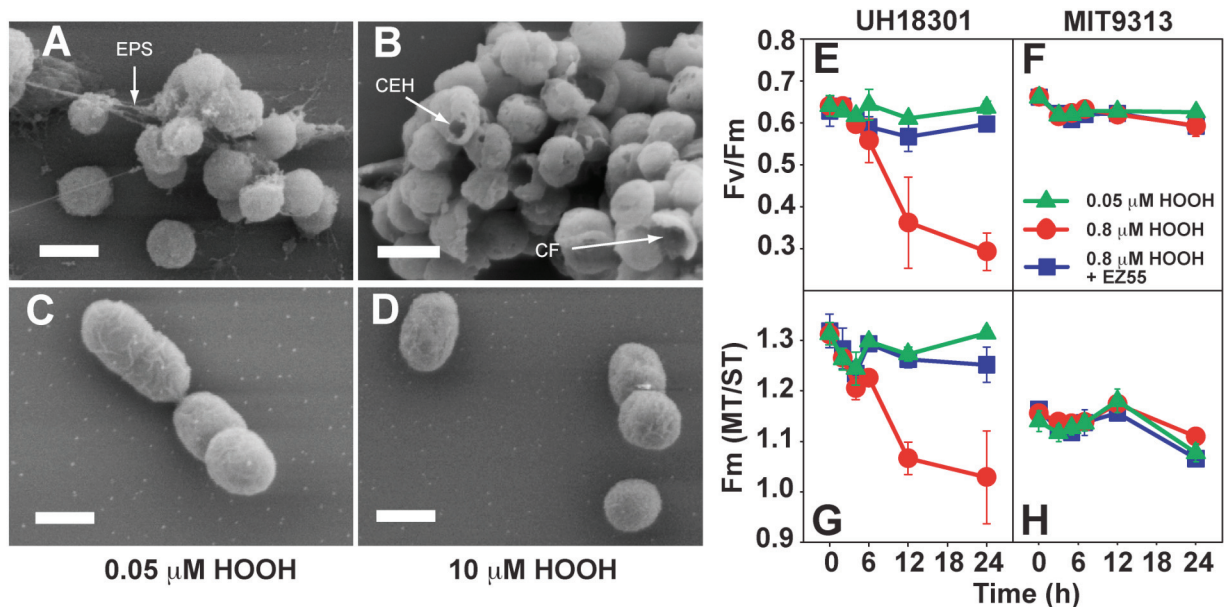


Figure 4.10. Cell envelope and photophysiological effects of HOOH on *Prochlorococcus* grown under low light. SEM of UH18301 (A and B) and MIT9313 (C and D) after 24 h exposure to 0.05 μM (A and C) or 10 μM (B and D) HOOH. All scale bars = 500 nm. EPS, extracellular polymeric substances; CEH, cell envelope hole; CF, cell fragment. Cells were exposed to ~10 fold higher HOOH to compensate for the ~100 fold higher cell concentrations necessary to recover sufficient biomass for imaging. Photophysiological parameters Fv/Fm and Fm(MT/ST) (see text) of *Prochlorococcus* UH18301 (E and G) and MIT9313 (F and H) were also measured during 24 h exposure to the indicated [HOOH] in sterile unamended seawater.

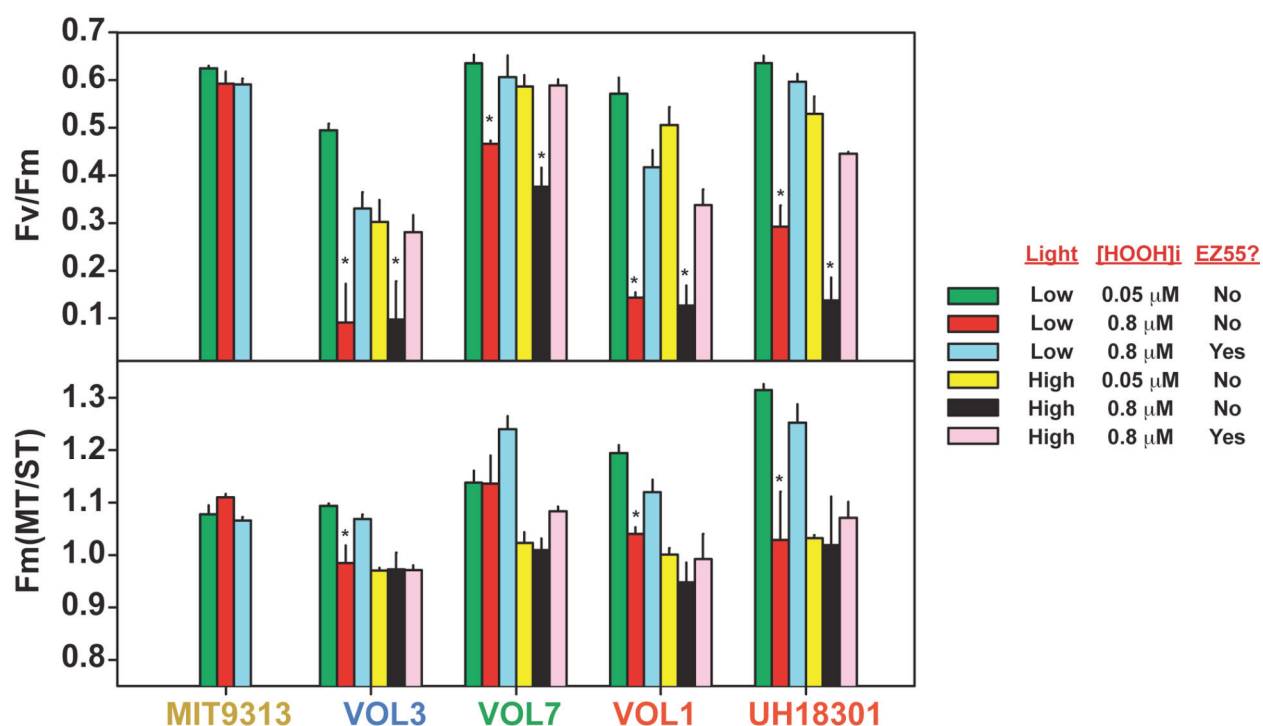


Figure 4.11. Photophysiological parameters of *Prochlorococcus* ecotypes following exposure to SMC (0.8 μ M) HOOH exposure. Fv/Fm and Fm(MT/ST) of representative strains of *Prochlorococcus* were measured after 24 h in sterile unamended seawater with 0.05 or 0.8 μ M HOOH. Low light, maximum 40 μ M quanta $\text{m}^{-2}\text{s}^{-1}$; high light, maximum 250 μ M quanta $\text{m}^{-2}\text{s}^{-1}$. All error bars are the standard error of three biological replicates. *, axenic, HOOH-treated cultures are significantly different (t-test, $\text{df}=4$, $p < 0.05$) than both untreated axenic cultures and HOOH-treated cultures containing EZ55.

representative eMED4 (VOL7) and eNATL2A (VOL3) strains, under high or low light conditions (Fig. 4.11). Fv/Fm of control cultures (no added HOOH) was also significantly lower in cells grown under high light than under low (Fig. 4.11). While the decrease in Fv/Fm resulting from elevated HOOH was largely eliminated by the presence of helpers, the decrease resulting from elevated light levels was not (Fig. 4.11), which may indicate that distinct mechanisms of inhibition are at play, with the high-light associated mechanism not involving a diffusible reactive oxygen intermediary that can be removed by helpers.

The FIRE protocol used in these studies measures two distinct types of variable fluorescence. Fm(ST) is obtained using a rapid series of short flashlets to induce a single turnover of the PSII pool, whereas Fm(MT) is measured during a much longer series of flashlets, during which multiple turnovers of the system completely reduce the plastoquinone (PQ) pool. The ratio of these two values, Fm(MT/ST), thus allows an estimate of the ratio of PQ and/or other downstream components of the photosynthetic electron transport chain to photosystem II reaction centers (RCIIs) [258]. Fm(MT/ST) was significantly greater in UH18301 cultures grown in low light than in high light (Fig. 4.10G), suggesting that under low light conditions, PQ availability increases, perhaps to maximize the efficiency of harnessing of scarce excitation energy. Following HOOH exposure, however, Fm(MT/ST) of low light acclimated cultures dropped to the level of high light acclimated cultures (Fig. 4.10G and 4.11). The target of this HOOH-mediated oxidative damage is not known, but whether it is the RCII itself and/or some downstream element (PQ, cytochrome b₆f, etc.), this damage appears to result in diminished PQ re-oxidation rates and depression of Fm(MT) in relation to Fm(ST). Like Fv/Fm, however, the HOOH-mediated decline in Fm(MT/ST) is suppressed in co-cultures with EZ55 (Fig. 4.10G and 4.11). Like UH18301, all other strains assayed that are able to grow at high light (i.e., all except MIT9313) had higher Fm(MT/ST) in low light conditions. Interestingly, HOOH exposure eliminated this difference in all strains *except* VOL7 (Fig. 4.11), suggesting that this strain may have unique, currently unknown, protective mechanisms.

D) Phylogenetic distribution of helper dependency. Interestingly, while the eMIT9313 ecotype has never been observed at high abundance in the surface mixed layer [177] – most likely due to its sensitivity to high light [191] – strain MIT9313 was significantly *more* resistant to HOOH than strains of the three ecotypes (eMIT9312, eMED4, and eNATL2A) that *are* found

in the HOOH-enriched surface mixed layer. MIT9313 was uniquely capable of growth at the SMC HOOH concentration (Fig. 4.8), and this concentration had minimal impact on photosynthetic efficiency (Fig. 4.10F, 4.10H, and 4.11) or cell integrity (Fig. 4.9C and 4.10C-D). Importantly, none of the strains tested were able to significantly lower the HOOH concentration of the medium within the time frame of the photophysiology experiments (24h, Fig. 4.1), suggesting that the greater resistance of MIT9313 relative to the other strains is not achieved via higher rates of HOOH scavenging.

The results with the SMC HOOH concentrations suggested that the genetically distinct ecotypes may have unique responses to oxidative stress. To explore this possibility at a finer scale, we compared their growth properties at a HOOH concentration permissive to all strains, and under a high and low light intensity that may be experienced within the surface mixed layer. Altogether, eight axenic strains of *Prochlorococcus* representing two HL and two LL ecotypes, as well as one strain of marine *Synechococcus* were tested. Invariably, axenic *Prochlorococcus* cultures inoculated at 100 cells mL⁻¹ into autoclaved Pro99 (containing ~0.2 µM HOOH) grew poorer relative to co-cultures with EZ55, with higher variability in growth rate (Fig. 4.12) and lag time (data not shown) between replicates. In contrast, there was no difference in the growth rate or lag of *Synechococcus* WH7803 in the presence or absence of EZ55. Light intensity had variable effects on the EZ55 helping phenotype for the *Prochlorococcus* strains (Fig. 4.12). Amongst the HL ecotypes, eMED4 strains VOL7 and VOL8 were more reliant on help in low light, whereas the members of the eMIT9312 ecotype did not share a single light preference: UH18301 was less dependent on help in high light, while VOL1 was incapable of axenic growth at this light intensity. The LL eNATL2A strain VOL3 was incapable of axenic growth under high light, whereas growth under high light was permissible in the presence of EZ55. While it exhibited relatively high HOOH resistance in low light (Fig. 4.9C), the LL strain MIT9313 exhibited no growth after 30 days in high light from an inoculum of 100 cells mL⁻¹, with or without EZ55, supporting our assertion in the prior section that photoinhibition of *Prochlorococcus* [191] includes a mechanism that is independent of diffusible HOOH.

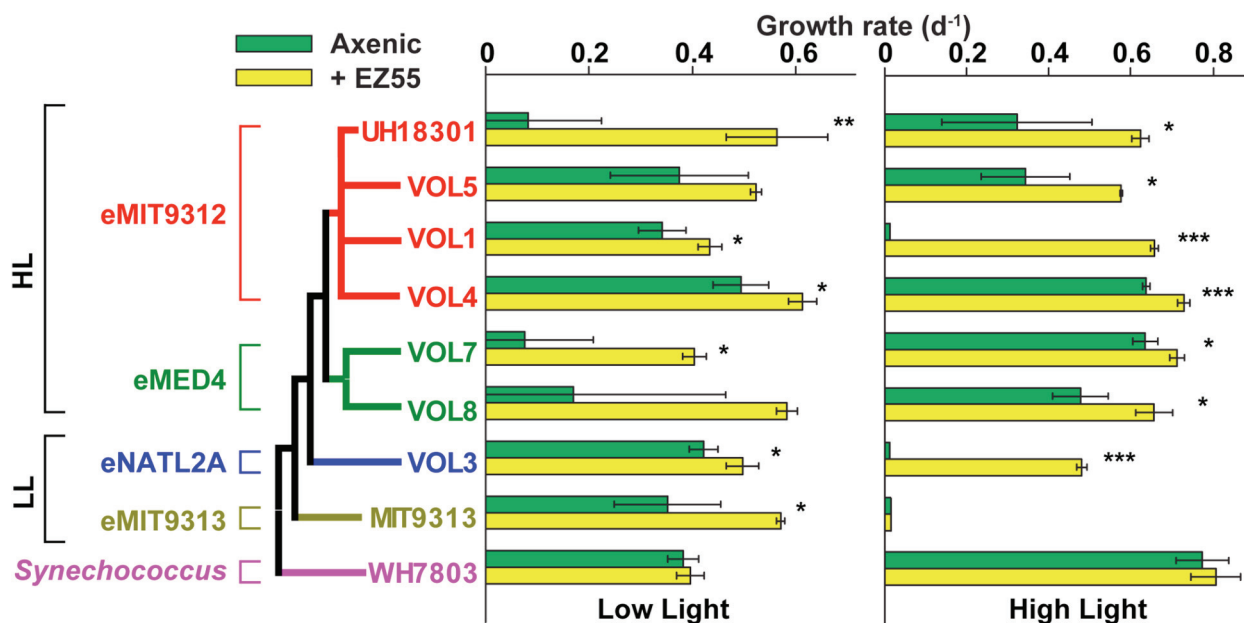


Figure 4.12. Growth at 0.2 μM HOOH as a function of helpers and light intensity for cultures with very dilute (~ 100 cells mL^{-1}) initial cell concentrations. Growth rates for strains representing 4 *Prochlorococcus* ecotypes and a marine *Synechococcus* ecotype with (yellow bars) or without (green bars) EZ55 helpers in low or high light (40 or 250 μM quanta $\text{m}^{-2} \text{s}^{-1}$, respectively). Consensus (100% support in 1000 bootstrap replicates) neighbor-joining tree was prepared using the entire rDNA operon (≈ 5.5 kb) and rooted on the *Synechococcus* branch. Only topology is shown; branch lengths do not represent genetic distance. Significance levels of t-tests (df=4): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. HL, high light adapted ecotypes; LL, low light adapted ecotypes.

III. Discussion

A) HOOH removal is the primary mechanism responsible for the helper phenotype.

In this work we have confirmed our initial report (Chapter 2) that heterotrophic bacteria help dilute *Prochlorococcus* cultures grow via removal of HOOH from the environment. HOOH diffuses freely across cellular membranes [26], and hence the heterotrophic HOOH degradation could occur in the cytoplasm, periplasm, or extracellular milieu, perhaps with the same overall effect. Density-dependence of HOOH resistance has also been observed in *Escherichia coli*, and catalase-positive *E. coli* has been shown to cross-protect more vulnerable catalase-negative mutants in co-culture [259]. Inter-species cross-protection from oxidative stress has been noted for heterotrophic bacterial communities involved in the degradation of aromatic compounds [260] and in oral biofilms [261], as well as in a benthic mat-forming cyanobacterium [262], but to our knowledge this is the first demonstration that such helping can occur between planktonic microbes of different trophic levels.

While HOOH removal is sufficient to explain the helping phenotype of heterotrophic bacteria (at least for the EZ55 strain), other types of interactions may also contribute. For instance, heterotrophs can help eukaryotic algae by improving carbon fixation, either via increasing DIC concentrations [123] or lowering oxygen concentrations (thus diminishing the competing oxygenase reaction of Rubisco [263]). However, the addition of heterotrophic helpers to the *Prochlorococcus* cultures either led to no observable difference in bulk bicarbonate, CO₂, or oxygen, or a change in the opposite direction to that expected for a beneficial role (Fig. 4.2). In other cases, heterotrophs help by supplying an essential metabolite (e.g. vitamin B12 [264] or indole acetic acid [265]) or trace nutrient (e.g., by producing iron-binding siderophores [266]). While we cannot rule out involvement of such cross-feeding interactions, our results suggest that any nutritional mechanism must play a secondary role relative to oxidative stress reduction, as adding HOOH back to helper-treated media was alone sufficient to restore the growth limitation of dilute *Prochlorococcus* cultures (Fig. 4.4A). Other than inorganic C, N, P, and metals, *Prochlorococcus* has no known nutritional requirements for growth [180,181], and in fact grows better when dense versus dilute, contrary to expectations for an organism with a nutritional deficiency. Additionally, that a dense strain of *Prochlorococcus* can help a dilute strain (Fig.

4.4B) argues against a requirement for a nutrient that *Prochlorococcus* itself cannot produce from the medium.

With the exception of MIT9313, exposure of all axenic cultures of *Prochlorococcus* to SMC levels of HOOH results in catastrophic loss of cell envelope integrity (Fig. 4.9B, 4.10B). Concomitant with this envelope damage is the loss of photosynthetic efficiency (Fig. 4.10G), and both of these effects may be consequences of lipid peroxidation. The slow decline of HOOH in sterile, light exposed media (Fig. 4.1) may be the result of the Fenton reaction, where photochemically produced Fe(II) reacts with HOOH to produce highly reactive $\cdot\text{OH}$ [38]. In turn, $\cdot\text{OH}$ can attack polyunsaturated fatty acids such as those found in most biological membranes, producing relatively stable lipid peroxide radicals that can spread via radical propagation if not countered by antioxidant defenses [26]. Both the cytoplasmic and the photosynthetic membranes may be susceptible to lipid peroxidation, and this may account for the coincident loss of cell envelope integrity and photosynthetic capacity we observed. Of note, dependence of HOOH-induced lethality on free Fe(II) could explain the greater resistance of MIT9313 to SMC challenge, as this is the only strain of *Prochlorococcus* to express the Fe-binding *dpsA* gene [231] which is linked to oxidative stress tolerance in other organisms [267]. Interestingly, the *Synechococcus* PCC7942 DpsA has a weak catalase activity [268] and if similar, the DpsA homolog of MIT9313 may be responsible for the cell's ability to eventually deplete the HOOH after 2 weeks under the SMC treatment (data not shown).

HOOH may also affect photosynthesis by impinging on the turnover of RCII protein D1. D1 proteins are continuously degraded in illuminated photosynthetic membranes, and cells must repair or replace these proteins to maintain photosynthetic activity [269]. In the freshwater cyanobacteria *Synechococcus* PCC7942 and *Synechocystis* PCC6803, exogenous HOOH inhibits protein synthesis by specific inactivation of elongation factor G [117], leading to net loss of photosystems containing functional D1. Hence, two non-mutually-exclusive mechanisms – lipid peroxidation and interruption of D1 turnover – may be responsible for the HOOH-dependent loss of photosynthetic efficiency in *Prochlorococcus*, and future studies should be aimed at testing these hypotheses.

B) Impact of helpers on *Prochlorococcus* ecology. Our results suggest that *Prochlorococcus* depends on the HOOH-degrading members of the microbial community to

grow in the surface mixed layer of the open ocean. Opposing photochemical production reactions [46,79] and microbial degradation reactions [97,98] maintain the HOOH concentration within the permissive range ($<0.2 \mu\text{M}$) for the three ecotypes of *Prochlorococcus* that exploit the mixed layer niche. Absent the mixed layer microbial community, photochemical production of HOOH yields concentrations that are lethal to all three ecotypes (Fig. 4.7B-C, 4.8). The surface monoculture (SMC) challenge (Fig. 4.7C, 4.8) demonstrated that, absent a counteracting microbial community, HOOH in surface seawater from the open ocean climbs to concentrations that ecologically relevant cell concentrations of these ecotypes are unable to survive. Curiously, the one strain that can tolerate the SMC challenge, MIT9313, belongs to an ecotype that is restricted from the mixed layer [177], probably due to its sensitivity to high light [191]. While the SMC challenge reflects a worst case scenario for un-helped *Prochlorococcus* (e.g. sustained presence within 5 m of the surface), we note that even modest increases of HOOH above the steady state mixed layer concentrations (e.g. $<0.2 \mu\text{M}$, Fig. 4.7A) are enough to significantly impact growth (Fig. 4.9), suggesting that the entire *Prochlorococcus* mixed layer population, not just cells at the very surface, benefit from the activity of helpers. Hence, an important but previously unrecognized role of the microbial community is to make the surface mixed layer permissive for the growth of *Prochlorococcus*, and by doing so, to facilitate the expansion of *Prochlorococcus*' habitat range. Whether this relationship between *Prochlorococcus* and its HOOH-consuming neighbors is commensal or mutualistic remains to be determined, but as the numerically-dominant primary producer in the surface mixed layer, it is certainly conceivable that the HOOH consumers may benefit indirectly through the release of organic carbon during the growth [270] or lysis of *Prochlorococcus*.

Our study contributes to a growing body of evidence that interspecies interactions can contribute significantly to niche expansion via stress reduction. For instance, land plants in high altitude alpine sites subjected to high abiotic stress (e.g., low moisture, low temperature, strong winds) experience pronounced losses in productivity as well as increased mortality in the absence of other members of the plant community (i.e., as a monoculture), whereas this helping effect does not occur in otherwise similar communities in less stressful, lower altitude habitats [271]. Likewise, lichenized algae and fungi gain vastly improved resistance to desiccation and oxidative stress in symbiosis, expanding the range of each to include subaerial habitats [272].

Similarly, cnidarians (e.g., corals) and zooxanthellae tolerate temperature and hypoxia extremes together that neither can tolerate separately [230]. Collectively, these studies support the broader “stress-gradient hypothesis” that cooperation should be stronger in more stressful environments [273], and emphasize the need to assess stress responses at the community level in order to understand their impacts on biological distributions in nature.

The microbial community in the surface mixed layer is genetically diverse [51], and identifying the microbes contributing most significantly to HOOH degradation is a major challenge for future studies. We know that *Prochlorococcus* degrades HOOH poorly (Fig. 4.1) and thus is not likely to contribute significantly to HOOH decomposition in the ocean. Intriguingly, the same may be true for the most abundant heterotroph in the oligotrophic ocean as well, as the genome of *Pelagibacter ubique*, the first cultured representative of the SAR11 cluster, also lacks homologs of catalase and other antioxidant defenses [10]. The lack of robust HOOH scavenging pathways in both the numerically dominant autotroph and heterotroph implies that this critical function may be performed by less abundant “keystone” species in the mixed layer. Given the high catalytic rate of catalase [30] and the efficiency of HOOH removal observed in culture (e.g. Fig. 4.1), it is conceivable that a relatively small number of catalase-expressing organisms could protect the entire surface mixed layer community from solar-generated HOOH. We note that many of the confirmed helpers of *Prochlorococcus* (Chapter 2) are catalase-positive members of the α - and γ -Proteobacteria whose genetic signatures appear at reasonable frequencies in the open ocean mixed layer [51,274], and are thus potential candidates for these keystone microbes. However, there are clearly other biological mechanisms for removing HOOH (e.g., the residual HOOH scavenging abilities of catalase mutants, Table 4.2), any one of which may contribute to the helper phenotype of surface mixed layer communities.

C) Evolutionary implications of the helper-*Prochlorococcus* interaction. Our results present an apparent paradox regarding the evolutionary history of the *Prochlorococcus* lineage: the eMIT9313 ecotype, which is restricted from the HOOH-enriched mixed layer by its sensitivity to high light, is more resistant to HOOH than the ecotypes found in high abundance in the mixed layer. eMIT9313 is the earliest branching lineage from the last common ancestor of *Prochlorococcus* and *Synechococcus* [247,275], and although it lacks catalase, may share other ROS defense mechanisms with *Synechococcus* (e.g. *dpsA*). Indirect defense mechanisms may

also be involved in eMIT9313. For example the peptidoglycan synthesis genes of MIT9313 are more similar to those of *Synechococcus* than those of the HL strain MED4, and the MIT9313 cell wall is intermediate in thickness between those of *Synechococcus* and MED4 [276]. A thicker cell wall may confer enhanced resistance by limiting HOOH diffusion into cells and/or preventing cell lysis (e.g., Fig. 4.10B versus Fig. 4.10D). It is not clear why eMIT9313 has retained the relatively high resistance to HOOH; perhaps the genes conferring resistance are also involved in other cellular functions that remain under selection. However, what is clear is that for the more recently derived lineages, including the LL ecotype eNATL2A, the net genomic reduction that has occurred relative to eMIT9313 [247] has coincided with a loss of HOOH resistance. Thus, the evolution of the genus leading to tolerance of temporary exposures to high light (eNATL2A) and true high light adaptation (eMED4 and eMIT9312) that allowed this lineage to exploit the surface mixed layer habitat did not coincide with a greater resistance to HOOH; in fact, the opposite occurred.

We believe that this paradox is resolved because the HOOH-consuming microbes present at the ocean's surface made the HOOH resistance genes in *Prochlorococcus* dispensable. *Prochlorococcus* evolved in the context of an extant HOOH-scavenging community, and thus as it developed tolerance to high light and possibly other environmental stresses in the surface mixed layer, it had no selection pressure to maintain the high level of resistance to HOOH. As the oligotrophic environment imposed pressure to reduce genome size [11,246,247,277,278], genes encoding these resistance mechanisms were amongst the pool of expendable genes, and were eventually lost. Similar mechanisms may have been at play during the genome streamlining of the ocean's most abundant heterotroph, *P. ubiquus* [12], although it is unknown as yet whether or not this organism benefits from co-culture with helpers. It is intriguing to note that both *Prochlorococcus* and *P. ubiquus* are conspicuously difficult to cultivate, leading to speculation that other, as-yet-uncultured organisms may be similarly dependant on helpers in nature (e.g., [266]). Hence, this study emphasizes the importance of community-level stress responses not only for the ecology, but also the evolutionary history of free-living microbes.

Finally, we note that while the catalase gene could have been lost from the *Prochlorococcus* lineage (either prior to or after the divergence from *Synechococcus*) as a consequence of neutral genetic drift, it may also have been lost as a selectively favorable event.

One reason may be economic: as hypothesized for the reduction of peptidoglycan in the HL strain MED4 [276] and the reduction of phospholipids in favor of sulfolipids throughout the *Prochlorococcus* lineage [233], the loss of catalase may have been selected to lower the cell quota for scarce nutrients in the oligotrophic ocean. The catalase-peroxidase found in most cyanobacteria is a large dimeric enzyme (160 kilodaltons) that has 4 iron-containing heme co-factors [30]. If we make certain assumptions about expression levels based on published data (see Section IV.H below), we may estimate that loss of catalase-peroxidase would result in a reduction of cell quotas for Fe, P, and N by 0.2%, 0.14%, and 0.05%, respectively, for *Prochlorococcus* MED4 (Table 4.4). As the primary selective pressure implicated in the genomic reductions for this oligotrophic lineage is to lower the cost of such scarce nutrients for cell production [11,246,247,277,278], it is reasonable that such a gene would be lost in the absence of selective pressure for its retention. Additionally, catalases in keratinocytes have been shown to generate an unidentified form of reactive oxygen species when exposed to UV-B [279]. If similar side reactions occur for bacterial catalases, cells in the UV-exposed surface mixed layer may experience a tradeoff, degrading HOOH while also producing another form of activated oxygen; under these conditions the negative consequences may outweigh the positive ones for *Prochlorococcus*. In the future, development of robust genetic tools for *Prochlorococcus* should allow us to ectopically express catalase and determine if it indeed has a positive or negative impact under mixed layer conditions.

IV. Methods

A) Field sites and methods. HOOH levels in the euphotic zone were measured on three cruises (Table 4.3): Jan-Feb 2007 in the Western Pacific (WP2), Jun-Jul 2008 in the Eastern Pacific (DCM08), and May-Jun 2009 in the Sargasso Sea (BC09). Seawater was collected in 20 L Niskin bottles and dispensed into lightproof polypropylene bottles rinsed three times with sample water. Care was taken to shield these samples from light prior to laboratory analysis. In situ HOOH levels were measured within 30 minutes of sample retrieval. HOOH measurements for WP2 were conducted using an acridinium ester chemiluminescence flow-injection method [213] implemented with a FeLume (Waterville Analytical, Waterville, ME). HOOH measurements for DCM08 and BC09 were performed using a similar protocol modified for use

Table 4.4. Estimated effects of *katG* loss from a hypothetical catalase-positive ancestor of *Prochlorococcus* MED4 on cell quotas for selected nutrients.

	C	N	P	Fe
Total cell quota¹	3.06 x 10 ⁹	3.85 x 10 ⁸	6.40 x 10 ⁶	1.51 x 10 ⁵
Requirements for KatG	350,230	187,620	8,784	300
% savings	0.01%	0.05%	0.14%	0.20%

¹ All values are numbers of atoms per cell. Estimates were obtained based on the assumptions described in Methods.

in an Orion-L microplate luminometer (Chapter 3), and restricted to surface mixed layer (≤ 10 m) depths. Flow cytometry or quantitative PCR [243] confirmed that *Prochlorococcus* was abundant ($\geq 10^4$ cells mL^{-1}) in all mixed layer samples analyzed (data not shown).

To measure rates of HOOH production in sterile seawater, approximately 15 ml of the above samples were passed through 0.22 μm Millex GV syringe filters (Millipore, Billerica, MA) into custom-built UVT acrylic cuvettes, and sealed with threaded nylon caps. These cuvettes permit about 90% transmission of photosynthetically active radiation, UV-A, and UV-B, as assessed using PAR (QSL2100, Biospherical Instruments, San Diego, CA) and UV sensors (Mannix UV340, General Tools, New York, NY). Both the cuvettes and the caps were sterilized prior to use using a UV germicidal lamp. Samples in cuvettes were exposed to ambient light in a UVT acrylic deck incubator, and HOOH levels were measured at 0 and 24 h. Samples received between 60-80% of surface irradiance (i.e., ~ 1200 - $1600 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ on clear days), and temperature was stabilized by continuous flow of surface water through the incubator. Control cuvettes were treated identically, only shielded from light.

B) Cultures and culture conditions. Strains used in this study are listed in Table 4.1. *Prochlorococcus* cultures were grown in Pro99 natural seawater medium [181] prepared using water collected from 15 m at station R2 in the Georgia Bight ($31^\circ 22.35' \text{ N}$, $80^\circ 33.68' \text{ W}$). Strain UH18301 was isolated from Station ALOHA ($22^\circ 45' \text{ N}$, 158° W) in the North Pacific Subtropical Gyre, at 125 m on July 14, 2006. Seawater was diluted onto 75% Pro99 plates pre-seeded with $\sim 10^6$ cells mL^{-1} of helper UH06001 (*Alteromonas* sp. and a close relative of EZ55) and incubated at 22°C in a natural diel light cycle incubator with a 12:12 light:dark cycle with a maximum irradiance of $50 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. A green *Prochlorococcus* colony that formed after ~ 2 months of incubation was picked and grown in Pro99 liquid medium and this culture was later rendered clonal and axenic through the antibiotic selection method described below. Axenic MIT9313 [280] was a generous gift of L. R. Moore, whereas the other strains (Table 4.1) were rendered axenic by a modified version of our previously described method (Chapter 2). Spontaneously streptomycin-resistant mutants of the *Prochlorococcus* strains were diluted to extinction in Falcon Microtest U-bottom 96-well microtiter plates (#35-3077, BD, Franklin Lakes, NJ) containing 200 μL Pro99 and approximately 2×10^5 cells of the streptomycin sensitive helper *Alteromonas* sp. EZ55. These plates were incubated at 24°C in a “Sunbox” growth

chamber (Percival, Perry, IA) designed to simulate a natural light regime by gradually raising light level from darkness to a “noon” maximum of $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and back to darkness again over a 12/12 light/dark photoperiod [216]. Microtiter cultures exhibiting growth of *Prochlorococcus* were tested for purity (apart from the streptomycin-sensitive helper) by placing aliquots into 1/10 ProAC broth (Chapter 2) containing streptomycin, followed by incubation in the dark. If this purity broth did not become turbid within a week, the Pro99 culture was diluted into fresh Pro99, grown until visibly green, and treated with $100 \mu\text{g mL}^{-1}$ streptomycin to remove the helpers. Purity of cultures was routinely confirmed by diluting aliquots of the culture into 1/10 ProAC or PLAG media (Chapter 2). Cultures were considered axenic if no turbidity formed after 60 d in purity broth, and no DAPI-stained cells other than *Prochlorococcus* were observed via fluorescence microscopy and flow cytometry.

Heterotrophic bacteria were grown in YT3 medium (per L, 10 g tryptone, 5 g yeast extract, 1 mM TAPS buffer pH 8.0, dissolved in Turk’s Island Sea Salts [181] and filter sterilized with a $0.22 \mu\text{m}$ Supor filter [Pall]). Helpers were harvested by centrifugation from 2 d cultures and washed twice with sterile Pro99 prior to addition to *Prochlorococcus* cultures.

All experiments were performed at 24°C in a Sunbox (see above). “Low light” experiments were conducted in a Sunbox fitted with blue theater gels (Roscolux #69, Rosco, Stamford, CT) and set to produce a noon maximum of about $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$; “high light” experiments used unfiltered white light with a noon maximum of about $250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. *Prochlorococcus* strains were acclimated to growth conditions over at least two passages into fresh media prior to the start of experiments. All growth experiments used inocula taken from mid-exponential phase cultures (between 10^7 and $10^8 \text{ cells mL}^{-1}$). Unless otherwise specified, all chemicals were Sigma Ultra grade (Sigma-Aldrich, St. Louis, MO).

C) Quantification of *Prochlorococcus* and helpers. *Prochlorococcus* concentrations were determined by flow cytometry, either on a Cytopeia Influx instrument (BD, Franklin Lakes, NJ) or a Guava EasyCyte 8HT (Millipore), using established protocols [188,243]. For some experiments, growth was tracked by bulk chlorophyll fluorescence using either a TD700 fluorometer (Turner Designs, Sunnyvale, CA, USA) equipped with an in vivo chl a filter set (excitation 340–500 nm; emission $\geq 665 \text{ nm}$) or a Synergy HT-1 96-well plate fluorometer (BioTek, Winooski, VT). In experiments using co-cultures of different *Prochlorococcus* strains,

concentrations of the different populations were distinguished using quantitative PCR (qPCR) as previously described [240]. Helper cell viable counts were determined on YTSS plates containing 50 U ml⁻¹ bovine liver catalase.

Exponential growth rates (μ) were calculated as the slope of the natural logarithms of at least three points during exponential phase; the highest of these measurements is C_{end} , taken at time t_{end} . Lag phase duration (LPD) was calculated using the equation

$$\text{LPD} = t_{\text{end}} - \mu^{-1} \ln \left(\frac{C_{\text{end}}}{C_0} \right) \quad (1)$$

where C_0 is the initial measurement at time 0. LPD data was then expressed as an inverse lag proportion (LP^{-1}), given by the ratio of LPD for a control culture ($\text{LPD}_{[\text{HOOH}]0}$) with <0.05 μM HOOH and a culture with an elevated HOOH concentration ($\text{LPD}_{[\text{HOOH}]x}$):

$$\text{LP}^{-1} = \frac{\text{LPD}_{[\text{HOOH}]0}}{\text{LPD}_{[\text{HOOH}]x}} \quad (2)$$

such that cultures that did not grow ($\text{LPD}_{[\text{HOOH}]x} = \text{infinity}$) could be assigned a plottable value of ~ 0 .

Prochlorococcus viability was determined by staining with 5 μM Sytox Green (Invitrogen, Carlsbad, CA) for at least 10 minutes prior to flow cytometry, following manufacturer's instructions. Dead cells were defined as events in the *Prochlorococcus* gate (forward angle light scatter and red fluorescence) that exhibited green fluorescence values similar to a glutaraldehyde-killed control.

D) Transposon mutagenesis. Genomic knockout mutants of *Alteromonas* sp. EZ55 and *Ruegeria lacuscaerulensis* were created using a transposon, mini-*Tn5*, delivered by conjugation (E. Webb, unpublished method). Insertional mutants containing the *Tn5* kanamycin resistance cassette were selected on LB agar with 25 $\mu\text{g mL}^{-1}$ kanamycin. Catalase-deficient mutants were discovered by screening colonies for absence of bubbling on exposure to HOOH. Independently, a second catalase mutant of *R. lacuscaerulensis* was identified in an *a priori* screen for mutants incapable of facilitating *Prochlorococcus* growth on semisolid media. An arbitrarily primed-PCR protocol was employed to discover the location of transposon insertion [281]. Amplicons were sequenced and analyzed by BLAST [282] to determine which gene was affected in the mutant (Table 4.1).

E) HOOH exposure experiments. The effects of elevated HOOH (i.e., different from the concentration obtained by simply autoclaving seawater) on *Prochlorococcus* were tested using cultures initially containing approximately 10^5 cells mL⁻¹ of *Prochlorococcus*, either with or without 10^6 cells mL⁻¹ of the helper heterotroph EZ55. These concentrations represent typical levels of *Prochlorococcus* and total bacteria, respectively, observed in the field [9,51]. Seawater for the Pro99 media was prepared by ultrafiltration with a Labscale tangential flow filtration device fitted with a Pellicon XL 30 kilodalton cutoff regenerated cellulose cartridge (Millipore), followed by passage through a washed 0.22 μ m Supor filter. This was necessary to produce a medium with a sufficiently low initial HOOH concentration to serve as a low HOOH control. Both autoclave and microwave sterilization led to similar HOOH levels of ~ 0.2 μ M. While filter-sterilized seawater had much lower HOOH levels ($\sim 40 \pm 1$ nM), the possible presence of *Prochlorococcus* phage in natural seawater necessitated either some form of heat sterilization prior to preparation of culture media, or else the ultrafiltration to remove virus size-class particles. *Prochlorococcus* was inoculated into Pro99 media made from this ultrafiltered seawater either unamended (< 0.05 μ M HOOH) or amended with HOOH (0.1 to 200 μ M), and if no growth was detectable after 60 d, a culture was determined to be dead.

Experiments involving exposure to accumulating HOOH were designed using the buffer HEPES that facilitates steady, light-driven production of HOOH (Chapter 3 and [211,212]). The rate of HOOH production is linear for at least 1 week, and varies linearly as a function of buffer concentration (Chapter 3). All cultures were set to pH 8.0 and contained 3.75 mM buffer, using a combination of HEPES and TAPS (a non-HOOH generating buffer, Fig. 4.4C) to produce a range of HOOH fluxes in a constant buffering capacity background. Cultures were acclimated to the presence of 3.75 mM buffer in TAPS-containing media before being transferred into media containing HEPES.

F) Chemical and enzymatic assays. All HOOH measurements in the laboratory were performed using the Orion-L luminometer and an acridinium ester chemiluminescence protocol (Chapter 2). Bulk water chemistry measurements followed standard protocols [283] using samples filtered through washed 0.22 μ m Millex GV filters. Dissolved inorganic carbon (DIC) concentrations were determined by calculation based on measurements of alkalinity (by the bromocresol green endpoint method) and pH (using an UltraBasic probe, Denver Instrument,

Bohemia, NY). Dissolved O₂ (DO) was measured using an Orion 4star probe (Fisher, Waltham, MA) calibrated in water-saturated air. Dissolved organic carbon (DOC) levels were measured using a TOC-VCP analyzer (Shimadzu, Kyoto, Japan). Alkaline phosphatase activity was measured colorimetrically, following the increase in absorbance at 420 nm after addition of p-nitrophenyl phosphate (final concentration 0.04% w/v) using a BioTek Synergy HT-1 plate reader [284]. Total hydroperoxidase activity was measured by adding 0.5 μ M HOOH to a sterile sample and measuring HOOH concentrations every 2 h for 6 h.

G) Effects of HOOH on *Prochlorococcus* photophysiology and morphology.

Photophysiological parameters were assessed using a Fast Induction and Relaxation (FIRE) fluorometer (Satlantic, Halifax, Nova Scotia) using the methodology described by Johnson [285]. Variable photosystem II fluorescence (Fv/Fm) was determined by curve fitting in MATLAB using both a rapid single turnover (ST) flash series and a longer multiple turnover (MT) series [286]. Photophysiological diel measurements were all performed beginning at the same point in the diel cycle (~3 h after subjective “sunrise”). Initial decreases in photophysiological parameters (Fig. 4.E-H) were observed in all cultures, including controls, and likely represent normal diel cycling of these characteristics, as observed in *Prochlorococcus* PCC 9511 [287].

Cell morphology was assessed by scanning electron microscopy (SEM). 20 mL cultures at approximately 10⁸ cells ml⁻¹ were concentrated by centrifugation at 6500 g for 15 min. Pellets were resuspended in 0.1 M cacodylate-buffered 3% glutaraldehyde for 60 min, and then washed three times with buffer water for 10 min. Cells were then post-fixed in 0.1 M cacodylate-buffered 2% osmium tetroxide for 60 min. After fixation cells were washed three times with water; during the final wash cells were allowed to settle onto a clean silicon chip. Samples were dehydrated in a graded ethanol series at 15 min intervals. Following dehydration samples were critical point dried with liquid CO₂ in a critical point dryer (Ladd, Williston, VT). Dried samples were sputtered with a thin layer of gold before examination with a LEO 1525 scanning electron microscope (Carl Zeiss SMT Inc, Peabody, MA). As higher concentrations of cells (10⁸ cells ml⁻¹) were required for the preparation of the SEM samples, we challenged the cells with 10 μ M HOOH rather than the usual 0.8 μ M SMC value. That an effect of this HOOH treatment was observed for the UH18301 cells but not for the more HOOH-resistant MIT9313 cells (Fig. 4.5) indicates that the concentrations used were appropriate for these assays.

H) Calculation of cell quota effects for catalase loss. Cell quotas for C, N, and P for *Prochlorococcus* MED4 were obtained from [288]. Fe quotas were obtained from [289]. By calculation the *Synechococcus* WH 7803 KatG polypeptide is 80 kD in size and contains two heme cofactors. This enzyme's active configuration is dimeric [30], so the holoenzyme is 160 kD and contains 2054 N and 4 Fe atoms. In *E. coli* there is an average of 2 mRNA copies/OTU and about 1270 copies of each expressed protein [290]. The volume of an average *E. coli* cell is $1.1 \mu\text{m}^3$ [291]; assuming that MED4 is spherical and 0.5 μm in diameter, its volume is $0.065 \mu\text{m}^3$, or about 1/17 that of *E. coli*. If we assume that the abundance ratio of an average *Prochlorococcus* protein to an average *E. coli* protein is proportional to the volume ratio of these organisms, then the average protein would be present in about 75 copies in MED4. If we further assume that catalase is present at average abundance, and that a MED4 cell contains a single chromosomal *katG* and 2 mRNA's, then loss of the *katG* gene from a putative catalase-positive MED4 ancestor would give the savings indicated in Table 4.4.

CHAPTER 5

The Ecological and Evolutionary Implications of the Helper Phenotype

Acknowledgements

We would like to note here that Marty Szul coined the term “beneficiary” as the counterpoint of “helper” for the foregoing theoretical discussion. Marty Szul (Firmicutes), Claire Campbell (Eukarya), and Jackson Gainer (*Synechococcus*) also assisted with the helper assays. Jeremy Chandler helped with field work. The field data presented in this chapter were collected on the WP2 cruise (January-February 2007) aboard the *R/V Kilo Moana*, and the DCM08 cruise (June-July 2008) aboard the *R/V New Horizon*, so we would like to thank the captains, crews, and chief scientists of those cruises for their help. We also thank Zackary Johnson and Anna Ritchie for help with flow cytometry of field samples.

ABSTRACT

Prochlorococcus, the numerically dominant photosynthetic organism in the oligotrophic oceans, shows the genomic hallmarks of reductive evolution, a process rarely observed in free-living organisms. One consequence of gene loss in this genus appears to be dependence on co-existing organisms for protection from naturally-occurring hydrogen peroxide (HOOH). Oxidative stress-resistance genes such as catalase are conspicuously absent not only from *Prochlorococcus*, but from other abundant marine organisms such as *Pelagibacter*. We show that catalase genes are in fact dramatically over-represented in cultured organisms in comparison to in situ ocean populations, suggesting that the assumption that all aerobic organisms are strongly protected against HOOH may be mistaken. Further, this observation suggests that dependency on helpers may be a common phenotype for bacteria, at least in the ocean, and that some catalase-enriched lineages (e.g., the Proteobacteria) may serve keystone roles in protecting euphotic zone microbial communities. We present here a theory of co-evolution, the Black Queen Hypothesis, which seeks to explain how such relationships may come to be.

I. Introduction

The conventional wisdom in microbiology maintains that all obligately aerobic organisms are strongly protected against reactive oxygen species (ROS), as these dangerous molecules are unavoidably generated whenever O₂ is present [292]. However, we showed in the preceding chapters that laboratory cultures of one abundant organism, *Prochlorococcus*, are incapable of tolerating environmentally-relevant concentrations of the ROS hydrogen peroxide (HOOH) unless they are cross-protected by other, more resistant organisms. This deficiency is particularly surprising in *Prochlorococcus*, which is routinely in the presence of super-saturating concentrations of O₂ as a consequence of photosynthetic oxygen evolution. In Chapter 4 we suggested that the ecology of *Prochlorococcus* could not be understood outside of a community context, but as yet we have only begun to explore the natural euphotic zone community implicated as helpers in the ocean. It is beyond the scope of this dissertation to exhaustively examine the interrelationships between natural populations of *Prochlorococcus* and their neighbors. However, in this concluding chapter we will review what is known about ROS resistance in the ocean. We will also present some compelling, if preliminary, findings that begin to shed light on the character and extent of the “helper” phenotype in the ocean, along with some speculation as to how such interactions could have evolved. It is our hope that these observations will drive future research on this important community interaction in marine (and perhaps other) environments.

II. Expression of oxidative stress-related genes in *Prochlorococcus*

Since *Prochlorococcus* appeared to be dependent on helpers for survival at the ocean’s surface (Chapter 4), we wanted to look at the genetic responses of marine microbes, including *Prochlorococcus*, to changes in the oxidative regime. Hypothesizing that oxidative stress would attend other stresses, we re-examined several existing data sets [216,293,294] for evidence of oxidative stress responses.

The expression of *Prochlorococcus*’ minimal complement of genes is strongly synchronized to the diel cycle, with about 80% of genes exhibiting periodicity, as shown in the transcriptome study of Zinser et al. [216]. These authors did not specifically look at the expression of stress related genes, except to note that there was evidence of a generalized stress

response during peak illumination. In the ocean, we would expect oxidative stress to reach a peak in the afternoon, as both photosynthetically generated ROS and UV-generated HOOH would be maximally abundant [87,217,254]. Therefore, we hypothesized that genes responsible for detoxifying these molecules would also have peak expression at, or slightly before, this time period. Since Zinser et al. used a 14:10 light:dark cycle, with the lights coming on at 06:00 and going off at 20:00, afternoon conditions occurred between 14:00 and 20:00. This study also probably inadvertently simulated the diel production of HOOH in natural waters, as their medium included 10 mM HEPES (see Chapter 3, but it should be noted that no measurements of HOOH were performed by Zinser et al.). This study monitored the transcriptome of a *Prochlorococcus* MED4 culture for 48 hours, so in the presence of HEPES we would expect any ROS response to be stronger on day 2 than on day 1. Using these criteria, we re-examined the published data from this experiment to draw some initial conclusions about the response of this important organism to HOOH exposure.

We began by looking across the entire transcriptome for genes that fit our criteria for involvement in oxidative stress response. We first looked for genes that had a significant diel cycle (false discovery rate < 0.05 , see [216]) and peak expression in the afternoon. Second, we looked for genes with stronger expression on day 2 than day 1. Expression was considered significantly higher if the day 2 maximum was more than 1 standard deviation higher than day 1, with the standard deviation calculated across all data points for that gene. About 25% of the genome fit into one or the other of these categories, and 7% fit into both. The stress-associated genes found in these groups are summarized in Table 5.1. A number of genes associated with DNA repair and recombination peaked in the target time period, which is perhaps unsurprising, since this is also the time period when *Prochlorococcus* replicates its genome. However, several such genes involved in base excision repair (e.g., *mutS*, *uvrA*) were also expressed more strongly on day 2, suggesting they may play a role in repair of oxidative damage from HOOH. Eleven of *Prochlorococcus* MED4's high-light-inducible (*hli*) genes were upregulated on day 2. Interestingly, 5 of these genes had expression peaks at night, suggesting they have a more general stress-protection role than suggested by their name. Indeed, genes for a number of different kinds of stress (osmotic, heavy metal, temperature) were upregulated on the second day. Most environmental stresses have an oxidative stress component, however (Chapter 1), so it is

Table 5.1. Genes implicated in the HOOH response of *Prochlorococcus* MED4.

Gene	Name/Function	Functional Class	Diel Cycle ¹	Peak ²	Day One	Day Two	Ratio ³
<u>Diel cycling genes peaking between 14:00 and 20:00</u>							
PMM0897	<i>dnaK</i>	Chaperone	yes	19.8	1.79	1.84	1.03
PMM1713	<i>recN</i>	DNA repair	yes	18	2.51	2.88	1.15
PMM1097	<i>recR</i>	DNA repair	yes	17.6	3.02	3.65	1.21
PMM0942	<i>ruvA</i>	DNA repair	yes	17.6	1.74	1.40	0.80
PMM1615	<i>ruvB</i>	DNA repair	yes	19.6	2.79	3.00	1.08
PMM0882	<i>uvrC</i>	DNA repair	yes	20	1.43	1.58	1.10
PMM0637	<i>fur</i>	Metal stress	yes	18.8	2.11	1.78	0.84
PMM0742	<i>clpP1</i>	Protease	yes	17	1.91	1.76	0.92
PMM1314	<i>clpP3</i>	Protease	yes	17.8	1.66	1.76	1.06
PMM1313	<i>clpP4</i>	Protease	yes	17.8	1.82	1.86	1.02
PMM1409	<i>rub</i> (rubredoxin)	Redox control	yes	19.6	1.53	1.65	1.08
PMM1061	<i>trxA</i> (thioredoxin)	Redox control	yes	17	1.22	1.11	0.90
<u>Genes more strongly expressed on day 2 than day 1</u>							
PMM0122	<i>dnaJ</i>	Chaperone	yes	20.6	1.26	1.53	1.22
PMM1704	<i>dnaK2</i>	Chaperone	yes	21.8	1.39	2.19	1.58
PMM1436	<i>groEL</i>	Chaperone	yes	20.6	1.46	1.68	1.15
PMM0452	<i>groEL2</i>	Chaperone	yes	22.6	1.20	1.74	1.45
PMM0016	<i>grpE</i>	Chaperone	yes	22	1.27	1.66	1.31
PMM0901	<i>htpG</i>	Chaperone	no	23	1.32	2.39	1.81
PMM1140	<i>polA</i>	DNA repair	yes	2.6	1.12	1.40	1.25
PMM0504	<i>cutA1</i> (Cu resistance)	Metal stress	no	23	1.12	1.36	1.22
PMM0804	ferritin	Metal stress	no	23.8	1.06	1.44	1.37
PMM1594	<i>ho1</i>	Metal stress	no	17.2	1.31	1.56	1.19
PMM1118	<i>hli4</i>	Light stress	yes	1.6	2.17	3.15	1.45
PMM1404	<i>hli5</i>	Light stress	no	19.4	1.03	1.81	1.76
PMM1397	<i>hli8</i>	Light stress	no	1.2	1.19	1.76	1.48
PMM1396	<i>hli9</i>	Light stress	no	1.4	1.11	1.62	1.45
PMM1385	<i>hli11</i>	Light stress	yes	21.6	1.53	1.86	1.21
PMM1384	<i>hli12</i>	Light stress	yes	22	1.42	1.89	1.33
PMM1135	<i>hli14</i>	Light stress	yes	1.8	1.42	2.60	1.83
PMM0818	<i>hli16</i>	Light stress	no	1.8	1.10	1.87	1.70
PMM0817	<i>hli17</i>	Light stress	no	1.4	1.18	1.78	1.51
PMM0689	<i>hli22</i>	Light stress	yes	1.4	1.25	1.60	1.28
PMM0580	<i>clpB1</i>	Protease	no	0.8	1.08	1.45	1.34
PMM0207	<i>clpB2</i>	Protease	yes	20.6	1.21	1.43	1.18
PMM0903	<i>prxQ1</i>	Redox control	yes	2.4	1.34	1.61	1.20

Table 5.1. (continued)

Gene	Name/Function	Functional Class	Diel Cycle ¹	Peak ²	Day One	Day Two	Ratio ³
<u>Genes fitting both categories</u>							
PMM0873	<i>dnaJ</i>	Chaperone	yes	17.6	1.36	2.16	1.58
PMM1645	<i>mutS</i>	DNA repair	yes	18	1.24	1.60	1.29
PMM0133	<i>radA</i>	DNA repair	yes	17.6	2.40	3.10	1.29
PMM1562	<i>recA</i>	DNA repair	yes	17.8	2.69	3.49	1.30
PMM1712	<i>uvrA</i>	DNA repair	yes	15.6	1.15	1.38	1.20
PMM1390	<i>hli10</i>	Light stress	yes	14.8	1.88	2.85	1.52
PMM1025	<i>osmC</i>	Osmotic stress	yes	18	1.18	1.62	1.37
PMM1656	<i>clpP2</i>	Protease	yes	16.8	1.56	2.14	1.37
PMM1657	<i>clpX</i>	Protease	yes	18	2.38	2.95	1.24
PMM0090	Serine protease	Protease	yes	18.4	1.38	2.20	1.60
PMM1425	Serine protease	Protease	yes	15.6	1.33	1.91	1.44
PMM1490	Serine protease	Protease	yes	16.2	1.43	1.69	1.18
PMM1150	<i>NTR</i>	Redox control	yes	18.8	1.64	2.34	1.43
PMM0336	<i>PTOX</i>	Redox control	yes	18.2	3.60	4.91	1.36

¹ Cycling was determined using Fourier transformation as described in [216].

² Time of peak expression on a 24-hour clock. Lights came on at 6.0 and went off at 20.0.

³ Maximum expression values were determined separately for the first and second 24 h period. The ratio is the degree of increase between day 1 and day 2.

quite possible that these genes may offer protection against HOOH through unknown mechanisms. Proteases and chaperones were also conspicuous, both in terms of afternoon peaks and stronger day 2 expression. For instance, ten of 15 annotated proteases were contained in one list or another (Table 5.1). ROS are capable of extensive modification of proteins (Chapter 1), leading to loss of function and denaturation. It is important that cells remove damaged proteins in order to prevent aggregation of insoluble proteins, and to free “stalled” ribosomes from damaged proteins [295]. Expression of these enzymes is a common feature of many stress responses.

Notably missing in Table 5.1 are the most obvious proteins for ROS-protection: the “peroxidases.” Despite misleading gene names (e.g., glutathione peroxidase, *gpx*), all cyanobacterial peroxidases are thought to use thioredoxin (Trx) as a substrate for reducing HOOH and other peroxides [126]. Amongst *Prochlorococcus* MED4’s peroxidases, only *prxQ1* appears in Table 5.1. The 2-Cys peroxiredoxin *tpx* (Fig. 5.1A) was weakly periodic, with a peak around midnight that was not significantly different on day 2 than on day 1. While *prxQ1* was more abundant on the second day, along with *prxQ3* it was only very weakly periodic (Fig. 5.1B). Expression of another peroxiredoxin, *prxQ2*, was more cyclical, albeit with high variability amongst replicates (Fig. 5.1B). Expression of the misnamed gene “glutathione peroxidase” (*gpx*) had strong periodicity (Fig. 5.1C), but peaked at the same time as *prxQ2*, in early morning. Thus, there is no clear connection between these enzymes and the most likely time period for elevated HOOH stress. However, the gene *tpx* was among the most abundant *Prochlorococcus* transcripts in a field metatranscriptome, and the only abundant transcript that was clearly stress-related [294], suggesting that peroxidases may be more important in the field than in culture.

Genes involved in the production and maintenance of Trx were found in our dataset, even if the enzymes that potentially could use it to detoxify HOOH were not. Peroxiredoxins use electrons from Trx to reduce peroxides, leaving an oxidized Trx that must be re-reduced by another enzyme. In *Prochlorococcus*, this is accomplished by an NADPH-dependent thioredoxin reductase (NTR) [231]. Both the gene encoding Trx and the *NTR* gene have peaks in the afternoon; NTR is very strongly cyclical, and was ~50% more abundant on day 2 than day 1 (Table 5.1, Fig. 5.1D). Thus, it is possible that the abundance of Trx, rather than the various

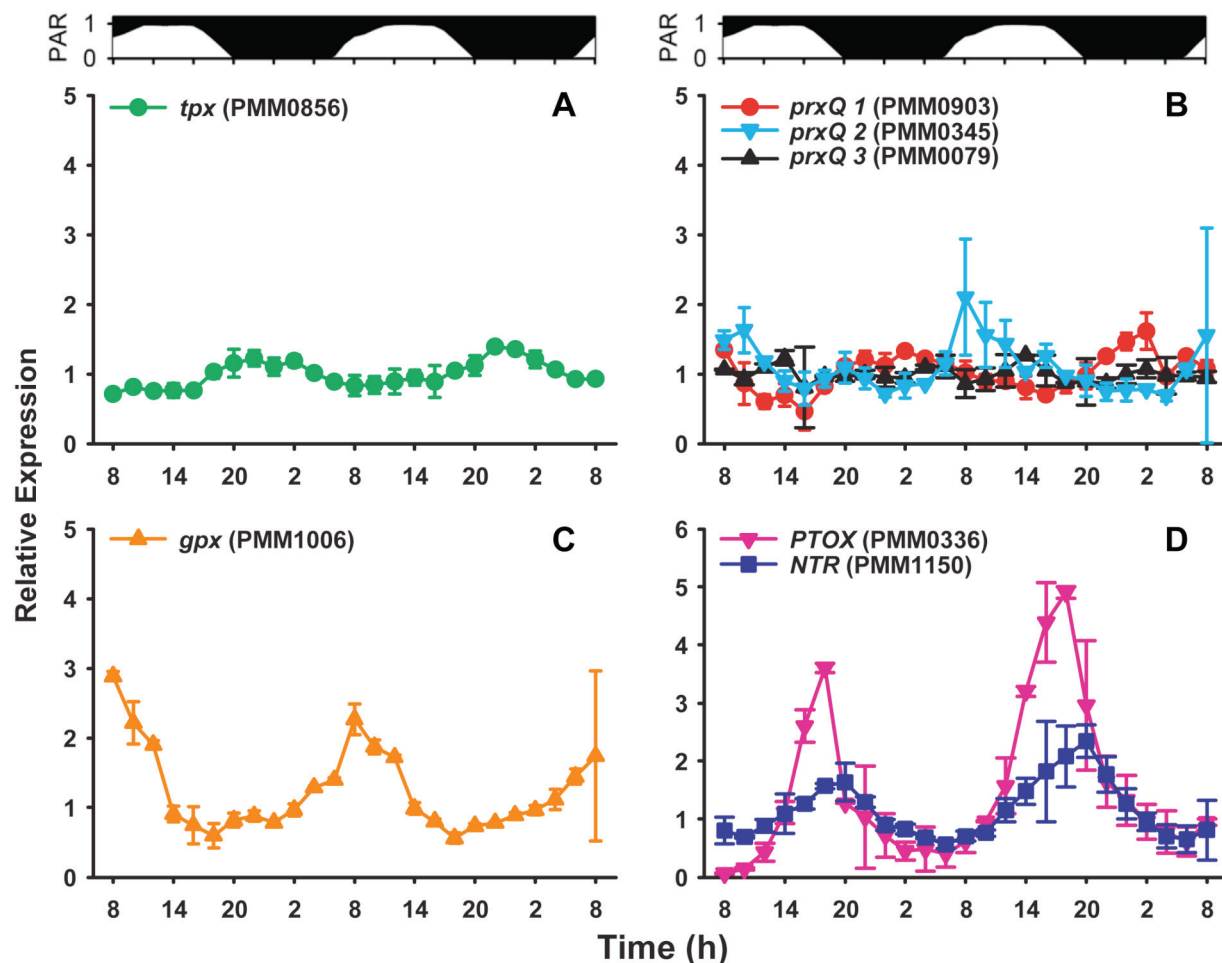


Figure 5.1. Diel expression of some oxidative stress-related genes for *Prochlorococcus* MED4. A) Thioredoxin peroxidase, the best-characterized anti-HOOH gene in the genome; B) other peroxiredoxins; C) “glutathione peroxidase;” D) *NTR*, NADPH-dependent Thioredoxin Reductase, and *PTOX*, Plastoquinol Terminal Oxidase. All data were re-analyzed from the transcriptome study of Zinser et al. [216]. Cultures were grown on a 14:10 light/dark cycle (see bars at the top of the figure) in a Percival Sunbox incubator (see Chapter 4). Error bars are the means of three replicate cultures.

peroxiredoxins, limits HOOH scavenging in *Prochlorococcus*. It is also possible that proteins other than the five peroxiredoxins considered here are responsible for using Trx as an antioxidant. Thioredoxin reductase, but not any peroxiredoxin, was also among the most upregulated proteins in a stationary phase *Pelagibacter ubique* culture (compared to an exponential phase control), suggesting that maintenance of the thioredoxin pool is of central importance for ROS-protection in heterotrophic marine bacteria as well [293].

Lastly, the gene for plastoquinol terminal oxidase (*PTOX*) not only fit into both categories, but also had the highest overall diel amplitude of any gene in the genome (Table 5.1, Fig. 5.1D). PTOX is thought to be a secondary electron acceptor for photosystem II [231], although its function is not yet known. PTOX might be able to use electrons to quench lipid peroxides, or oxidize pigments that have scavenged hydroxyl radicals. A homologous enzyme in the green alga *Haematococcus pluvialis* was found to be involved in resistance to multiple stresses, including oxidative stress [296]. If PTOX has a strong antioxidant role in *Prochlorococcus*, it could help explain the counterintuitive observation that some strains (e.g., MED4, the strain used in this diel study) are more resistant to oxidative stress in high light than in low (Chapter 4).

It must be noted that these observations were based solely on the re-interpretation of data from an experiment whose purpose was to look at the interaction of light and gene expression. While the authors effectively simulated natural light conditions using Sunbox incubators (Chapter 4), other parameters were necessarily not ecologically relevant. Notably, the cells were maintained at very high concentrations ($>10^7$ cells mL⁻¹, E. Zinser, personal communication) in order to have sufficient biomass for RNA extraction. At these concentrations, *Prochlorococcus* is much more resistant to HOOH than at ecologically relevant concentrations of $\sim 10^5$ cells mL⁻¹ (Chapter 4 and data not shown). However, we can draw some new hypotheses from this re-analysis. First, it appears that thioredoxin cycling stimulated by NTR may be more important for HOOH defense than production of peroxiredoxins themselves. Second, either *Prochlorococcus* responds to oxidative stress with a general stress response, or else various other stresses affect *Prochlorococcus* mainly by causing oxidative damage. Third, the strong expression of PTOX suggests it may be quite important in ROS defense, at least in MED4. Future experiments should focus on *NTR* and *PTOX* to further elucidate the response of *Prochlorococcus* to oxidative stress.

III. Field acclimation experiments.

We conducted a number of field experiments to measure rates of HOOH degradation in natural communities. Most of this data is summarized in Appendix I, along with more explicit methods, and in general follows observations made by other researchers: HOOH dark decomposition is largely performed by organisms between 0.2 and 5 μm in size and approximately follows first-order kinetics. One surprising finding was that addition of an HOOH spike prior to incubation often caused samples to transiently accumulate more HOOH than we added (data not shown). This suggests that physiological stress (ironically produced by HOOH in this case) can lead to metabolic production of HOOH, followed by accumulation of HOOH in the medium. Another unexpected finding was that some waters had pronounced dark production of HOOH. Fig. 5.2A demonstrates this phenomenon with samples from the Eastern Pacific, where HOOH concentrations in raw seawater did not consistently decrease during dark incubations, in contrast to previously studies [98,217,255]. If the water was first passed through a sterile 5 μm filter, however, HOOH decay was first-order as expected. When the water was passed through a 0.2 μm filter, HOOH was again stable. This suggested that, in these waters, something in the $> 5 \mu\text{m}$ size class was producing HOOH in a light-independent manner. Smaller organisms, on the other hand, were responsible for degradation at rates roughly equal to dark production. One likely explanation is that larger organisms are leaking HOOH into the seawater, perhaps as a consequence of some metabolic imbalance related to the absence of light.

Since HOOH and light are positively correlated in the water column (Chapter 1), we also wanted to know if communities would alter their hydroperoxidase capacity when transferred to a different light regime. To test this, we collected samples from several depths in the water column at two stations in the South Pacific: near surface (5m), sub-surface mixed layer (25m), and deep chlorophyll maximum (DCM, about 90 m at both stations). Duplicate samples were placed in clear polycarbonate bottles and incubated in flow-through deck incubators at ambient seawater temperature either at near-surface light intensity ($\sim 80\%$ surface irradiance) or at DCM-like conditions ($\sim 6\%$ surface irradiance). After 24 h incubation, aliquots were removed from the bottles, spiked with HOOH to 0.5 μM , and incubated either in the dark (only for the first station, Fig. 5.2B) or in the light (Fig. 5.2C/D) for 24 h. HOOH was measured by acridinium ester chemiluminescence [213] at the beginning and end of this incubation period. HOOH

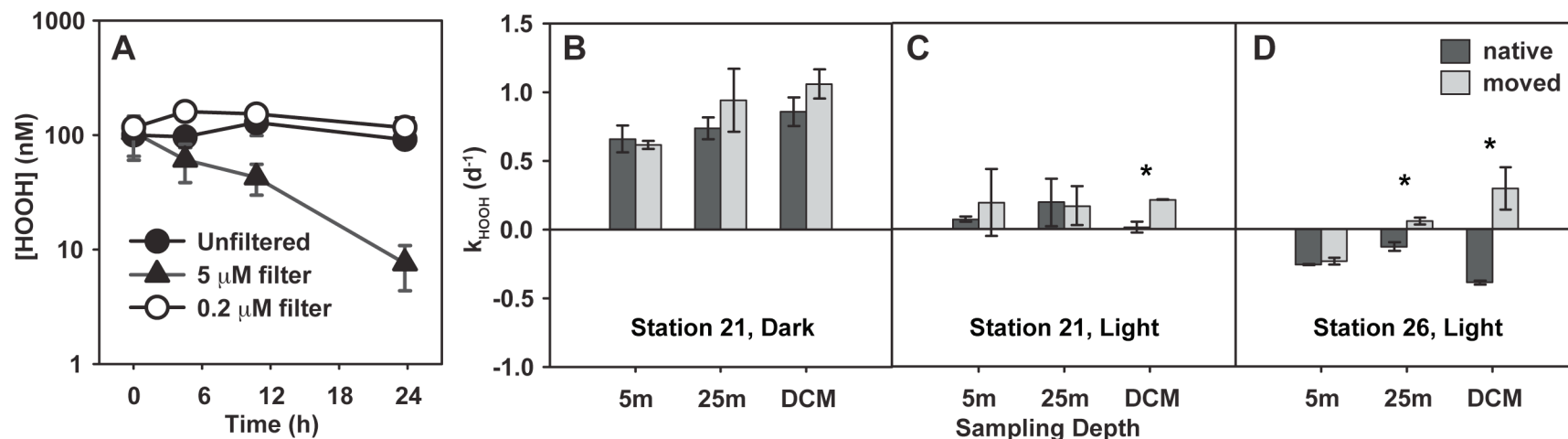


Figure 5.2. Influences on HOOH degradation rates in the ocean. A) In the Eastern Pacific (24° 59.93' N, 117° 59.82' W), HOOH concentrations were stable in unfiltered seawater incubated in the dark as well as in sterile-filtered seawater, whereas 5 µm filtered samples degraded HOOH with first-order kinetics. This is consistent with the hypothesis that larger organisms were generating HOOH in a light-independent fashion in these waters. Error bars are the standard error of three replicates. B) Duplicate seawater samples from the Western Pacific (29° 2.45' S, 164° 20.27' E) were incubated in the light for 24h, either at their native light intensity (dark bars) or under a different light intensity (light bars). After this initial incubation, samples were spiked with HOOH and incubated in the dark for an additional 24h, at which time the remaining HOOH was measured. The rate of change of HOOH (k_{HOOH}) was calculated assuming first-order kinetics. "Moved" samples from surface and 25m depths were incubated at DCM conditions; DCM samples incubated at surface conditions. Positive values indicate net decay of HOOH; negative values indicate net accumulation. C) As B, but following addition of the HOOH spike, samples were placed back under the same light regime instead of being incubated in the dark. D) As C, but with seawater taken from a different station (32° 25.29' S, 159° 5.33' E). For B-D: *, treatments significantly different (t-test, df=2, $p < 0.05$); DCM, deep chlorophyll maximum. Error bars are the standard error of duplicate samples.

concentrations decreased in all dark-incubated samples (Fig. 5.2B). Rates of dark decomposition (calculated assuming first-order kinetics) correlated well with bacterial abundance of the samples measured by flow cytometry using the DNA stain SYBR green (Spearman Rank Order correlation, $n=3$, $r = 0.99$, $p=0.1$, data not shown). There was no significant difference, however, between “native” and “moved” samples in dark degradation rates.

HOOH change in light-exposed samples, however, was much less straightforward. The rate of HOOH decay was much lower in light-exposed samples than in dark samples, even for samples incubated at very low (DCM) light intensities. At the second station, HOOH actually accumulated in light-exposed samples incubated at their native light intensity, probably because bacterial abundance at this site was quite low (data not shown). The discrepancy between light and dark HOOH kinetics indicates that photochemical HOOH production occurs in these samples. However, the greatest amount of photochemical HOOH production at both stations was in the DCM samples incubated in their native, low light conditions. Since the DCM is defined as a peak in abundance of chlorophyll (and hence, photosynthetic biomass), one clear possibility is that HOOH generation is caused by phototrophs. The apparent insensitivity of photochemical HOOH generation to light intensity suggests that HOOH may originate from a source other than photosynthesis, or that communities may use photosynthetically derived electrons to reduce ROS (see the discussion above on PTOX).

No surface samples appeared to modulate their hydroperoxidase activity when shifted from high light to low. At station 26 (Fig. 5.2D), there was a moderate, but significant, increase in HOOH decomposition in 25m samples moved to low light, but no similar change was observed at station 21 (Fig. 5.2C). On the other hand, DCM samples strongly responded to being moved to surface light intensities (Fig. 5.2C/D). At both stations, DCM samples at low light generated substantial amounts of HOOH, but after 24h incubation at high light, the discrepancy between rates of dark and light HOOH removal was significantly reduced. If our conclusion that phytoplankton are responsible for HOOH formation in these samples is accurate, this finding suggests that DCM phytoplankton, or their surrounding community of “helpers,” are capable of responding to high light in some way to minimize HOOH leakage.

Collectively, the observations in this section suggest that the majority of decomposition of HOOH at our study sites was accomplished by picoplankton – mostly by heterotrophic

bacteria, since *Prochlorococcus* comprised the majority of the picophytoplankton community (data not shown) and it clearly has little HOOH scavenging capacity (Chapter 4). Further, phytoplankton, particularly larger organisms, may be responsible for a substantial amount of the HOOH formed, contrary to the accepted paradigm that abiotic reactions are the source of most oceanic HOOH. This is consistent with recent work showing active superoxide production by microorganisms, potentially to facilitate Fe acquisition [297]. Lastly, mixed layer communities appear to constitutively express their HOOH scavenging systems, as they neither lost nor gained hydroperoxidase capacity when transferred to low light; however, deep communities can acclimate to higher light by upregulating antioxidant systems. Future experiments should focus on discovering which taxa are the predominant agents responsible for both biotic production and degradation of HOOH.

IV. The catalase-deficient biosphere

In Chapter 2 we related the results of experiments that explored the phylogenetic breadth of the helper phenotype. One conclusion was that all strains of *Prochlorococcus* tested could be helped by at least some of the bacteria tested. Perhaps more surprisingly, *none* of the several dozen bacterial strains we tested were unable to help at least one strain of *Prochlorococcus*. Since then, we have explored the helper phenotypes of even more organisms, both in liquid and on solid media. Tested organisms represent a phylogenetically broad sampling of microbial life, including not only heterotrophic bacteria but also eukaryotes and even other cyanobacteria (Fig. 5.3). *Every organism tested* was able to facilitate significantly superior growth of *Prochlorococcus* than that obtained with axenic cultures.

The surprising universality of the helper phenotype drove our early hypotheses for the mechanism of helping: it had to involve some activity that was absent in *Prochlorococcus*, but was common enough that our culture collection contained no strains that could not do it. Working within the classic paradigm that all aerobic organisms are resistant to ROS, we queried the numerous sequenced *Prochlorococcus* genomes and found no evidence of robust anti-ROS systems, such as catalase and heme peroxidase, in any of them. In contrast, all of the bacterial strains we initially tested for the helper phenotype were at least weakly catalase positive (Chapter 2). Later, the work described in Chapter 4 confirmed the key hypothesis of *Prochlorococcus*'

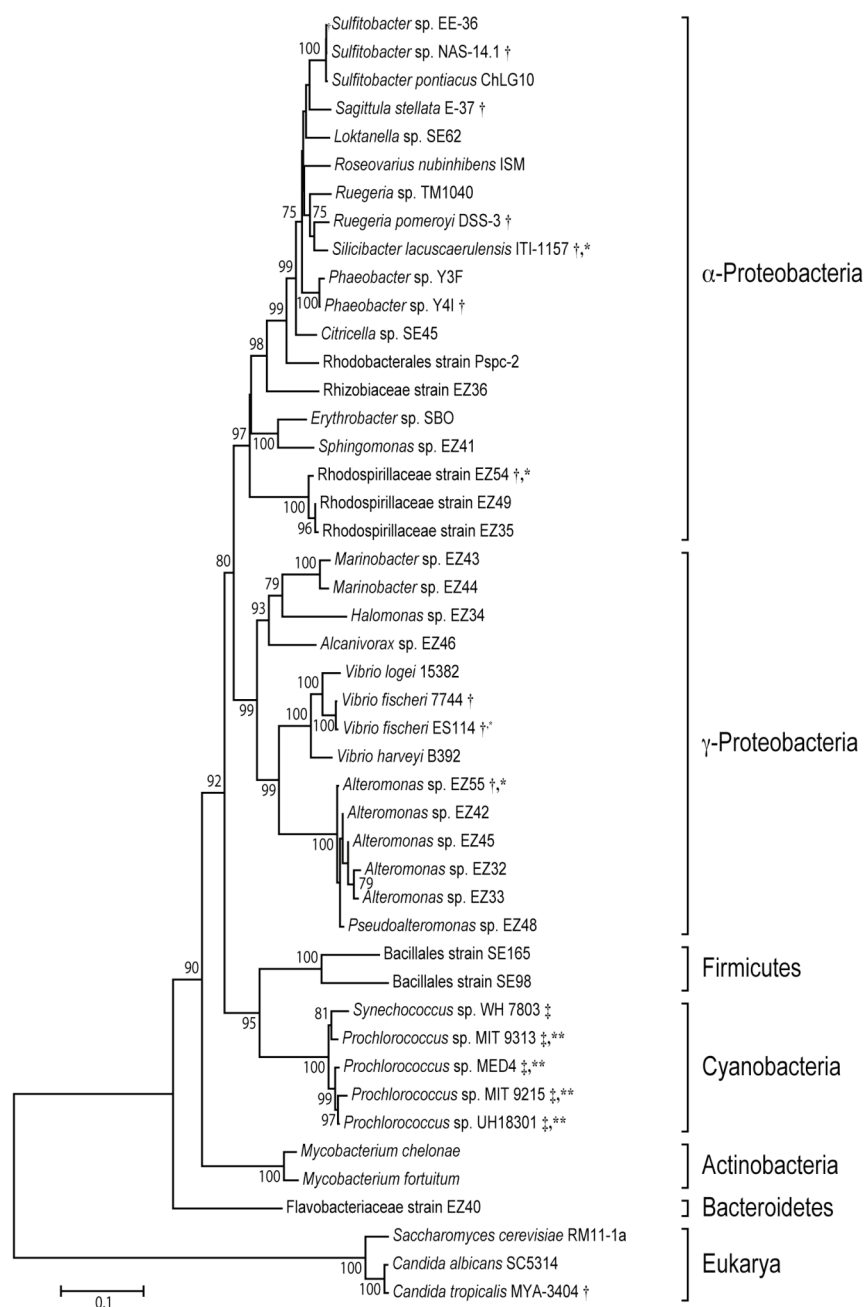


Figure 5.3. SSU-rDNA phylogeny of microbes with the helper phenotype. Sequences were aligned using SILVA [298]. Unrooted neighbor-joining tree was produced in MEGA 4 [299] with pairwise deletion of gaps, using the Tamura-Nei evolutionary model to infer phylogeny. Bootstrap values (1000 replicates) less than 75% were omitted. Unless otherwise noted, all helper assays were performed using the streak plate assay (Chapter 2). Selected helpers were tested in liquid media (‡, see Chapter 4), or using both methods (†). In liquid assays, helpers were added at 10^6 cells mL^{-1} unless otherwise noted. Selected strains were tested at lower inocula; *, 10^2 cells mL^{-1} inoculum was effective for helping; **, lower inocula were not effective.

vulnerability. Based on the universality of the helper phenotype in our experiments, and the absolute dependence of cultured *Prochlorococcus* on help, we naturally assumed that these catalase-positive organisms were also ubiquitous in the ocean.

As we began to extend our focus from laboratory cultures of *Prochlorococcus* to natural populations, we began to question this assumption. Specifically, we wondered if our culture collections were truly representative of the natural flora in *Prochlorococcus*-rich waters. While we tested a broad variety of microorganisms for their helper phenotype, they were not specifically isolated for any particular attribute and merely represented the breadth of strains in our freezer. Closer consideration of this assortment revealed that most organisms were members of clades known for their ease of cultivation. For instance, we tested numerous marine Roseobacters, which are well-known for being more amenable to cultivation than other marine bacteria [300]. Similarly, the strains we isolated from *Prochlorococcus* cultures were merely diluted to extinction on rich agar, the most basic of all cultivation techniques (Chapter 2). Thus, the large majority of strains we tested fell into the category of organisms colloquially known as “weeds,” i.e., fast-growing, copiotrophic organisms with simple nutritional requirements and a wide range of physiological tolerances [301]. This raised two questions: first, how common is the helper phenotype in field populations, and second, are other collections of cultivated bacteria as skewed toward catalase-positive organisms as ours? In other words, has ignorance of ROS-sensitivity in natural bacteria led to a gross oversampling of ROS-resistant bacteria, and led to the attendant, erroneous conclusion that all aerobes are robust ROS-degraders? Are these easily cultivable, catalase positive organisms in any way representative of the diversity of life?

In order to estimate the level of ROS-resistance in cultured organisms, we looked for catalase genes in all sequenced bacterial and archaeal genomes in the JCVI-CMR database (<http://cmr.jcvi.org>). We chose catalase for several reasons. Foremost is the ease of detection of catalases. Trivially, this is because the colloquial name “catalase” is so well established that even the first sequenced genomes use the same terminology as those most recently sequenced. Other anti-ROS genes, such as glutathione peroxidase, are more recently characterized and have contradictory nomenclature. Thus, a simple keyword search was sufficient to return almost all enzymes that are homologous to known catalases, whereas a much greater sampling effort would be required to uncover other likely genes. Catalase is also a desirable target because of the

unambiguous nature of its function – it degrades HOOH in order to protect cells from oxidative stress. Other peroxidases have a wider range of substrates and different functionalities, such as the manipulation of secondary metabolites using HOOH as an electron donor, or the repair of lipid peroxides. While these enzymes *probably* provide protection from HOOH, it is not clear how well they do this in relation to other functions, nor is it certain that they are capable of robust defense against exogenous HOOH (as opposed to metabolically-generated ROS). Lastly, the only clearly helper-negative organism we tested was a catalase-deficient mutant of the *Roseobacter Ruegeria lacuscaerulensis* (Chapter 4).

Figure 5.4A shows the results of this search. Out of 770 genomes searched, >60% contained at least one annotated catalase, and many contained more than one type of catalase (average 2.4 catalases per catalase-positive genome). If we assume that these genomes are representative of the scope of culture collections, then we must conclude that the majority of cultured organisms are catalase-positive. Additionally, the median genome size of catalase-positive organisms was more than twice as large as that of catalase-negative genomes. We have argued that the lack of ROS defenses in *Prochlorococcus* spp. is a result of adaptive gene loss (Chapter 4); this finding is consistent with a general trend toward loss of catalase during the process of genome size reduction across a phylogenetically broad assortment of bacteria. Also of note, all 9 of the *Candidatus* species contained on JCVI-CMR were catalase-negative. “*Candidatus*” is a stopgap designation for bacterial species that have been identified by their 16S DNA signature, either in the field or in enrichment cultures, but have not been isolated in pure culture. This also suggests analogy with *Prochlorococcus*; perhaps the inability of these organisms to grow in axenic culture is also the result of oxidative stress following removal of their helpers.

In order to quantify the prevalence of catalase genes in the open ocean, we queried the 21 stations ($\sim 2.1 \times 10^9$ bp) marked as “Open Ocean” in the Global Ocean Sampling database [8], excluding the first 2 Sargasso Sea stations [302], with a database containing all of the unique catalase sequences identified in the aforementioned JCVI-CMR genomes (1093 sequences, Supp. File 1) using *blastn* [282] implemented through CAMERA (<http://camera.calit2.net>). We also queried the same stations with a database of all unique *rpsL* sequences from the JCVI-CMR genomes (677 sequences, Supp. File 2). This gene encodes a critical ribosomal protein and was

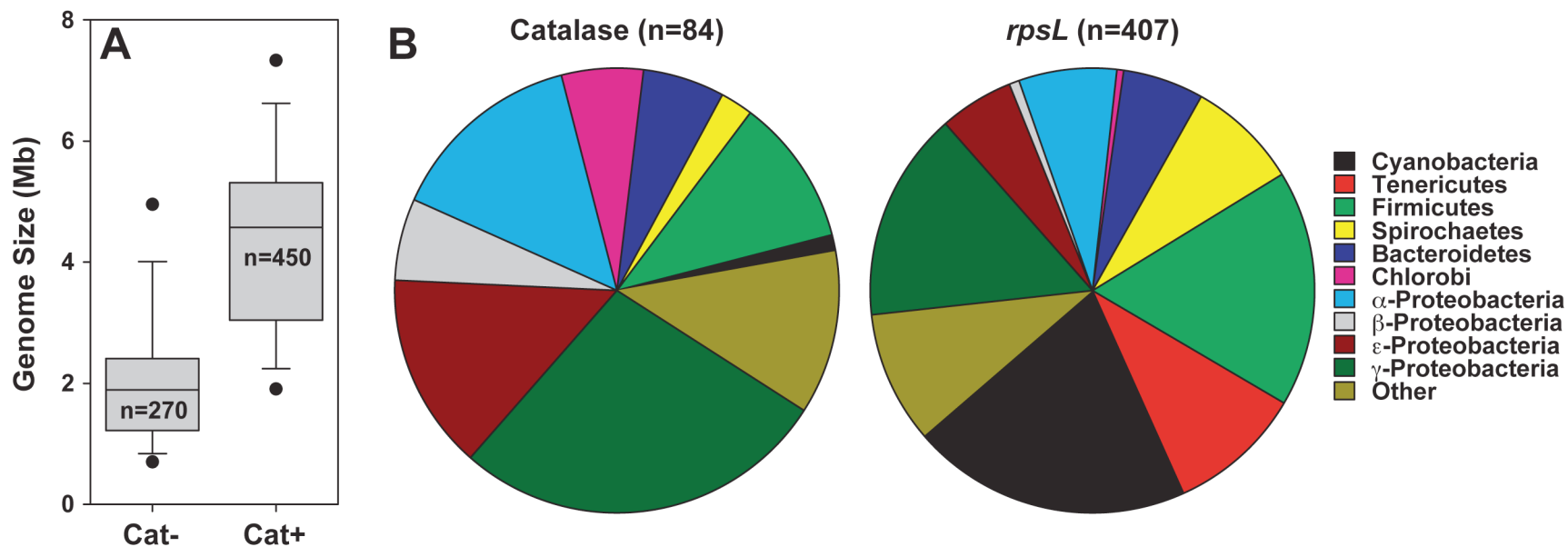


Figure 5.4. Catalase is much more common in cultured organisms compared to the open ocean. A) Box plot of genome sizes for catalase-positive and -negative sequenced genomes. ~60% of sequenced bacterial and archaeal genomes from the JCVI-CMR website contained at least one annotated catalase. Catalase-containing genomes were significantly larger than others (t-test, $df=718$, $p < 0.001$). B) In open ocean stations of the global ocean sampling (GOS) dataset [8], catalase-like sequences were ~20% as frequent as sequences resembling the ubiquitous ribosomal protein *rpsL* (found in a single copy in almost all sequenced bacterial genomes). The GOS metagenome was queried using blastn [282] with a database of all annotated catalases or *rpsL* genes from sequenced genomes at JCVI-CMR. Only reads from open ocean stations ($\sim 2 \times 10^9$ bp) with alignments > 100 bp and E-values < 0.05 were retained for the analysis. Most (~68%) catalases grouped with the Proteobacteria, which accounted for only ~31% of *rpsL* sequences. Complete data for the catalase and *rpsL* GOS hits, including metadata and sequence alignments, can be found in Supp. Files 4 and 5, respectively.

found in >95% of genomes examined, usually in a single copy (average 1.1 copies per genome). Thus, *rpsL* abundance is a reasonable proxy for mean abundance, and can be used to normalize the catalase data. BLAST hits were curated to remove all but the best matches: any hits with E values > 0.05 or alignments < 100 bp in length were removed. The results are summarized in Figure 5.4B. 407 *rpsL* hits were recovered. Assuming the average genome is between 1.5 and 4 Mbp in size, we would expect between about 500 and 1400 “genomes” to be represented in a 2.1×10^9 bp dataset, so the abundance of *rpsL* hits is reasonable for the size of the database searched. Strikingly, almost five times as many *rpsL* sequences as catalase sequences were discovered, suggesting that catalase-negative organisms outnumber catalase-positive organisms by a significant degree. This gives a low estimate of the discrepancy between catalase-positive and -negative organisms, however, as many sequenced genomes contained multiple analogous catalases (see above), but only a single *rpsL*. Further, catalase-encoding sequences are generally 5 times longer than those of *rpsL*, so they should be better represented in random shotgun clone libraries like those used to generate the GOS dataset. Hence, catalase-negative organisms may be more numerous by more than an order of magnitude. Based on their nearest hit in the query database, catalase sequences were also less diverse than *rpsL* sequences, both in terms of sequence richness (40 vs. 140) and evenness (Shannon index, 3.14 vs. 4.48). From these data it would seem obvious that catalase-positive organisms are over-represented in culture collections in comparison to the ocean.

We also assigned taxonomic identities to all catalase and *rpsL* hits gathered from the GOS data based on their closest match in our database (Fig. 5.4B, Supp. File 3). We chose to group these hits at the highest-level taxon for several reasons. First, while our query databases were constructed with many hundreds of sequences, they were still entirely derived from cultured organisms, so inference of low-level taxa from homology with these sequences is problematic at best. Second, this problem is amplified by the possibility of horizontal gene transfer, especially of functional genes like catalase. Inter-phylum gene transfer is uncommon [303], so we felt it was reasonably safe to assume that homology to an α -Proteobacterial catalase implied that a sequence came from an α -proteobacterium (but see [304]). If we accept this assumption, our analysis shows that some lineages are much more likely to be catalase producers than others, at least in the open ocean. More than 2/3 of the catalase hits were assigned to the

various Proteobacteria, whereas these organisms represented less than 1/3 of the *rpsL* hits. Conversely, Cyanobacteria (mostly *Prochlorococcus*) constituted >20% of the *rpsL* dataset, but only 1 catalase was recovered from this group. Somewhat surprisingly, many *rpsL* sequences were assigned to the phylum Tenericutes, which amongst cultured organisms is solely represented by obligate parasites such as *Mycoplasma* spp. No catalases are known from these organisms, which have undergone reductive evolution [305] similar to *Prochlorococcus* and *Pelagibacter* (Chapter 4).

Several caveats should be mentioned here. First, it is possible that there are catalases in the ocean that have no homologs in our BLAST database (e.g., from as-yet unknown free-living Tenericutes). This seems unlikely, however, because of the strong conservation of regions of these enzymes through all three domains of life [30]. Second, it is possible that other enzymes or molecules are responsible for HOOH detoxification in the ocean. *Escherichia coli*, for instance, is thought to rely primarily on the peroxiredoxin AhpC for front-line defense against *endogenous* HOOH [125], but our analysis in section II produced little evidence that similar enzymes are important in defending *Prochlorococcus* from oxidative stress. Recent work has suggested that some helpers facilitate growth by the production of siderophores [266]; these authors suggest that the siderophores help provide Fe to bacteria, but an equally good explanation is that they sequester Fe and prevent Fenton-catalyzed production of $\cdot\text{OH}$ (Chapter 1). Catalases, however, appear to be the weapons of choice against externally generated HOOH [140]. Third, it is possible that catalase production, or HOOH degradation in general, is the purview of larger organisms; the GOS sampling regime at these stations only sequenced organisms passing a 0.8 μm filter [8]. However, organisms in the <0.8 μm size class numerically dominate open ocean waters, so the conclusion that HOOH scavenging in the ocean is accomplished by a small subset of the population as a whole would still be sound even if *all* larger organisms were strong catalase producers. The same argument would hold if some organisms over-expressed their catalase genes, producing more mRNA per gene than other organisms. Moreover, data presented in Section III showed that larger organisms are net *producers* of HOOH in some waters, but picoplankton were net *consumers*. Lastly, it cannot be ignored that many of the cultured organisms used to generate our catalase database are pathogens of human beings. As the mammalian immune system uses ROS as one of its defenses, it is possible that over-sampling of

pathogens could explain the prevalence of catalase in sequenced organisms. This bias should be diminished by the fact that many of the intracellular pathogens that have been sequenced (e.g., the Tenericutes) are catalase-negative.

What, then, can be said about the catalase-deficient lineages represented in Figure 5.4B? Are they all as HOOH-vulnerable as *Prochlorococcus*? This will require experimentation to discover, but if so, we must conclude that they, too, are dependant on helpers for survival in the ocean. The Proteobacteria are clear candidates for this role, although the task may be the purview of an even narrower phylogenetic group. If these preliminary observations stand up to rigorous experimentation, we must conclude that those organisms expressing catalase in the ocean occupy a keystone position, disproportionately affecting the state of their community in relation to their share of the biomass. Such a finding would have consequences for our understanding of the evolution and ecology of the picoplankton as well as the marine food chain they support. It would also inform efforts for conservation and bioremediation, as the health of the generally ignored “weeds” would have to be considered alongside that of the conspicuous primary producers.

V. Mutualists, commensals, or parasites?

So far we have only discussed the benefits of the helper phenotype to *Prochlorococcus*, and potentially to other HOOH-sensitive microbes. This is sufficient to classify the helper interaction as a form of symbiosis, broadly defined as the “living together” of different species where there is a measurable benefit for at least one of the partners. It is not clear, however, whether this interaction is good for *both* partners. In this section we will consider the question of where the helper interaction fits in the spectrum ranging from mutualism to parasitism. We will approach this issue first with empirical observation, and then from a theoretical perspective.

Many of the helpers we tested were isolated from mixed cultures of *Prochlorococcus* and “contaminating” heterotrophic bacteria (Chapter 2). These contaminants were almost certainly of marine origin (Alteromonads, Marinobacters, etc), isolated along with *Prochlorococcus* rather than being introduced accidentally during culture propagation in the laboratory. Since *Prochlorococcus* was cultured in natural seawater media containing no added carbon source, it would be reasonable to conclude that the survival of these organisms in mixed culture for over a

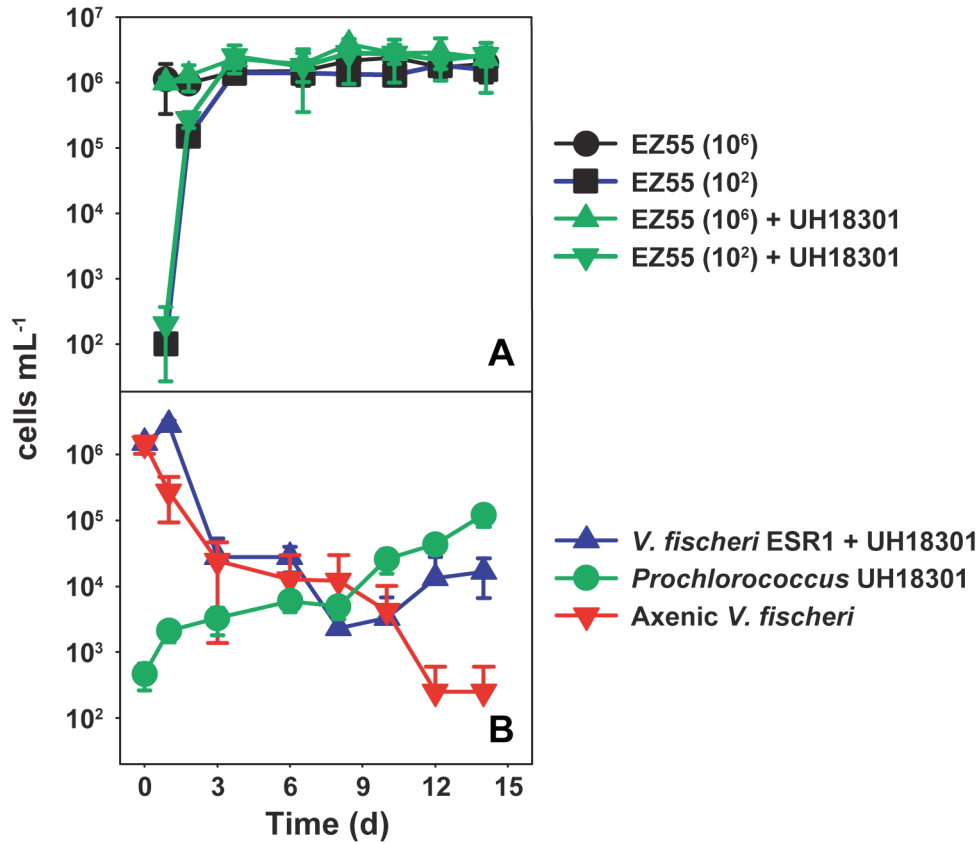


Figure 5.5. Growth kinetics of helpers in *Prochlorococcus* seawater media. A) *Alteromonas* sp. EZ55 was not dependant on co-culture with *Prochlorococcus* to grow in “carbon-free” media. When inoculated at 100 cells mL⁻¹, this strain rapidly grew to 10⁶ cells mL⁻¹ whether or not *Prochlorococcus* was also present. B) *Vibrio fischeri* required *Prochlorococcus* to grow in seawater. Axenic *V. fischeri* gradually died off, eventually becoming completely nonculturable. In co-culture, however, cells dropped off until *Prochlorococcus* exceeded them by about an order of magnitude, and then began to grow at about the same rate as *Prochlorococcus*.

decade means that they are deriving energy from *Prochlorococcus*' primary production. However, this does not appear to be the case, at least for some strains. *Alteromonas* sp. EZ55 was able to grow when inoculated into sterile seawater at sufficiently low cell density (Fig. 5.5A), reaching stationary phase at approximately 10^6 cells mL⁻¹, a concentration that was reasonably stable for at least a month. This was the case in all sources of seawater we tested, and was also observed for several other heterotrophic species (*R. latuscaerulensis* and Rhodobacteraceae sp. EZ54, data not shown). Whether or not EZ55 was co-cultured with *Prochlorococcus* did not significantly affect either growth rate or cell yield (Fig. 5.5A). It is unclear what carbon source these organisms are exploiting, or whether each species uses the same one. Indeed, there was no evidence that EZ55 significantly drew down the dissolved organic carbon content of the medium in the process of growing from 10^5 to 10^6 cells mL⁻¹ in sterile Pro99 medium (Fig. 4.2G). Even more surprisingly, these strains were also able to grow to 10^6 cells mL⁻¹ in *artificial* seawater, even after the cells had been rinsed four times in the medium before inoculation to remove any rich growth media that might have attended the inoculum (data not shown). Therefore, it would appear that these copiotrophic organisms are extremely resourceful in exploiting growth substrates (e.g., trace carbon contamination from medium salts, culture vessels, etc.), and are not dependant on co-culture with autotrophs to persist in natural seawater media under typical cultivation conditions. However, we cannot conclude from these experiments that no cross-feeding between helpers and *Prochlorococcus* is occurring. *Prochlorococcus* is known to secrete C compounds into the medium [270], and some marine heterotrophs have been shown to be chemotactically attracted to these exudates [306]. Future experiments to address cross-feeding should focus on eliminating the obscuring effects of media C contamination.

In contrast to the above strains, *Vibrio fischeri* ESR1 was incapable of surviving in seawater media without *Prochlorococcus*. When inoculated axenically at 10^6 cells mL⁻¹, *V. fischeri* cell counts steadily dropped until they fell below the limit of detection (100 cells mL⁻¹, Fig. 5.5B). In axenic culture, *V. fischeri* eventually lost viability, and late-stage addition of rich medium failed to recover the culture (data not shown). However, in co-culture with dilute *Prochlorococcus*, some *V. fischeri* cells survived, and eventually re-entered exponential growth, always lagging behind *Prochlorococcus* by approximately an order of magnitude (Fig. 5.5B).

We can make two conclusions from this. First, whatever the other helpers were growing on in the seawater medium is unavailable to *V. fischeri*. Second, they *are* able to grow using some exudate of *Prochlorococcus*. In this case, there is a clear benefit to *V. fischeri* of co-culture with *Prochlorococcus*, indicating that helping in this case is mutualistic. It is unclear if this relationship is of any ecological interest, however, since *V. fischeri* concentrations in the ocean are very low [307].

Thus, empirical data from our *Prochlorococcus*:helper dyads suggest that helping is either commensal or mutualistic. We now consider helping from a theoretical ecological perspective. In Chapter 4 we discussed one scenario for the reductive evolution of *Prochlorococcus*: that adaptation to the ultra-oligotrophic open ocean has encouraged gene loss and concomitant resource savings in terms of N and P demand for cell division. As such, it would seem that helpers are getting the short end of the stick: they retain costly ROS-scavenging functions so that *Prochlorococcus* (or other “beneficiaries”) can improve its growth rate. However, the actual outcome of the relationship will likely differ depending on the trophic strategy of the partners (Table 5.2). Keeping in mind that the following discussion is probably a gross oversimplification, let us consider individually the four possible combinations of heterotrophs and autotrophs.

In the case of autotrophic organisms acting as helpers, the most relevant consideration is that all autotrophs are theoretically limited by inorganic nutrients, at least in the dilute conditions of the open ocean. Thus, if an autotroph were a helper of another autotroph, this would clearly be a parasitic relationship, since both partners would be limited by the same resources and an increase in the fitness of one must manifest as a cost to the other. If an autotroph were to help a heterotroph, however, the issue is not so certain. If the heterotrophs are limited by inorganic nutrients, helping could be seen as parasitic. However, primary production limits heterotrophic production in the ocean [51]. Thus, there is no direct competition between the autotrophic helpers and the heterotrophic beneficiaries, so the relationship is commensal.

Now we consider the case where heterotrophs are helpers, as in the experiments described in this dissertation. If heterotrophs help heterotrophs, the situation is *probably* the same as when autotrophs help autotrophs: parasitism. However, this assumes that the helper and beneficiary heterotrophs are competing for the same substrate. If this is not the case, then there

Table 5.2. Nature of the helper:beneficiary symbiosis for various trophic pairings.

		Trophic Strategy of Beneficiaries	
		Heterotroph	Autotroph
Trophic Strategy of Helpers	Heterotroph	Parasitic/Commensal ¹	Mutualistic
	Autotroph	Commensal/Parasitic ²	Parasitic

¹ The relationship is parasitic only if the heterotrophs are growing on the same carbon source; otherwise, it is commensal.

² The relationship is commensal if the heterotrophs are C-limited; otherwise it is probably parasitic.

is no direct competition, so the interaction is commensal. Lastly, consider the case of heterotrophs helping autotrophs, as in the *Prochlorococcus*:helper interaction. Let us assume that the helpers are not limited by inorganic nutrients, but rather by primary production of the beneficiary. If *Prochlorococcus* increases its rate of production as a consequence of genome reduction, this raises the productivity of the *entire community*. Therefore, helping would be equivalent to a beneficial “division of labor” in economics, improving the productivity of all members of the population. Thus, it is likely that this last possible interaction would be mutualistic for all possible combinations of helper heterotrophs and beneficiary autotrophs.

Note that there is no special reason to assume that the benefit (or lack thereof) to the helper will influence the development of this relationship. All organisms probably have their parasites, and the deleterious nature of this interaction to the host is neither an impediment for the parasite, nor usually strong enough in its own right to lead to the host’s extinction. As we will consider in the following section, it may be that the nature of some functions (such as HOOH scavenging) makes the development of helper/beneficiary interactions inevitable.

VI. The Black Queen Hypothesis

Let us now consider the evolution of helping. In popular thought, evolution is a relentless progression toward increasing complexity. However, the persistence of “simple” life forms, as well as countless examples where “simple” organisms have “complex” ancestors, demonstrates the naiveté of this conception (e.g., [308]). As has been mentioned, the genomes of *Prochlorococcus* and several other marine bacteria show signs of reductive evolution, a process common in symbionts and parasites but rare amongst free-living organisms [11,246,247]. In the previous chapters we presented evidence that, for *Prochlorococcus*, gene loss has resulted in an inability to grow at the ocean’s surface in the absence of helper organisms. Because HOOH is as membrane permeable as water [26], it is essentially impossible for an organism to protect itself from HOOH without simultaneously protecting its neighbors. We have further hypothesized that *Prochlorococcus* (and perhaps other “streamlined” marine bacteria such as *Pelagibacter ubique* [12]) has gained a growth advantage by losing costly antioxidant genes such as catalase, at the expense of a minority of HOOH-degrading helpers that protect the entire community from HOOH. Moreover, we have alluded to the fact that this process of gene loss may represent a

previously unknown trajectory of co-evolution with implications beyond the system studied herein.

One well-known theory of co-evolution, the Red Queen Hypothesis [309], uses a metaphor derived from Lewis Carroll's *Through the Looking-Glass* [310] – “it takes all the running you can do, to keep in the same place” – to describe the evolutionary “arms race” between ecological antagonists such as parasites and their hosts. This hypothesis sought to explain the surprising constancy of extinction rates in the fossil record, and posited that all species experience a constantly deteriorating fitness landscape as a consequence of co-evolution with other species in their environment. In contrast, evolution toward dependency on “helpers” favors commensal or cooperative interactions. We present here a theory of co-evolution called “the Black Queen Hypothesis” (BQH). Similar to the Red Queen, the “Black Queen” refers to a playing card, in this case the queen of spades in the card game Hearts. Unlike most games, the goal of Hearts is to score as few points as possible. The queen of spades, however, is worth more points than all other cards combined, and therefore the predominant strategy of the game is to *not* be the player that ends up with that card. BQH predicts that there are genes, or more broadly, biological functions, that are analogous to the queen of spades. Such a function is costly and therefore undesirable, leading to a selective advantage for organisms that stop performing it (i.e., the winner of a hand of Hearts will certainly be one of the players who *doesn't* end up with the queen of spades). At the same time, the function must be indispensable for community survival, necessitating its retention by at least a subset of the individuals comprising the community (i.e., one can't play Hearts without a queen of spades). HOOH scavenging fits both of these criteria, and therefore BQH predicts that it will be performed by a small, phylogenetically narrow subset of species in any stable community.

In order to quantitatively model BQH, we must consider under what conditions it is advantageous for an organism to stop performing a function. Clearly, any functional gene loss comes with a potential cost in fitness; therefore, to be selected, function loss must also provide a gain in fitness over the ancestor that outweighs this cost. Both gain and cost may be quantified empirically. Fitness *gain* is roughly equal to the savings in resources from gene loss, relative to the total resource demand for producing a new cell. Fitness *cost* is based on the fitness of the organism in an environment where the lost function is not performed at all (S), modified by the

degree to which other members of the community provide the service – i.e., how much “help” the surrounding community provides. This last factor may be described by a “leakiness coefficient” L , which measures how well an organism can selfishly retain the products of the function in question. While the criteria for quantifying L will differ greatly for different functions, some generalizations can be made. At low L , helping must be an “intentional” activity, as products of the function are easily withheld from the community at large. At high L , however, “helping” is essentially unavoidable. BQH predicts that evolution will favor communities in which poorly partitionable functions (high values of L) are carried out by relatively few taxa (probably those with the lowest mutation rates) to the benefit of the entire community. Moreover, functions with low S and high L will tend to encourage the development of symbiotic interactions, as fast evolvers become dependent on slow evolvers to perform essential functions. When the sensitive strains also provide essential products (as in the case of sensitive photoautotrophs), this mutualistic interaction could be both mutualistic and extremely stable.

As an example, let us consider a simple competition between modern *Prochlorococcus*, with essentially no capacity to destroy HOOH, and a putative ancestor that can perform this task but is otherwise genetically identical. In the absence of HOOH, the mutant *should* win this competition, because its smaller genome requires fewer resources to produce the next generation. However, in the presence of HOOH, both strains must survive. Since they are otherwise isogenic, the ancestor is just as vulnerable to any unscavenged HOOH as the mutant. Because HOOH is membrane permeable, L is close to 1, and intracellular degradation of HOOH must lead to a net HOOH loss for the whole community. Therefore, there is no HOOH flux where the ancestor, but not the mutant, can persist. If the ancestor scales back degradation enough to harm the mutants, it too will begin to accumulate damage. Thus, based on BQH, the hypothesis that loss of antioxidant capacity was adaptive for *Prochlorococcus* as a member of its natural community is reasonable. Further, BQH is consistent with the relative scarcity, and lack of diversity, of catalases in the ocean, as described in section IV above.

As a final note, BQH makes very specific predictions that are amenable to mathematical modeling. Equations have previously been developed to describe the conditions under which antibiotic-sensitive and -resistant bacteria can coexist [311]. This model could easily be

modified to include the variables *S* and *L* described above, creating a framework to test BQH both in vitro and in silico.

VII. Final thoughts

In this dissertation, we have provided compelling evidence that the important cyanobacterium *Prochlorococcus* is dependant on “helpers” to survive in its natural habitat at the ocean’s surface. While many studies have looked at symbiotic interactions between microbes, this is one of the first to thoroughly document such a relationship that does not obviously entail intimate contact between the organisms. Further, we have provided preliminary evidence supporting the idea that this could be the “tip of the iceberg,” and that much of the uncultured diversity in the microbial world could be “unculturable” because of requirements for help from other members of the community [183,266]. Whether such help comes in the form of H₂O₂ removal or other as yet uncharacterized activities remains to be seen.

In closing, we would like to point out that the discovery of H₂O₂ sensitivity in *Prochlorococcus* began with simple logic. At first, all we had were a collection of confusing observations:

- *Prochlorococcus* does not grow from dilute cultures, or on solid media as colonies, without helpers.
- *Prochlorococcus* has a very small genome.
- Axenic *Prochlorococcus* cultures grow well when cell concentrations are high, but poorly when cells are dilute.
- Essentially all easily cultivated heterotrophic microbes are able to help.

From these observations, logic drew us inexorably to oxidative stress as an explanation. The helping mechanism had to be ubiquitous in cultured microbes, making the invocation of special growth factors or autoinducers unlikely. The helping mechanism also had to be more important when cells were extremely dilute than when they were concentrated, making helper influence on most bulk medium properties, such as pH, O₂, and nutrient limitation, equally unlikely. This left us with very few options, among them the possibility that helpers cross-

protect *Prochlorococcus* against a toxin found in the medium. A search of the numerous small *Prochlorococcus* genomes confirmed the absence of catalase and other common antioxidant enzymes, and early measurements confirmed that culture media had higher HOOH concentrations than natural seawater. Hence, we concluded that protection from oxidative stress was a reasonable hypothesis. It should be stressed that when we began this project we had *no* direct evidence supporting this hypothesis, only this series of logical assumptions.

We bring this up because the trend in modern science seems to be to approach microbiological problems using impressive technologies and massive funding. One method of looking for a helping mechanism would have been to use microarrays, or deep sequencing of the transcriptome, to measure the genetic response of *Prochlorococcus* and helpers to each other's presence. This would have been quite expensive, and probably would not have uncovered the correct answer since the genetics of oxidative stress resistance are superficially similar to many other stress responses. Instead, we used relatively inexpensive, but logical and elegant, experimental designs to test a hypothesis that we arrived at through simple reasoning. It is important, we believe, for we scientists to remember that many of the great discoveries in our fields have come about in this manner, rather than as a consequence of impressive, taxpayer-draining technological overkill. More of us should reflect on the fundamental differences between the research styles of Joshua Lederberg and Craig Venter, and find solace and inspiration in the former.

BIBLIOGRAPHY

1. Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science* 281: 237-240.
2. Elser JJ, Bracken MES, Cleland EE, Gruner DS, Harpole WS, et al. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters* 10: 1135-1142.
3. Smith SV (1984) Phosphorus versus nitrogen limitation in the marine environment. *Limnology and Oceanography* 29: 1149-1160.
4. Landry MR, Constantinou J, Latasa M, Brown SL, Bidigare RR, et al. (2000) Biological response to iron fertilization in the eastern equatorial Pacific (IronEx II). III. Dynamics of phytoplankton growth and microzooplankton grazing. *Marine Ecology-Progress Series* 201: 57-72.
5. Behrenfeld MJ, Bale AJ, Kolber ZS, Aiken J, Falkowski PG (1996) Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. *Nature* 383: 508-511.
6. Timmermans KR, van Leeuwe MA, de Jong JTM, McKay RML, Nolting RF, et al. (1998) Iron stress in the Pacific region of the Southern Ocean: evidence from enrichment bioassays. *Marine Ecology-Progress Series* 166: 27-41.
7. Garrison T (2005) *Oceanography: An Invitation To Marine Science*. Toronto: Brooks/Cole. 522 p.
8. Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, et al. (2007) The Sorcerer II Global Ocean Sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. *PLoS Biology* 5: 398-431.
9. Partensky F, Hess WR, Vaulot D (1999) *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. *Microbiol Mol Biol Rev* 63: 106-127.
10. Morris RM, Rappe MS, Connon SA, Vergin KL, Siebold WA, et al. (2002) SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* 420: 806-810.
11. Dufresne A, Garczarek L, Partensky F (2005) Accelerated evolution associated with genome reduction in a free-living prokaryote. *Genome Biology* 6: 10.
12. Giovannoni SJ, Tripp HJ, Givan S, Podar M, Vergin KL, et al. (2005) Genome streamlining in a cosmopolitan oceanic bacterium. *Science* 309: 1242-1245.

13. Sarthou G, Baker AR, Kramer J, Laan P, Laes A, et al. (2007) Influence of atmospheric inputs on the iron distribution in the subtropical Northeast Atlantic Ocean. *Marine Chemistry* 104: 186-202.
14. Duce RA, Tindale NW (1991) Atmospheric transport of iron and Its deposition in the ocean. *Limnology and Oceanography* 36: 1715-1726.
15. Waniek JJ (2003) The role of physical forcing in initiation of spring blooms in the northeast Atlantic. *Journal of Marine Systems* 39: 57-82.
16. Hewson I, Poretsky RS, Beinart RA, White AE, Shi T, et al. (2009) In situ transcriptomic analysis of the globally important keystone N₂-fixing taxon *Crocospaera watsonii*. *ISME J* 3: 618-631.
17. Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ (1997) *Trichodesmium*, a globally significant marine cyanobacterium. *Science* 276: 1221-1229.
18. May RM, Beddington JR, Clark CW, Holt SJ, Laws RM (1979) Management of multispecies fisheries. *Science* 205: 267-277.
19. Redfield AC, Ketchum BH, Richards FA (1963) The influence of organisms on the composition of sea-water. In: Hill MN, editor. *The Sea*. New York: Academic Press. pp. 26-77.
20. Madsen EL (2008) *Environmental Microbiology*. Malden, MA: Blackwell Publishing. 479 p.
21. Bekker A, Holland HD, Wang PL, Rumble D, Stein HJ, et al. (2004) Dating the rise of atmospheric oxygen. *Nature* 427: 117-120.
22. Falkowski PG, Godfrey LV (2008) Electrons, life and the evolution of Earth's oxygen cycle. *Philosophical Transactions of the Royal Society B-Biological Sciences* 363: 2705-2716.
23. Schopf JW (1993) Microfossils of the early Archean apex chert - new evidence of the antiquity of life. *Science* 260: 640-646.
24. Imlay JA (2003) Pathways of oxidative damage. *Annu Rev Microbiol* 57: 395-418.
25. Sawyer DT (1991) *Oxygen Chemistry*. Oxford: Oxford University Press. 223 p.
26. Halliwell B, Gutteridge JMC (2007) *Free Radicals In Biology and Medicine*. New York: Oxford University Press. 851 p.
27. Niyogi KK (2009) Photoprotection and high light responses. pp. 847-870.

28. Draper WM, Crosby DG (1983) Photochemical generation of superoxide radical anion in water. *J Agric Food Chem* 31: 734-737.
29. Kellogg EW, III, Fridovich I (1975) Superoxide, hydrogen peroxide, and single oxygen in lipid peroxidation by a xanthine oxidase system. *The Journal of Biological Chemistry* 250: 8812-8817.
30. Chelikani P, Fita I, Loewen PC (2004) Diversity of structures and properties among catalases. *Cell Mol Life Sci* 61: 192-208.
31. Welinder KG (1992) Superfamily of plant, fungal, and bacterial peroxidases. *Current Opinion in Structural Biology* 2: 388-393.
32. Fenton HJH (1894) Oxidation of tartaric acid in presence of iron. *Journal of the Chemical Society, Transactions* 65: 899-910.
33. Johnson KS, Gordon RM, Coale KH (1997) What controls dissolved iron concentrations in the world ocean? *Marine Chemistry* 57: 137-161.
34. King DW, Lounsbury HA, Millero FJ (1995) Rates and mechanism of Fe(II) oxidation at nanomolar total iron concentrations. *Environmental Science & Technology* 29: 818-824.
35. Canfield DE (1998) A new model for Proterozoic ocean chemistry. *Nature* 396: 450-453.
36. Rue EL, Bruland KW (1995) Complexation of iron(III) by natural organic ligands in the central North Pacific as determined by a new competitive ligand equilibration adsorptive cathodic stripping voltammetric method. *Marine Chemistry* 50: 117-138.
37. Southworth BA, Voelker BM (2003) Hydroxyl radical production via the photo-Fenton reaction in the presence of fulvic acid. *Environ Sci Technol* 37: 1130-1136.
38. Zepp RG, Faust BC, Hoigne J (2002) Hydroxyl radical formation in aqueous reactions (pH 3-8) of iron(II) with hydrogen peroxide: the photo-Fenton reaction. *Environmental Science & Technology* 26: 313-319.
39. Liochev SI, Fridovich I (2002) The Haber-Weiss cycle - 70 years later: an alternative view. *Redox Report* 7: 55-57.
40. Koppenol WH (2001) The Haber-Weiss cycle - 70 years later. *Redox Report* 6: 229-234.
41. Pignatello JJ, Oliveros E, MacKay A (2006) Advanced oxidation processes for organic contaminant destruction based on the Fenton reaction and related chemistry. *Critical Reviews in Environmental Science and Technology* 36: 1-84.

42. Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annu Rev Plant Physiol Plant Mol Biol* 50: 601-639.
43. Milligan AJ, Berman-Frank I, Gerchman Y, Dismukes GC, Falkowski PG (2007) Light-dependent oxygen consumption in nitrogen-fixing cyanobacteria plays a key role in nitrogenase protection. *Journal of Phycology* 43: 845-852.
44. Helman Y, Tchernov D, Reinhold L, Shibata M, Ogawa T, et al. (2003) Genes encoding A-type flavoproteins are essential for photoreduction of O₂ in cyanobacteria. *Current Biology* 13: 230-235.
45. Van Baalen C, Marler JE (1966) Occurrence of hydrogen peroxide in sea water. *Nature* 211: 951.
46. Cooper WJ, Zika RG, Petasne RG, Plane JMC (1988) Photochemical formation of H₂O₂ in natural waters exposed to sunlight. *Environ Sci Technol* 22: 1156-1160.
47. Taiz L, Zeiger E (2002) *Plant Physiology*. Sunderland, MA: Sinauer. 690 p.
48. Eisenhut M, Ruth W, Haimovich M, Bauwe H, Kaplan A, et al. (2008) The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiontically to plants. *Proceedings of the National Academy of Sciences of the United States of America* 105: 17199-17204.
49. Frederick SE, Gruber PJ, Tolbert NE (1973) Occurrence of glycolate dehydrogenase and glycolate oxidase in green plants - evolutionary survey. *Plant Physiology* 52: 318-323.
50. Latifi A, Jeanjean R, Lemeille S, Havaux M, Zhang C-C (2005) Iron starvation leads to oxidative stress in *Anabaena* sp. strain PCC 7120. *Journal of Bacteriology* 187: 6596-6598.
51. Kirchman DL, editor (2008) *Microbial Ecology of the Oceans*. 2nd ed. Hoboken, NJ: Wiley. 620 p.
52. Ross C, Alstyne KLV (2007) Intraspecific variation in stress-induced hydrogen peroxide scavenging by the ulvoid macroalga *Ulva lactuca*. *Journal of Phycology* 43: 466-474.
53. Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *The Plant Cell* 6: 65-74.

54. Kong IS, Bates TC, Hulsmann A, Hassan H, Smith BE, et al. (2004) Role of catalase and oxyR in the viable but nonculturable state of *Vibrio vulnificus*. FEMS Microbiology Ecology 50: 133-142.
55. Higuchi T, Fujimura H, Arakaki T, Oomori T (2009) The synergistic effects of hydrogen peroxide and elevated seawater temperature on the metabolic activity of the coral *Galaxea fascicularis*. Marine Biology 156: 589-596.
56. Suggett DJ, Warner ME, Smith DJ, Davey P, Hennige S, et al. (2008) Photosynthesis and production of hydrogen peroxide by *Symbiodinium* (Pyrrophyta) phylotypes with different thermal tolerances. J Phycol 44: 948-956.
57. Franca MB, Panek AD, Eleutherio ECA (2007) Oxidative stress and its effects during dehydration. Comparative Biochemistry and Physiology A - Molecular & Integrative Physiology 146: 621-631.
58. Rhee SG (2006) H₂O₂, a necessary evil for cell signaling. Science 312: 1882-1883.
59. Visick KL, Ruby EG (1998) The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. Journal of Bacteriology 180: 2087-2092.
60. Babior BM (2000) Phagocytes and oxidative stress. American Journal of Medicine 109: 33-44.
61. Orozco-Cardenas M, Ryan CA (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proceedings of the National Academy of Sciences of the United States of America 96: 6553-6557.
62. Lagrimini LM (1991) Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. Plant Physiology 96: 577-583.
63. Levine A, Tenhaken R, Dixon R, Lamb C (1994) H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583-593.
64. Ross C, Kupper FC, Vreeland V, Waite JH, Jacobs RS (2005) Evidence of a latent oxidative burst in relation to wound repair in the giant unicellular chlorophyte *Dasycladus vermicularis*. Journal of Phycology 41: 531-541.

65. Twiner MJ, Trick CG (2000) Possible physiological mechanisms for production of hydrogen peroxide by the ichthyotoxic flagellate *Heterosigma akashiwo*. *Journal of Plankton Research* 22: 1961-1975.
66. Oda T, Akaike T, Sato K, Ishimatsu A, Takeshita S, et al. (1992) Hydroxyl radical generation by red tide algae. *Archives of Biochemistry and Biophysics* 294: 38-43.
67. Oda T, Nakamura A, Shikayama M, Kawano I, Ishimatsu A, et al. (1997) Generation of reactive oxygen species by raphidophycean phytoplankton. *Bioscience Biotechnology and Biochemistry* 61: 1658-1662.
68. Darehshouri A, Affenzeller M, Lutz-Meindl U (2008) Cell death upon H₂O₂ induction in the unicellular green alga *Micrasterias*. *Plant Biology* 10: 732-745.
69. Ross C, Santiago-Vázquez L, Paul V (2006) Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcystis aeruginosa*. *Aquatic Toxicology* 78: 66-73.
70. Bidle KD, Falkowski PG (2004) Cell death in planktonic, photosynthetic microorganisms. *Nature Reviews Microbiology* 2: 643-655.
71. Segovia M, Haramaty L, Berges JA, Falkowski PG (2003) Cell death in the unicellular chlorophyte *Dunaliella tertiolecta*. A hypothesis on the evolution of apoptosis in higher plants and metazoans. *Plant Physiology* 132: 99-105.
72. Evans C, Malin G, Mills GP, Wilson WH (2006) Viral infection of *Emiliana huxleyi* (Prymnesiophyceae) leads to elevated production of reactive oxygen species. *Journal of Phycology* 42: 1040-1047.
73. Draper WM, Crosby DG (1983) The photochemical generation of hydrogen peroxide in natural waters. *Arch Environ Contam Toxicol* 12: 121-126.
74. Cooper WJ, Zika RG (1983) Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight. *Science* 220: 711-712.
75. Wilson CL, Hinman NW, Sheridan RP (2000) Hydrogen peroxide formation and decay in iron-rich geothermal waters: the relative roles of abiotic and biotic mechanisms. *Photochemistry and Photobiology* 71: 691-699.
76. Obernosterer I, Ruardij P, Herndl GJ (2001) Spatial and diurnal dynamics of dissolved organic matter (DOM) fluorescence and H₂O₂ and the photochemical oxygen demand of

- surface water DOM across the subtropical Atlantic Ocean. *Limnology and Oceanography* 46: 632-643.
77. Mopper K, Zhou X (1990) Hydroxyl radical photoproduction in the sea and its potential impact on marine processes. *Science* 250: 661-664.
78. White EM, Vaughan PP, Zepp RG (2003) Role of the photo-Fenton reaction in the production of hydroxyl radicals and photobleaching of colored dissolved organic matter in a coastal river of the southeastern United States. *Aquatic Sciences* 65: 402-414.
79. Gerringa LJA, Rijkenberg MJA, Timmermans KR, Buma AGJ (2004) The influence of solar ultraviolet radiation on the photochemical production of H₂O₂ in the equatorial Atlantic Ocean. *J Sea Res* 51: 3-10.
80. Kasting JF, Holland HD, Pinto JP (1985) Oxidant abundances in rainwater and the evolution of atmospheric oxygen. *Journal of Geophysical Research-Atmospheres* 90: 10497-10510.
81. Liang M-C, Hartman H, Kopp RE, Kirschvink JL, Yung YL (2006) Production of hydrogen peroxide in the atmosphere of a Snowball Earth and the origin of oxygenic photosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* 103: 18896-18899.
82. Kieber RJ, Cooper WJ, Willey JD, Avery GB, Jr. (2001) Hydrogen peroxide at the Bermuda Atlantic Time Series Station. Part 1: Temporal variability of atmospheric hydrogen peroxide and its influence on seawater concentrations. *Journal of Atmospheric Chemistry* 39: 1-13.
83. Yuan J, Shiller AM (2000) The variation of hydrogen peroxide in rainwater over the South and Central Atlantic Ocean. *Atmos Environ* 34: 3973-3980.
84. Hanson AK, Tindale NW, Abdel-Moati MAR (2001) An equatorial Pacific rain event: influence on the distribution of iron and hydrogen peroxide in surface waters. *Mar Chem* 75: 69-88.
85. Willey JD, Kieber RJ, Avery GB, Jr. (2004) Effects of rainwater iron and hydrogen peroxide on iron speciation and phytoplankton growth in seawater near Bermuda. *J Atmos Chem* 47: 209-222.
86. Kieber RJ, Peake B, Willey JD, Jacobs B (2001) Iron speciation and hydrogen peroxide concentrations in New Zealand rainwater. *Atmos Environ* 35: 6041-6048.

87. Avery GBJ, Cooper WJ, Kieber RJ, Willey JD (2005) Hydrogen peroxide at the Bermuda Atlantic Time Series Station: temporal variability of seawater hydrogen peroxide. *Mar Chem* 97: 236-244.
88. Willey JD, Paerl HW, Go M (1999) Impact of rainwater hydrogen peroxide on chlorophyll a content of surface Gulf Stream seawater off North Carolina, USA. *Marine Ecology-Progress Series* 178: 145-150.
89. Choo K-s, Snoeijs P, Pedersen M (2004) Oxidative stress tolerance in the filamentous green algae *Cladophora glomerata* and *Enteromorpha ahlneriana*. *Journal of Experimental Marine Biology and Ecology* 298: 111-123.
90. Palenik B, Zafiriou OC, Morel FMM (1987) Hydrogen peroxide production by a marine phytoplankter. *Limnology and Oceanography* 32: 1365-1369.
91. Stevens SE, Jr., Patterson COP, Myers J (1973) The production of hydrogen peroxide by blue-green algae: a survey. *Journal of Phycology* 9: 427-430.
92. Marshall JA, de Salas M, Oda T, Hallegraeff G (2005) Superoxide production by marine microalgae. *Marine Biology* 147: 533-540.
93. Vermilyea AW, Hansard SP, Voelker BM (2010) Dark production of hydrogen peroxide in the Gulf of Alaska. *Limnology and Oceanography* 55: 580-588.
94. Harvey HW (1925) Oxidation in sea water. *Journal of the Marine Biological Association of the United Kingdom* 13: 953-969.
95. Moffett JW, Zafiriou OC (1993) The photochemical decomposition of hydrogen peroxide in surface waters of the Eastern Caribbean and Orinoco River. *Journal of Geophysical Research* 98: 2307-2313.
96. Moffett JW, Zika RG (1987) Reaction kinetics of hydrogen peroxide with copper and iron in seawater. *Environ Sci Technol* 21: 804-810.
97. Cooper WJ, Zepp RG (1990) Hydrogen peroxide decay in waters with suspended soils: evidence for biologically mediated processes. *Can J Fish Aquat Sci* 47: 888-893.
98. Petasne RG, Zika RG (1997) Hydrogen peroxide lifetimes in south Florida coastal and offshore waters. *Mar Chem* 56: 215-225.
99. Moffett JW, Zafiriou OC (1990) An investigation of hydrogen peroxide chemistry in surface waters of Vineyard Sound with H₂¹⁸O₂. *Limnology and Oceanography* 35: 1221-1229.

100. Naclerio G, Baccigalupi L, Caruso C, Felice MD, Ricca E (1995) *Bacillus subtilis* vegetative catalase is an extracellular enzyme. *Applied and Environmental Microbiology* 61: 4471-4473.
101. Hassett DJ, Alsabbagh E, Parvatiyar K, Howell ML, Wilmot RW, et al. (2000) A protease-resistant catalase, KatA, released upon cell lysis during stationary phase is essential for aerobic survival of a *Pseudomonas aeruginosa oxyR* mutant at low cell densities. *Journal of Bacteriology* 182: 4557-4563.
102. Eremin AN, Mikhailova RV, Metelitsa DI (2000) Comparative kinetic characterization of catalases of the genus *Penicillium*. *Applied Biochemistry and Microbiology* 36: 221-226.
103. Angel DL, Fiedler U, Eden N, Kress N, Adelung D, et al. (1999) Catalase activity in macro- and microorganisms as an indicator of biotic stress in coastal waters of the eastern Mediterranean Sea. *Helgol Mar Res* 53: 209-218.
104. Coale KH, Johnson KS, Fitzwater SE, Blain SPG, Stanton TP, et al. (1998) IronEx-I, an *in situ* iron-enrichment experiment: Experimental design, implementation and results. *Deep-Sea Research Part II-Topical Studies in Oceanography* 45: 919-945.
105. Sulzberger B, Durisch-Kaiser E (2009) Chemical characterization of dissolved organic matter (DOM): A prerequisite for understanding UV-induced changes of DOM absorption properties and bioavailability. *Aquatic Sciences* 71: 104-126.
106. Mopper K, Zhou X, Kieber RJ, Kieber DJ, Sikorski RJ, et al. (1991) Photochemical degradation of dissolved organic carbon and its impact on the oceanic carbon cycle. *Nature* 353: 60-62.
107. Pullin MJ, Bertilsson S, Goldstone JV, Voelker BM (2004) Effects of sunlight and hydroxyl radical on dissolved organic matter: bacterial growth efficiency and production of carboxylic acids and other substrates. *Limnology and Oceanography* 49: 2011-2022.
108. Scully NM, Cooper WJ, Tranvik LJ (2003) Photochemical effects on microbial activity in natural waters: the interaction of reactive oxygen species and dissolved organic matter. *FEMS Microbiology Ecology* 46: 353-357.
109. Goldstone JV, Pullin MJ, Bertilsson S, Voelker BM (2002) Reactions of hydroxyl radical with humic substances: bleaching, mineralization, and production of bioavailable carbon substrates. *Environ Sci Technol* 36: 364-372.

110. Benner R, Biddanda B (1998) Photochemical transformations of surface and deep marine dissolved organic matter: Effects on bacterial growth. *Limnology and Oceanography* 43: 1373-1378.
111. Tranvik L, Kokalj S (1998) Decreased biodegradability of algal DOC due to interactive effects of UV radiation and humic matter. *Aquatic Microbial Ecology* 14: 301-307.
112. Gutteridge JMC (1984) Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. *Biochem J* 224: 761-767.
113. Hofer T, Badouard C, Bajak E, Ravanat JL, Mattsson A, et al. (2005) Hydrogen peroxide causes greater oxidation in cellular RNA than in DNA. *Biological Chemistry* 386: 333-337.
114. Tanaka M, Chock PB, Stadtman ER (2007) Oxidized messenger RNA induces translation errors. *Proceedings of the National Academy of Sciences of the United States of America* 104: 66-71.
115. Keyer K, Imlay JA (1996) Superoxide accelerates DNA damage by elevating free iron levels. *Proc Natl Acad Sci USA* 93: 13635-13640.
116. Bolann BJ, Ulvik RJ (1987) Release of iron from ferritin by xanthine oxidase. *Biochem J* 243: 55-59.
117. Kojima K, Oshita M, Nanjo Y, Kasai K, Tozawa Y, et al. (2007) Oxidation of elongation factor G inhibits the synthesis of the D1 protein of photosystem II. *Mol Microbiol* 65: 936-947.
118. Tamarit J, Cabiscol E, Ros J (1998) Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *Journal of Biological Chemistry* 273: 3027-3032.
119. Gutteridge JMC, Halliwell B (1990) The measurement and mechanism of lipid peroxidation in biological systems. *Trends in Biochemical Sciences* 15: 129-135.
120. Fay P (1992) Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiological Reviews* 56: 340-373.

121. Schramm A, De Beer D, Gieseke A, Amann R (2000) Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. *Environmental Microbiology* 2: 680-686.
122. Malfatti F, Azam F (2009) Atomic force microscopy reveals microscale networks and possible symbioses among pelagic marine bacteria. *Aquatic Microbial Ecology* 58: 1-14.
123. Paerl HW, Pinckney JL (1996) A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. *Microbial Ecology* 31: 225-247.
124. Hofmann B, Hecht HJ, Flohe L (2002) Peroxiredoxins. *Biological Chemistry* 383: 347-364.
125. Seaver LC, Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *Journal of Bacteriology* 183: 7173-7181.
126. Perez-Perez ME, Mata-Cabana A, Sanchez-Riego AM, Lindahl M, Florencio FJ (2009) A comprehensive analysis of the peroxiredoxin reduction system in the cyanobacterium *Synechocystis* sp. strain PCC 6803 reveals that all five peroxiredoxins are thioredoxin dependent. *J Bacteriol* 191: 7477-7489.
127. Grant RA, Filman DJ, Finkel SE, Kolter R, Hogle JM (1998) The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nature Structural Biology* 5: 294-303.
128. Maeda H, DellaPenna D (2007) Tocopherol functions in photosynthetic organisms. *Current Opinion in Plant Biology* 10: 260-265.
129. Niyogi KK, Björkman O, Grossman AR (1997) The roles of specific xanthophylls in photoprotection. *Proc Natl Acad Sci USA* 94: 14162-14167.
130. Yoshpepur Y, Henis Y (1976) Factors affecting catalase level and sensitivity to hydrogen peroxide in *Escherichia coli*. *Applied and Environmental Microbiology* 32: 465-469.
131. Arana I, Muela A, Iriberry J, Egea L, Barcina I (1992) Role of hydrogen peroxide in loss of culturability mediated by visible light in *Escherichia coli* in a fresh water ecosystem. *Applied and Environmental Microbiology* 58: 3903-3907.
132. Eisenstark A, Miller C, Jones H, Leven S (1992) *Escherichia coli* genes involved in cell survival during dormancy: role of oxidative stress. *Biochemical and Biophysical Research Communications* 188: 1054-1059.

133. Schnell S, Steinman HM (1995) Function and stationary-phase induction of periplasmic copper-zinc superoxide dismutase and catalase/oxidase in *Caulobacter crescentus*. *Journal of Bacteriology* 177: 5924-5929.
134. Zeller T, Klug G (2004) Detoxification of hydrogen peroxide and expression of catalase genes in *Rhodobacter*. *Microbiology* 150: 3451-3462.
135. Arana I, Orruno M, Perez-Pascual D, Seco C, Muela A, et al. (2007) Inability of *Escherichia coli* to resuscitate from the viable but nonculturable state. *FEMS Microbiology Ecology* 62: 1-11.
136. Oliver JD (1995) The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. *FEMS Microbiology Letters* 133: 203-208.
137. Samuilov VD, Bezryadnov DV, Gusev MV, Kitashov AV, Fedorenko TA (1999) Hydrogen peroxide inhibits the growth of cyanobacteria. *Biochemistry-Moscow* 64: 47-53.
138. Drabkova M, Admiraal W, Marsalek B (2007) Combined exposure to hydrogen peroxide and light – selective effects on cyanobacteria, green algae, and diatoms. *Environ Sci Technol* 41: 309-314.
139. Drabkova M, Matthijs HCP, Admiraal W, Marsalek B (2007) Selective effects of H₂O₂ on cyanobacterial photosynthesis. *Photosynthetica* 45: 363-369.
140. Tichy M, Vermaas W (1999) In vivo role of catalase-peroxidase in *Synechocystis* sp. Strain PCC 6803. *J Bacteriol* 181: 1875-1882.
141. Xenopoulos MA, Bird DF (1997) Effect of acute exposure to hydrogen peroxide on the production of phytoplankton and bacterioplankton in a mesohumic lake. *Photochem Photobiol* 66: 471-478.
142. Skurlatov YI, Ernestova LS (1998) The impact of human activities on freshwater aquatic systems. *Acta Hydrochim Hydrobiol* 26: 5-12.
143. Qiao R-P, Li N, Qi X-H, Wang Q-S, Zhuang Y-Y (2005) Degradation of microcystin-RR by UV radiation in the presence of hydrogen peroxide. *Toxicon* 45: 745-752.
144. Bandala ER, Martinez D, Martinez E, Dionysiou DD (2004) Degradation of microcystin-LR toxin by Fenton and photo-Fenton processes. *Toxicon* 43: 829-832.
145. Quimby PC, Jr. (1981) Preliminary evaluation of hydrogen peroxide as a potential herbicide for aquatic weeds. *J Aquat Plant Manage* 19: 53-55.

146. Drabkova M, Marsalek B, Admiraal W (2007) Photodynamic therapy against cyanobacteria. *Environmental Toxicology* 22: 112-115.
147. Kay SH, Quimby PC, Jr., Ouzts JD (1983) Control of coontail with hydrogen peroxide and copper. *J Aquat Plant Manage* 21: 38-40.
148. Barroin G, Feuillade M (1986) Hydrogen peroxide as a potential algicide for *Oscillatoria rubescens* D.C. *Wat Res* 20: 619-623.
149. Barrington DJ, Ghadouani A (2008) Application of hydrogen peroxide for the removal of toxic cyanobacteria and other phytoplankton from wastewater. *Environmental Science & Technology* 42: 8916-8921.
150. Häder D-P (2001) Ultraviolet radiation and aquatic ecosystems. In: Cockell CS, Blaustein AR, editors. *Ecosystems, Evolution, and Ultraviolet Radiation*. New York: Springer. pp. 221.
151. Herndl GJ (1997) Role of ultraviolet radiation on bacterioplankton activity. In: Häder D-P, editor. *The Effects of Ozone Depletion On Aquatic Ecosystems*. Austin: R. G. Landes Company. pp. 143-154.
152. Karentz D, Bothwell ML, Coffin RB, Hanson A, Herndl GJ, et al. (1994) Impact of UV-B radiation on pelagic freshwater ecosystems: Report of working group on bacteria and phytoplankton. *Ergebn Limnol* 43: 31-69.
153. Müller-Niklas G, Heissenberger A, Puskarić S, Herndl GJ (1995) Ultraviolet-B radiation and bacterial metabolism in coastal waters. *Aquatic Microbial Ecology* 9: 111-116.
154. Llabres M, Agusti S (2006) Picophytoplankton cell death induced by UV radiation: evidence for oceanic Atlantic communities. *Limnology and Oceanography* 51: 21-29.
155. Häder D-P, Worrest RC (1997) Consequences of the effects of increased solar ultraviolet radiation on aquatic ecosystems. In: Häder D-P, editor. *The Effects of Ozone Depletion on Aquatic Ecosystems*. Austin: R. G. Landes Company. pp. 11-30.
156. Zepp RG, Callaghan TV, Erickson DJ (1998) Effects of enhanced solar ultraviolet radiation on biogeochemical cycles. *Journal of Photochemistry and Photobiology B: Biology* 46: 69-82.

157. Shiu C-T, Lee T-M (2005) Ultraviolet-B-induced oxidative stress and responses of the ascorbate-glutathione cycle in a marine macroalga *Ulva fasciata*. *Journal of Experimental Botany* 56: 2851-2865.
158. Gomez I, Orostegui M, Huovinen P (2007) Morpho-functional patterns of photosynthesis in the South Pacific kelp *Lessonia nigrescens*: effects of UV radiation on ^{14}C fixation and primary photochemical reactions. *Journal of Phycology* 43: 55-64.
159. He Y-Y, Häder D-P (2002) UV-B-induced formation of reactive oxygen species and oxidative damage of the cyanobacterium *Anabaena* sp.: protective effects of ascorbic acid and *N*-acetyl-L-cysteine. *Journal of Photochemistry and Photobiology B: Biology* 66: 115-124.
160. Karentz D, Cleaver JE, Mitchell DL (1991) Cell survival characteristics and molecular responses of Antarctic phytoplankton to ultraviolet-B radiation. *Journal of Phycology* 27: 326-341.
161. Rai PK, Rai LC (1997) Interactive effects of UV-B and Cu on photosynthesis, uptake and metabolism of nutrients in a green alga *Chlorella vulgaris* under simulated ozone column. *The Journal of General and Applied Microbiology* 43: 281-288.
162. Moharikar S, D'Souza JS, Kulkarni AB, Rao BJ (2006) Apoptotic-like cell death pathway is induced in unicellular chlorophyte *Chlamydomonas reinhardtii* (Chlorophyceae) cells following UV irradiation: detection and functional analyses. *Journal of Phycology* 42: 423-433.
163. Rai LC, Tyagi B, Mallick N (1996) Alternation in photosynthetic characteristics of *Anabaena doliolum* following exposure to UVB and Pb. *Photochemistry and Photobiology* 64: 658-663.
164. Dillon JG, Miller SR, Castenholz RW (2003) UV-acclimation responses in natural populations of cyanobacteria (*Calothrix* sp.). *Environmental Microbiology* 5: 473-483.
165. Sobrino C, Neale PJ (2007) Short-term and long-term effects of temperature on photosynthesis in the diatom *Thalassiosira pseudonana* under UVR exposures. *Journal of Phycology* 43: 426-436.

166. Song YF, Qiu BS (2007) The CO₂-concentrating mechanism in the bloom-forming cyanobacterium *Microcystis aeruginosa* (Cyanophyceae) and effects of UVB radiation on its operation. *Journal of Phycology* 43: 957-964.
167. Llabres M, Agusti S, Alonso-Laita P, Herndl GJ (2010) *Synechococcus* and *Prochlorococcus* cell death induced by UV radiation and the penetration of lethal UVR in the Mediterranean Sea. *Marine Ecology-Progress Series* 399: 27-37.
168. Pinto E, Sigaud-Kutner TCS, Leitaó MAS, Okamoto OK, Morse D, et al. (2003) Heavy metal-induced oxidative stress in algae. *Journal of Phycology* 39: 1008-1018.
169. Encrenaz T, Bezard B, Greathouse TK, Richter MJ, Lacy JH, et al. (2004) Hydrogen peroxide on Mars: Evidence for spatial and seasonal variations. *Icarus* 170: 424-429.
170. Cockell CS, Catling DC, Davis WL, Snook K, Kepner RL, et al. (2000) The ultraviolet environment of Mars: Biological implications past, present, and future. *Icarus* 146: 343-359.
171. Andreini C, Banci L, Bertini I, Elmi S, Rosato A (2007) Non-heme iron through the three domains of life. *Proteins-Structure Function and Bioinformatics* 67: 317-324.
172. McKay CP, Hartman H (1991) Hydrogen peroxide and the evolution of oxygenic photosynthesis. *Origins of Life and Evolution of the Biosphere* 21: 157-163.
173. Bernroitner M, Zamocky M, Furtmüller PG, Peschek GA, Obinger C (2009) Occurrence, phylogeny, structure, and function of catalases and peroxidases in cyanobacteria. *J Exp Bot* 60: 423-440.
174. Blankenship RE, Hartman H (1998) The origin and evolution of oxygenic photosynthesis. *Trends in Biochemical Sciences* 23: 94-97.
175. Hutterli MA, McConnell JR, Stewart RW, Jacobi HW, Bales RC (2001) Impact of temperature-driven cycling of hydrogen peroxide (H₂O₂) between air and snow on the planetary boundary layer. *Journal of Geophysical Research-Atmospheres* 106: 15395-15404.
176. Odum EP, Finn JT, Franz EH (1979) Perturbation theory and the subsidy-stress gradient. *BioScience* 29: 349-352.

177. Zinser ER, Johnson ZI, Coe A, Karaca E, Veneziano D, et al. (2007) Influence of light and temperature on *Prochlorococcus* ecotype distributions in the Atlantic Ocean. *Limnol Oceanogr* 52: 2205-2230.
178. Morris JJ, Kirkegaard R, Szul MJ, Johnson ZI, Zinser ER (2008) Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by "helper" heterotrophic bacteria. *Appl Environ Microbiol* 74: 4530-4534.
179. Moore LR, Post AF, Rocap G, Chisholm SW (2002) Utilization of different nitrogen sources by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. *Limnology and Oceanography* 47: 989-996.
180. Rippka R, Coursin T, Hess W, Lichtle C, Scanlan DJ, et al. (2000) *Prochlorococcus marinus* Chisholm et al. 1992 subsp *pastoris* subsp nov strain PCC 9511, the first axenic chlorophyll a(2)/b(2)-containing cyanobacterium (Oxyphotobacteria). *International Journal of Systematic and Evolutionary Microbiology* 50: 1833-1847.
181. Moore LR, Coe A, Zinser ER, Saito MA, Sullivan MB, et al. (2007) Culturing the marine cyanobacterium *Prochlorococcus*. *Limnol Oceanogr: Methods* 5: 353-362.
182. Tolonen AC, Aach J, Lindell D, Johnson ZI, Rector T, et al. (2006) Global gene expression of *Prochlorococcus* ecotypes in response to changes in nitrogen availability. *Molecular Systems Biology*: 1-15.
183. Kaeberlein T, Lewis K, Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* 296: 1127-1129.
184. Alavi M, Miller T, Erlandson K, Schneider R, Belas R (2001) Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures. *Environmental Microbiology* 3: 380-396.
185. Ferrier M, Martin JL, Rooney-Varga JN (2002) Stimulation of *Alexandrium fundyense* growth by bacterial assemblages from the Bay of Fundy. *J Appl Microbiol* 92: 706-716.
186. Waterbury JB, Willey JM (1988) Isolation and growth of marine planktonic cyanobacteria. *Methods in Enzymology* 167: 100-105.
187. Giovannoni SJ (1991) The polymerase chain reaction. In: Stackebrandt E, Goodfellow M, editors. *Nucleic Acid Techniques in Bacterial Systematics*. New York, NY: Wiley and Sons. pp. 177-201.

188. Cavender-Bares KK, Frankel SL, Chisholm SW (1998) A dual sheath flow cytometer for shipboard analyses of phytoplankton communities from oligotrophic seas. *Limnol Oceanogr* 43: 1383-1388.
189. Dusenberry JA, Frankel SL (1994) Increasing the sensitivity of a FACScan flow cytometer to study oceanic picoplankton. *Limnology and Oceanography* 39: 206-209.
190. Ahlgren NA, Rocap G, Chisholm SW (2006) Measurement of *Prochlorococcus* ecotypes using real-time polymerase chain reaction reveals different abundances of genotypes with similar light physiologies. *Environmental Microbiology* 8: 441-454.
191. Moore LR, Chisholm SW (1999) Photophysiology of the marine cyanobacterium *Prochlorococcus*: ecotypic differences among cultured isolates. *Limnol Oceanogr* 44: 628-638.
192. Moore LR, Goericke R, Chisholm SW (1995) Comparative physiology of *Synechococcus* and *Prochlorococcus* - influence of light and temperature on growth, pigments, fluorescence and absorptive properties. *Mar Ecol: Prog Ser* 116: 259-275.
193. Marthi B, Shaffer BT, Lighthart B, Ganio L (1991) Resuscitation effects of catalase on airborne bacteria. *Applied and Environmental Microbiology* 57: 2775-2776.
194. Martin SE, Flowers RS, Ordal ZJ (1976) Catalase - its effect on microbial enumeration. *Applied and Environmental Microbiology* 32: 731-734.
195. Mizunoe Y, Wai SN, Takade A, Yoshida S (1999) Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using H₂O₂-degrading compounds. *Archives of Microbiology* 172: 63-67.
196. Olson JB, McCarthy PJ (2005) Associated bacterial communities of two deep-water sponges. *Aquatic Microbial Ecology* 39: 47-55.
197. Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM, Breznak JA (2004) New strategies for cultivation and detection of previously uncultured microbes. *Applied and Environmental Microbiology* 70: 4748-4755.
198. Bogosian G, Aardema ND, Bourneuf EV, Morris PJJ, O'Neil JP (2000) Recovery of hydrogen peroxide-sensitive culturable cells of *Vibrio vulnificus* gives the appearance of resuscitation from a viable but nonculturable state. *Journal of Bacteriology* 182: 5070-5075.

199. Regelsberger G, Jakopitsch C, Plasser L, Schwaiger H, Furtmüller PG, et al. (2002) Occurrence and biochemistry of hydroperoxidases in oxygenic phototrophic prokaryotes (cyanobacteria). *Plant Physiol Biochem* 40: 479-490.
200. Feierabend J, Engel S (1986) Photoinactivation of catalase *in vitro* and in leaves. *Archives of Biochemistry and Biophysics* 251: 567-576.
201. Mitchell RL, Anderson IC (1965) Catalase photoinactivation. *Science* 150: 74.
202. Tytler EM, Wong T, Codd GA (1984) Photoinactivation *in vivo* of superoxide dismutase and catalase in the cyanobacterium *Microcystis aeruginosa*. *FEMS Microbiology Letters* 23: 239-242.
203. Snoeyink VL, Jenkins D (1980) *Water Chemistry*. New York: John Wiley & Sons. 463 p.
204. Badger MR, Price GD (2003) CO₂ concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. *J Exp Bot* 54: 609-622.
205. Falkowski PG, Raven JA (2007) *Aquatic Photosynthesis*. Princeton, NJ: Princeton University Press. 484 p.
206. Maberly SC (1990) Exogenous sources of inorganic carbon for photosynthesis by marine macroalgae. *Journal of Phycology* 26: 439-449.
207. Slonczewski JL, Foster JW (1996) pH-regulated genes and survival at extreme pH. In: Neidhardt FC, editor. *Escherichia coli* and *Salmonella*. Washington, D.C.: American Society for Microbiology. pp. 1539-1550.
208. Zilberstein D, Agmon V, Schuldiner S, Padan E (1984) *Escherichia coli* intracellular pH, membrane potential, and cell growth. *Journal of Bacteriology* 158: 246-252.
209. Irvin RT, MacAlister TJ, Costerton JW (1981) Tris(hydroxymethyl)aminomethane buffer modification of *Escherichia coli* outer membrane permeability. *Journal of Bacteriology* 145: 1397-1403.
210. Good NE, Winget GD, Winter W, Connolly TN, Izawa S, et al. (1966) Hydrogen ion buffers for biological research. *Biochemistry* 5: 467-477.
211. Zigler JS, Jr., Lepe-Zuniga JL, Vistica B, Gery I (1985) Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. *In Vitro Cell Dev Biol* 21: 282-287.
212. Lepe-Zuniga JL, Zigler JS, Jr., Gery I (1987) Toxicity of light-exposed HEPES media. *Journal of Immunological Methods* 103: 145.

213. King DW, Cooper WJ, Rusak SA, Peake BM, Kiddle JJ, et al. (2007) Flow injection analysis of H₂O₂ in natural waters using acridinium ester chemiluminescence: method development and optimization using a kinetic model. *Anal Chem* 79: 4169-4176.
214. Miller WL, Kester DR (1988) Hydrogen peroxide measurement in seawater by (*p*-hydroxyphenyl)acetic acid dimerization. *Anal Chem* 60: 2711-2715.
215. Scully NM, McQueen DJ, Lean DRS, Cooper WJ (1996) Hydrogen peroxide formation: the interaction of ultraviolet radiation and dissolved organic carbon in lake waters along a 43-75° N gradient. *Limnology and Oceanography* 41: 540-548.
216. Zinser ER, Lindell D, Johnson ZI, Futschik ME, Steglich C, et al. (2009) Choreography of the transcriptome, photophysiology, and cell cycle of a minimal photoautotroph, *Prochlorococcus*. *PLoS ONE* 4: e5135.
217. Yuan J, Shiller AM (2001) The distribution of hydrogen peroxide in the southern and central Atlantic ocean. *Deep-Sea Res* 48: 2947-2970.
218. Kieber RJ, Helz GR (1995) Temporal and seasonal variations of hydrogen peroxide levels in estuarine waters. *Estuarine, Coastal, and Shelf Science* 40: 495-503.
219. Cooper WJ, Lean DRS (1989) Hydrogen peroxide concentration in a northern lake: photochemical formation and diel variability. *Environ Sci Technol* 23: 1425-1428.
220. Blanchemain A, Grizeau D, Guary JC (1994) Effect of different organic buffers on the growth of *Skeletonema costatum* cultures - further evidence for an autoinhibitory effect. *Journal of Plankton Research* 16: 1433-1440.
221. Wong GTF, Dunstan WM, Kim D-B (2003) The decomposition of hydrogen peroxide by marine phytoplankton. *Oceanol Acta* 26: 191-198.
222. McFadden GI, Melkonian M (1986) Use of HEPES buffer for microalgal culture media and fixation for electron microscopy. *Phycologia* 25: 551-557.
223. Hidalgo E, Dimple B (1996) Adaptive responses to oxidative stress: the *soxRS* and *oxyR* regulons. In: Lin ECC, Lynch AS, editors. *Regulation of Gene Expression in Escherichia coli*. Austin, Texas: R.G. Landes Company. pp. 433-452.
224. Godon C, Lagniel G, Lee J, Buhler J-M, Kieffer S, et al. (1998) The H₂O₂ stimulon in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry* 273: 22480-22489.

225. Dummermuth AL, Karsten U, Fisch KM, König GM, Wiencke C (2003) Responses of marine macroalgae to hydrogen peroxide stress. *J Exp Mar Biol Ecol* 289: 103-121.
226. Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55.
227. Latifi A, Ruiz M, Zhang CC (2009) Oxidative stress in cyanobacteria. *FEMS Microbiology Reviews* 33: 258-278.
228. Cuny C, Lesbats M, Dukan S (2007) Induction of a global stress response during the first step of *Escherichia coli* plate growth. *Applied and Environmental Microbiology* 73: 885-889.
229. Andersen R, editor (2005) *Algal Culturing Techniques*. Burlington, MA: Academic Press. 596 p.
230. Richier S, Furla P, Plantivaux A, Merle PL, Allemand D (2005) Symbiosis-induced adaptation to oxidative stress. *Journal of Experimental Biology* 208: 277-285.
231. Scanlan DJ, Ostrowski M, Mazard S, Dufresne A, Garczarek L, et al. (2009) Ecological genomics of marine picocyanobacteria. *Microbiol Mol Biol Rev* 73: 249-299.
232. Fogg GE, Thake B (1987) *Algal Cultures and Phytoplankton Ecology*. Madison, WI: University of Wisconsin Press. 269 p.
233. Van Mooy BAS, Rocap G, Fredricks HF, Evans CT, Devol AH (2006) Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. *Proc Natl Acad Sci USA* 103: 8607-8612.
234. Goericke R, Welschmeyer NA (1993) The marine prochlorophyte *Prochlorococcus* contributes significantly to phytoplankton biomass and primary production in the Sargasso Sea. *Deep-Sea Research I* 40: 2283-2294.
235. DiTullio GR, Geesey ME, Jones DR, Daly KL, Campbell L, et al. (2003) Phytoplankton assemblage structure and primary productivity along 170°W in the South Pacific Ocean. *Mar Ecol Prog Ser* 255: 55-80.
236. Partensky F, Blanchot J, Lantoin F, Neveux J, Marie D (1996) Vertical structure of picophytoplankton at different trophic sites of the subtropical northeastern Atlantic. *Deep-Sea Res I* 43: 1191-1213.

237. Ras J, Claustre H, Uitz J (2008) Spatial variability of phytoplankton pigment distributions in the subtropical South Pacific Ocean: Comparison between in situ and predicted data *Biogeosciences* 5: 353-369.
238. Veldhuis MJW, Kraay GW (2004) Phytoplankton in the subtropical Atlantic Ocean: towards a better assessment of biomass and composition. *Deep-Sea Res I* 51: 507-530.
239. West NJ, Scanlan DJ (1999) Niche-partitioning of *Prochlorococcus* populations in a stratified water column in the eastern North Atlantic Ocean. *Applied and Environmental Microbiology* 65: 2585-2591.
240. Zinser ER, Coe A, Johnson ZI, Martiny AC, Fuller NJ, et al. (2006) *Prochlorococcus* ecotype abundances in the North Atlantic Ocean as revealed by an improved quantitative PCR method. *Appl Environ Microbiol* 72: 723-732.
241. Malmstrom RR, Coe A, Kettler GC, Martiny AC, Frias-Lopez J, et al. (In Press) Temporal dynamics of *Prochlorococcus* ecotypes in the Atlantic and Pacific Oceans. *The ISME Journal*.
242. Bouman HA, Ulloa O, Scanlan DJ, Zwirgmaier K, Li WKW, et al. (2006) Oceanographic basis of the global surface distribution of *Prochlorococcus* ecotypes. *Science* 312: 918-921.
243. Johnson ZI, Zinser ER, Coe A, McNulty NP, Woodward EMS, et al. (2006) Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. *Science* 311: 1737-1740.
244. Zwirgmaier K, Jardillier L, Ostrowski M, Mazard S, Garczarek L, et al. (2008) Global phylogeography of marine *Synechococcus* and *Prochlorococcus* reveals a distinct partitioning of lineages among oceanic biomes. *Environmental Microbiology* 10: 147-161.
245. Rocap G, Larimer FW, Lamerdin J, Malfatti S, Chain P, et al. (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature* 424: 1042-1047.
246. Partensky F, Garczarek L (2010) *Prochlorococcus*: advantages and limits of minimalism. *Annual Review of Marine Science* 2: 305-331.

247. Kettler GC, Martiny AC, Huang K, Zucker J, Coleman ML, et al. (2007) Patterns and implications of gene gain and loss in the evolution of *Prochlorococcus*. PLoS Genet 3: 2515-2528.
248. Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. Annu Rev Physiol 68: 253-278.
249. Graf J, Dunlap PV, Ruby EG (1994) Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. Journal of Bacteriology 176: 6986-6991.
250. Petursdottir SK, Kristjansson JK (1997) *Silicibacter lacuscaerulensis* gen. nov., sp. nov., a mesophilic moderately halophilic bacterium characteristic of the Blue Lagoon geothermal lake in Iceland. Extremophiles 1: 94-99.
251. Dufresne A, Ostrowski M, Scanlan DJ, Garczarek L, Mazard S, et al. (2008) Unraveling the genomic mosaic of a ubiquitous genus of marine cyanobacteria. Genome Biology 9: 16.
252. Hebrard M, Viala JPM, Meresse S, Barras F, Aussel L (2009) Redundant hydrogen peroxide scavengers contribute to *Salmonella* virulence and oxidative stress resistance. J Bacteriol 191: 4605-4614.
253. Park S, You X, Imlay JA (2005) Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of *Escherichia coli*. Proc Natl Acad Sci USA 102: 9317-9322.
254. Zika RG, Saltzman ES, Cooper WJ (1985) Hydrogen peroxide concentrations in the Peru upwelling area. Mar Chem 17: 265-275.
255. Yuan J, Shiller AM (2005) Distribution of hydrogen peroxide in the northwest Pacific Ocean. Geochemistry Geophysics Geosystems 6: 1-3.
256. Miller WL, Kester DR (1994) Peroxide variations in the Sargasso Sea. Marine Chemistry 48: 17-29.
257. Johnson KS, Willason SW, Wiesenburg DA, Lohrenz SE, Arnone RA (1989) Hydrogen peroxide in the western Mediterranean Sea: a tracer for vertical advection. Deep-Sea Research 36: 241-254.
258. Schreiber U, Krieger A (1996) Two fundamentally different types of variable chlorophyll fluorescence in vivo. FEBS Lett 397: 131-135.

259. Ma M, Eaton JW (1992) Multicellular oxidant defense in unicellular organisms. *Proc Natl Acad Sci USA* 89: 7924-7928.
260. Fazzini RAB, Preto MJ, Quintas ACP, Bielecka A, Timmis KN, et al. (2010) Consortia modulation of the stress response: proteomic analysis of single strain versus mixed culture. *Environ Microbiol* 12: 2449.
261. Jakubovics NS, Gill SR, Vickerman MM, Kolenbrander PE (2008) Role of hydrogen peroxide in competition and cooperation between *Streptococcus gordonii* and *Actinomyces naeslundii*. *Fems Microbiology Ecology* 66: 637-644.
262. Dubinin AV, Gerasimenko LM, Venetskaya SL, Gusev MV (1992) Cyanobacterium *Microcoleus-Chthonoplastes* Fails to Grow in Pure Culture. *Microbiology* 61: 41-46.
263. Mouget JL, Dakhama A, Lavoie MC, de la Noije J (1995) Algal growth enhancement by bacteria: is consumption of photosynthetic oxygen involved? *FEMS Microbiol Ecol* 18: 35-44.
264. Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B₁₂ through a symbiotic relationship with bacteria. *Nature* 438: 90-93.
265. de Bashan LE, Antoun H, Bashan Y (2008) Involvement of indole-3-acetic-acid produced by the growth-promoting bacterium *Azospirillum* spp. in promoting growth of *Chlorella vulgaris*. *J Phycol* 44: 938-947.
266. D'Onofrio A, Crawford JM, Stewart EJ, Witt K, Gavrish E, et al. (2010) Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chemistry & Biology* 17: 254-264.
267. Halsey TA, Vazquez-Torres A, Gravdahl DJ, Fang FC, Libby SJ (2004) The ferritin-like dps protein is required for *Salmonella enterica* serovar *typhimurium* oxidative stress resistance and virulence. *Infect Immun* 72: 1155-1158.
268. Pena MMO, Bullerjahn GS (1995) The dpsA protein of *Synechococcus* sp. strain PCC7942 is a DNA-binding hemoprotein - linkage of the dps and bacterioferritin protein families. *Journal of Biological Chemistry* 270: 22478-22482.
269. Blankenship RE (2002) *Molecular Mechanisms of Photosynthesis*. London: Blackwell. 321 p.

270. Bertilsson S, Berglund O, Pullin MJ, Chisholm SW (2005) Release of dissolved organic matter by *Prochlorococcus*. *Vie Et Milieu-Life and Environment* 55: 225-231.
271. Choler P, Michalet R, Callaway RM (2001) Facilitation and competition on gradients in alpine plant communities. *Ecology* 82: 3295-3308.
272. Kranner I, Cram WJ, Zorn M, Wornik S, Yoshimura I, et al. (2005) Antioxidants and photoprotection in a lichen as compared with its isolated symbiotic partners. *Proceedings of the National Academy of Sciences of the United States of America* 102: 3141-3146.
273. Bertness MD, Callaway R (1994) Positive interactions in communities. *Trends in Ecology & Evolution* 9: 191-193.
274. DeLong EF, Preston CM, Mincer T, Rich V, Hallam SJ, et al. (2006) Community genomics among stratified microbial assemblages in the ocean's interior. *Science* 311: 496-503.
275. Rocap G, Distel DL, Waterbury JB, Chisholm SW (2002) Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. *Applied and Environmental Microbiology* 68: 1180-1191.
276. Ting CS, Hsieh C, Sundararaman S, Mannella C, Marko M (2007) Cryo-electron tomography reveals the comparative three-dimensional architecture of *Prochlorococcus*, a globally important marine cyanobacterium. *Journal of Bacteriology* 189: 4485-4493.
277. Vieira-Silva S, Touchon M, Rocha EPC (2010) No evidence for elemental-based streamlining of prokaryotic genomes. *Trends in Ecology & Evolution* 25: 319-320.
278. Marais GAB, Calteau A, Tenaillon O (2008) Mutation rate and genome reduction in endosymbiotic and free-living bacteria. *Genetica* 134: 205-210.
279. Heck DE, Vetrano AM, Mariano TM, Laskin JD (2003) UVB light stimulates production of reactive oxygen species - Unexpected role for catalase. *Journal of Biological Chemistry* 278: 22432-22436.
280. Moore LR, Ostrowski M, Scanlan DJ, Feren K, Sweetsir T (2005) Ecotypic variation in phosphorus acquisition mechanisms within marine picocyanobacteria. *Aquatic Microbial Ecology* 39: 257-269.
281. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, et al. (1999) Genetic approaches to study of biofilms. *Methods Enzymol* 310: 91-109.

282. Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-3402.
283. Eaton AD, Clesceri LS, Rice EW, Greenberg AE, Franson MAH, editors (2005) *Standard Methods for the Examination of Water and Wastewater*. 21st ed: American Public Health Association.
284. Brickman E, Beckwith J (1975) Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ 80 transducing phages. *J Mol Biol* 96: 307-316.
285. Johnson ZI (2004) Description and application of the background irradiance gradient-single turnover fluorometer (BIG-STf). *Marine Ecology-Progress Series* 283: 73-80.
286. Marchetti A, Varela DE, Lance VP, Johnson Z, Palmucci M, et al. (2010) Iron and silicic acid effects on phytoplankton productivity, diversity, and chemical composition in the central equatorial Pacific Ocean. *Limnology and Oceanography* 55: 11-29.
287. Bruyant F, Babin M, Genty B, Prasil O, Behrenfeld MJ, et al. (2005) Diel variations in the photosynthetic parameters of *Prochlorococcus* strain PCC 9511: Combined effects of light and cell cycle. *Limnology and Oceanography* 50: 850-863.
288. Bertilsson S, Berglund O, Karl DM, Chisholm SW (2003) Elemental composition of marine *Prochlorococcus* and *Synechococcus*: Implications for the ecological stoichiometry of the sea. *Limnology and Oceanography* 48: 1721-1731.
289. Thompson AW (2009) *Iron and Prochlorococcus*. Cambridge, MA: Massachusetts Institute of Technology. 251 p.
290. Neidhardt FC, Umbarger HE (1996) Chemical composition of *Escherichia coli*. In: Neidhardt FC, editor. *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. 2nd ed. Washington, D.C.: ASM Press. pp. 13-16.
291. Kubitschek HE, Friske JA (1986) Determination of bacterial cell volume with the Coulter counter. *Journal of Bacteriology* 168: 1466-1467.
292. Madigan MT, Martinko JM, Parker J (1997) *Brock Biology of Microorganisms*. Upper Saddle River, NJ: Prentice-Hall. 986 p.

293. Sowell SM, Norbeck AD, Lipton MS, Nicora CD, Callister SJ, et al. (2008) Proteomic analysis of stationary phase in the marine bacterium "*Candidatus Pelagibacter ubique*". Appl Environ Microbiol 74: 4091-4100.
294. Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, et al. (2008) Microbial community gene expression in ocean surface waters. Proceedings of the National Academy of Sciences of the United States of America 105: 3805-3810.
295. Dougan DA, Mogk A, Bukau B (2002) Protein folding and degradation in bacteria: To degrade or not to degrade? That is the question. Cellular and Molecular Life Sciences 59: 1607-1616.
296. Wang JX, Sommerfeld M, Hu Q (2009) Occurrence and environmental stress responses of two plastid terminal oxidases in *Haematococcus pluvialis* (Chlorophyceae). Planta 230: 191-203.
297. Rose AL, Webb EA, Waite TD, Moffett JW (2008) Measurement and implications of nonphotochemically generated superoxide in the equatorial Pacific Ocean. Environmental Science & Technology 42: 2387-2393.
298. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig WG, et al. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35: 7188-7196.
299. Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics 9: 299-306.
300. Buchan A, Gonzalez JM, Moran MA (2005) Overview of the marine Roseobacter lineage. Applied and Environmental Microbiology 71: 5665-5677.
301. Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, et al. (2009) The genomic basis of trophic strategy in marine bacteria. Proc Natl Acad Sci USA 106: 15527-15533.
302. Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304: 66-74.
303. Cohan FM, Koeppel AF (2008) The origins of ecological diversity in prokaryotes. Current Biology 18: R1024-R1034.

304. Klotz MG, Loewen PC (2003) The molecular evolution of catalatic hydroperoxidases: evidence for multiple lateral transfer of genes between Prokaryota and from bacteria into Eukaryota. *Molecular Biology and Evolution* 20: 1098-1112.
305. McCutcheon JP The bacterial essence of tiny symbiont genomes. *Current Opinion in Microbiology* 13: 73-78.
306. Seymour JR, Ahmed T, Durham WM, Stocker R (2010) Chemotactic response of marine bacteria to the extracellular products of *Synechococcus* and *Prochlorococcus*. *Aquatic Microbial Ecology* 59: 161-168.
307. Ruby EG, Lee KH (1998) The *Vibrio fischeri/Euprymna scolopes* light organ association: Current ecological paradigms. *Appl Environ Microbiol* 64: 805-812.
308. Gould SJ (1996) *Full House: The Spread of Excellence from Plato to Darwin*. New York, NY: Random House. 244 p.
309. Van Valen L (1973) A new evolutionary law. *Evol Theory* 1: 1-30.
310. Carroll L (1872) *Through the Looking-Glass*. online-literature.com.
311. Lenski RE, Hattingh SE (1986) Coexistence of two competitors on one resource and one inhibitor - a chemostat model based on bacteria and antibiotics. *Journal of Theoretical Biology* 122: 83-93.
312. Vermilyea AW, Dixon TC, Voelker BM (2010) Use of H_2O_2 - ^{18}O to measure absolute rates of dark H_2O_2 production in freshwater systems. *Environmental Science & Technology* 44: 3066-3072.
313. Abele D, Ferreyra GA, Schloss I (1999) H_2O_2 accumulation from photochemical production and atmospheric wet deposition in Antarctic coastal and off-shore waters of Potter Cove, King George Island, South Shetland Islands. *Antarctic Science* 11: 131-139.

APPENDIX

Testing the Paradigm of Natural Hydrogen Peroxide Dynamics

Acknowledgments

We would like to thank the captains and crews of the vessels used for the field work mentioned in this appendix: *R/V Kilo Moana*, *R/V Hugh R. Sharp*, *CCGS Limnos*, *R/V New Horizon*, and *R/V Cape Hatteras*. Also, we thank Zackary Johnson, Anna Ritchie, Jeremy Chandler, and Marty Szul for assistance in the field. Lastly, we thank Whitney King for his patience with our endless emails about the FeLume during the WP2 cruise.

I. Introduction

The ubiquity of hydrogen peroxide (HOOH) in light exposed waters is well established across all aquatic ecosystems (Chapter 1 and references therein). Studies have shown that it has a number of sources, including both biotic and abiotic generators, and that it similarly has several sinks, including both chemical and enzymatic reactions (Chapter 1). Since the discovery of photochemical HOOH generation in natural waters in the 1980's, a paradigm has arisen that maintains that the predominant *source* of HOOH is photochemical and abiotic, whereas the predominant *sink* is microbial. While the paradigm neither excludes biological HOOH production nor abiotic removal (e.g., by Fenton chemistry), it suggests that these processes are, in general, of much lesser importance, particularly in open-water environments. The paradigm maintains, further, that these countervailing processes maintain a steady state HOOH concentration that explains the very similar HOOH concentrations ($\sim 0.1 \mu\text{M}$) detected at most study sites worldwide.

However, some recent studies have called this paradigm into question. Using stable isotopes, researchers demonstrated that surface communities in both freshwater and seawater generated significant amounts of HOOH in the dark, presumably as a consequence of biological activity [93,312]. By calculation they concluded that competing rates of biological production and degradation were sufficient to maintain the steady state in situ HOOH concentration at their study site, independently of any photochemical HOOH generation. Other studies have shown substantial rates of Fenton chemistry in pelagic waters [76], and some have detected cell free, enzyme-like decomposition of HOOH [103]. Precipitation contains very high concentrations of HOOH, and can locally create dramatic increases for in situ HOOH [85,87,88,313], which could be much higher in the wake of large (and therefore research vessel-proof) storms. Thus, evidence exists that the prevailing paradigm of HOOH dynamics may be overly simplistic.

Studies that simultaneously measured rates of HOOH production and decay in natural waters are rare; those that measure these rates in both dark and lighted samples are even less common. In this section we present a large data set, spanning 10 time zones and four distinct aquatic environments, in which we measured both in situ HOOH concentrations throughout the euphotic zone, as well as rates of production and degradation in the light and the dark, with or

without the biological community. Using this data, we approach the steady state from two perspectives. First, we look at HOOH dynamics over a diel cycle at several stations to test the paradigm's predictions that in situ HOOH will cycle over the course of the day. Second, we present a mathematical model of the paradigm, and then examine how well it predicts measured in situ HOOH concentrations. Finally, we consider several possible sources of deviation from the steady-state predictions made by the prevailing paradigm.

II. Methods

Sampling was performed on 5 cruises, ranging from January 2007 to June 2009 (Table A.1). Water collection on station as well as HOOH measurements were performed as described in Chapter 4. HOOH on the WP2 cruise was measured using the FeLume method [213]; on the other cruises, it was measured using the Orion-L luminometer (Chapter 4).

HOOH dynamics were estimated using bottle incubations. Bottles were thoroughly acid-washed before each cruise [181] and soaked overnight in micro-Q detergent followed by six rinses with milli-Q water between samples while at sea. Dark incubations were conducted in polycarbonate bottles double-wrapped with black electrical tape or, when sterility was required, in black, acid-washed, autoclaved conical bottom tubes (Argos, Elgin, IL). Light exposure experiments were performed either in clear polycarbonate bottles (WP2 and AB1 cruises) or in UV transparent vessels (MELEE XII, DCM08, and BC09, described in Chapter 4). Samples taken from the mixed layer were incubated at ~80% surface light intensity; samples from below this depth were incubated at <5% surface intensity. Bottles were incubated at surface water temperatures in flow-through deck incubators. When UV transparent bottles were used, they were either placed under neutral density screening exposed to sunlight (MELEE XII cruise) or else placed in an incubator made from UV-transparent Plexiglas (Chapter 4). Incubations were either performed with no modifications (i.e., with an initial [HOOH] identical to the in situ value) or with an added spike of 0.5 – 1.0 μM HOOH. Most incubations were performed in duplicate, although in several cases contamination or other problems required one replicate to be discarded.

In several cases we took numerous measurements of incubations to obtain relatively high-resolution estimated of the kinetics of HOOH production and degradation. Generally, HOOH

Table A.1. Locations and dates of sampling.

Cruise	Station	Latitude	Longitude	Date
WP2 ¹	5	12.47	-167.69	1/7/07
WP2	6	10.08	-170.14	1/8/07
WP2	7	7.94	-172.28	1/9/07
WP2	8	5.65	-174.53	1/10/07
WP2	11	-2.30	177.44	1/14/07
WP2	12	-4.72	174.73	1/15/07
WP2	13	-7.07	172.31	1/16/07
WP2	14	-9.25	170.00	1/17/07
WP2	15	-12.58	169.86	1/20/07
WP2	18	-19.50	170.21	1/22/07
WP2	19	-21.62	169.66	1/23/07
WP2	20	-25.67	165.42	1/26/07
WP2	21	-29.04	164.34	1/27/07
WP2	22	-31.92	163.36	1/28/07
WP2	23	-34.16	162.52	1/29/07
WP2	24	-36.17	161.77	1/30/07
WP2	25	-34.23	160.35	2/2/07
WP2	26	-32.42	159.09	2/5/07
AB1 ²	2	38.47	-76.39	7/15/07
AB1	6	37.10	-75.72	7/16/07
AB1	CTD19	37.39	-76.08	7/18/07
AB1	CTD20	37.97	-76.27	7/18/07
AB1	CTD21	38.07	-76.22	7/18/07
MELEE XII ³	557	41.65	-82.81	8/20/07
MELEE XII	971	41.95	-83.05	8/20/07
MELEE XII	972	41.86	-83.20	8/20/07
MELEE XII	973	41.79	-83.32	8/20/07
MELEE XII	357	41.82	-82.97	8/21/07
MELEE XII	478	41.66	-82.82	8/21/07
MELEE XII	882	41.47	-82.72	8/21/07
MELEE XII	885	41.52	-82.64	8/21/07
MELEE XII	968	41.74	-82.73	8/21/07
MELEE XII	969	41.60	-82.92	8/21/07
MELEE XII	974	41.72	-83.15	8/21/07
MELEE XII	1163	41.47	-82.72	8/21/07
MELEE XII	84	41.94	-81.65	8/22/07
MELEE XII	958	41.52	-81.71	8/22/07
MELEE XII	23	42.50	-79.89	8/23/07
MELEE XII	589	42.44	-79.83	8/23/07
MELEE XII	937	42.71	-80.24	8/23/07
MELEE XII	948	41.96	-80.64	8/23/07
MELEE XII	956	41.69	-81.44	8/23/07

Table A.1. Continued.

Cruise	Station	Latitude	Longitude	Date
DCM08 ⁴	CTD03	30.12	-118.41	6/16/08
DCM08	CTD04	28.38	-117.27	6/17/08
DCM08	CTD05	27.22	-116.53	6/17/08
DCM08	CTD06	26.07	-115.78	6/17/08
DCM08	CTD07	24.99	-113.41	6/18/08
DCM08	CTD10	25.01	-118.02	6/20/08
DCM08	CTD13	19.69	-113.09	6/23/08
DCM08	CTD16	18.72	-110.36	6/23/08
DCM08	CTD18	17.94	-108.10	6/24/08
DCM08	CTD48	17.35	-107.48	6/29/08
DCM08	CTD53	18.01	-105.00	7/1/08
DCM08	CTD60	21.30	-107.80	7/5/08
DCM08	CTD61	22.20	-108.80	7/6/08
BC09 ⁵	CTD2	33.00	-72.00	5/23/09
BC09	CTD5	30.17	-72.00	5/24/09
BC09	CTD39	30.17	-72.01	5/29/09
BC09	CTD60	31.08	-73.53	5/31/09
BC09	CTD61	31.67	-74.43	5/31/09
BC09	CTD62	32.22	-75.38	6/1/09
BC09	CTD63	33.03	-76.71	6/1/09
BC09	CTD64	33.59	-77.08	6/1/09

¹ Cruise WP2, *R/V Kilo Moana*, Jan-Feb 2007, transect from Honolulu, HI to Brisbane, Australia, Chief Scientists (CS) ER Zinser and ZI Johnson

² Cruise AB1, *R/V Hugh R. Sharp*, Jul 2007, Chesapeake Bay, CS A Buchan

³ Cruise MELEE XII, *CCGS Limnos*, August 2007, Lake Erie, CS S Wilhelm

⁴ Cruise DCM08, *R/V New Horizon*, Jun-Jul 2008, transect from San Diego, CA to La Paz, Mexico, CS K Barbeau and E Mann

⁵ Cruise BC09, *R/V Cape Hatteras*, May-Jun 2009, transect out from Beaufort, NC, CS B Binder

degradation in the dark was pseudo-first order in [HOOH], whereas HOOH production in the light was essentially zero-order. Therefore, we calculated either first-order or zero-order rate constants for dark or light incubations, respectively, when fewer time points were collected for given samples.

Where indicated, seawater samples were filtered prior to incubation. Osmonics polycarbonate filters (0.2 μm or 2 μm , General Electric) were used on the WP2 cruise, whereas Millipore PVDF filters (0.2 μm or 5 μm) were used for the other cruises. For sterile incubations, bottles were either autoclaved or exposed to UV radiation prior to use.

III. In situ HOOH dynamics

We measured in situ HOOH concentrations at 63 different stations on 5 cruises (Table A.1). At most stations, multiple depths were sampled, ranging from surface waters to the bottom of the euphotic zone (~ 200 m on the three open ocean cruises), although HOOH concentrations below the mixed layer rapidly dropped below the limit of detection (<10 nM). These cruises represented several very different pelagic regimes, including open ocean waters of the Pacific and Atlantic (Figs A.1-3), coastal waters (Figs A.2, 3-4), an estuary (Fig. A.4), and a Great Lake (Fig. A.5). Sample sites also ranged from extremely oligotrophic (North Pacific Gyre, Fig. A.1) to eutrophic (the western basin of Lake Erie, Fig. A.5), and spanned a surface temperature range from $\sim 20^\circ\text{C}$ to $\sim 30^\circ\text{C}$. Despite this wide physicochemical range, however, in situ HOOH concentrations were remarkably consistent at all stations. Surface concentrations occupied a surprisingly narrow range, averaging 95.9 ± 29.3 nM. Rates of production (in sterile filtered, UV exposed seawater) and degradation (in unfiltered, dark incubated samples), measured at 15 and 42 stations, respectively, were significantly more variable, averaging 35.6 ± 27.4 nM h^{-1} and $0.07 \pm .06$ h^{-1} . There was no significant correlation either between these rates and each other, or between either rate and the in situ HOOH concentration (Pearson Correlation, $P > 0.05$).

As mentioned above, the paradigm of HOOH dynamics predicts that there should be a diel pulse in HOOH consistent with formation during the day against a constant background of degradation. To test this hypothesis, we followed changes in HOOH concentration at three stations over a 24 h period. We also simultaneously measured dark decomposition rates using bottle incubations set up at each time point. At both a coastal station (Fig. A.6A) and one open

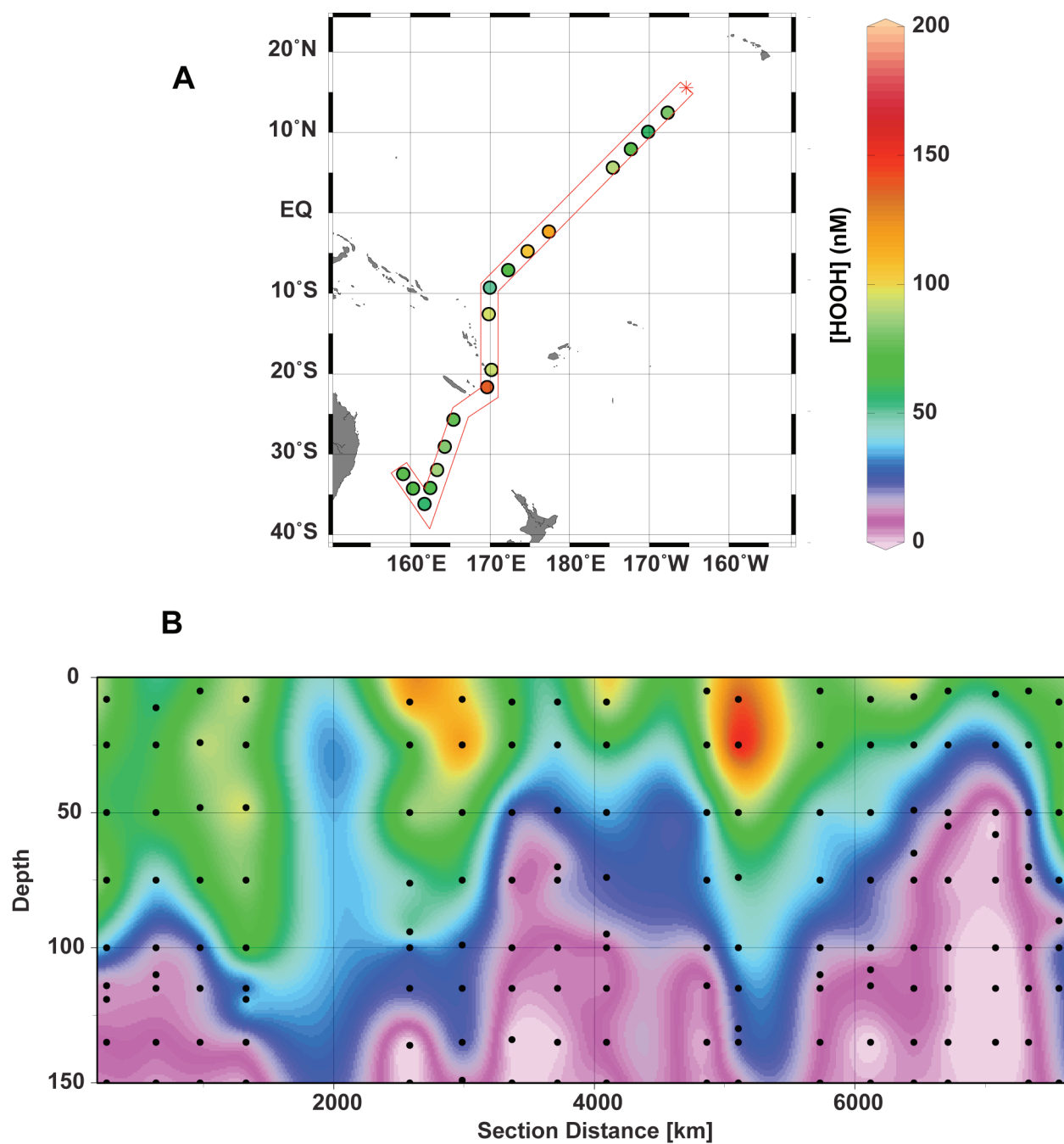


Figure A.1. In situ HOOH concentrations along the WP2 transect. Samples were collected from Jan-Feb 2007. A) Station locations, showing surface HOOH concentrations. B) HOOH concentrations throughout the water column at each station. Stations are in order along the transect outlined in panel A, with the leftmost station represented by the red asterisk. Dots, sampled points. The color bar in panel A applies to both panels.

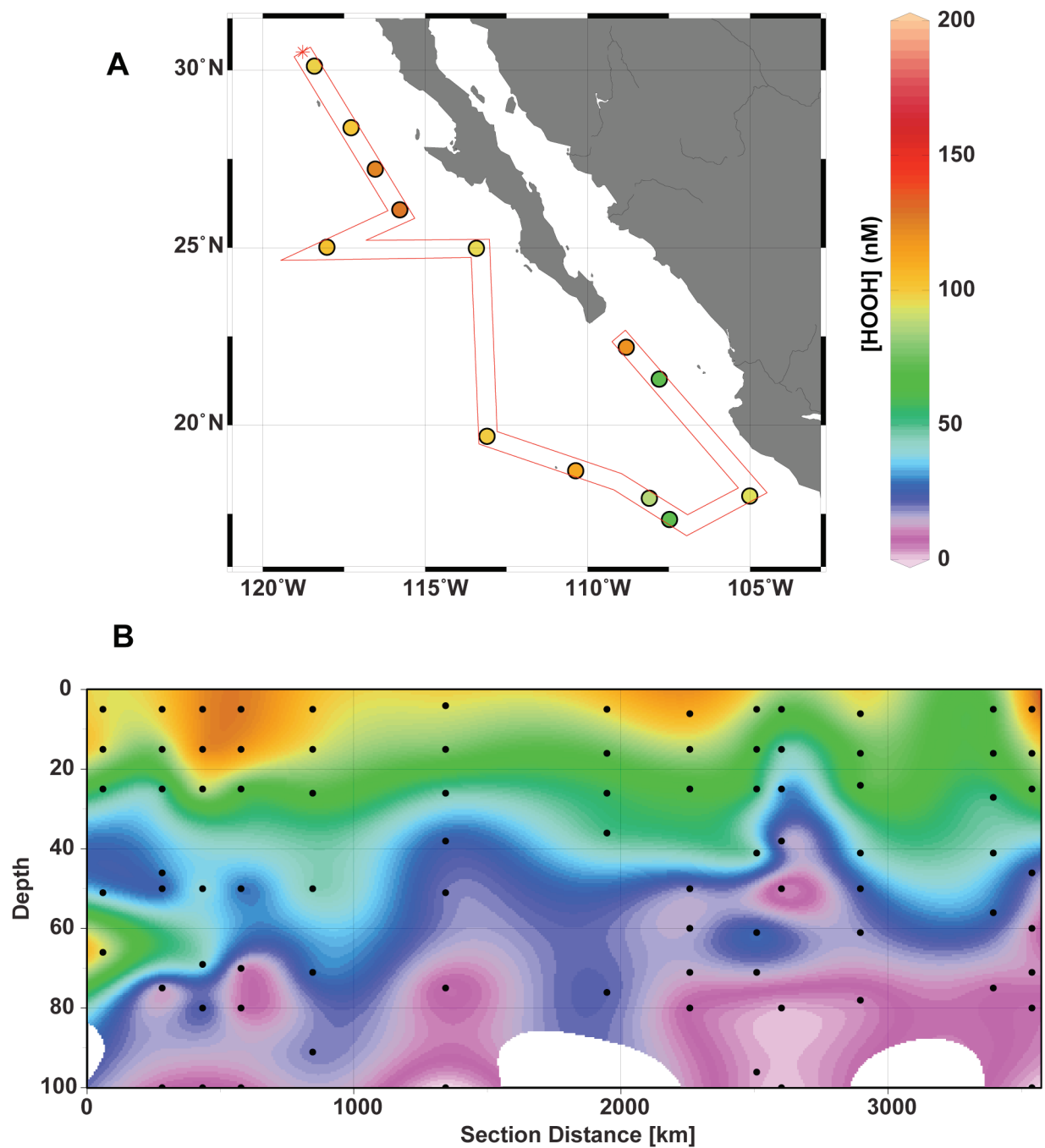


Figure A.2. In situ HOOH concentrations along the DCM08 transect. Samples were collected Jun-Jul 2008. See legend for Figure A.1 for interpretation. Empty spaces on the plot indicate absence of data, not bathymetry.

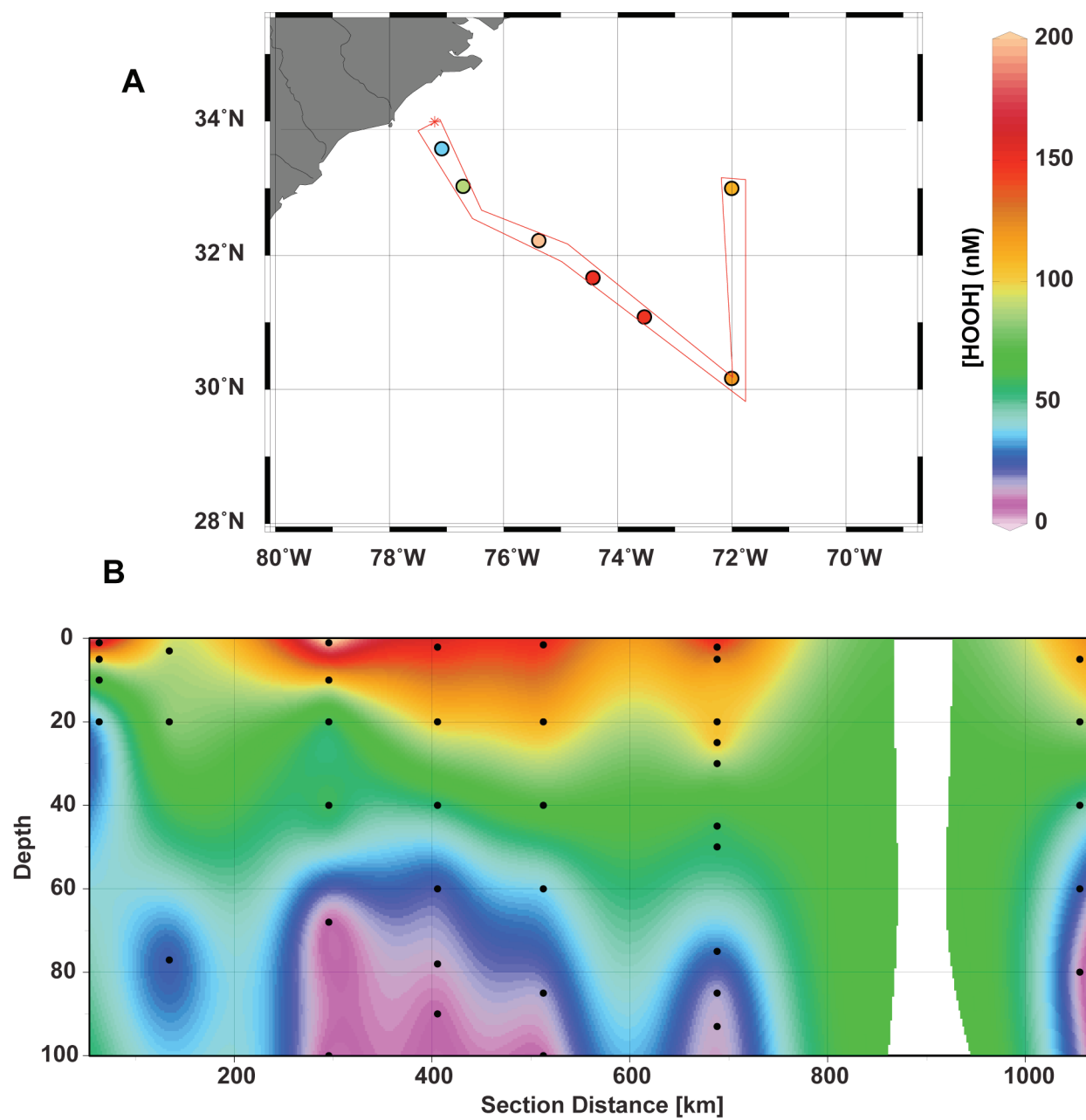


Figure A.3. In situ HOOH concentrations along the BC09 transect. Samples were collected May-Jun 2009. See legend for Figure A.1 for interpretation. Empty spaces on the plot indicate absence of data, not bathymetry.

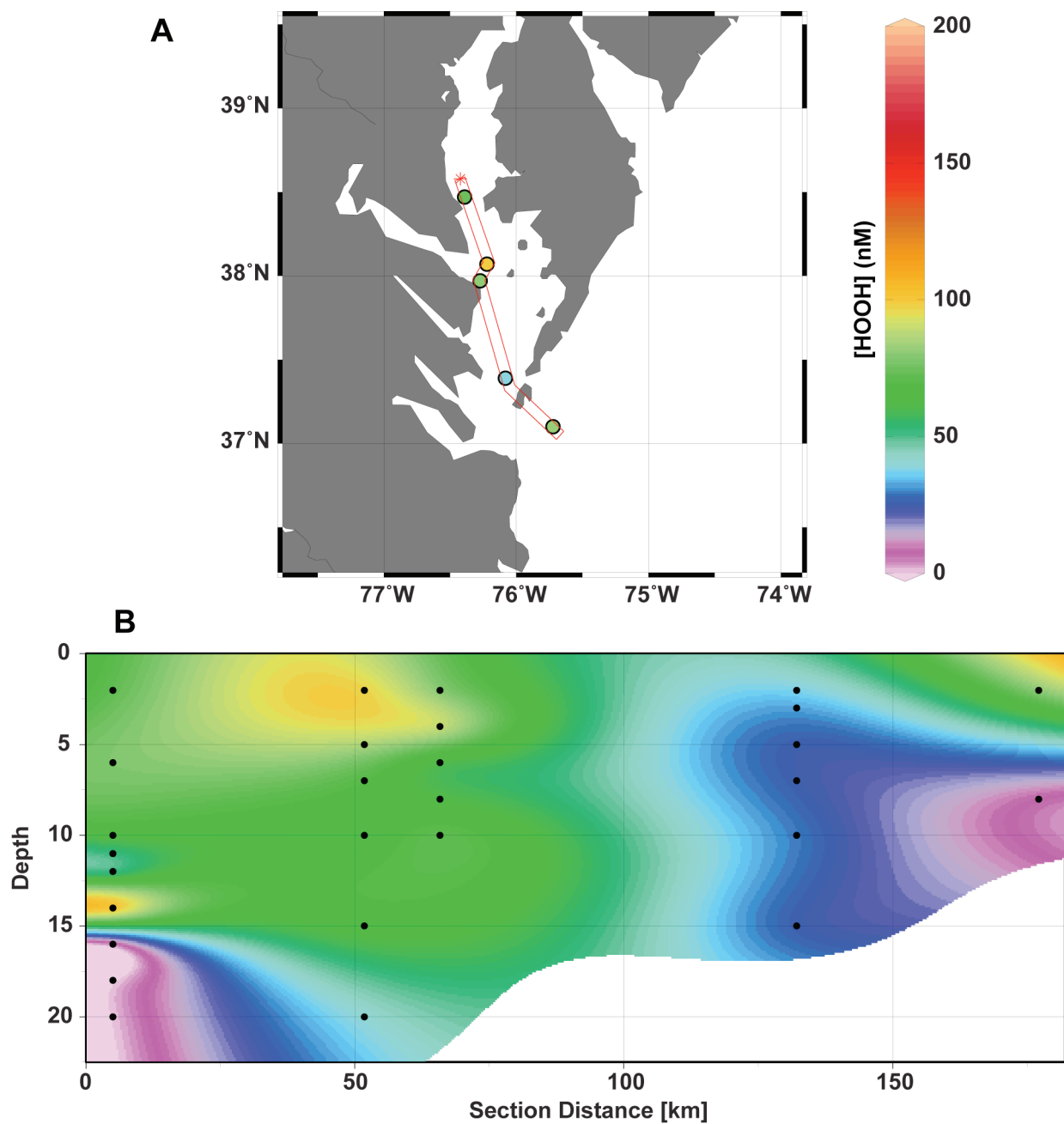


Figure A.4. In situ HOOH concentrations in Chesapeake Bay. Samples were collected in July 2007. See legend for Figure A.1 for interpretation. Empty spaces on the plot indicate absence of data, not bathymetry.

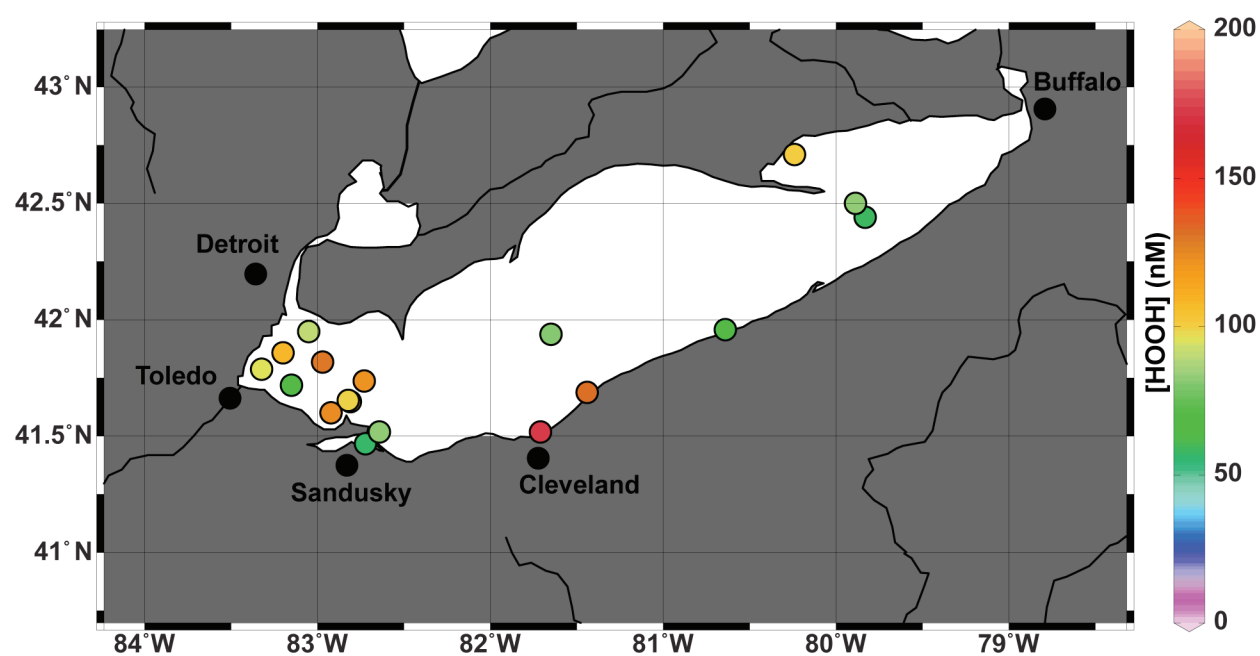


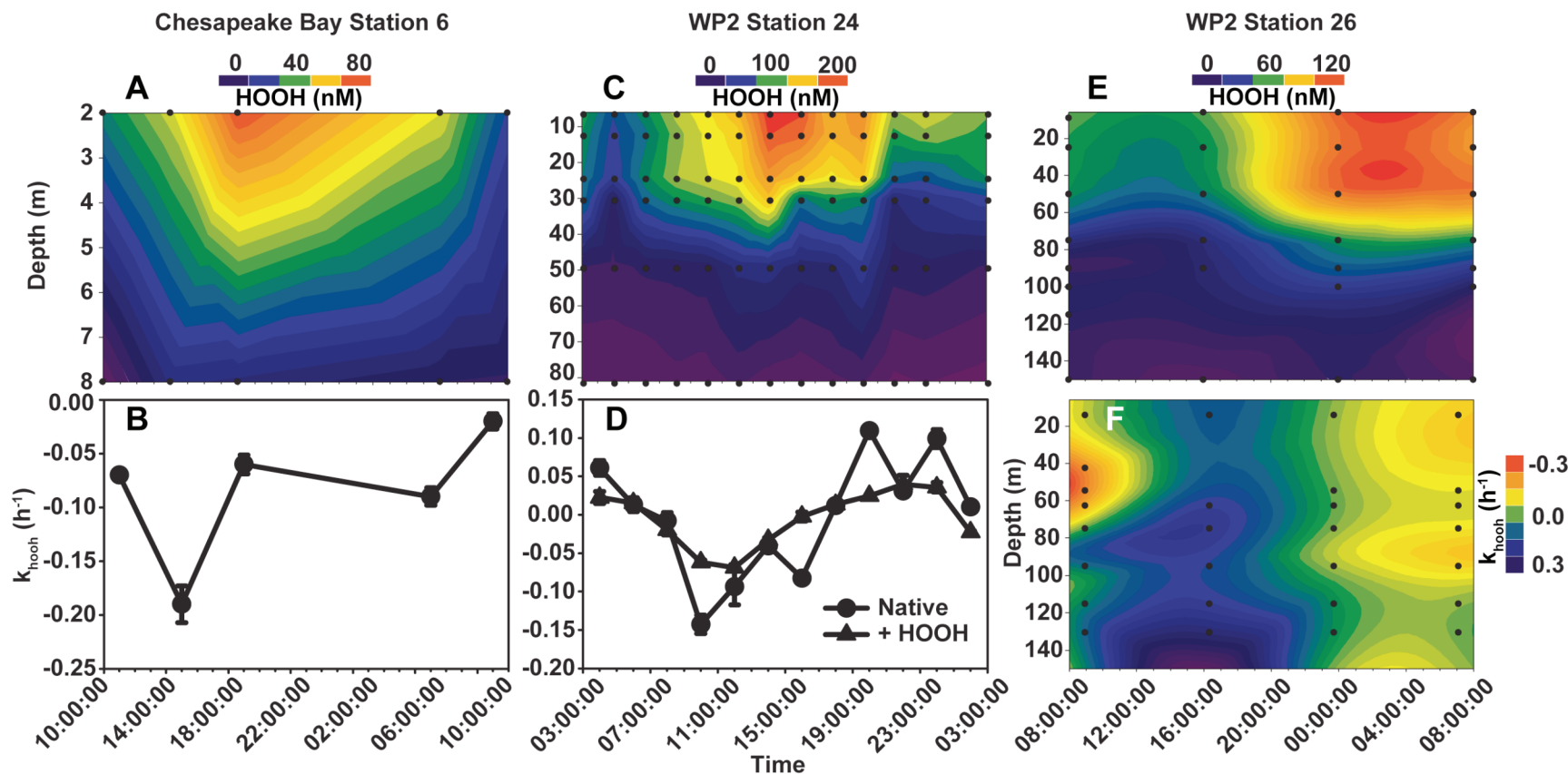
Figure A.5. In situ surface HOOH concentrations in Lake Erie. Samples were collected in August 2007.

ocean station in the Western Pacific (Fig. A.6C) in situ HOOH followed the paradigm's prediction, peaking in the afternoon and gradually returning to roughly the initial concentration by the next morning. At a third station, however, also in the Western Pacific, HOOH levels did not appear to be in steady state (Fig. A.6E). Concentrations by the second morning were almost twice as high as on the first.

Interestingly, rates of change in HOOH concentration in dark incubations also showed a clear diel cycle at each of these stations. In incubations of surface water from Chesapeake Bay (Fig. A.6B), degradation rates peaked in early afternoon, just as the in situ HOOH concentration began to rise. Similarly, degradation was most pronounced in the afternoon at Station 24 in the Western Pacific (Fig. A.6D), also coinciding with the beginning of net HOOH accumulation. Interestingly, at both of these stations increasing HOOH was attended with a corresponding *decrease* in the HOOH degradation rates. If microbes predominantly facilitated degradation in these samples, this suggests that the population does not modulate its HOOH defenses in response to increasing HOOH. One possible explanation is that exposure to HOOH either damages the organisms themselves, or else aggressively inactivates elements of their antioxidant defense systems. Catalase, for instance, is known to be inactivated by prolonged exposure to HOOH [26]. This hypothesis is partially supported by the observation that dark incubations spiked with 0.5 μM HOOH had lower peak degradation rates than native, unamended incubations (Fig. A.6D).

Unlike Chesapeake Bay incubations, some dark-incubated samples collected at night at Station 24 experienced net increases in HOOH concentration (Fig. A.6D). Since HOOH was not accumulating in the water column during these hours, it is likely that these generally small increases were experimental artifacts. In many cases, we observed increases in HOOH at the onset of incubations containing microbes, but not in sterile incubations (data not shown), suggesting that sample collection and processing created stress for these populations that manifested in leakage of HOOH. However, the possibility that these increases represented ecologically relevant biological HOOH production cannot be excluded.

HOOH dynamics at Station 26 were quite different than the other two diel stations (Fig. A.6F). At this station, net HOOH accumulation occurred in dark incubations of samples taken in the afternoon from throughout the water column. In samples collected at night, net decay of



HOOH was measured, with degradation rates peaking early in the morning. It is noteworthy that this apparently biological generation of HOOH during the day preceded the buildup of in situ HOOH, which peaked in early evening and remained high throughout the night (Fig. A.6E). The HOOH dynamics at this station suggest that the primary source of HOOH is biological, and is not directly related to either photochemistry or photosynthesis.

These experiments support the hypothesis that HOOH has a diel cycle in nature. However, they suggest that the current paradigm of formation and degradation of HOOH is overly simplistic. For instance, at Station 26, solar irradiation of the water column does not appear to be an important source of HOOH. Rather, dark production, probably biological, appears to be more important, leading to an overnight HOOH peak. It is unclear what process is responsible for HOOH production in these waters, since both biotic and abiotic photochemical reactions are excluded. Future work at this station, or other stations near the Southern Ocean, should be directed toward discovering what makes these waters different from others that have been studied.

The paradigm of biological HOOH degradation suggests that biological decomposition of HOOH is more or less constant, whereas generation only occurs during the day. Our evidence suggests that decomposition is in fact strongly cyclical, and becomes essentially non-existent during certain parts of the day. At Station 26, HOOH accumulated prior to the onset of net HOOH degradation in incubations (Fig. AI6.E-F), suggesting that microorganisms may be responding to elevated HOOH by upregulating defense mechanisms. However, at the other diel stations, peak degradation preceded peak HOOH (Fig. AI6.A-D), suggesting perhaps that upregulation of defensive mechanisms is a circadian function, and as such “anticipates” daytime accumulation of HOOH. However, it is also possible that the decreasing degradation rates through the afternoon are caused by HOOH- or UV-induced damage, and that expression of antioxidant systems continues to increase with HOOH. Future work should examine expression of key genes such as catalase in similar diel experiments, to better understand how these communities are responding to, and creating, the diel pulse of HOOH.

IV. Empirical tests of a mathematical model of the HOOH paradigm

The current HOOH paradigm is simple enough to be quite amenable to mathematical modeling. Essentially, the paradigm predicts that in situ HOOH concentrations will be constrained by two competing reactions: abiotic, photochemical formation and biological, enzyme-mediated decomposition. Based on purely theoretical considerations as well as our empirical observations, we may assign rate laws to each reaction. If we assume that HOOH formation is slow compared to production of dissolved organic matter in the water column, then abiotic formation of HOOH should be limited by light. Since the supply of light is unaffected by the concentration of reactants, light-limited photochemical reactions should exhibit zero-order kinetics. On the other hand, enzymatic reactions will obey Michaelis-Menten kinetics and will be sensitive to the concentrations of reactants, products, and the enzyme itself. If we assume that catalase is the primary enzyme responsible for HOOH elimination [99], then the concentration of HOOH is so far below this enzyme's K_m that pseudo-first order reaction kinetics should be observed. Thus, we may assign the following rate law for the HOOH paradigm:

$$\frac{d[\text{HOOH}]}{dt} = k_L - k_D[\text{HOOH}]$$

where k_L is the zero-order rate constant for HOOH accumulation in sterile, light-exposed seawater, and k_D is the pseudo-first order rate constant for dark decomposition in unfiltered seawater. From this law we may integrate to derive an equation for predicting the concentration of HOOH at time t based on initial concentration H_0 :

$$\begin{aligned} d[\text{HOOH}] &= (k_L - k_D[\text{HOOH}])dt \\ [\text{HOOH}] &= \int (k_L - k_D[\text{HOOH}])dt \\ [\text{HOOH}] &= k_L t - k_D t[\text{HOOH}] + H_0 \\ [\text{HOOH}] &= \frac{k_L t + H_0}{1 + k_D t} \end{aligned}$$

Additionally, we may calculate a stable steady-state HOOH concentration using this equation for any combination of rate constants by solving for the conditions where $[\text{HOOH}]$ and H_0 are identical. If we separate t into a “day component” and a “day+night” component for calculating the numerator and denominator respectively, and assume equinoctial conditions, steady-state HOOH is given by

$$[HOOH]_{ss} = \frac{k_L}{2k_D}$$

This model assumes that unfiltered samples in the dark *always* have net HOOH loss, and that light-exposed samples always have net HOOH gain. If either rate constant deviates from this assumption, the equation rapidly yields meaningless results, either yielding negative [HOOH] or else increasing to infinity. However, inside the assumptions of the paradigm, the equation yields plots that resemble the diel experiments described above (Fig. A.7). In this figure, we began with averages taken from our in situ field measurements: $H_0 = 100$ nM, and $k_L = 35$ nM h⁻¹. Each curve represents a different value of k_D within the range of our field measurements. Dotted lines represent steady-state values calculated for each value of k_D . Clearly, each curve approaches its respective steady-state value as time progresses.

While this model is necessarily simplified in relation to the complex physicochemical environment of real aquatic systems, it does suggest that rates within the ranges we measured are capable of creating a steady state similar to that found in all published studies of aquatic HOOH. The model predicts steady states that are somewhat high in comparison to the actual ocean, but this is to be expected, since the rates of production we measured are almost certainly overestimates. Our goal was to measure *maximum* production values (as a proxy for the lethality of a “helper-free” ocean for *Prochlorococcus*, Chapter 4), so we focused on sterile surface seawater exposed to full UV radiation. In nature, much of this rapid near-surface HOOH production would diffuse into lower strata, leading to a lower production rate when integrated across the entire water column. Since we rarely measured production with high depth resolution, it would be pointless for our purposes to add this level of complexity to our model. We also made no attempt to model the parabolic diel dynamics of solar irradiation that exist in nature. If we tentatively accept these reservations, we may conclude that the paradigm model can adequately relate in situ HOOH concentrations with rates of HOOH change.

We next attempted to fit competing rates measured in the field to this model, with the aim of comparing the steady state prediction to measured in situ HOOH concentrations. Where more than one measurement was made at any give station, we selected the highest rates of production and degradation. Stations where either value was outside of the paradigm’s predictions (i.e., net dark production or net light degradation) were excluded from the analysis. After this curation, we were left with 23 measurements, taken from 15 different stations (Table A.2). Of these, only

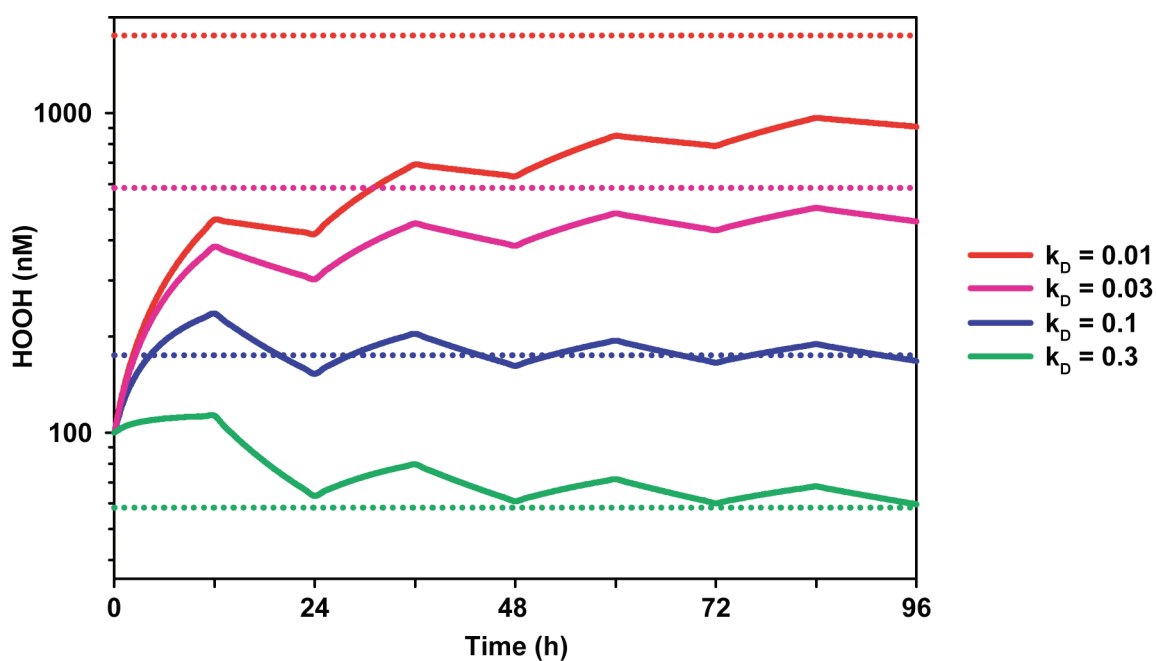


Figure A.7. Mathematical model of the HOOH paradigm. Each iteration of the model was calculated with initial [HOOH] $H_0 = 100$ nM and $k_L = 35$ nM h⁻¹. Different colored lines represent different values of k_D . Dotted lines, steady-state prediction from the model for the given k_D .

Table A.2. Comparison of model predictions with in situ rate data. Gray cells indicate predicted values within the natural range (~0-250 nM); the box indicates samples where model predictions and in situ concentrations were within 2-fold of each other.

Cruise, Station	Depth	Steady State	In situ	Ratio
DCM08, CTD18	5	5.35	84.76	0.06
WP2, 20	110	1.67	13.66	0.12
WP2, 20	5	31.86	74.73	0.43
DCM08, CTD10	5	48.40	101.00	0.48
DCM08, CTD61	5	67.91	118.31	0.57
DCM08, CTD13	36	32.84	49.50	0.66
WP2, 13	134	0.80	1.00	0.80
DCM08, CTD13	5	106.55	97.29	1.10
DCM08, CTD16	71	19.62	13.48	1.46
BC09, CTD05	5	238.69	121.61	1.96
WP2, 22	25	133.50	49.66	2.69
WP2, 22	7	245.81	86.07	2.86
DCM08, CTD16	5	451.86	108.75	4.15
WP2, 21	8	422.41	81.40	5.19
DCM08, CTD06	5	728.56	126.35	5.77
WP2, 21	25	338.93	53.75	6.31
MELEE XII, 84	1	700.49	80.37	8.72
WP2, 22	65	158.26	15.82	10.01
DCM08, CTD07	5	1507.80	93.43	16.14
BC09, CTD02	5	2699.94	110.47	24.44
WP2, 21	108	226.43	7.09	31.95
WP2, 15	95	548.18	13.21	41.50
DCM08, CTD10	101	539.75	11.83	45.63

7 (~30%) had predictions within 2-fold of the actual in situ value. 14 (~60%) had predicted values within the range of reported natural HOOH values, although some were conspicuously high for the depths where the samples were collected. In the other samples, steady state estimates ranged as high as 2.7 μM , an order of magnitude above any concentration measured in pelagic waters. These high estimates were replicated, and were detected at select stations on each cruise, arguing against any systemic methodological errors leading to overestimation. Instead, we conclude that the 70% of samples where in situ concentrations and steady state predictions are out of sync represent actual deviations from the paradigm, and may represent ongoing “oxidative anomalies” or the lingering effects of previous such events. Another possibility is that the time of sample collection led to deviations from calculated rates. For instance, most WP2 samples were collected early in the morning, so if degradation fluctuated on a diel basis, as was shown to be the case for the diel experiments we reported in the previous section, this could lead to erroneously high or low estimates of community HOOH removal. Future studies of degradation should carefully measure rates over a diel cycle and integrate them to more accurately test the predictions of the paradigm model. Unfortunately, we did not measure light production rates during our diel studies.

V. Potential sources of deviation from the model

A comprehensive analysis of deviations from paradigm predictions will require much additional work. We have already discussed the diel variation in degradation rates that may be a primary source of disagreement between in situ rates and model predictions based on our snapshot estimates of HOOH dynamics. While not observed in our studies, precipitation, algal blooms, viral lysis events, or sporadic influxes of organic matter (e.g., from macrofaunal death) could lead to stochastic, but non-artifactual, increases or decreases in HOOH concentrations. Our model predicts, however, that such transient changes in H_0 will not affect the ultimate steady state, and within a few days without further disturbance the effects of these perturbations will be eliminated. Still, such changes may explain some of the deviations from steady state we detected. Our data also suggest two further sources of disagreement with the model. First, we have good evidence that larger organisms are important sources of HOOH in natural waters.

Second, there is reason to believe that, in some waters, extracellular enzymes may be involved in HOOH elimination.

At 29 oceanic stations we performed incubations at multiple depths using both raw seawater and seawater pre-filtered through large pore size (2-5 μm) polycarbonate membranes. This treatment effectively removes most eukaryotic plankton from the treatments, including larger phytoplankton such as diatoms, dinoflagellates, and coccolithophores. Also, most colonial cyanobacteria such as *Trichodesmium* should be excluded. Out of 54 such incubations, 40 (74%) had higher dark degradation rates after removal of the larger organisms (Fig. A.8A). In a smaller subset of these samples where light incubations were also performed, 12 of 18 (67%) also had lower rates of HOOH production after filtration (Fig. A.8B). For the dark incubations this difference was statistically significant (Wilcoxon Signed Rank test, $n=54$, $p<0.01$). These results strongly suggest that larger organisms are active generators of HOOH.

Another possible source of deviation from the paradigm is the existence of “abiotic,” or at least extracellular, sources of HOOH removal. Angel et al. [103] reported extracellular catalase activity in waters of the Mediterranean Sea, and numerous organisms are known to secrete catalases into their environments (Chapter 1). If such enzymes were present in seawater, they could lead to erroneously low estimates of steady state HOOH using our model. We checked for the presence of extracellular hydroperoxidases using dark incubations of sterile seawater. In the majority of samples, no significant changes from initial conditions were observed, consistent with the prevailing paradigm. In several samples, however, early decreases in HOOH were observed in sterile, dark incubations, but degradation did not continue past the first few hours. Because of the high risk of contamination of sterile samples at sea, we adopted strict criteria for accepting that degradation in such incubations was caused by actual extracellular enzymes present in situ. First, the sample had to display clear first-order degradation kinetics ($r^2 > 0.9$ for a log-linear plot of HOOH vs. time) starting at the first sample point. This was important, since we assumed a) an extracellular enzyme would not experience a “lag phase,” and b) any contaminants should be inoculated at fairly small concentrations and should require at least a few hours to begin affecting the sample’s HOOH concentration. Second, the finding had to be replicated with a minimal difference between the calculated rates of the replicates. Third, the amount of HOOH degraded in 24h had to be higher than the

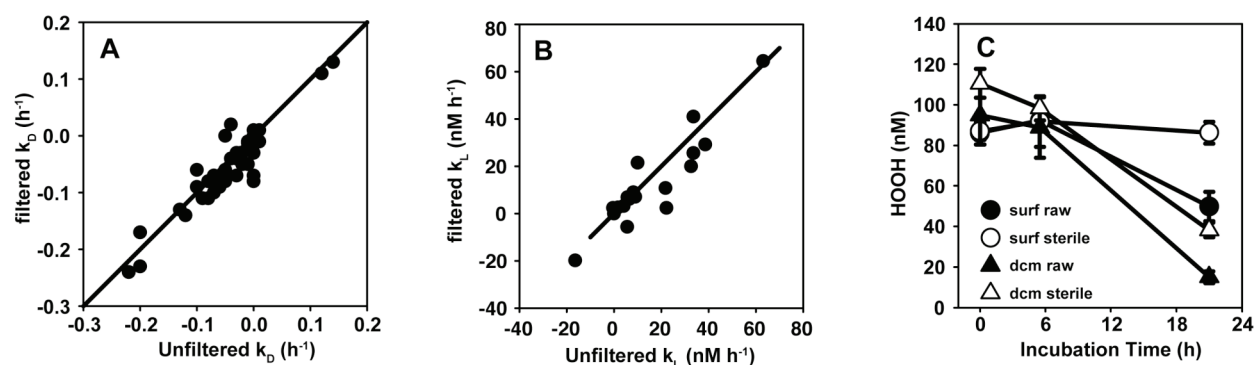


Figure A.8. Potential causes of conflict with the steady-state model. Attempts to fit field data into the paradigm model described in the text assumed that rates of change in HOOH in sterile incubations are purely abiotic/photochemical, and rates of change in dark incubations of raw water are purely a result of microbial HOOH degradation. We saw ample evidence that larger organisms generated HOOH in our samples. A) In dark incubations, unfiltered samples had significantly lower HOOH degradation rates than samples passed through a large (2-5 μ m) pore size membrane filter prior to incubation. This is visualized in this chart as points lying beneath the unity line representing equal rates in filtered and unfiltered samples. B) In light incubations, rates of HOOH production were often lower in filtered than in unfiltered samples, although this difference was not significant. C) At DCM08 station 16, we observed evidence of enzyme-like (pseudo-first order) decay of HOOH in sterile-filtered water from the deep chlorophyll maximum (DCM, open triangles), whereas this was not observed in sterile water from the surface (open circles).

standard deviation of the initial and final samples. Only one sample from all our stations fit these strict criteria. At DCM08 station 16 in the Eastern Pacific near Socorro Island, water from the deep chlorophyll maximum at 74 m depth showed clear signs of extracellular HOOH degradation (Fig. AI8.C). Incubations of raw surface seawater from this station showed strong degradation kinetics, whereas HOOH in sterile filtered surface water was stable. When deep water was incubated, HOOH degradation was only moderately affected by filtration. Degradation in both the raw and sterile deep samples fit a log-linear line well ($r^2 = 0.94$). Of course, it is impossible to say conclusively that extracellular proteins were involved, since no proteins were recovered. Further, these results do not suggest that any proteins present were secreted; they could have been released by lysis. However, this observation does support the hypothesis that extracellular hydroperoxidases exist, and provides incentive for further investigation into the possibility that some organisms may actively release HOOH degrading enzymes into the water column.

VI. Conclusions

Our results for the most part support the established HOOH paradigm. In the majority of cases where enough data was collected to test the paradigm model, predicted steady states were within the range of HOOH concentrations observed in the field. However, several samples deviated significantly from predicted values, and our diel studies suggest that rates of microbial HOOH removal are as dynamic as the in situ concentrations themselves. Additionally, biological HOOH production, particularly by larger organisms, and extracellular hydroperoxidase enzymes potentially confound measurements of rates, and could lead to either long- or short-term deviations from model predictions.

One conclusion we may draw is simply that not enough is known about HOOH dynamics in nature. Studies to date have narrowly focused on specific chemical questions about HOOH, and have made very simplistic assumptions about the nature of the microbial populations implicated in removing HOOH. Our studies show that degradation displays circadian cycles, consistent with most microbial processes in the ocean, rather than straightforward pseudo-first order kinetics like those found in acellular chemical reactions. Much more work is necessary to

piece together the story of HOOH dynamics, and this effort will require cross-disciplinary cooperation from physical, chemical, and biological scientists to be successful.

Another conclusion, apropos to this dissertation, is that the dynamics of HOOH in nature may be such as to lead to at least temporary stress for microbial populations. In Chapter 4 we concluded that *Prochlorococcus* could not survive on its own at the ocean's surface. However, at some of these stations we observed HOOH in excess of 200 nM, and moreover measured rates tending toward steady states in the micromolar range. The growth rate of axenic *Prochlorococcus* cultures was impaired when exposed to 200 nM HOOH (Chapter 4), suggesting that at these field sites growth could be inhibited for this important species. Certainly, any stochastic event leading to local concentrations higher than this will be deleterious for *Prochlorococcus* and potentially other similarly “streamlined” organisms. The observation that dark degradation rates *decreased* with increasing HOOH during the Station 24 diel (Fig. A.6C-D) gives tentative support to the hypothesis that this community was suffering oxidative damage. While data are not robust enough to incorporate oxidative stress into multifactorial models of microbial growth in natural waters, our data suggest that it should be considered along with grazing and viral lysis as an important source of mortality amongst smaller organisms in the pelagic environment.

The data presented in this chapter show that HOOH concentrations in the field are variable, and deviations from steady state are common. The prevailing paradigm of HOOH dynamics is accurate, but incomplete. Further field studies are necessary to understand the extent and ecological importance of variations in HOOH concentrations in natural ecosystems. Toward these ends, improved collaboration between biological, chemical, and physical oceanographers is imperative. Hopefully, this study will provide an incentive for workers in these fields to pay more attention to HOOH and its importance for the microbial ecology of the oceans.

VITA

J. Jeffrey Morris was born in 1974 in Shelbyville, Tennessee, son of James M. Morris and Sally E. Morris. He grew up in the small town of Fayetteville, Tennessee, where he was early exposed to environmental science at the Fayetteville Junior High Environmental Center, administered by veteran teacher Flavil Hatcher. He graduated in the top 10% of his class at Lincoln County High School in 1992. Attending the University of Alabama at Huntsville on a full tuition scholarship, Jeff attempted a double major in Biochemistry and Philosophy, but left without a degree in 1998 to pursue a career as a musician. His band, d’hiver mort, released two independent full-length albums, *Elegium in Obitum Veriis* in 1999 and *Palindrome* in 2002. Returning to university in 2004, Jeff studied Biology at Kennesaw State University in northern Georgia, graduating with honors in 2006. While at KSU, he pursued independent research in the laboratory of Joseph Dirnberger, studying the effects of water current velocity on the productivity and community composition of epilithic algae in a small stream.

Jeff met his future wife, Laura Weathers, in 2000, when she photographed one of d’hiver mort’s performances in Huntsville, Alabama. The two were finally married in 2004, and have two children together: Elizabeth Hippolyta (2006) and Eirik Milton (2009). In addition to science, Jeff enjoys cooking, brewing and drinking beer, and plotting world conquest. The latter is expected to be facilitated by the time travel device he is building in his garage, in his spare time, with his own funds.

