Exploring the pathogenic interaction of marine roseobacter *Phaeobacter inhibens* and its coccolithophore host *Emiliania huxleyi*

by

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Abstract

Among phytoplankton, coccolithophores are the main calcifiers, taking up inorganic carbon and calcium to make delicate calcite scales called: coccoliths. The model coccolithophore, *Emiliania huxleyi*, is the most ubiquitous extant species. *E. huxleyi* is a species complex with three morphologically distinct cell types: calcifying diploid, non-calcifying haploid, and non-calcifying diploid. *E. huxleyi* has a bloom—bust lifestyle in which the dominant calcifying cells rapidly form expansive blooms (>100,000 km^2), then precipitously collapse. Rapid coccolithophore bloom collapse is commonly attributed to highly specific *E. huxleyi* viruses (EhVs), while the potential bacterial pathogens are frequently overlooked.

Coccolithophore blooms are surrounded with bacteria and have a notably strong correlation with several members of the roseobacter clade. The frequent co-occurrence of roseobacters with algae has been attributed to several shared phenotypic traits such as: chemotaxis toward and metabolism of algal exudates, rapid surface colonization, and their repertoire of secretion systems, which are all postulated to enhance roseobacter-algal interactions. Roseobacters also produce a myriad of chemical signals (antibiotics, vitamins, algicides, etc.) that likely accumulate to functional concentrations within the algal phycosphere—the area immediately surrounding an algal cell, suggesting the potential for complex chemical interactions with algal hosts. In fact, several roseobacters, including *Phaeobacter inhibens,* interact directly with *E. huxleyi*, but a mechanistic understanding of this interaction has not been established.

To further investigate the mechanism of this *P. inhibens*—coccolithophore interaction I developed a miniaturized microtiter plate assay to rapidly screen algal photosynthetic health, cell counts, and a suite of other desired parameters. Using this bioassay, I discovered that *P. inhibens* has a dynamic biphasic interaction with its algal host, initially interacting beneficially, and then

switching into a deadly pathogen. This switch was initially attributed to the process of *E. huxleyi* aging, during which the alga releases a signaling molecule called *p*-coumaric acid (*p*CA). In response to *p*CA, *P. inhibens* produces bioactive algicides called roseobacticides, which directly kill a representative strain of the non-calcifying diploid *E. huxleyi* cell type. Given the rapid way in which roseobacticides induce algal death, they were implicated as the key bacterial bioactives responsible for killing *E. huxleyi*. However, the efficacy of roseobacticides on the dominant calcifying and haploid flagellated strains has not yet been tested.

The central goal of this thesis is to elucidate the mechanism of the pathogenic interaction between *P. inhibens* and its coccolithophore host *E. huxleyi*. This was first done by testing representative members of the three *E. huxleyi* cell types (calcifying diploid, non-calcifying haploid, and non-calcifying diploid) in co-culture with the pathogen *P. inhibens*. Surprisingly, *P. inhibens* is a selective pathogen, killing the tested calcifying and haploid flagellated strains, but not killing several tested non-calcifying diploid strains. Additionally, the implicated roseobacticides were not lethal to either of the sensitive *E. huxleyi* cell types (calcifying or haploid), suggesting that an alternate virulence factor was responsible for *P. inhibens* pathogenesis.

The viral pathogen of *E. huxleyi* kills its algal host by hijacking algal metabolic pathways and producing bioactive viral-glycosphingolipids (vGSLs), which induce algal programmed cell death (PCD). This pathogenic interaction is a highly sophisticated viral manipulation of algal metabolic and death pathways, and it was hypothesized that the bacterial pathogen might also manipulate algal PCD networks to induce host death. I next established (using biochemical, morphological, and physiological parameters) that *P. inhibens* initiates algal apoptosis-like PCD (AL-PCD) in the calcifying *E. huxleyi* cell type. This is the first time a marine bacterial pathogen

has been shown to induce AL-PCD of an algal host. To further describe the mechanism of this interaction I obtained transposon mutants in various genes in the *P. inhibens* type IV secretion system (T4SS) and established that several mutants in the genes required for a functional T4SS were avirulent to the algal host. This is the first time a functional effector T4SS has been demonstrated to be required for roseobacter pathogenesis of an algal host. Together, these findings strengthen our current understanding of how marine pathogens manipulate and kill algal host cells by demonstrating both a required bacterial effector T4SS as well as the manner in which the algal host undergoes rapid AL-PCD.

Preface

Some of the research conducted for this thesis is part of collaborative work (detailed below).

Chapter 2 has been published as: **Bramucci, A.R.**, Labeeuw, L., Mayers, T., Saby, J., and R. J. Case. 2015. A small volume bioassay to assess bacterial-phytoplankton co-culture using WATER-pulse-amplitude-modulated (WATER-PAM) fluorometry. *Journal of Visual Experimentation*. **97**. doi:10.3791/52455.

ARB, LL, and RJC designed the method. **ARB** contributed Figures 2.1 and 2.2. **ARB** and TM contributed Figure 2.3 and Appendix B. LL contributed Figure 2.4. **ARB**, LL, TM, and RJC contributed to the writing of the manuscript.

Chapter 3 was a collaborative effort involving: **Bramucci, A.R.**, Labeeuw, L., Orata F.D., Malmstrom R., and R.J. Case.

ARB, LL, and RJC designed the experiments. LL and **ARB** contributed preliminary experiments. LL contributed Table 3.1. RM contributed *Phaeobacter inhibens* transposon mutants. **ARB** contributed Figures 3.1-3.8 and Appendix C. **ARB**, LL, and RJC contributed to the writing of the manuscript.

Chapter 4 was a collaborative effort involving: **Bramucci, A.R.** and R. J. Case.

ARB and RJC designed the experiments. **ARB** contributed Figures 4.1-4.9. **ARB** and RJC contributed to the writing of the manuscript.

Chapter 5 was a collaborative effort involving: **Bramucci, A.R.,** Malmstrom R., and R. J. Case.

ARB and RJC designed the experiments. **ARB** contributed Figures 5.1-5.5 and Appendix D. RM contributed *Phaeobacter inhibens* transposon mutants. **ARB** and RJC contributed to the writing of the manuscript.

Other collaborative works that resulted in publications are listed in Appendix A.

Dedication

I would like to dedicate this work to my parents Mary and Ralph Bramucci who have always modeled perseverance, hard work, and love of learning.

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I would like to thank Dr. Rebecca Case for giving me the freedom to take my research in new unexplored directions to complete this story. And I would like to thank Rebecca for encouraging me to apply to CMORE at University of Hawaii; the CMORE course was an educational experience I will never forget, and it was instrumental in molding the way I contemplate how marine microbes shape global processes.

I would also like to thank my committee members for their unwavering guidance and support throughout this academic journey. To Dr. Vincent St. Louis for always encouraging me to think about the marine ecosystem from the bottom up by contemplating the complex ways nutrient cycles control marine ecosystems. To Dr. Sally Leys for ensuring that I was thinking about the big picture marine concepts and for letting me partake in the Leys Lab's Hecate Strait and Strait of Georgia glass sponge reef expeditions. And finally, to Dr. Lisa Stein for encouraging me to contemplate interactions from different microbial perspectives. Throughout my PhD I have learned that communicating science effectively should be one of the main goals of every scientist, and so I am also thankful to all of my mentors and colleagues for coming to practice talks, offering their scientific perspectives, and engaging in scientific debates, all of which have highlighted to me the importance of scientific communication.

Throughout my years at the University of Alberta several of my fellow graduate students have supported me with friendship and become essential to my success in the Case Lab. First, I would like to thank all of my lab mates, past and present, for making coming into the laboratory in rainy, sunny, and negative-40-degree days, both enjoyable and scientifically fruitful. Thank you to Dr. Leen Labeeuw who first taught me the critical Case Lab methodologies and who always motivated me to keep at it, even on days when 'experiments seemed sentient'. And to Teaghan Mayers who always knew when it was time for a margarita or a little fun playing games outside of the lab, to help keep those scientific juices bubbling. And thank you to the current Case Lab students Yue Xu and Cat Bannon who have made the last hurdle of my PhD so enjoyable. They both motivated me to continue to work hard right to the end and then encouraged me to stop for ping pong breaks when they were most needed.

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eternally grateful. And of course, all my fellow biological sciences graduate students, whether we passed quietly in the long nights, played micro-themed Pictionary games, or chatted at one of the several coffee corners, have made this long endeavor infinitely more fun and for that I am very thankful.

My family have also been extremely supportive of me throughout the long years of my education. My mother and father have always inspired me to be curious about the natural world and reminded me to *never turn your back on the ocean*. My sister Gina and brother Stephen have also always championed my successes and supported me during the most difficult periods of my studies from kindergarten through to final days of my PhD. Also to my brother-in-law Jöel and sister-in-law Nikta thank you both for all of the great family times and for bringing my wonderful nieces and nephews into my life: Circé, Esmée Alma, Zani Rafael, and Julien River. (All of them joined the family during my PhD and brought more love and laughter to us all!). Finally, I want to thank Aaron, my partner in life and adventure, for helping me through the long nights of research and for never tiring of hearing about the very small organisms that I have spent so long contemplating in such excessive detail. To all of these people, thank you for your encouragement, your support, and your endless love. I appreciate you all more than I can say. Thank you.

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Abbreviations

½MA	½ marine agar	JGI	Joint Genome Institute
½MB	½ marine broth	F_m	maximum algal fluorescence
ANG	accessory nidamental gland	NCBI	National Centre for Biotechnology Information
AL-PCD	Apoptosis-like-PCD	NCMA	National Centre for Marine Algae and Microbiota
ATG	Autophagy-related genes	ORF	open-reading frame
IETDase	Caspase-8-like (cleavage of substrates at: Ile-Glu-Thr-Asp)	OM	outer membrane
cfu	colony forming unit	PBS	phosphate buffer solution
CV	crystal violet	*p*CA	*p*-coumaric acid
caspases	cysteine aspartic proteases	PS	periplasmic space
cyt *c*	cytochrome *c*	PSII	Photosystem II
DUB	deubiquitinating	F_v/F_m	potential quantum yield
DIC	differential interference contrast	PCD	programmed cell death
DOM	dissolved organic matter	PAM	pulse-amplitude-modulation
DMSO	dimethyl sulfoxide	ROS	reactive oxygen species
DMS	dimethyl sulphide	RTX	repeat-in-toxin
DMSP	dimethylsulfoniopropionate	SSC-A	side scatter area
T4SSA	effector T4SS (*virB/D4* gene arrangement)	ssT-DNA	single stranded transfer-DNA
T4SSB	effector T4SS (*dot/Icm* gene arrangement)	SAR	stramenopiles-alveolates-Rhizaria
EhV	*Emiliania huxleyi virus*	FSC-A	forward scatter area
ER	endoplasmic reticulum	TDA	tropodithetic acid
HGT	horizontal gene transfer	Ti-plasmid	tumour inducing plasmid
hGSL	host-glycosphingolipid	T(1-9)SS	type (I-IX) secretion system
IAA	indole-3-acetic acid	VPE	vacuolar processing enzyme (caspase-1-like)
F_0	initial algal fluorescence	vGSL	viral-glycosphingolipid
IN	inner membrane	WT	wild type

Chapter 1. Introduction

1.1. Beneficial bacterial-algal interactions

At the microscale, the marine ecosystem is a vast seascape of microorganisms interacting with each other and their environment. Despite its superficial homogeneous appearance, the clear blue ocean is sprinkled with occasional nutrient 'hot-spots' (i.e., zooplankton waste, dying algae, marine snow, etc.) and chemical gradients (i.e., plumes of organic matter exuding from leaky algae, sloppy grazing, or sinking particulates) (Azam *et al.*, 2007; Stocker, 2012; Smriga *et al.*, 2016). This microscale patchiness of the marine ecosystem, as well as the underlying variability of ocean currents, light penetration, etc., all influence the dynamic distribution patterns of marine microorganisms (Mitchell *et al.*, 2008). Photosynthetic microbes, collectively called phytoplankton, are a diverse group of freely floating or weakly swimming photosynthetic prokaryotes and eukaryotic algae that encompassing an estimated 25,000 unicellular species (Costello *et al.*, 2013; Klais *et al.*, 2016). Photosynthetic phytoplankton use photosynthesis to convert solar energy and inorganic carbon (CO_2) into sugars like glucose ($C_6H_{12}O_6$), releasing the by-product oxygen (O_2). Phytoplankton biomass is a biological reservoir of bioavailable organic carbon in the marine ecosystem, feeding and sustaining primary consumers, which go on to feed higher trophic levels. However, nearly half of the carbon fixed by marine phytoplankton is released as dissolved and particulate organic matter (DOM and POM), which is then predominantly metabolized by heterotrophic bacteria (Azam, 1998; Durham *et al.*, 2014). Heterotrophic bacteria are astoundingly abundant in the marine environment; every milliliter of ocean water contains an estimated 10^5 to 10^6 bacterial cells (Whitman *et al.*, 1998). And despite their tiny size these bacteria interact directly with nearby phytoplankton in a plethora of ways.

1.1.1. The roseobacter clade

The monophyletic roseobacter clade, within the Rhodobacteraceae family of the alphaproteobacteria class, is a ubiquitous group of bacteria, with members found in marine habitats all over the world (Buchan *et al.*, 2005; Wagner-Döbler *et al.*, 2006). Roseobacters have been identified from coastal regions to open ocean, and from the sea ice to sea floor (Brinkhoff *et al.*, 2008). They also have variable lifestyles, thriving as free-living and associated (e.g., to particulates (Dang *et al.*, 2000), microalgae (Miller and Belas, 2004; Segev *et al.*, 2016),

macroalgae (Fernandes *et al.*, 2011), and crustaceans (Buddruhs *et al.*, 2013)). Additionally, members of the roseobacter clade dominate the microbial consortia of coastal regions (Luo, Swan, *et al.*, 2014) and have a strong correlation with phytoplankton (González *et al.*, 2000; Zubkov *et al.*, 2001; Sapp *et al.*, 2007; Mayali *et al.*, 2008; Green *et al.*, 2015). The frequent co-occurrence of roseobacters and various phytoplankton is attributed to shared traits, like motility (Smriga *et al.*, 2016), sensing and moving toward algal exudates (Miller and Belas, 2004), metabolism of algal exudates (Moran *et al.*, 2003, 2012), and rapid surface colonization (Dang *et al.*, 2000), which are all suspected to enhance roseobacter-algal interactions. Interactions between roseobacters and their algal hosts can be beneficial, detrimental, or neutral to the algal host. Beneficial interactions are classified as either obligate or non-obligate mutualistic (both parties benefit) or commensal (one party benefits) interactions. The following sections highlight a few beneficial roseobacter-algal interactions suspected to occur in the marine environment.

1.1.2. Nutrient exchanges

A portion of the carbon fixed by marine phytoplankton is effectively consumed by primary consumers, and passed through the marine food web to higher trophic levels. However, phytoplankton are notably leaky cells, leaking or excreting organic carbon and other metabolites as they photosynthesize (Borchard *et al.*, 2012; Thornton, 2014). Moreover, phytoplankton injury and/or death due to gradual senescence, sloppy grazing (Lampert, 1978), viral lysis (Wilhelm *et al.*, 1999; Suttle *et al.*, 2005), etc. accelerate the release of DOM and POM from phytoplankton, which then feeds the microbial loop (Buchan *et al.*, 2014) (Figure 1.1).

Heterotrophic bacteria utilize algal organic matter (free floating sugars and exudates), subsequently becoming another microbial reservoir of organic matter (i.e., bacterial biomass) that feeds higher trophic levels (Azam *et al.*, 2007). During respiration, heterotrophic bacteria actively facilitate the remineralization of organic molecules back into their inorganic forms, making them bioavailable to the surrounding phytoplankton and bacteria (Figure 1.1) (Buchan *et al.*, 2014). Other sources of new inorganic nutrients are also plentiful in the marine system, such as unicellular heterotrophic eukaryotes (protists) releasing inorganic waste or upwelling events (Moore *et al.*, 2013). In more nutrient limited waters, however phytoplankton growth is likely to directly benefit from bacterial regeneration of nitrogen and phosphorus (Cole, 1982), making this a beneficial non-obligate mutualistic interaction.

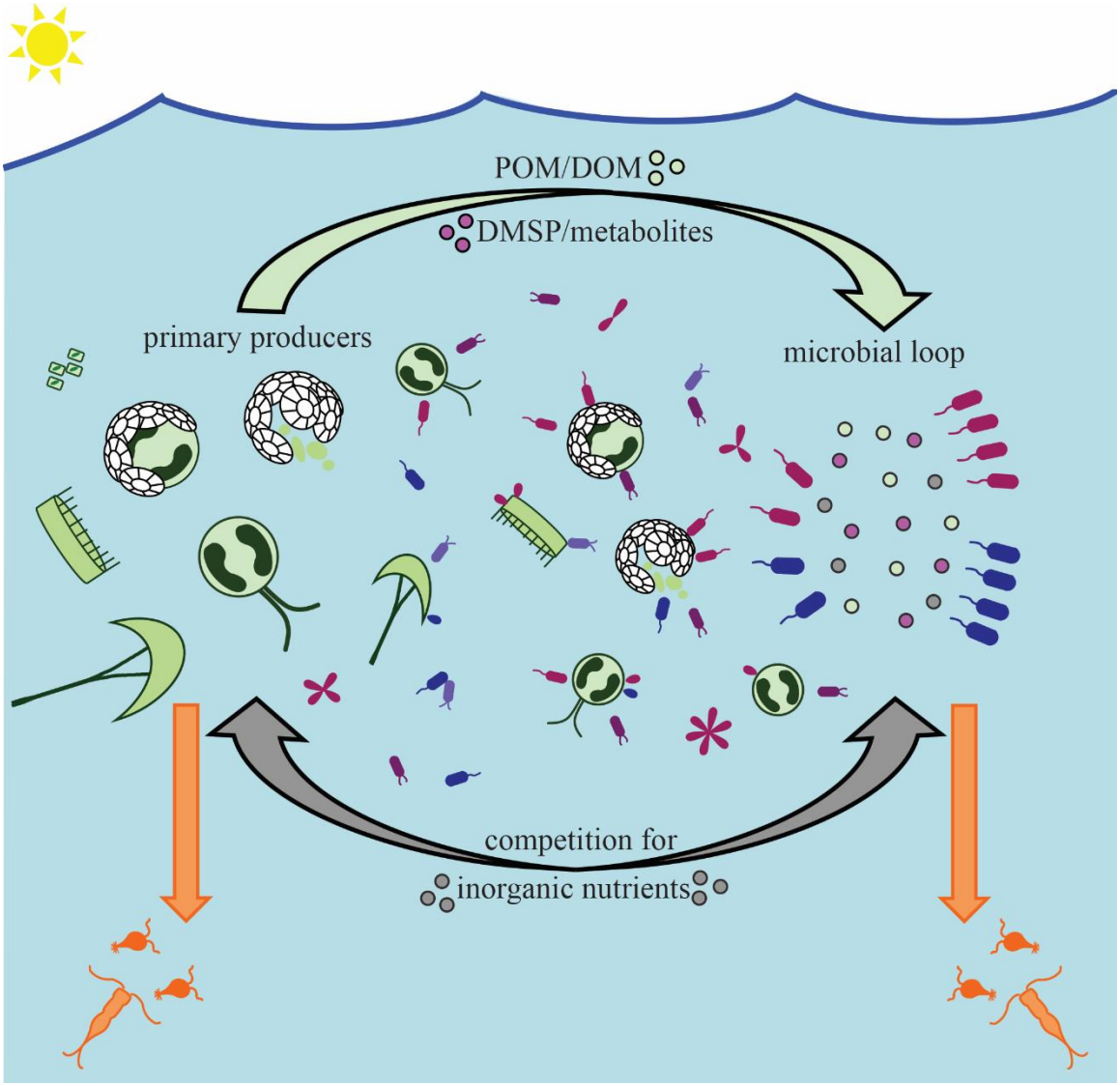

Figure 1.1. **Primary producers and the microbial loop: sustaining each other through the exchange of metabolites and nutrients.** As phytoplankton divide, die, are lysed by viruses, or are consumed by sloppy grazers stores of algal organic matter (POM/DOM, DMSP, metabolites, etc.) are released, feeding the heterotrophic bacteria of the microbial loop. Dissolved inorganic nutrients from viral lysis, consumer waste, or upwelling events (grey circles), stimulate both phytoplankton and bacteria with required inorganic nutrients. Marine bacteria are eventually consumed by primary consumers (orange), which in turn are consumed by secondary consumers, and the energy transformed by marine phytoplankton and the microbial loop is transferred up to higher marine trophic levels. Inspired in part by (Seymour *et al.*, 2017).

1.1.3. Chemosensory metabolites

From a microbial perspective, even dense algal blooms have a patchy distribution. This patchy seascape means that bacteria have a relatively low probability of randomly encountering an algal cell (~0.0035 cells/day) (Seymour *et al.*, 2017). However, 20-60% of marine bacteria are motile (Stocker, 2012), enhancing their likelihood of coming into contact with leaky phytoplankton (~9 cells/day) (Seymour *et al.*, 2017). Chemotaxis (the ability to sense and move toward chemical gradients) further improves a microbe's ability to move toward a particle or algal cell (Stocker *et al.*, 2008; Stocker, 2012). One of the important chemotactic molecules linking marine phytoplankton and surrounding heterotrophic bacteria is the algal metabolite and info-chemical dimethylsulfopropionate (DMSP).

DMSP is a multi-use molecule, playing important roles as an algal osmolyte, antioxidant, and cryoprotectant (Sunda *et al.*, 2002). Upon release from phytoplankton, dissolved DMSP serves as a major nutritional link between phytoplankton and the heterotrophic microbial population. In fact, it is estimated that marine bacteria may obtain up to 15% of their carbon and most of their sulfur from algal DMSP (Zubkov *et al.*, 2001). DMSP is also a strong info-chemical and chemoattractant for marine roseobacters, many of which are capable of incorporating DMSP directly into the amino acid methionine (Moran *et al.*, 2012). Alternatively, bacteria can cleave DMSP, generating the bad taste molecule acrylate and dimethyl sulphide (DMS), which rapidly volatilizes, increasing cloud cover and acting as a foraging cue to seabirds (Nevitt *et al.*, 1995). In this way, DMSP-degrading bacteria might be interacting with phytoplankton in either a non-obligate commensal interaction (where bacteria benefit by producing methionine) or a non-obligate mutualistic interaction (where the bacteria benefit from resources and the algae benefit from decreased grazing pressure due to acrylate accumulation (Strom *et al.*, 2003)).

1.1.4. Vitamins and antibiotics

The study of bacterial-algal interactions is still in its infancy, but it is beginning to be appreciated that a diverse consortia of bacteria can reside within the algal phycosphere—the space immediately surrounding the algal cell (Bell, 1983; Ramanan *et al.*, 2015) (Figure 1.2a). Additionally, attachment of bacteria directly to algal hosts further ensures that both parties gain access to trace nutrients and vitamins they do not otherwise produce themselves. For example, the diatom *Thalassiosira pseudonana* harbors a cyanocobalamin (vitamin B_{12}) requiring version

of the methionine synthase gene (*metH*), but cannot synthesize B_{12} (Durham *et al.*, 2014). In fact, recent surveys suggest that over half of algae require, but are unable to synthesize, one or more essential vitamins (e.g., B_1, B_{12}, etc.) (Croft *et al.*, 2006). Importantly, the ability to synthesize B_{12} is widespread, and potentially uniform, throughout the marine roseobacter clade (Wagner-Döbler *et al.*, 2006; Newton *et al.*, 2010; Luo and Moran, 2014). In the case of *T. pseudonana*, the diatom takes part in a mutually beneficial interaction with the roseobacter *Ruegeria pomeroyi* DSS-3, which is one of a limited number of marine bacteria capable of catabolizing the *T. pseudonana* sulfur metabolite: 2,3- dihydroxypropane-1-sulfonate (Durham *et al.*, 2014) (Figure 1.2b). In return, the bacterial symbiont supplies the diatom with vitamin B_{12} (Durham *et al.*, 2014). Another mutually beneficial interaction is exemplified by dinoflagellate *Prorocentrum minimum* providing *Dinoroseobacter shibae* with organic matter and vitamin nicotinamide (B_3) (Wagner-Döbler *et al.*, 2010). The roseobacter symbiont, seemingly to benefit its algal host, then releases vitamins its host is unable to synthesize (B_1 and B_{12}) (Wagner-Döbler *et al.*, 2010), connecting them in a mutually beneficial interaction (Figure 1.2d).

The potential exchange of nutrients is not the only way bacteria and algae interact beneficially during close symbiosis. Bacteria can also influence the bacterial assemblage composition of the algal phycosphere as they often produce antibiotics. The production of antibiotics by phycosphere bacteria might be a means of ensuring exclusive access to the alga's nutrient-rich exudates, while also protecting the algal host from pathogenic or fouling bacteria (Rao *et al.*, 2007). Bacteria in the roseobacter clade produce several antibiotics, which might potentially protect their host from pathogens (Bentzon-Tilia *et al.*, 2017). One example, is the potent antibiotic tropodithietic acid (TDA) (Brinkhoff *et al.*, 2004; Grotkjær *et al.*, 2015). TDA-producing roseobacters, for instance, protect their various hosts (e.g., the macroalga *Ulva australis* (Rao *et al.*, 2007), cod larvae (D'Alvise *et al.*, 2012), and turbot larvae (Grotkjær *et al.*, 2015)) from TDA-sensitive pathogens. Though a similar interaction has not yet been identified on the microbial scale (i.e., roseobacter protection of a microalga host) this kind of antibiotic exchange, should it occur, might benefit the TDA-producing symbionts as well as their microalga hosts (Figure 1.2c).

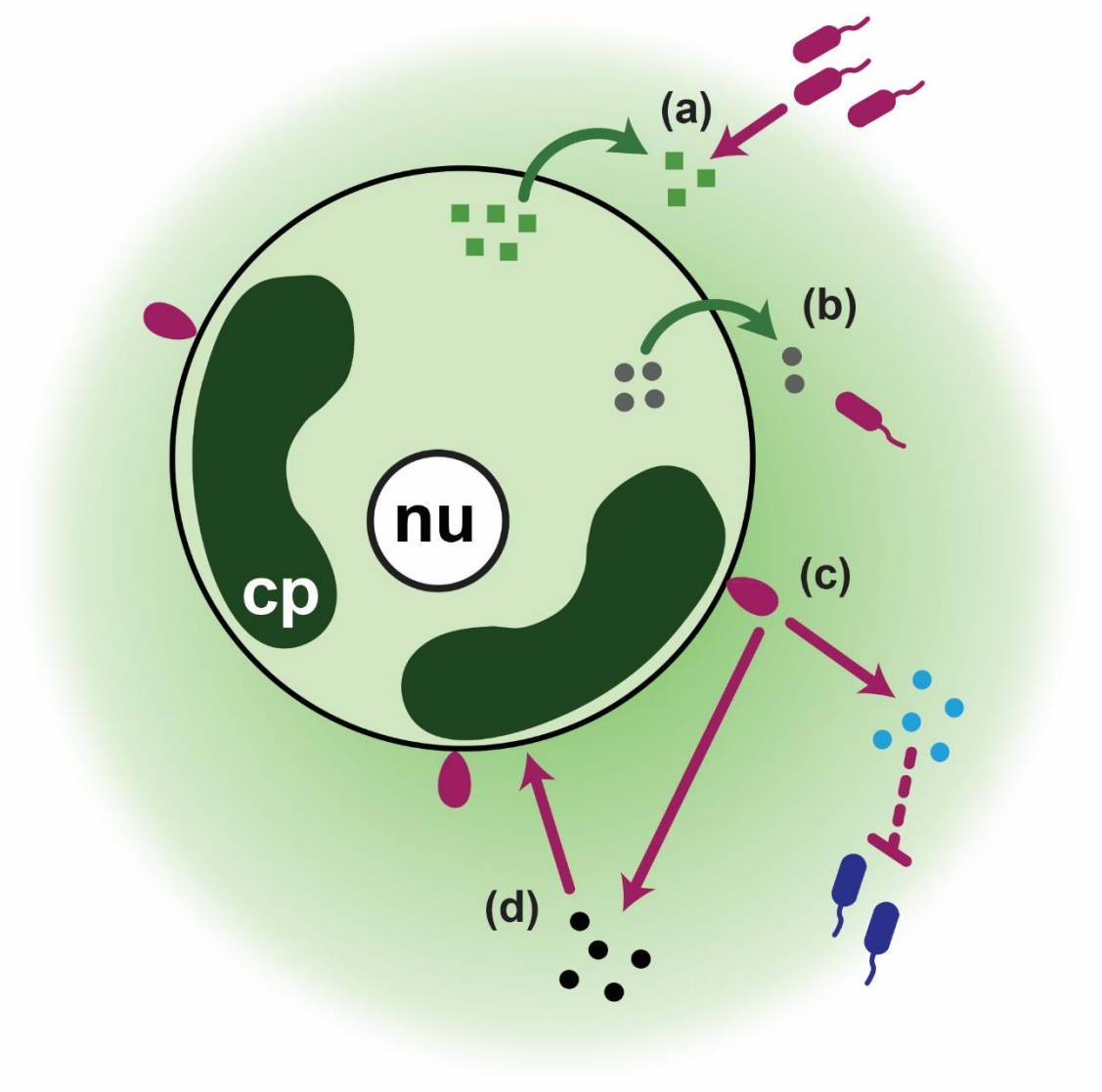

Figure 1.2**. Beneficial phytoplankton-algal interactions mediated by the exchange of bioactive molecules within the algal phycosphere.** Host algal cell (green), two chloroplasts (cp), nucleus (nu) (other organelles left off for simplicity), surrounded by cell wall (black outline), and green haze representing the phycosphere. Beneficial interactions between attached and non-associated bacteria (pink) and the algal host: (a) leaky algae release organic matter, secondary metabolites, and/or chemosensory molecules like, DSMP (green squares); (b) algal hosts release vitamins or trace nutrients that are required or beneficial to bacterial symbionts (grey circles); (c) Antibiotic-producing phycosphere bacteria release antibiotics (i.e., TDA), that might inhibit various TDA-sensitive pathogenic bacteria (blue). (d) Some bacteria produce vitamins or growth factors (black circles) that can enhance or prolong algal health.

1.2. Antagonistic bacterial-algal interactions

Antagonistic interactions between a bacteria and phytoplankton can be divided into two main categories: parasitic and pathogenic. Parasitic bacteria live off their algal host, negatively affecting the health or fecundity of the alga without causing death, thereby ensuring their own survival. Parasites have been recognized as important drivers of macroalgal population dynamics (for example, ~10% of red algae are parasitic to other red algae (Hancock *et al.*, 2010)), but little investigation into microalgal parasites has taken place. Instead, studies have focused primarily on pathogenic interactions resulting in the lysis and death of the algal host, which will be the focus of the remainder of this work as well.

1.2.1. Traits enhancing roseobacter pathogenesis

Gram-negative bacteria, like the roseobacter clade, have evolved a sophisticated cell envelope made up of two concentric membranes composed of a complex array of integral and peripheral proteins separated by the periplasmic space (PS). The outer membrane (OM) is an asymmetric bilayer comprised of an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharides intercalated with transmembrane proteins (Silhavy *et al.*, 2010). Next, there is a thin layer of ridged peptidoglycan and an array of polysaccharides within the periplasmic space, adding structure and stability to the membranes (Chang *et al.*, 2014). Finally, the cytoplasmic or inner membrane (IM) is a phospholipid bi-layer intercalated with proteins, which contains the cellular material (Silhavy *et al.*, 2010). For the bacterium, the primary function of these membranes is to act as a highly selective permeability barrier, ensuring orchestrated diffusion and/or transport of nutrients into and wastes out of the cell. Several pathogenic strategies depend on the successful transport of proteins (virulence factors, bioactive molecules, etc.) across these concentric bacterial envelopes, thereby permitting chemical interaction with nearby or adjacent cells. To this end, pathogens have evolved a variety of multifaceted protein secretion systems to facilitate these complex (potentially cross-kingdom) pathogenic interactions.

Bacterial secretion systems can be differentiated based on the number of membranes the transferred substrate crosses: one membrane, two membranes (both bacterial), or three membranes (where two are bacterial and one is the membrane of the target cell) (Chang *et al.*, 2014) (Table A-1). The architecture of these secretion systems can range from as simple as three interacting proteins to multicomponent systems containing over 20 distinct protein subunits that

span from the cytoplasm of the bacterial cell to the cytoplasm of the target cell (crossing 3 membranes) (Gunasinghe *et al.*, 2017). Distinct among these secretion systems are the type III, type IV, and type VI secretion systems (T3SS, T4SS, and T6SS, respectively) that are capable of transferring proteins, and in the case of T4SS, protein-DNA complexes, directly into target cells (Christie, 2001; Russell *et al.*, 2014; Buttner, 2016).

1.2.2. Targeted substrate secretion into cells

A wide range of Gram-negative symbionts and pathogens utilize T3SS to inject effector proteins directly into the cytoplasm of eukaryotic target cells (Persson *et al.*, 2009). Both intracellular pathogens (residing in a vacuole) and extracellular pathogens utilize a T3SS to interact directly with host cell (animal or plant) (Table A-1). For example, during *Pseudomonas syringae* infection of apple and pear trees, the bacteria utilizes T3SS to transfer effector proteins that localize to the chloroplasts suppressing photosynthesis, thereby causing fireblight disease (Li *et al.*, 2014). In another T3SS mediated infection strategy *P. syringae* injects cysteine protease effectors into host plant cells where they target and cleave integral plant proteins, eventually inducing plant cell death (Shao, 2003).

The T6SS is one of the most recently identified secretion systems (Pukatzki *et al.*, 2006). So far, it has been identified that T6SS can translocate proteins into adjacent prokaryotic cells, eukaryotic cells (Pukatzki *et al.*, 2007), or into the extracellular milieu (Si *et al.*, 2017). For example, the human pathogen *Vibrio cholera*, utilizes T6SS during competition (i.e., killing off bacterial competitors within the human gut) (MacIntyre *et al.*, 2010). Additionally, *V. cholera* relies on T6SS for pathogenic infection of phagocytic eukaryotes (such as murine macrophages and *Dictyostelium discoideum*) (Pukatzki *et al.*, 2006). The remarkable flexibility of T6SS is mirrored in the even more widespread and exceptionally versatile T4SS (Christie *et al.*, 2014).

As opposed to other secretion systems, which are dedicated to the secretion of certain types of substrates (Table A-1), T4SSs are promiscuous transporters, transferring an array of substrates including: monomeric proteins, multimeric toxic proteins, ssDNA-protein complexes, and even plasmids (Wallden *et al.*, 2010). T4SSs are so multipurpose that they are further subdivided into three subfamilies: 1) conjugation machines, 2) DNA release/uptake systems, and 3) effector translocator systems (Table A-1). The main T4SS subfamily is comprised of T4SSs dedicated to conjugal DNA transfers (Christie, 2001). Conjugation T4SS play the critical role of shaping bacterial genomes by facilitating plasmid conjugation into bacteria of the same bacterial species,

across species (Christie *et al.*, 2014), or even across genera (Patzelt *et al.*, 2016). The DNA release/uptake systems are less widespread contact independent systems that release or take up DNA into/from the extracellular milieu. DNA release/uptake T4SS are present in specialized naturally competent pathogens and, like conjugal T4SSs, this system also increases both intra and inter-species horizontal gene transfer (HGT).

Finally, some pathogens have adapted T4SS systems for the purpose of delivering substrates from the bacterial cell directly into eukaryotic cells (effector T4SS). Typically, effector T4SSs deliver protein substrates directly through the pilus unit into eukaryotic cells, making these systems critical to cross-kingdom pathogenesis. Effector T4SSs are further subdivided (based on protein homology) into two main subgroups (T4SSA or T4SSB) (Table 1.1), depending on whether the structural components resemble the VirB/D4 complex of the plant pathogen *Agrobacterium tumefaciens* (T4SSA) or the conjugal transfer system of the IncI plasmid (T4SSB) (Voth *et al.*, 2012). The T4SSB type system have extensive similarity to the conjugal transfer system of the self-transmissible IncI plasmid (Voth *et al.*, 2012). In fact, the T4SSB of *Legionella pneumophila* even retained its ability to transfer plasmids (Hubber *et al.*, 2010).

Pathogens using an effector T4SSA, which are structurally similar to the prototypical *A. tumefaciens* VirB/D4 system, on the other hand, are much more widespread. The diversity of effector T4SSAs is so great that even bacteria sharing the basic *virB/virD* gene organization, can have distinct differences in required genes and/or final number of required proteins in their T4SSA apparatus. For instance, the *Bordetlla pertussis* causes whooping cough by releasing T4SSA toxins directly into the extracellular milieu without requiring a functional pilus unit (Table 1.1). Similarly, *Brucella* spp. T4SSA lacks two proteins that are typically required for other T4SSA systems, and yet this pathogen infects several mammalian host macrophages causing brucellosis (Table 1.1). Even so, the majority of T4SSA containing pathogens have homologous proteins to all the prototypically required proteins of *A. tumefaciens* VirB/D4 type T4SSA (VirB1-VirB11 and VirD4) and share a similar final structure of their T4SSA apparatus (Figure 1.3). Because of this, and due to the protein similarities further discussed in Chapter 4, the *A. tumefaciens* VirB/D4 type T4SSA will be used as a comparative model when discussing the less comprehensively understood roseobacter T4SS.

Table 1.1. **Effector T4SS subclasses and key examples of pathogens relying on T4SS directed excretion of effectors for pathogenesis.**

Type	Name	Known effector(s)	Pathogen	Destination of secreted effectors and associated disease(s)
T4SSA	VirB/ D4	ssT-DNA/VirD2, VirE2, VirE3, VirF, VirD5	*Agrobacterium tumefaciens*	Plant root cells; causes crown gall tumors (Zechner *et al.*, 2012)
	Ptl	Pertussis toxin	*Bordetella pertussis*	Extracellular milieu of ciliated epithelial lung cells; causes whooping cough (Locht *et al.*, 2011)
	Cag	CagA, VacA cytotoxin	*Helicobacter pylori*	Gastric epithelial cells; causes gastritis and ulcers (Fischer, 2011)
	Trw	7 Bep proteins	*Bartonella* spp.	Human endothelial cells; causes cat scratch disease (Schulein *et al.*, 2005)
	Trw	15 proteins	*Brucella spp.*	Host (i.e., cattle, swine, humans) macrophages, causes brucellosis (Ke *et al.*, 2015)
T4SSB	Dot/ Icm	~300 proteins and transfers IncQ plasmid DNA	*Legionella pneumophila*†	Location: within host alveolar macrophages (Secreted into alveolar macrophages) Dot/Icm facilitates intracellular replication (causes Legionnaires disease) (Hubber *et al.*, 2010; Nagai *et al.*, 2011)
	Dot/ Icm	~100 proteins	*Coxiella burnetii*†	Location: within an acidified vacuole derived from host lysosomes (Secreted into cytosol of host lysosomes) Dot/Icm facilitates intracellular replication (causes Q fever) (Newton *et al.*, 2014).

†intracellular pathogen

1.2.3. *Agrobacterium tumefaciens* model effector T4SSA

A. tumefaciens T4SSA is organized in two operons on the tumour inducing plasmid (Ti-plasmid) and once constructed it spans from the bacterial inner membrane (IM), crosses the periplasmic space (PS), and passes through the bacterial outer membrane (OM) (Juhas *et al.*, 2008; Christie *et al.*, 2014) (Figure 1.3). A functional VirB/VirD4 T4SSA consist of 11 genes *virB2-virB11* and *virD4*. These genes encode for proteins that facilitate the construction of and structurally form the T4SSA apparatus (Figure 1.3). VirB1 is a periplasmic protein, that is critical to the assembly and biogenesis of the T4SSA, additionally VirB1, a lytic transglycosylase, locally lyses the bacterial peptidoglycan to allow insertion of bulky T4SSA proteins into the membrane (Zupan *et al.*, 2007). VirB3, is an inner membrane protein that is proposed to aid VirB1 in pilus assembly (Mossey *et al.*, 2010). Starting from the bacterial inner membrane: three ATPases (VirB4, VirB11 and VirD4) transect the inner membrane, 14 copies each of VirB7, VirB9, and VirB10 together with VirB8 form a complex channel that spans the PS (Christie *et al.*, 2014). Finally, the extracellular pilus, formed by VirB2 and the pilus-tip adhesin protein VirB5, extends through the OM and out from the bacterial cell into the target cell membrane (Christie *et al.*, 2014).

The *Agrobacterium*—plant interaction is a complex process involving bacterial effector proteins and DNA/protein complexes as well as host proteins. The soil pathogen *A. tumefaciens*, relies on a functional VirB/D4 T4SSA to facilitate the translocation of a segment of the bacterial Ti-plasmid or single stranded transfer-DNA (ssT-DNA), along with several effector proteins, into root nodule cells of the host plants (Vergunst, 2000). The portion of bacterial ssT-DNA is excised between a specific border of repeats and a single VirD2 protein associates with its 5' end (Păcurar *et al.*, 2011). Then the ssT-DNA/VirD2 complex is translocated to the ATPase VirD4, which recruits it into the T4SS channel (Wallden *et al.*, 2010). Once in the plant cell, the bacterial ssT-DNA/VirD2 complex is coated with VirE2 (ssDNA-binding protein), which is independently transferred into the plants cell via the same T4SSA (Păcurar *et al.*, 2011). VirE2 protects the DNA from being degraded by host nucleases and (along with VirD2) translocate the DNA into the plant nucleus (Gelvin, 1998). Besides VirD2 and VirE2, additional Vir proteins are also imported into host cells via the T4SSA, including: VirD5, VirE, and VirF (Table 1.1). A critical step in this process is the VirF directed proteolysis of VirE2, which releases the ssT-DNA allowing it to be integrated into the host chromosomal DNA (Tzfira *et al.*, 2004).

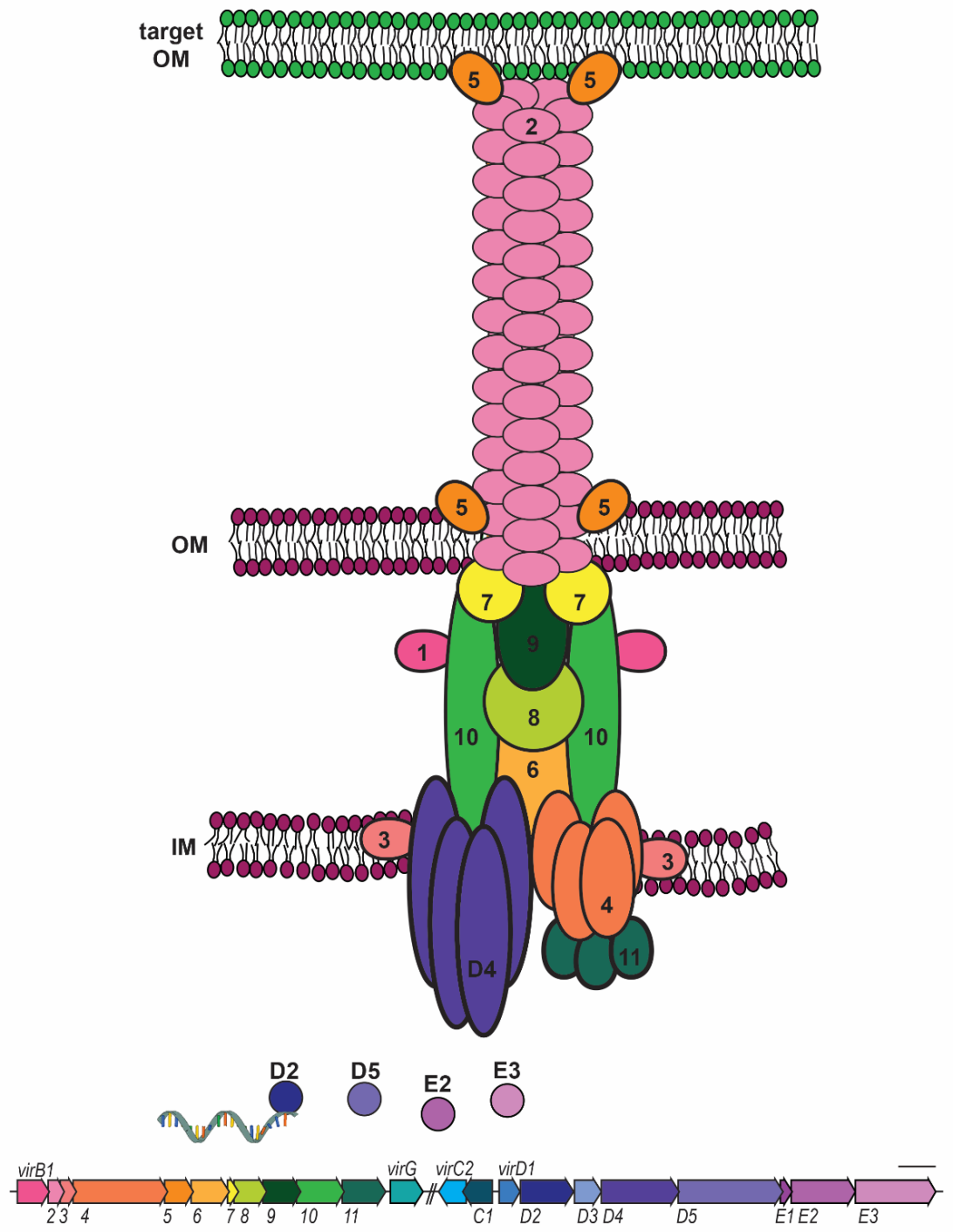

Figure 1.3. **Schematic of the *A. tumefaciens* VirB/D4 T4SSA components determined to date.** VirB/D4 protien subunits, color coded to match the genes encoding them (genetic layout of *A. tumefaciens* operons shown below for context: *virB1-virB11, virG, virC2, virC1, virD1-virD5, virE1-virE3*). This representation was created using the combined findings of (Juhas *et al.*, 2008; Christie *et al.*, 2014). Encoded T4SSA effectors (VirD2, VirD5, VirE2, and VirE3), drawn as circles. Scale bar shows the length of 900 bp.

There are two general classes of genes encoded on the segment of transferred bacterial T-DNA: opine-related genes and oncogenes. Opine-related genes induce host directed production of specialized plant amino acids or opines (i.e., octopine, agropine, etc. (Escobar *et al.*, 2003)). *A. tumefaciens* also possesses Ti-plasmid encoded genes for opine uptake and catabolism, in this way the bacteria hijacks the plant cells to provide a growth substrate that preferentially benefits *A. tumefaciens* cells (Escobar *et al.*, 2003). Oncogenes, on the other hand are responsible for inducing production of various enzymes that catalyze the synthesis of plant hormones (i.e., indole-3-acetic acid (IAA)), which stimulates root cell growth resulting in gall formation (Pitzschke *et al.*, 2010).

1.2.4. Roseobacter secretion systems in pathogenesis

Roseobacters are versatile marine bacteria with an array of bacterial secretion systems, several of which might facilitate pathogenic (or symbiotic) interactions with algal hosts, but this research is still in its infancy. To date, roseobacter secretion systems have not been directly connected with roseobacter pathogenesis, but their likely importance in the roseobacter pathogenesis is impossible to ignore. For instance, roseobacter *R. pomeroyi* DSS-3, along with 11 other roseobacters (Christie-Oleza *et al.*, 2012), all utilize T1SS to secrete repeat-in-toxin (RTX) proteins (Christie-Oleza *et al.*, 2010), which are potentially toxic to nearby algae. T2SS transporters, which are key to *Vibrio* pathogenesis (Sikora, 2013), are not commonly found in roseobacters (Slightom *et al.*, 2009). Generally, T3SS transporters are common in gammaproteobacteria, but are far less frequent in alphaproteobacteria, like members of the roseobacter clade (Persson *et al.*, 2009). *Silicibacter* TM1040, a roseobacter isolated from a culture of the *Pfiestera piscicida*, contains both T3SS and T4SS biosynthesis pathways (Worden *et al.*, 2006), but these systems have yet to be associated with pathogenesis of its dinoflagellate host. Similarly, *Dinoroseobacter shibae*, is also a pathogen of its dinoflagellate host *Prorocentrum minimum* (Wang *et al.*, 2015), but T4SS-dependent pathogenesis was not demonstrated, instead *D. shibae*'s apparatus appears to be a conjugation T4SS (Patzelt *et al.*, 2016). Finally, T6SS genes were estimated to be in roughly 31% of the genome sequenced marine species, but were more prevalent in gammaproteobacteria than among the sequenced roseobacters at the time (Persson *et al.*, 2009). Interestingly, T6SS are notably overrepresented in roseobacters identified within the accessory nidamental gland (ANG) of a bobtail squid, compared to roseobacters identified in other environments (Collins *et al.*, 2015). Given the wide

array of likely important roseobacter secretion systems it is perhaps surprising that these systems have not yet been linked directly to roseobacter pathogenesis, highlighting a large area of research that requires more investigation.

1.3. Coccolithophores

Coccolithophores are an ideal microalga for investigating bacterial-algal interactions for several reasons. First, coccolithophores annually form massive recurrent (almost annually) globally distributed blooms, occurring in upwelling regions and the open-ocean (Tyrrell *et al.*, 2004; Signorini *et al.*, 2009; Winter *et al.*, 2014). Additionally, coccolithophore blooms are easily identified and visible from space (Holligan *et al.*, 1993; Tyrrell *et al.*, 2009), as the light scattering properties of their ornate calcite coverings or coccoliths turn the ocean a bright milky-turquoise blue (Poulton *et al.*, 2011). Finally, coccolithophore blooms have a notably strong co-occurrence with various members of the roseobacter clade (González *et al.*, 2000; Zubkov *et al.*, 2001; Segev *et al.*, 2016). In fact, two roseobacters have already been shown to interact directly with the most globally dominant coccolithophore in modern oceans: *Emiliania huxleyi* (Mayers *et al.*, 2016; Segev *et al.*, 2016).

1.3.1. Emiliania huxleyi

Emiliania huxleyi (Lohmann) Hay and Mohler (Prymnesiophyceae, Haptophyta) (Hay *et al.*, 1967), is widely considered a model coccolithophore for several reasons, the first being that it is by far the most ubiquitous coccolithophore species in modern oceans (Young, Geisen, *et al.*, 2003). *E. huxleyi* also has a pronounced ability to adapt to a wide range of environments and to form blooms in a number of environments from eutrophic temperate regions, to subpolar waters, and even the subtropical gyres (Monteiro *et al.*, 2016). This wide distribution has been predominantly attributed to *E. huxleyi*'s extensive genetic (Read *et al.*, 2013) and ecophenotypic plasticity (Young, Geisen, *et al.*, 2003; Langer *et al.*, 2009). *E. huxleyi* is a vast species complex with a pan genome, meaning various strains share a set of core genes and additional set variable genes, which enhances its pronounced intraspecies variability (Read *et al.*, 2013). This genetic plasticity enhances *E. huxleyi*'s niche partitioning and the variation between strains and even cell types (von Dassow *et al.*, 2014). Interestingly, *E. huxleyi* is the only coccolithophore with calcifying cells that regularly overproduce and shed coccoliths (Paasche, 2002), making it of particular interest in terms of the marine carbon cycle (Suchéras-Marx *et al.*, 2014). In short, *E.*

huxleyi is an exemplary model system and has been used in a wide array of scientific studies, including those focusing on potential *E. huxleyi* pathogens (bacterial and viral) (Frada *et al.*, 2008; Seyedsayamdost *et al.*, 2011).

E. huxleyi exhibits a haplo-diplontic life cycle, during which cells alternate between calcifying diploid (2N) cells and flagellated haploid (1N) cells (Klaveness, 1972a). The third cell type appears to be a variant cell that originates from calcifying diploid cells, but has lost the ability to produce coccoliths: non-calcifying diploid (2N) (Klaveness, 1972a). Coccolithophore blooms (defined as marine waters having $>10^6$ cells/mL coccolithophores (Tyrrell *et al.*, 2004)) are typically dominated by the fast growing diploid calcifying cells (Baumann *et al.*, 2008). However, several coccolithophore species (e.g., *Coccolithus pelagicus* (Poulton *et al.*, 2010)) as well as *E. huxleyi's* non-calcifying flagellated haploid and non-calcifying diploid cell types are also present within *E. huxleyi* blooms (Frada *et al.*, 2012). Blooms of calcified cells remain in the upper ocean for several weeks; during which time some cells die as a result of cellular damage, gradual senescence, and microzooplankton grazing (Harvey *et al.*, 2015). Eventually the remaining calcified cells undergo rapid bloom collapse event, during which the majority of the cells rapidly die. Rapid collapse of the calcifying *E. huxleyi* population has been primarily attributed to lytic double-stranded-DNA containing coccolithoviruses called *E. huxleyi* viruses (EhVs) (Bratbak *et al.*, 1993; Brussaard *et al.*, 1996; Vardi *et al.*, 2012), which hijack host metabolic pathways and induce activation of host programmed cell death (PCD) pathways (Bidle *et al.*, 2007; Rosenwasser *et al.*, 2014; Schatz *et al.*, 2014).

1.3.2. Programmed cell death of phytoplankton

PCD is the genetically programmed process of self-deconstruction and is both regulated and implemented by cellular proteases. Apoptotic-PCD (or apoptosis (Kerr *et al.*, 1972)) is defined as having 1) a strict reliance on the biochemical activity of highly specific metazoan cysteine aspartic proteases or caspases and 2) conserved death morphologies (cell shrinkage, chromatin condensation, nuclear blebbing, apoptotic bodies, etc.) (Lawen, 2003; Kroemer *et al.*, 2009). Caspases are synthesized as innocuous zymogens, but once activated they rapidly cleave substrate proteins containing the corresponding cleavage motif (4-5 amino acid motif) (Stennicke *et al.*, 1999). The identification of caspase-like cleavage of peptides (i.e., cleavage at DEVD, caspase-3-like; cleavage at IETD, caspase-8-like, etc.) in organisms lacking caspases, led to a new PCD classification: apoptosis-like-PCD (AL-PCD) (Danon *et al.*, 2000).

AL-PCD is defined as, an organism dying with (some but not necessarily all) apoptosis morphologies, but lacking the hallmark caspases that are required for metazoan apoptosis (Danon *et al.*, 2000). Instead, AL-PCD relies on the cleavage activity of either distantly related metacaspases or caspase-like proteases to orchestrate and execute AL-PCD (Danon *et al.*, 2000). These caspase-like proteases have sometimes evolved to either cleave substrate proteins at a similar cleavage motif to caspases (Coffeen *et al.*, 2004; Woltering, 2004; Xu *et al.*, 2009) or target substrate proteins with similar biological activities to those commonly targeted by caspases (Madeo *et al.*, 2002; Sundström *et al.*, 2009). In plant systems, a few caspase-like proteases have been identified; for instance, vacuolar processing enzyme (VPE) (Hatsugai *et al.*, 2015) and some serine proteases (i.e., saspases, phytaspases (Vartapetian *et al.*, 2011)), can cleave substrates at a caspase-like cleavage motifs.

This overlap in targeted substrate proteins, has led to some overlapping morphologies and physiologies in organisms undergoing apoptosis and those dying via AL-PCD. However, some death morphologies and physiologies required of metazoan apoptosis are typically (or always) absent in plants or phytoplankton dying via AL-PCD (Table A-2, and sources therein). For instance, late-stage membrane blebbing into apoptotic bodies is considered one of the hallmark required morphologies of organisms dying via apoptosis (Kerr *et al.*, 1972), but is not a common feature of plants undergoing AL-PCD (Fath *et al.*, 2000; Reape, Molony, *et al.*, 2008). Similarly, late-stage laddering of DNA, which occurs non-uniformly as a result of metazoan apoptosis (Oberhammer *et al.*, 1993; Kroemer *et al.*, 2009), is not considered a required marker for plant AL-PCD (Danon *et al.*, 2000). Additionally, (apart from the characterized metacaspases (Bidle *et al.*, 2007)) the identities of the genes responsible for these caspase-like activities in phytoplankton are still largely unknown (Bidle, 2016), which greatly limits understanding and prediction of likely AL-PCD death morphologies. Nonetheless, AL-PCD requires caspase-like activities, so biochemical inhibition of the proteases responsible for the caspase-like activities should inhibit and abolish AL-PCD (Franklin *et al.*, 2006).

Another destruction mechanism, which can be induced instead of or during apoptosis, is the process of autophagy. Autophagy is traditionally defined as a cellular survival mechanism without dependence on caspase involvement (Jones, 2000; Wyllie *et al.*, 2001). Additionally, autophagy is engaged to enhance survival during periods of starvation (Avila-Ospina *et al.*, 2014), recycle damaged cellular components (Ishida *et al.*, 2014), and can play a protective role

by degrading intracellular pathogens and bacterial virulence factors (Cemma *et al.*, 2012). In terms of its role in PCD, autophagy describes the mechanism of removing cellular constituents, and as such can occur in conjunction with caspase-dependent apoptosis or caspase-independent PCD (Kroemer *et al.*, 2009). Instead of caspases, autophagy requires early-stage lysosome vesicles, which engulf cytoplasmic constituents or fuse with organelles to break down cellular components (Bursch *et al.*, 2000; Yu *et al.*, 2004). Alternatively, proteins and bacterial virulence factors can be tagged for degradation by the ubiquitin proteasome system and directed to either the proteasome or autophagasome for degradation (Zhang *et al.*, 2005). In fact, in some organisms these complex processes are intertwined, such that if apoptosis fails, then autophagy is engaged to dismantle the cell (Shimizu *et al.*, 2004; Yu *et al.*, 2004).

Several unicellular phytoplankton lineages lacking caspases, undergo versions of either caspase-like AL-PCD or autophagy-PCD (e.g., cyanobacteria (Berman-Frank *et al.*, 2004), diatoms (Berges *et al.*, 1998), dinoflagellates (Franklin *et al.*, 2004), chlorophytes (Segovia *et al.*, 2003), and haptophytes (Bidle *et al.*, 2007; Schatz *et al.*, 2014)). The widespread existence of both AL-PCD and autophagy processes in several phytoplankton lineages highlights just how important understanding the mechanisms of activating these death processes might be for better understanding phytoplankton turnover and DOM release (Thornton, 2014; Bidle, 2016).

1.3.3. EhV-induced PCD of *E. huxleyi*

Highly specific EhVs are members of *Phycodnaviridae*, a group of giant dsDNA viruses (~407-kb genomes) (Wilson *et al.*, 2015), that infect the dominant diploid calcifying and diploid non-calcifying *E. huxleyi* cells types, while the haploid escapes viral lysis (Bidle *et al.*, 2007, 2012). EhVs utilize host-specific glycosphingolipid (hGSL) lipid rafts, which extend from the algal membrane into the extracellular environment, for entering and exiting the algal cell by budding through the algal lipid rafts (Mackinder *et al.*, 2009; Bidle, 2015). During viral infection, EhVs, which possess a suite of glycosphingolipid (GSL) biosynthetic genes in their genome (Wilson *et al.*, 2015), rewire host lipid metabolism pathways to generate viral GSLs (vGSLs) (Rosenwasser *et al.*, 2014). Initially, vGSLs induce intracellular ROS production in infected *E. huxleyi*, which might be critical in the propagation of the PCD process (Bidle *et al.*, 2011). Then other PCD hallmarks such as increasing caspase-like activities and upregulation of metacaspase genes (Bidle *et al.*, 2007), arise in infected cells and infected diploid cells die via either AL-PCD or autophagy (Vardi *et al.*, 2009, 2012).

Today, EhVs are considered the major pathogens responsible for decimating *E. huxleyi* blooms (Vardi *et al.*, 2012); but viral resistance is presently on the rise in both diploid calcifying and non-calcifying *E. huxleyi* (Kendrick *et al.*, 2014). And *E. huxleyi* resistance to viral pathogens increases dramatically with slight elevations in temperature, suggesting that as ocean temperatures increase so will the rates of viral resistance (Kendrick *et al.*, 2014). This potential loss of a vital *E. huxleyi* pathogen might mean that new pathogens could begin to play a more important role in natural bloom scenarios. Recently, studies have directed more attention to the bacteria frequently associated with *E. huxleyi* blooms (González *et al.*, 2000; Green *et al.*, 2015; Ramanan *et al.*, 2016), and it is becoming clear, that some members of the roseobacter clade are opportunistic pathogens of *E. huxleyi* (Mayers *et al.*, 2016; Segev *et al.*, 2016).

1.4. *Phaeobacter-E. huxleyi* interactions

This thesis work aims to identify how a model roseobacter from the *Phaeobacter* genus, called *Phaeobacter inhibens*, interacts with the microalga *E. huxleyi.* The genus *Phaeobacter* was first introduced by Martens *et al.* 2006, and was chosen for studying bacterial-*E. huxleyi* interactions for several reasons. First, *Phaeobacter* strains are widespread in marine environments, they have been isolated from ocean water (Park *et al.*, 2015), marine sediments (Zhang *et al.*, 2008), as well as the surface of the green alga *Ulva australis* (Rao *et al.*, 2007) and the scallop *Pecten maximus* (Buddruhs *et al.*, 2013). Secondly, *Phaeobacters* have also been frequently identified within *E. huxleyi* blooms (Segev *et al.*, 2016) and non-axenic culture collections of calcifying *E. huxleyi* (Green *et al.*, 2015). Phenotypically this genus has several of the previously discussed roseobacter traits thought to enhance interactions with algal hosts (i.e., motility, chemotaxis, DMSP metabolism, rapid attachment, etc. (Moran *et al.*, 2003, 2012). Additionally, they readily attach to other *P. inhibens* cells at the poles, forming star-like multicellular structures called rosettes (Frank *et al.*, 2015; Segev *et al.*, 2015; Michael *et al.*, 2016). Finally, and of importance to the current work, *P. inhibens* DSM17395, hereafter called *P. inhibens*, is one of the only roseobacters that has been successfully genetically manipulated to generate an extensive transposon mutant library (Wetmore *et al.*, 2015).

1.4.1. Identified and proposed *Phaeobacter-E. huxleyi* interactions

Certain roseobacters, including *P. inhibens*, are deadly pathogens of *E. huxleyi* (Mayers *et al.*, 2016; Segev *et al.*, 2016). *P. inhibens* has a novel interaction with the coccolithophore *E.*

huxleyi. Initially, *P. inhibens* quickly attaches to the alga and directly benefits from metabolizing the algal metabolite DMSP (Segev *et al.*, 2016) (Figure 1.4). However, as the algal cells enter gradual senescence *E. huxleyi* cells release a chemical senescent cue (degradation product of a cell wall component) called *p*-coumaric acid (*p*CA) (Seyedsayamdost *et al.*, 2011). *P. inhibens* responds to algal *p*CA by switching from a symbiont to a deadly pathogen of *E. huxleyi* and producing algicidal bioactives called roseobacticides (Seyedsayamdost *et al.*, 2011). Previous work has demonstrated that purified roseobacticides kill one representative non-calcifying diploid *E. huxleyi* strain (Seyedsayamdost *et al.*, 2011) (Figure 1.4c). Additionally, *P. inhibens* produces bioactives called roseochelins, which kill a representative type of non-calcifying diploid cells as well as one calcifying diploid *E. huxleyi* strain (Wang *et al.*, 2017). Both bioactives have since been proposed to be the killing molecules responsible for this interaction, but neither suggestion has been conclusively demonstrated (Figure 1.4c).
Finally, another group suggested that bacterial production of the plant hormone IAA might kill *E. huxleyi* cells (Segev *et al.*, 2016) (Figure 1.4d). IAA is produced by several roseobacters (Fernandes *et al.*, 2011), including *P. inhibens*, and can kill CCMP3266 at high concentrations (1,000 μM) (Labeeuw *et al.*, 2016). However, the precursor to IAA, tryptophan, was also found to be lethal at such a high concentration (Labeeuw *et al.*, 2016). Additionally, the lethal IAA concentration reported by Segev *et al.* is much higher than the concentration bacteria are known to produce (~0.4-10 nM) (2016). Finally, this group tested several mutants in the IAA pathway and all of them remained virulent, despite the mutation interrupting the synthesis of IAA (Segev *et al.*, 2016). The authors suggest that there are several pathways to synthesize IAA (Segev *et al.*, 2016), but I propose instead that perhaps another bacterial virulence factor or bioactive molecule might be critically important for pathogenesis of *E. huxleyi*.

To date, the mechanism of *P. inhibens* mediated killing of calcifying *E. huxleyi* remains unresolved and the involvement of the bacterially produced roseobacticides, roseochelins, and/or IAA in this process remains hypothetical (Seyedsayamdost *et al.*, 2011; Segev *et al.*, 2016; Wang *et al.*, 2017). Furthermore, the possible interaction between *P. inhibens* and the flagellated haploid cell type has never been assessed. Surprisingly, a related roseobacter was recently shown to cause activation of algal caspase-like molecules (Mayers *et al.*, 2016). In light of this discovery, I propose that perhaps *P. inhibens* utilizes a novel mechanism of pathogenesis, during which the pathogen induces *E. huxleyi* AL-PCD.

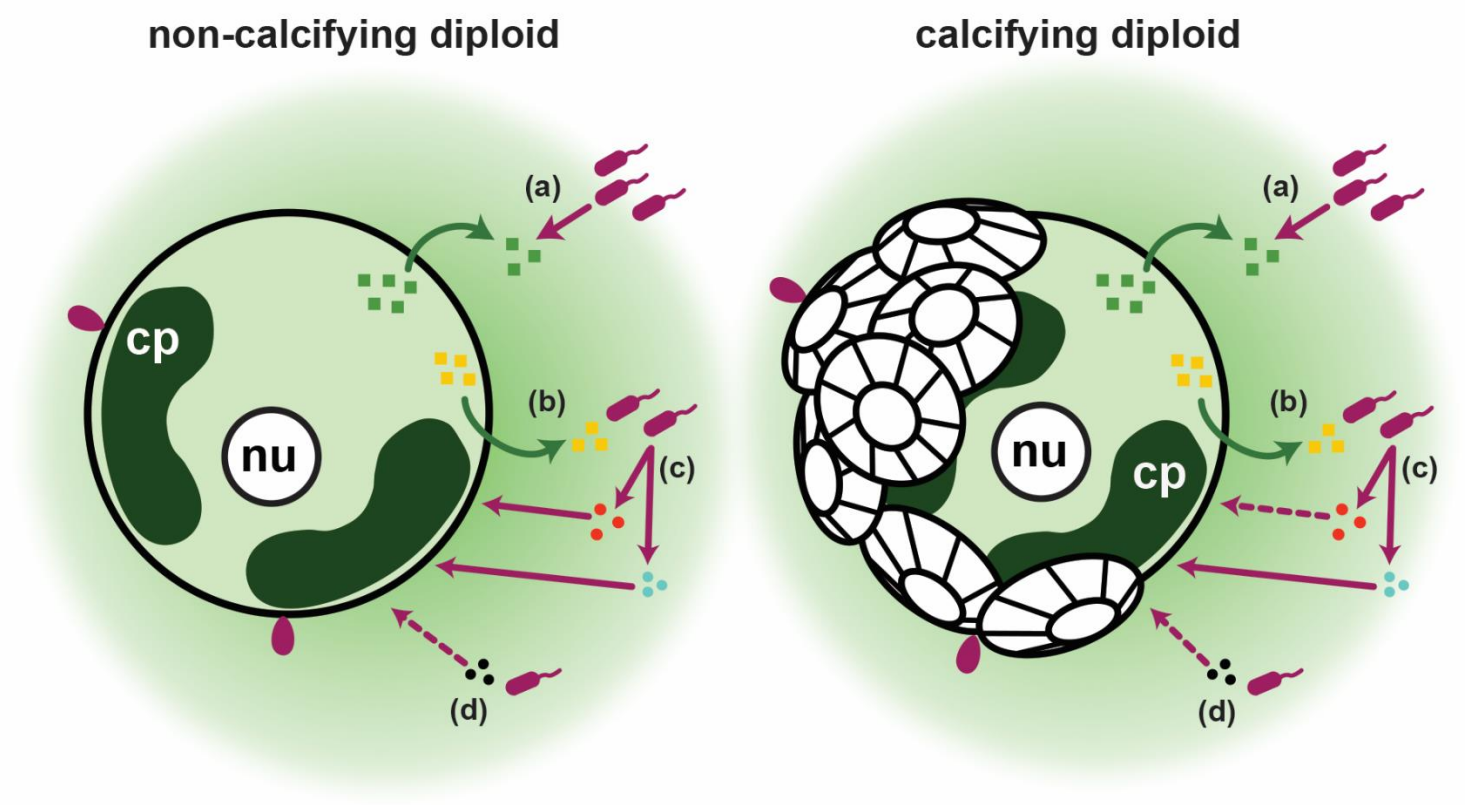

Figure 1.4. ***Phaeobacter-E. huxleyi* interactions mediated by the exchange of bioactive molecules within the algal phycosphere.** Known (solid arrows) and suspected (dashed arrows) interactions between *P. inhibens* and *E. huxleyi* non-calcifying cell (left) and *E. huxleyi* calcifying cell (right): (a) *P. inhibens* chemotaxing toward DSMP (green squares), (b) aging algae release the senescent cue pCA (yellow squares), pCA induces production of roseobacticides (red circles). Purified roseobacticides kill specific non-calcifying *E. huxleyi* strains (Seyedsayamdost et al., 2011). (c) *P. inhibens* produces algicidal roseochelins (blue circles) in response to synapic acid, purified roseochelins kill respresentative strains of both diploid cell types (Wang et al., 2017). (d) IAA (black circles) was proposed (without experimental evidence) to kill both cell types (Segev et al., 2016). Haploid motile *E. huxleyi* cells are not shown as interactions between *P. inhibens* and haploid cells have not yet been examined.

1.4.2. Proposed *P. inhibens* induction of *E. huxleyi* AL-PCD

A highly sophisticated method of bacterial pathogenesis is to hijack control of the host cell. The pathogen can then benefit from prolonging host health (while slowly draining nutrients) or benefit instantly by killing the host cell quickly. One-way bacterial pathogens do this is by translocating effector molecules that trigger the hosts' genetically programed, biochemically orchestrated PCD pathway (Gao *et al.*, 2000). This mechanism of pathogenesis is a very effective way of gaining control of the host cell and has been demonstrated for mammalian pathogens (Amieva *et al.*, 2008), but our understanding how this process may work in plants or unicellular algae is still in its infancy.

1.4.2.1. *Looking to other systems for insights about haptophyte AL-PCD*

Before comparing the processes of pathogen induced PCD of a unicellular phytoplankton to pathogen induced PCD of an animal or plant cell, it is first important to briefly evaluate the phylogenetic relationship of phytoplankton to animals and plants. Eukaryotic algae are a diverse polyphyletic group with a complex and highly contested phylogeny, complicated by several endosymbiosis acquisition events and losses of plastids (Keeling, 2013). In brief, the first endosymbiosis event involved a mitochondrion containing eukaryote engulfing a coccoid cyanobacterial cell, which retained the ability to perform oxygenic photosynthesis and became the membrane bound organelle known as a plastid (Gray *et al.*, 1982). The primary plastid containing alga led to the lineages of glaucophytes, red algae, and green algae (which later evolved into plants), all part of the supergroup Archeaplastida (Keeling, 2013). During the secondary endosymbiosis events, different eukaryotes engulfed a primary plastid containing alga (this occurred twice: one green and one red alga), leading to the production of other lineages containing either a green plastid (i.e., euglenids) or a red plastid (i.e., haptophytes, cyptomonads, SAR (stramenopiles, alveolates, and Rhizaria)) (Keeling, 2013). The precise phylogenetic relationship of haptophytes and cryptophytes to the other eukaryote supergroups (Archaeplastida, Excavata, SAR, and Unikonts) remains a matter of scientific debate (Keeling, 2013; Derelle *et al.*, 2015; Burki, 2016). This lack of understanding is important to note here, because during the discussion of coccolithophore (haptophyte) AL-PCD, it is necessary to compare this uncharacterized system to better investigated systems such as bacterial induction of apoptosis in mammalian cells and bacterial induction of AL-PCD in plant cells. Phylogenetically speaking,

haptophytes are not closely related to animals (Unikonts), plants (green algae), or algae (red algae or glaucophytes), making cross-kingdom comparisons more relevant (Burki, 2016).

1.4.2.2. *Mammalian control of PCD and how pathogens hijack that pathway*

Mammalian PCD is highly controlled by the biochemical specificity of the responsible proteases. These proteases are synthesized in the cell as inactive precursors (or pro-caspase) of the active enzyme (or caspase) (Stennicke *et al.*, 1999). When inactive they are innocuous in the cell, but once activated these caspases rapidly propagate the activation of other caspases, triggering a cascade-like process that commits the host cell to rapid death (Stennicke *et al.*, 1999). Commandeering the highly controlled process of activating inactive caspases is therefore one way in which pathogens can manipulate host cells (Hilbi *et al.*, 1998; Hersh *et al.*, 1999), triggering host directed apoptotic-PCD (apoptosis).

Pathogens have a plethora of strategies for infecting and killing host cells, some of which enable them to directly control the fate of a host cell by hijacking the PCD process at several steps along the caspase cascade (Figure 1.5). However, the first hurdle to overcome for any pathogen is gaining access to the host cell. There are two main ways for pathogens to gain access to a eukaryotic host: external access and internal access. External access to a host cell is commonly achieved by: 1) breaking down the eukaryotic cell wall or membrane (frequent strategy of pytopathogens (Chang *et al.*, 2014)), 2) translocating pore forming proteins (Podack *et al.*, 2016), or 3) using (T3SS, T4SS, or T6SS) to directly translocate effectors into the host cell (Green *et al.*, 2016). Alternatively, the microbe can become engulfed and survive intracellularly to directly manipulate the host cell from within (Isberg *et al.*, 2009). Specialized pathogens with either T3SS or T4SSs are adept at translocating effector molecules or proteins from the bacterial cytoplasm into the adjoining eukaryotic cell. For instance, *Shigella flexneri* and *Salmonella typhimurium* utilize a T3SS to inject bacterial proteins into the host macrophage, which can directly activate specific caspases (Hilbi *et al.*, 1998; Hersh *et al.*, 1999) (i.e., caspase-1) (Figure 1.5b). A similar mechanism is used by various *Yersinia* species that use T3SS to inject several proteins that can both activate host caspases and inhibit host survival pathways, resulting in apoptosis (Bleves *et al.*, 2000) (Figure 1.5a,b). Intracellular pathogen *Helicobacter pylori*, relies on T4SS to translocate VacA, which pops holes in the mitochondria causing release of cytochrome *c* (cyt *c*), which induces apoptosis (Amieva *et al.*, 2008) ((Figure 1.5f). *Legionella pneumophila*, causative agent of Legionnaires' disease, delivers over 200 T4SSB effectors into

human monocytes and alveolar macrophages, several of which directly manipulate host cell death pathways (Hubber *et al.*, 2010). For instance, at least five of them can interrupt protein synthesis in the endoplasmic reticulum (ER) thereby triggering apoptosis (Hubber *et al.*, 2010). Similarly, enteric pathogens *S. dysenteriae* and *Escherichia coli*, release Shiga toxins that inhibit ER protein synthesis, which triggers the ER to release Ca^{2+} stores; the release of Ca^{2+}then induces and propagates apoptosis of the host mammalian cell (Lee *et al.*, 2008) ((Figure 1.5g).

1.4.2.3. *Plant cell AL-PCD and how pathogens might hijack that pathway*

Plant AL-PCD is similar to apoptosis in several ways, but AL-PCD lacks the hallmark characteristic of apoptosis: dependence on caspases. As in apoptosis, the enzymes responsible for AL-PCD (metacaspases and/or caspase-like proteases) are synthesized in an innocuous form and activated by various death stimuli. Once active caspase-like proteases propagate activation of other proteases committing the cell to AL-PCD (Danon *et al.*, 2000). However, a cascade-like process of activation, reminiscent of caspase cascade, has yet to be elucidated in plants (Vartapetian *et al.*, 2011).

Plants are continuously challenged by pathogens. The bacterial ability to infect plant cells frequently depends on bacterial secretion systems, specifically T3SS, in order to inhibit host cell wall defenses (i.e., stop the host from closing stoma to impede the entrance of pathogens), while also injecting various adhesins, toxins, and degradative enzymes into the host (Abramovitch *et al.*, 2004). *P. syringae* injects two T3SS effectors, both of which suppress host immunity and inhibit plant AL-PCD (Abramovitch *et al.*, 2004), enabling the pathogen to delay host detection and establish infection (Figure 1.6a). Specialized bacterial virulence factors or effector molecules can initiate host AL-PCD pathways by prematurely activating caspase-like enzymes, thereby inducing host directed AL-PCD. For instance, *Erwinia amylovora* injects T3SS effectors that either directly activate plant VPEs (likely stored in vacuoles, vesicles, or chloroplasts (Ambastha *et al.*, 2015)) or induce ROS in the cell, both of which cause AL-PCD of apple leaf cells (Iakimova *et al.*, 2013) (Figure 1.6b). In plant systems, attempted infection by avirulent bacteria can lead to the PCD of the plant cell. This phenomenon is due to the host defense mechanism of inducing an oxidative burst at the site of infection (Levine *et al.*, 1996). This burst, and subsequent accumulation of ROS, induces an influx of Ca^{2+} at the infection site, which propagates AL-PCD (Levine *et al.*, 1996). *Cochliobolus victoriae* secretes a host-selective toxin that triggers an oxidative burst in the mitochondria, resulting localized ROS and AL-PCD

morphologies and death of oat leaf cells (Yao *et al.*, 2002) (Figure 1.6c). Unlike metazoans, plants have a second ROS-generating organelle (the chloroplasts), which have also been associated with triggering AL-PCD, though bacterial initiation of the process remains hypothetical (Figure 1.6e). Similarly, the plant ER is another potential source of Ca^{2+} which has been associated with propagating AL-PCD in plant cells (Martínez-Fábregas *et al.*, 2014), though again bacterial involvement in such processes has not yet been established (Figure 1.6).

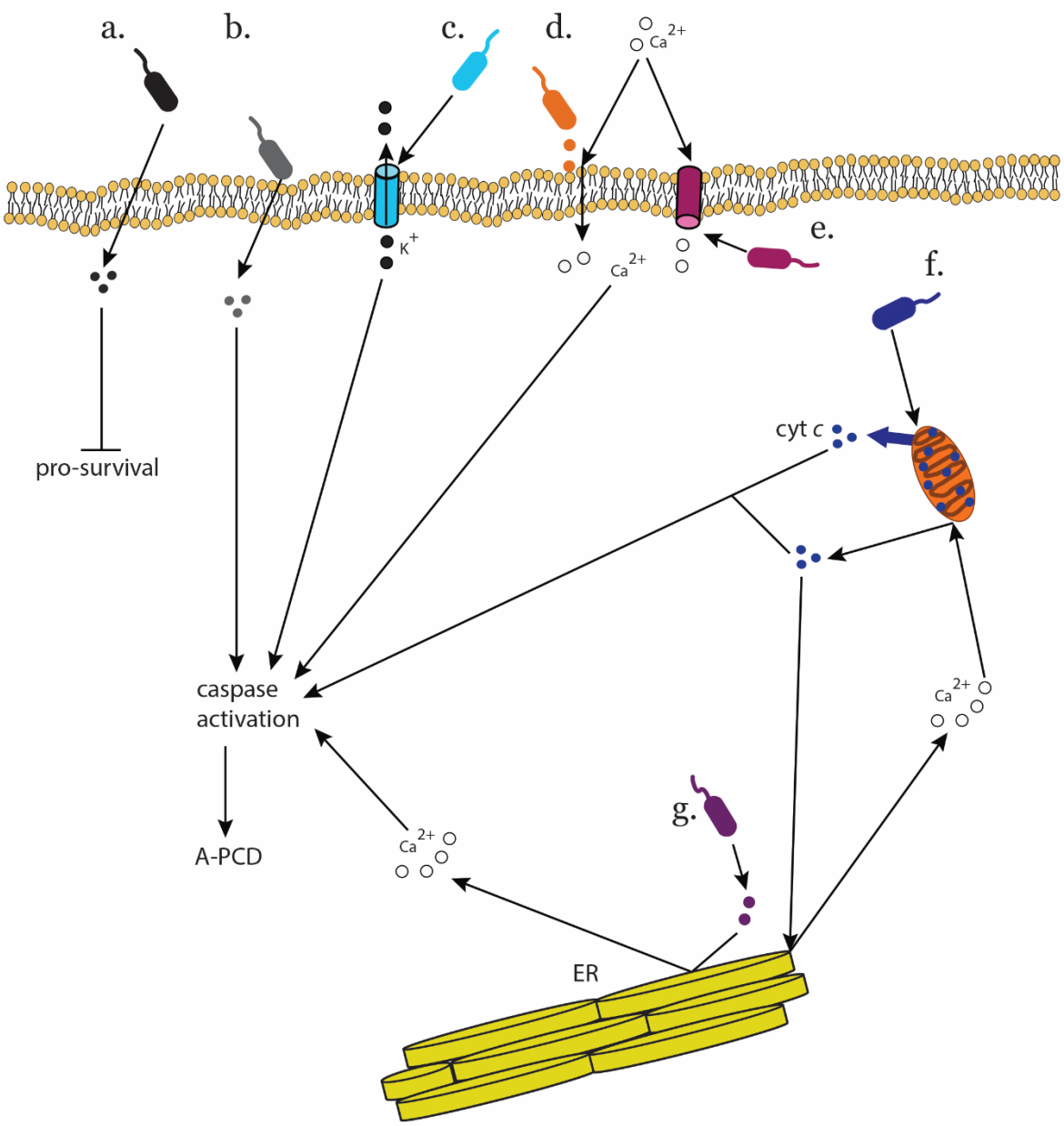

Figure 1.5. **Bacterial induction of apoptosis in mammalian cells**. (a) *Yersinia* T3SS effectors inhibit host pro-survival pathways, (b) *S. flexneri*, *S. typhimur*, and others inject T3SS effector proteins that activate specific caspases (Hilbi *et al.*, 1998; Hersh *et al.*, 1999). (c) *S. aureus* secretes pore forming toxins that cause efflux of K^+ from the cell (Kloft *et al.*, 2009), while other toxins (d) cause influx of Ca^{2+}, both of which activate caspases (Kwak *et al.*, 2012). (e) Intracellular, *Neisseria* translocates a pore forming protein from the bacterial membrane into the membrane of the host cell, causing Ca^{2+} influx (Mu *et al.*, 1999). (f) Several pathogens target the mitochondria, releaseing cyt *c* (blue circles) to induce apoptosis (Jan *et al.*, 2008), and some pathogens (g) target other organelles such as the ER, causing a stress response that inhibits protien synthesis and triggers apoptosis (Isberg *et al.*, 2009). ER stress response also releases Ca^{2+}, which directly activates apoptosis and propogates apoptotic response by releasing cyt c from the mitochondria (Rutkowski *et al.*, 2004), conversley cyt *c* also causes ER disfunction.

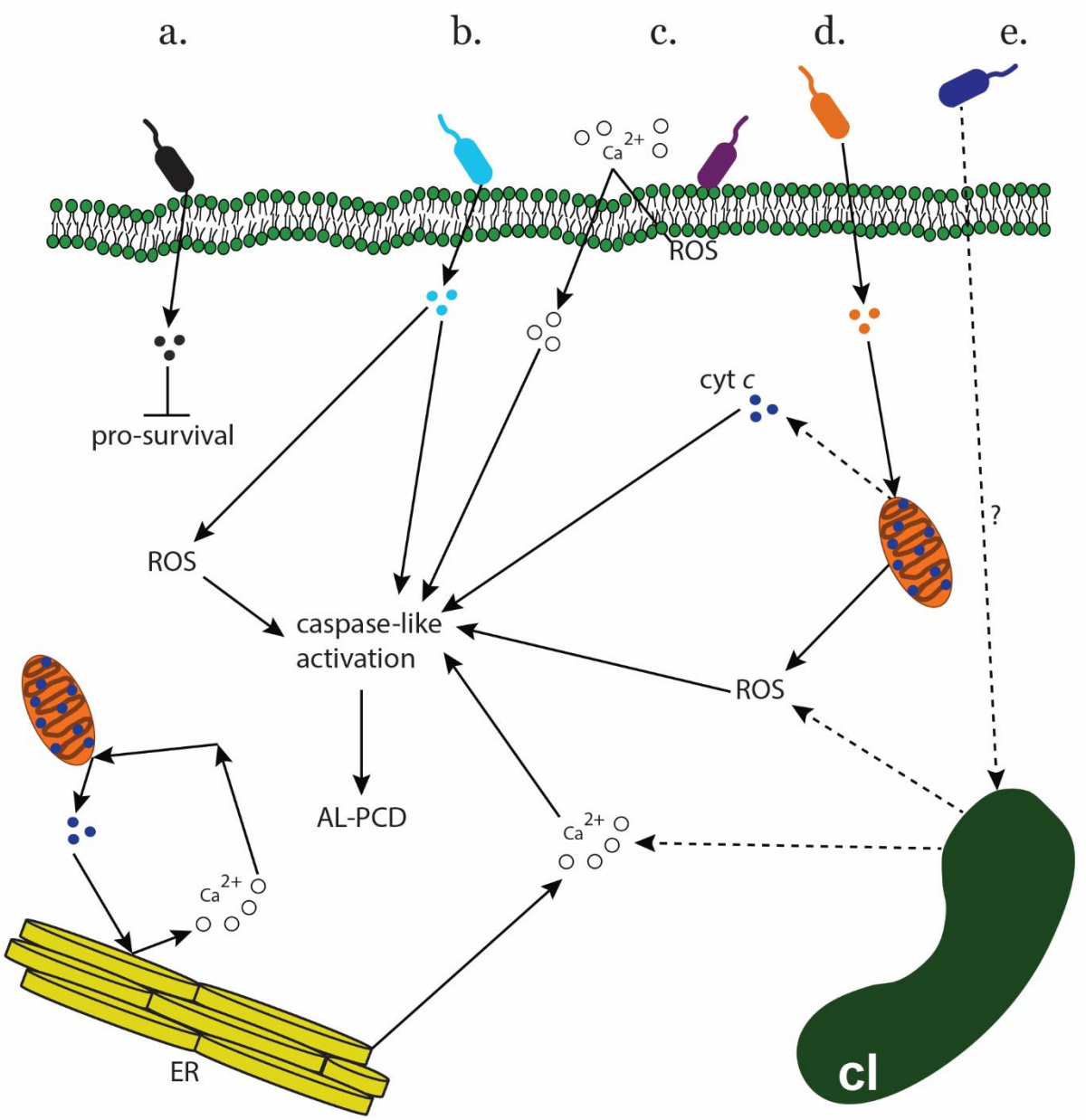

Figure 1.6. **Bacterial induction of apoptosis-like PCD in plant cells**. (a) *P. syringae* injects two known T3SS effectors, both of which suppress host immunity and inhibit plant AL-PCD, (b) *E. amylovora* uses a T3SS to inject effector proteins that either directly activate caspase-like molecules (i.e., VPEs) or induce ROS accumulation (Iakimova *et al.*, 2013), (c) host defense oxidative bust at the site of infection, causes an accumulation of ROS that induces an influx of Ca^{2+} at the infection site that can induce AL-PCD (Levine *et al.*, 1996), (d) *Cochliobolus victoriae* secretes a host-selective toxin that triggers an oxidative bust of the mitochondria, resulting localized ROS and AL-PCD (Yao *et al.*, 2002), (e) chloroplasts induce AL-PCD by releasing ROS, Ca^{2+}, proteases, and bioactives (Ambastha *et al.*, 2015), but pathogen involvement has not yet been established. Likewise, plant ER have been shown to release Ca^{2+}, which propogates AL-PCD (Martínez-Fábregas *et al.*, 2014), but again bacterial involvement is not known.

1.5. Thesis objectives

The aim of this thesis is to zoom in on the microscale interactions occurring between two main organisms commonly identified together in the marine environment: the dominant calcifying microalga *E. huxleyi* and the roseobacter *P. inhibens*. By zooming in on the interactions occurring between these two organisms in a controlled laboratory setting I hope to elucidate the mechanism involved in *P. inhibens* pathogenesis of the dominant calcifying microalga *E. huxleyi*.

1.5.1. Hypotheses

Based on current understanding of the interactions occurring between *P. inhibens* and *E. huxleyi* cell types (summarized in Figure 1.4 for diploid non-calcifying and calcifying cell types), I developed my specific hypotheses.

1) I hypothesized that the algicidal roseobacticides produced by *P. inhibens* were responsible for *E. huxleyi* cell death (Chapter 3).

2) I hypothesized that *P. inhibens* might hijack and/or induce apoptosis-like programmed cell death (AL-PCD) in the calcifying microalga *E. huxleyi* (Chapter 4).

3) Finally, I hypothesized that if two closely related *P. inhibens* strains presented differential pathogenesis of *E. huxleyi*, then a required virulence factor or secretion system might absent in the avirulent strain (Chapter 5).

1.5.2. Thesis outline

Chapter 2, will present a novel miniaturized bioassay to assess phytoplankton physiology when an alga is grown either alone, with a bacterium of interest, or is exposed to a bioactive molecule. This miniaturized format dramatically increases the number of possible independent replicates and eliminates the need for subsampling. This chapter also tests the miniaturized growth format on the dominant calcifying microalga *E. huxleyi*, to ensure this alga can grow for an extended period of time (60 d) in the small volume (1 mL) microtiter plate format.

Chapter 3 investigates the interaction between roseobacter *P. inhibens* and each of the three cell types of its algal host *E. huxleyi* (calcifying diploid, non-calcifying diploid, and flagellated haploid). *P. inhibens* demonstrates highly specific pathogenesis, killing both the calcifying diploid strain and the flagellated haploid strain of *E. huxleyi*, but not the tested diploid non-calcifying strains. The bacterial production of known algicides (roseobacticides) was postulated

to be responsible for this pathogenic interaction. However, Chapter 3 examines this possibility and establishes that roseobacticides are not the bioactives responsible for *P. inhibens* pathogenesis.

Chapter 4 establishes that the bacterial pathogen *P. inhibens* induces AL-PCD of the calcifying *E. huxleyi* strain CCMP3266. Chapter 4 relies on a series of AL-PCD morphological and biochemical features to confirm that calcifying *E. huxleyi* is undergoing AL-PCD instead of another algal death process.

Chapter 5 investigates a frequent marine pathogen strategy involving the use of a T4SS to damage or kill eukaryotic host cells. To test if *P. inhibens* DSM17395 employs this cross-kingdom pathogenesis strategy during pathogenesis of calcifying *E. huxleyi*, I tested a closely related *P. inhibens* strain DSM24588, that shares >97% genome similarity to *P. inhibens* DSM17395, but lacks a functional T4SS, in co-culture with the calcifying alga *E. huxleyi.* Only one strain, *P. inhibens* DSM17395, containing a fully functional copy of the T4SS, killed the calcified alga. Chapter 5 next tested *P. inhibens* DSM17395 transposon mutants in genes within the *virB/virD* T4SS operons in co-culture with the calcifying alga *E. huxleyi*.

Finally, Chapter 6 concludes by discussing the possible ecological importance of bacterial induced *E. huxleyi* population demise. This chapter also highlights the gaps of knowledge this thesis has filled by presenting an updated model of *P. inhibens*-*E. huxleyi* interactions.

1.6. Works Cited

Abramovitch, R. B. and Martin, G. B. (2004) 'Strategies used by bacterial pathogens to suppress plant defenses', *Current Opinion in Plant Biology*, 7(4), pp. 356–364. doi: 10.1016/j.pbi.2004.05.002.

Ambastha, V., Tripathy, B. C. and Tiwari, B. S. (2015) 'Programmed Cell Death in Plants: a chloroplastic connection', *Plant Signaling & Behavior*, 10(2), p. e989752. doi: 10.4161/15592324.2014.989752.

Amieva, M. R. and El-Omar, E. M. (2008) 'Host-bacterial interactions in *Helicobacter pylori* infection', *Gastroenterology*. doi: 10.1053/j.gastro.2007.11.009.

Avila-Ospina, L., Moison, M., Yoshimoto, K. and Masclaux-Daubresse, C. (2014) 'Autophagy, Plant senescence, and nutrient recycling', *Journal of Experimental Botany*, 65(14), pp. 3799–3811. doi: 10.1093/jxb/eru039.

Azam, F. (1998) 'Microbial Control of oceanic carbon flux: the plot thickens', *Science*, (280), pp. 694–696.

Azam, F. and Malfatti, F. (2007) 'Microbial structuring of marine ecosystems', *Nature reviews. Microbiology*, 5(10), pp. 782–791. doi: 10.1038/nrmicro1798.

Baumann, K., Boeckel, B. and Čepek, M. (2008) 'Spatial Distribution of living coccolithophores along an East-West transect in the subtropical South Atlantic', *Journal of Nannoplankton Research*, 30(1), pp. 9–21.

Bell, W. H. (1983) 'Bacterial utilization of algal extracellular products. 3. The specificity of algal-bacterial interaction', *Limnology and Oceanography*, 28(6), pp. 1131–1143. doi: 10.4319/lo.1983.28.6.1131.

Bentzon-Tilia, M. and Gram, L. (2017) 'Biotechnological applications of the roseobacter clade', in Paterson, R. and Lima, N. (eds) *Bioprospecting: Success, Potential and Constraints*. Cham: Springer International Publishing, pp. 137–166. doi: 10.1007/978-3-319-47935-4_7.

Berges, J. A. and Falkowski, P. G. (1998) 'Physiological Stress and cell death in marine phytoplankton: induction of proteases in response to nitrogen or light limitation', *Limnology and Oceanography*, 43(1), pp. 129–135. doi: 10.4319/lo.1998.43.1.0129.

Berman-Frank, I., Bidle, K. D., Haramaty, L. and Falkowski, P. G. (2004) 'The Demise of the marine cyanobacterium, *Trichodesmium* Spp., Via An Autocatalyzed Cell Death Pathway', *Limnology and Oceanography*, 49(4), pp. 997–1005. doi: 10.4319/lo.2004.49.4.0997.

Bidle, K. D. (2015) 'The molecular ecophysiology of Programmed Cell Death in marine phytoplankton', *Annual Review of Marine Science*, 7, p. 24.1–24.35. doi: 10.1146/annurev-marine-010213-135014.

Bidle, K. D. (2016) 'Programmed Cell Death in unicellular phytoplankton', *Current Biology*. Elsevier Ltd, 26(13), pp. R594–R607. doi: 10.1016/j.cub.2016.05.056.

Bidle, K. D., Haramaty, L., Barcelos E Ramos, J. and Falkowski, P. (2007) 'Viral Activation And Recruitment Of Metacaspases In The Unicellular Coccolithophore, *Emiliania Huxleyi*', *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), pp. 6049–6054. doi: 10.1073/pnas.0701240104.

Bidle, K. D. and Kwityn, C. J. (2012) 'Assessing The role of caspase activity and metacaspase expression on viral susceptibility of the coccolithophore, *Emiliania Huxleyi* (Haptophyta).', *Journal of Phycology*, 48(5), pp. 1079–1089. doi: 10.1111/j.1529-8817.2012.01209.x.

Bidle, K. D. and Vardi, A. (2011) 'A Chemical arms race at sea mediates algal host-virus interactions', *Current opinion in microbiology*. Elsevier Ltd, 14(4), pp. 449–57. doi: 10.1016/j.mib.2011.07.013.

Bleves, S. and Cornelis, G. R. (2000) 'How to survive in the host: the *Yersinia* lesson', *Microbes and Infection*, (2), pp. 1451–1560.

Borchard, C. and Engel, A. (2012) 'Organic matter exudation by *Emiliania huxleyi* under simulated future ocean conditions', *Biogeosciences*, 9(8), pp. 3405–3423. doi: 10.5194/bg-9-3405-2012.

Bratbak, G., Egge, J. K. and Heldal, M. (1993) 'Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms', *Marine Ecology Progress Series*, 93(1–2), pp. 39–48. doi: 10.3354/meps093039.

Brinkhoff, T., Bach, G., Heidorn, T., Liang, L., Schlingloff, A. and Simon, M. (2004) 'Antibiotic Production by a roseobacter clade-affiliated species from the German Wadden Sea and its antagonistic effects on indigenous isolates', *Applied and Environmental Microbiology*, 70(4), pp. 2560–2565. doi: 10.1128/AEM.70.4.2560-2565.2003.

Brinkhoff, T., Giebel, H.-A. and Simon, M. (2008) 'Diversity, ecology, and genomics of the roseobacter clade: A short overview', *Archives of microbiology*, 189(6), pp. 531–9. doi: 10.1007/s00203-008-0353-y.

Brussaard, C. P. D., Kempers, R. S., Kop, A. J., Riegman, R. and Heldal, M. (1996) 'Virus-like Particles in a summer bloom of *Emiliania huxleyi* in the North Sea', *Aquatic Microbial Ecology*, 10, pp. 105–113.

Buchan, A., González, J. M., Moran, M. A. and Gonza, M. (2005) 'Overview Of the marine roseobacter lineage', *Society*, 71(10). doi: 10.1128/AEM.71.10.5665.

Buchan, A., LeCleir, G. R., Gulvik, C. a and González, J. M. (2014) 'Master Recyclers: Features and functions of bacteria associated with phytoplankton blooms', *Nat Rev Microbiol*. Nature Publishing Group, 12(August), pp. 686–698. doi: 10.1038/nrmicro3326.

Buddruhs, N., Chertkov, O., Petersen, J., Fiebig, A., Chen, A., Pati, A., Ivanova, N., Lapidus, A., Goodwin, L. A., Chain, P., *et al.* (2013) 'Complete genome sequence of the marine methyl-halide oxidizing *Leisingera methylohalidivorans* type strain (DSM 14336T), a representative of the roseobacter clade', *Standards in Genomic Sciences*, 9(1), pp. 128–141. doi: 10.4056/sigs.4297965.

Burki, F. (2016) 'The Eukaryotic Tree of life from a global phylogenomic perspective', *Cold Spring Harbor perspectives in biology*, pp. 1–18. doi: 10.1101/cshperspect.a016147.

Bursch, W., Ellinger, a, Gerner, C., Fröhwein, U. and Schulte-Hermann, R. (2000) 'Programmed Cell Death (PCD). Apoptosis, Autophagic PCD, or others?', *Annals of the New York Academy of Sciences*, 926, pp. 1–12. doi: 10.1111/j.1749-6632.2000.tb05594.x.

Buttner, D. (2016) 'Behind the lines-actions of bacterial Type III effector proteins in plant cells', *FEMS Microbiology Reviews*, 40(6), pp. 894–937. doi: 10.1093/femsre/fuw026.

Cemma, M. and Brumell, J. H. (2012) 'Interactions Of pathogenic bacteria with autophagy systems', *Current biology : CB*. Elsevier, 22(13), pp. R540-5. doi: 10.1016/j.cub.2012.06.001.

Chang, J. H., Desveaux, D. and Creason, A. L. (2014) 'The ABCs and 123s of bacterial secretion systems in plant pathogenesis', *Annual Review of Phytopathology*. doi: 10.1146/annurev-phyto-011014-015624.

Christie-Oleza, J. A. and Armengaud, J. (2010) 'In-depth Analysis Of Exoproteomes From Marine Bacteria By Shotgun Liquid Chromatography-tandem Mass Spectrometry: The Ruegeria Pomeroyi DSS-3 Case-study', *Marine Drugs*. doi: 10.3390/md8082223.
Christie-Oleza, J. A., Pina-Villalonga, J. M., Bosch, R., Nogales, B. and Armengaud, J. (2012) 'Comparative Proteogenomics Of Twelve Roseobacter Exoproteomes Reveals Different Adaptive Strategies Among These Marine Bacteria', *Molecular & Cellular Proteomics*. doi: 10.1074/mcp.M111.013110.
Christie, P. J. (2001) 'Type IV Secretion: Intercellular Transfer Of Macromolecules By Systems Ancestrally Related To Conjugation Machines', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2001.02302.x.
Christie, P. J., Whitaker, N. and González-Rivera, C. (2014) 'Mechanism And Structure Of The Bacterial Type IV Secretion Systems', *Biochimica et Biophysica Acta - Molecular Cell Research*. doi: 10.1016/j.bbamcr.2013.12.019.
Coffeen, W. C. and Wolpert, T. J. (2004) 'Purification And Characterization Of Serine Proteases That Exhibit Caspase-Like Activity And Are Associated With Programmed Cell Death In Avena Sativa', *The Plant Cell Online*, 16(4), pp. 857–873. doi: 10.1105/tpc.017947.
Cole, J. J. (1982) 'Interactions Between Bacteria And Algae In Aquatic Ecosystems', *Annual review of ecological systems*, (13), pp. 291–314.
Collins, A. J., Fullmer, M. S., Gogarten, J. P. and Nyholm, S. V. (2015) 'Comparative Genomics Of Roseobacter Clade Bacteria Isolated From The Accessory Nidamental Gland Of *Euprymna Scolopes*', *Frontiers in Microbiology*, 6(FEB), pp. 1–14. doi: 10.3389/fmicb.2015.00123.
Costello, M. J., Bouchet, P., Boxshall, G., Fauchald, K., Gordon, D., Hoeksema, B. W., Poore, G. C. B., van Soest, R. W. M., Stöhr, S., Walter, T. C., *et al.* (2013) 'Global Coordination And Standardisation In Marine Biodiversity Through The World Register Of Marine Species (WoRMS) And Related Databases', *PLoS ONE*, 8(1). doi: 10.1371/journal.pone.0051629.
Croft, M. T., Warren, M. J. and Smith, A. G. (2006) 'Algae Need Their Vitamins', *Eukaryotic Cell*, 5(8), pp. 1175–1183. doi: 10.1128/EC.00097-06.
D'Alvise, P. W., Lillebø, S., Prol-Garcia, M. J., Wergeland, H. I., Nielsen, K. F., Bergh, Ø. and Gram, L. (2012) '*Phaeobacter Gallaeciensis* Reduces Vibrio Anguillarum In Cultures Of Microalgae And Rotifers, And Prevents Vibriosis In Cod Larvae', *PLoS ONE*, 7(8). doi: 10.1371/journal.pone.0043996.
Dang, H. and Lovell, C. R. (2000) 'Bacterial Primary Colonization And Early Succession On Surfaces In Marine Waters As Determined By Amplified RRNA Gene Restriction Analysis And Sequence Analysis Of 16S RRNA Genes', *Applied and Environmental Microbiology*, 66(2), pp. 467–475. doi: 10.1128/AEM.66.2.467-475.2000.
Danon, A., Delorme, V., Mailhac, N. and Gallois, P. (2000) 'Plant Programmed Cell Death: A Common Way To Die', *Plant Physiology and Biochemistry*, 38(9), pp. 647–655. doi: 10.1016/S0981-9428(00)01178-5.
von Dassow, P., John, U., Ogata, H., Probert, I., Bendif, E. M., Kegel, J. U., Audic, S., Wincker, P., Da Silva, C., Claverie, J.-M., *et al.* (2014) 'Life-cycle Modification In Open Oceans Accounts For Genome Variability In A Cosmopolitan Phytoplankton', *The ISME Journal*, 9(6), pp. 1–13. doi: 10.1038/ismej.2014.221.
Derelle, R., Torruella, G., Klimeš, V., Brinkmann, H., Kim, E., Vlček, Č., Lang, B. F. and Eliáš, M. (2015) 'Bacterial Proteins Pinpoint A Single Eukaryotic Root', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.1420657112.

Durham, B. P., Sharma, S., Luo, H., Smith, C. B., Amin, S. a, Bender, S. J., Dearth, S. P., Van Mooy, B. a S., Campagna, S. R., Kujawinski, E. B., *et al.* (2014) 'Cryptic Carbon And Sulfur Cycling Between Surface Ocean Plankton', *Proc Natl Acad Sci U S A*, in review(2), pp. 453–457. doi: 10.1073/pnas.1413137112.

Escobar, M. A. and Dandekar, A. M. (2003) 'Agrobacterium Tumefaciens As An Agent Of Disease', *Trends in Plant Science*. doi: 10.1016/S1360-1385(03)00162-6.

Fath, A., Bethke, P., Lonsdale, J., Meza-Romero, R. and Jones, R. (2000) 'Programmed Cell Death In Cereal Aleurone', *Plant Molecular Biology*, 44(3), pp. 255–266. doi: 10.1023/A:1026584207243.

Fernandes, N., Case, R. J., Longford, S. R., Seyedsayamdost, M. R., Steinberg, P. D., Kjelleberg, S. and Thomas, T. (2011) 'Genomes And Virulence Factors Of Novel Bacterial Pathogens Causing Bleaching Disease In The Marine Red Alga *Delisea Pulchra*', *PloS one*, 6(12), p. e27387. doi: 10.1371/journal.pone.0027387.

Fischer, W. (2011) 'Assembly And Molecular Mode Of Action Of The *Helicobacter pylori* Cag Type IV Secretion Apparatus', *FEBS Journal*. doi: 10.1111/j.1742-4658.2011.08036.x.

Frada, M. J., Bidle, K. D., Probert, I. and de Vargas, C. (2012) 'In Situ Survey Of Life Cycle Phases Of The Coccolithophore *Emiliania Huxleyi* (Haptophyta)', *Environmental Microbiology*, 14(6), pp. 1558–1569. doi: 10.1111/j.1462-2920.2012.02745.x.

Frada, M., Probert, I., Allen, M. J., Wilson, W. H. and de Vargas, C. (2008) 'The "Cheshire Cat" escape Strategy Of The Coccolithophore *Emiliania Huxleyi* In Response To Viral Infection.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(41), pp. 15944–15949. doi: 10.1073/pnas.0807707105.

Frank, O., Michael, V., Pauker, O., Boedeker, C., Jogler, C., Rohde, M. and Petersen, J. (2015) 'Plasmid Curing And The Loss Of Grip - The 65-kb Replicon Of *Phaeobacter Inhibens* DSM 17395 Is Required For Biofilm Formation, Motility And The Colonization Of Marine Algae', *Systematic and Applied Microbiology*. Elsevier GmbH., 38(2), pp. 120–127. doi: 10.1016/j.syapm.2014.12.001.

Franklin, D. J. and Berges, J. a (2004) 'Mortality In Cultures Of The Dinoflagellate Amphidinium Carterae During Culture Senescence And Darkness.', *Proceedings. Biological sciences / The Royal Society*, 271(1553), pp. 2099–107. doi: 10.1098/rspb.2004.2810.

Franklin, D. J., Brussaard, C. P. D. and Berges, J. a. (2006) 'What Is The Role And Nature Of Programmed Cell Death In Phytoplankton Ecology?', *European Journal of Phycology*, 41(2006), pp. 1–14. doi: 10.1080/09670260500505433.

Gao, L. Y. and Abu Kwaik, Y. (2000) 'Hijacking Of Apoptotic Pathways By Bacterial Pathogens', *Microbes and Infection*, 2(14), pp. 1705–1719. doi: 10.1016/S1286-4579(00)01326-5.

Gelvin, S. B. (1998) 'Agrobacterium VirE2 Proteins Can Form A Complex With T Strands In The Plant Cytoplasm', *Journal of Bacteriology*.

González, J. M., Simó, R., Massana, R., Covert, J. S., Casamayor, E. O., Pedrós-Alió, C. and Moran, M. a (2000) 'Bacterial Community Structure Associated With A Dimethylsulfoniopropionate-producing North Atlantic Algal Bloom.', *Applied and environmental microbiology*, 66(10), pp. 4237–46. doi: 10.1128/AEM.66.10.4237-4246.2000.

Gray, M. W. and Doolittle, W. F. (1982) 'Has The Endosymbiont Hypothesis Been Proven?', *Microbiological reviews*, 46(1), pp. 1–42.

Green, D. H., Echavarri-Bravo, V., Brennan, D. and Hart, M. C. (2015) 'Bacterial Diversity Associated With The Coccolithophorid Algae *Emiliania Huxleyi* And *Coccolithus Pelagicus F. Braarudii*', *BioMed Research International*, 2015. doi: 10.1155/2015/194540.

Green, E. R. and Mecsas, J. (2016) 'Bacterial Secretion Systems: An Overview', in *Virulence Mechanisms of Bacterial Pathogens, Fifth Edition*. doi: 10.1128/microbiolspec.VMBF-0012-2015.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P., Dourala, N., Nielsen, K. F. and Gram, L. (2015) 'Isolation Of TDA-producing *Phaeobacter* Strains From Sea Bass Larval Rearing Units And Their Probiotic Effect Against Pathogenic *Vibrio* Spp. In *Artemia* Cultures', *Systematic and Applied Microbiology*, 39, pp. 180–188. doi: 10.1016/j.syapm.2016.01.005.

Gunasinghe, S. D., Webb, C. T., Elgass, K. D., Hay, I. D. and Lithgow, T. (2017) 'Super-Resolution imaging of protein secretion systems and the cell surface of Gram-negative bacteria', *Frontiers in cellular and infection microbiology*. doi: 10.3389/fcimb.2017.00220.

Hancock, L., Goff, L. and Lane, C. (2010) 'Red Algae lose key mitochondrial genes in response to becoming parasitic', *Genome biology and evolution*, 2(September), pp. 897–910. doi: 10.1093/gbe/evq075.

Harvey, E. L., Bidle, K. D. and Johnson, M. D. (2015) 'Consequences Of strain variability and calcification in *Emiliania huxleyi* on microzooplankton grazing', *Journal of Plankton Research*, 0(0), pp. 1–12. doi: 10.1093/plankt/fbv081.

Hatsugai, N., Yamada, K., Goto-Yamada, S. and Hara-Nishimura, I. (2015) 'Vacuolar Processing enzyme in plant progammed cell death', *Frontiers in Plant Science*, 6(April), pp. 1–11. doi: 10.3389/fpls.2015.00234.

Hay, W. W., Mohler, H. P., Roth, P. H., Schmidt, R. R. and Boudreau, J. E. (1967) 'Calcareous Nannoplankton zonation of the Cenozoic of the Gulf Coast and Caribbean-Antillean area, and Transoceanic', *Planktonic zonation of tertiary*, XVII.

Hersh, D., Monack, D., Smith, M., Ghori, N., Falkw, S. and Sychlisnski, A. (1999) 'The *Salmonella invasin* SipB induces macrophage apoptosis by binding to Caspase-1', *Proc Natl Acad Sci U S A*, 96(March), pp. 2396–2401.

Hilbi, H., Moss, J. E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., Flavell, R. A., Yuan, J., Sansonetti, P. J. and Zychlinsky, A. (1998) '*Shigella*-induced apoptosis is dependent on Caspase-1 which binds to IpaB*', 273(49), pp. 32895–32900.

Holligan, P. M., Fernandez, E., Aiken, J., Balch, W. M., Boyd, P., Burkill, P., Finch, M., Groom, S., Malin, G., Muller, K., *et al.* (1993) 'A Biogeochemical study of the coccolithopore, *Emiliania huxleyi*, in the North Atlantic', 7(4), pp. 879–900.

Hubber, A. and Roy, C. R. (2010) 'Modulation of host cell function by *Legionella pneumophila* Type IV Effectors'. doi: 10.1146/annurev-cellbio-100109-104034.

Iakimova, E. T., Sobiczewski, P., Michalczuk, L., Wegrzynowicz-Lesiak, E., Mikiciński, A. and Woltering, E. J. (2013) 'Morphological and biochemical characterization of *Erwinia amylovora*-induced hypersensitive cell death in apple leaves', *Plant Physiology and Biochemistry*, 63, pp. 292–305. doi: 10.1016/j.plaphy.2012.12.006.

Isberg, R. R., O'Connor, T. J. and Heidtman, M. (2009) 'The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells', *Nature Reviews Microbiology*, 7(1), pp. 13–24. doi: 10.1038/nrmicro1967.

Ishida, H., Izumi, M., Wada, S. and Makino, A. (2014) 'Roles of autophagy in chloroplast recycling', *Biochimica et Biophysica Acta - Bioenergetics*. doi: 10.1016/j.bbabio.2013.11.009.

Jan, N., Hussain, M. and Andrabi, K. (2008) 'Programmed Cell Death or apoptosis: do animals and plants share anything in common', *Biotechnol Mol Biol Rev*, 3(5), pp. 111–126.
Jones, A. R. (2000) 'Does the plant mitochondrion integrate cellular stress and regulate Programmed Cell Death?', *Trends in Plant Science*, 5(5), pp. 225–230. doi: 10.1016/S1360-1385(00)01605-8.
Juhas, M., Crook, D. W. and Hood, D. W. (2008) 'Type IV Secretion Systems: Tools of bacterial horizontal gene transfer and virulence', *Cellular Microbiology*. doi: 10.1111/j.1462-5822.2008.01187.x.
Ke, Y., Wang, Y., Li, W. and Chen, Z. (2015) 'Type IV Secretion System of *Brucella* spp. and its effectors', *Front Cell Infect Microbiol*.
Keeling, P. J. (2013) 'The number, speed, and impact of plastid endosymbioses in Eukaryotic evolution', *Annual Review of Plant Biology*, 64(1), pp. 583–607. doi: 10.1146/annurev-arplant-050312-120144.
Kendrick, B. J., DiTullio, G. R., Cyronak, T. J., Fulton, J. M., Van Mooy, B. a. S. and Bidle, K. D. (2014) 'Temperature-induced viral resistance in *Emiliania huxleyi* (Prymnesiophyceae)', *PLoS ONE*, 9(11), p. e112134. doi: 10.1371/journal.pone.0112134.
Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) 'Apoptosis: A basic biological phenomenon with wide- ranging implications in tissue kinetics', *Journal of Internal Medicine*, 258(6), pp. 479–517. doi: 10.1111/j.1365-2796.2005.01570.x.
Klais, R., Norros, V., Lehtinen, S., Tamminen, T. and Olli, K. (2016) 'Community assembly and drivers of phytoplankton functional structure', *Functional Ecology*, pp. 760–767. doi: 10.1111/1365-2435.12784.
Klaveness, D. (1972) '*Coccolithus Huxleyi* (Lohm.) Kamptn II. The flagellate cell, aberrant cell types, vegetative propagation and life cycles', *British Phycological Journal*, 7(3), pp. 309–318. doi: 10.1080/00071617200650321.
Kloft, N., Busch, T., Neukirch, C., Weis, S., Boukhallouk, F., Bobkiewicz, W., Cibis, I., Bhakdi, S. and Husmann, M. (2009) 'Biochemical and biophysical research communications pore-forming toxins activate MAPK P38 by causing loss of cellular potassium', *Biochemical and Biophysical Research Communications*. Elsevier Inc., 385(4), pp. 503–506. doi: 10.1016/j.bbrc.2009.05.121.
Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E., Baehrecke, E., Blagosklonny, M., El-Deiry, W., Golstein, P., Green, D., *et al.* (2009) 'Classification of cell death: recommendations of the nomenclature committee on cell death 2009', *Cell death and differentiation*, 16(1), pp. 3–11. doi: 10.1038/cdd.2008.150.
Kwak, Y., Vikström, E., Magnusson, K., Vécsey-semjén, B., Colque-navarro, P. and Möllby, R. (2012) 'The *Staphylococcus aureus* alpha-toxin perturbs the barrier function in Caco-2 epithelial cell monolayers by altering junctional integrity', pp. 1670–1680. doi: 10.1128/IAI.00001-12.
Labeeuw, L., Khey, J., Bramucci, A. R., Atwal, H., De La Mata, A. P., Harynuk, J. and Case, R. J. (2016) 'Indole-3-acetic acid is produced by *Emiliania huxleyi* coccolith-bearing cells and triggers a physiological response in bald cells', *Frontiers in Microbiology*, 7(JUN). doi: 10.3389/fmicb.2016.00828.
Lampert, W. (1978) 'Release Of Dissolved Organic Carbon By Grazing Zooplankton', *Limnology and Oceanography*, 23(4), pp. 831–834. doi: 10.4319/lo.1978.23.4.0831.

Langer, G., Nehrke, G., Probert, I., Ly, J. and Ziveri, P. (2009) 'Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry', *Biogeosciences Discussions*, 6(2), pp. 4361–4383. doi: 10.5194/bgd-6-4361-2009.

Lawen, A. (2003) 'Apoptosis: An introduction', *BioEssays*, 25(9), pp. 888–896. doi: 10.1002/bies.10329.

Lee, S., Lee, M., Cherla, R. P. and Tesh, V. L. (2008) '*Shiga* toxin 1 induces apoptosis through the Endoplasmic Reticulum stress response in human monocytic cells', (2007), pp. 770–780. doi: 10.1111/j.1462-5822.2007.01083.x.

Levine, A., Pennell, R. I., Alvarez, M. E., Palmer, R. and Lamb, C. (1996) 'Calcium-mediated apoptosis in a plant hypersensitive disease resistance response', *Current biology : CB*, 6(4), pp. 427–437. doi: 10.1016/S0960-9822(02)00510-9.

Li, G., Froehlich, J. E., Elowsky, C., Msanne, J., Ostosh, A. C., Zhang, C., Awada, T. and Alfano, J. R. (2014) 'Distinct *Pseudomonas* Type-III Effectors use a cleavable transit peptide to target chloroplasts', *Plant Journal*. doi: 10.1111/tpj.12396.

Locht, C., Coutte, L. and Mielcarek, N. (2011) 'The ins and outs of *Pertussis* toxin', *Febs Journal*. doi: 10.1111/j.1742-4658.2011.08237.x.

Luo, H. and Moran, M. A. (2014) 'Evolutionary ecology of the marine roseobacter clade', *Microbiology and Molecular Biology Reviews*, 78(4), pp. 573–587. doi: 10.1128/MMBR.00020-14.

Luo, H., Swan, B. K., Stepanauskas, R., Hughes, A. L. and Moran, M. A. (2014) 'Comparing effective population sizes of dominant marine alphaproteobacteria lineages', *Environmental Microbiology Reports*, 6(2), pp. 167–172. doi: 10.1111/1758-2229.12129.

MacIntyre, D. L., Miyata, S. T., Kitaoka, M. and Pukatzki, S. (2010) 'The *Vibrio cholerae* Type VI Secretion System displays antimicrobial properties', *Proc. Natl. Acad. Sci. USA*. doi: 10.1073/pnas.1012931107.

Mackinder, L. C. M., Worthy, C. A., Biggi, G., Hall, M., Ryan, K. P., Varsani, A., Harper, G. M., Wilson, W. H., Brownlee, C. and Schroeder, D. C. (2009) 'A unicellular algal virus, *Emiliania huxleyi* virus 86, exploits an animal-like infection strategy', *Journal of General Virology*. doi: 10.1099/vir.0.011635-0.

Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S., *et al.* (2002) 'A caspase-related protease regulates apoptosis in yeast', *Molecular Cell*, 9(4), pp. 911–917. doi: 10.1016/S1097-2765(02)00501-4.

Martínez-Fábregas, J., Díaz-Moreno, I., González-Arzola, K., Díaz-Quintana, A. and De la Rosa, M. A. (2014) 'A common signalosome for programmed cell death in humans and plants', *Cell Death and Disease*. doi: 10.1038/cddis.2014.280.

Mayali, X., Franks, P. J. S. and Azam, F. (2008) 'Cultivation and ecosystem role of a marine roseobacter clade-affiliated cluster bacterium', *Applied and Environmental Microbiology*, 74(9), pp. 2595–2603. doi: 10.1128/AEM.02191-07.

Mayers, T., Bramucci, A., Yakimovich, K. and Case, R. (2016) 'A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*', *Frontiers in Microbiology*, 7(JUN). doi: 10.3389/fmicb.2016.00892.

Michael, V., Frank, O., Bartling, P., Scheuner, C., Göker, M., Brinkmann, H. and Petersen, J. (2016) 'Biofilm Plasmids With A Rhamnose Operon Are Widely Distributed Determinants Of The "swim-or-stick" Lifestyle In Roseobacters', *The ISME Journal*, pp. 1–16. doi: 10.1038/ismej.2016.30.

Miller, T. R. and Belas, R. (2004) 'Dimethylsulfoniopropionate Metabolism By Pfiesteria - Associated Roseobacter Spp .', *Applied and environmental microbiology*, 70(6), pp. 3383–3391. doi: 10.1128/AEM.70.6.3383.

Mitchell, J. G., Yamazaki, H., Seuront, L., Wolk, F. and Li, H. (2008) 'Phytoplankton Patch Patterns: Seascape Anatomy In A Turbulent Ocean', *Journal of Marine Systems*, 69, pp. 247–253. doi: 10.1016/j.jmarsys.2006.01.019.

Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P., Rickaby, R. E. M., Poulton, A. J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., Gibbs, S., *et al.* (2016) 'Why marine phytoplankton calcify', *Science Advances*, 2(7), pp. e1501822–e1501822. doi: 10.1126/sciadv.1501822.

Moore, C. M., Mills, M. M., Arrigo, K. R., Berman-Frank, I., Bopp, L., Boyd, P. W., Galbraith, E. D., Geider, R. J., Guieu, C., Jaccard, S. L., *et al.* (2013) 'Processes And Patterns Of Oceanic Nutrient Limitation', *Nature Geoscience*. Nature Publishing Group, 6(9), pp. 701–710. doi: 10.1038/ngeo1765.

Moran, M. A., González, J. M. and Kiene, R. P. (2003) 'Linking A Bacterial Taxon To Sulfur Cycling In The Sea: Studies Of The Marine Roseobacter Group', *Geomicrobiology Journal*, 20(4), pp. 375–388. doi: 10.1080/01490450303901.

Moran, M. A., Reisch, C. R., Kiene, R. P. and Whitman, W. B. (2012) 'Genomic Insights Into Bacterial DMSP Transformations', *Annual Review of Marine Science*, 4(1), pp. 523–542. doi: 10.1146/annurev-marine-120710-100827.

Mossey, P., Hudacek, A. and Das, A. (2010) 'Agrobacterium Tumefaciens Type IV Secretion Protein VirB3 Is An Inner Membrane Protein And Requires VirB4, VirB7, And VirB8 For Stabilization', *Journal of Bacteriology*, 192(11), pp. 2830–2838. doi: 10.1128/JB.01331-09.

Mu, A., Gu, D., Du, F., Naumann, M., Meyer, T. F. and Rudel, T. (1999) '*Neisserial* Porin (PorB) Causes Rapid Calcium Influx In Target Cells And Induces Apoptosis By The Activation Of Cysteine Proteases', 18(2), pp. 339–352.

Nagai, H. and Kubori, T. (2011) 'Type IVB Secretion Systems Of Legionella And Other Gram-negative Bacteria', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2011.00136.

Nevitt, G. A., Veit, R. R. and Kareiva, P. (1995) 'Dimethyl Sulphide As A Foraging Cue For Antarctic *Procellariiform* Seabirds', *Nature*, pp. 680–682. doi: http://dx.doi.org/10.1038/376680a0.

Newton, H. J., Kohler, L. J., McDonough, J. A., Temoche-Diaz, M., Crabill, E., Hartland, E. L. and Roy, C. R. (2014) 'A Screen Of Coxiella Burnetii Mutants Reveals Important Roles For Dot/Icm Effectors And Host Autophagy In Vacuole Biogenesis', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1004286.

Newton, R. J., Griffin, L. E., Bowles, K. M., Meile, C., Gifford, S., Givens, C. E., Howard, E. C., King, E., Oakley, C. A., Reisch, C. R., *et al.* (2010) 'Genome Characteristics Of A Generalist Marine Bacterial Lineage.', *The ISME journal*. Nature Publishing Group, 4(6), pp. 784–98. doi: 10.1038/ismej.2009.150.

Oberhammer, F., Wilson, J., Dive, C., Morris, I., Hickman, J., Wakeling, A., Walker, P. and Sikorska, M. (1993) 'Apoptotic Death In Epithelial Cells: Cleavage Of DNA To 300 And/or 50 Kb Fragments Prior To Or In The Absence Of Internucleosomal Fragmentation', *The EMBO journal*, 12(9), pp. 3679–3684.

Paasche, E. (2002) 'A Review Of The Coccolithophorid *Emiliania Huxleyi*.pdf', *Phycologia*, 40(6), pp. 503–529.

Păcurar, D. I., Thordal-Christensen, H., Păcurar, M. L., Pamfil, D., Botez, M. L. and Bellini, C. (2011) 'Agrobacterium Tumefaciens: From Crown Gall Tumors To Genetic Transformation',

Physiological and Molecular Plant Pathology, 76(2), pp. 76–81. doi: 10.1016/j.pmpp.2011.06.004.

Park, S., Park, J. M., Jung, Y. T., Won, S. M. and Yoon, J. H. (2015) '*Primorskyibacter Insulae* Sp. Nov., Isolated From The Junction Between The Ocean And A Freshwater Spring', *International Journal of Systematic and Evolutionary Microbiology*, 65(11), pp. 3971–3976. doi: 10.1099/ijsem.0.000522.

Patzelt, D., Michael, V., Päuker, O., Ebert, M., Tielen, P., Jahn, D., Tomasch, J., Petersen, J. and Wagner-Döbler, I. (2016) 'Gene Flow Across Genus Barriers - Conjugation Of *Dinoroseobacter Shibae*'s 191-kb Killer Plasmid Into *Phaeobacter Inhibens* And AHL-mediated Expression Of Type IV Secretion Systems', *Frontiers in Microbiology*, 7(MAY), pp. 1–12. doi: 10.3389/fmicb.2016.00742.

Persson, O. P., Pinhassi, J., Riemann, L., Marklund, B. I., Rhen, M., Normark, S., González, J. M. and Hagström, Å. (2009) 'High abundance of virulence gene homologues in marine bacteria', *Environmental Microbiology*, pp. 1348–1357. doi: 10.1111/j.1462-2920.2008.01861.x.

Pitzschke, A. and Hirt, H. (2010) 'New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation', *EMBO*.

Podack, E. R. and Munson, G. P. (2016) 'Killing of microbes and cancer by the immune system with three mammalian pore-forming killer proteins', *Frontiers in Immunology*. doi: 10.3389/fimmu.2016.00464.

Poulton, A. J., Charalampopoulou, A., Young, J. R., Tarran, G. a., Lucas, M. I. and Quartly, G. D. (2010) 'Coccolithophore dynamics in non-bloom conditions during late summer in the central iceland basin (July-August 2007)', 55(4), pp. 1601–1613. doi: 10.4319/lo.2010.55.4.1601.

Poulton, A. J., Young, J. R., Bates, N. R. and Balch, W. M. (2011) 'Biometry of detached *Emiliania huxleyi* along the Patagonian Shelf', *Marine Ecology Progress Series*, 443, pp. 1–17. doi: 10.3354/meps09445.

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D. and Mekalanos, J. J. (2007) 'Type VI Secretion System Translocates A Phage Tail Spike-like Protein Into Target Cells Where It Cross-links Actin', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0706532104.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F. and Mekalanos, J. J. (2006) 'Identification Of A Conserved Bacterial Protein Secretion System In Vibrio Cholerae Using The Dictyostelium Host Model System.', *Proceedings of the National Academy of Sciences of the United States of America*, 103(5), pp. 1528–1533. doi: 10.1073/pnas.0510322103.

Ramanan, R., Kang, Z., Kim, B.-H., Cho, D.-H., Jin, L., Oh, H.-M. and Kim, H.-S. (2015) 'Phycosphere Bacterial Diversity In Green Algae Reveals An Apparent Similarity Across Habitats', *Algal Research*. Elsevier B.V., 8, pp. 140–144. doi: 10.1016/j.algal.2015.02.003.

Ramanan, R., Kim, B. H., Cho, D. H., Oh, H. M. and Kim, H. S. (2016) 'Algae-bacteria Interactions: Evolution, Ecology And Emerging Applications', *Biotechnology Advances*. doi: 10.1016/j.biotechadv.2015.12.003.

Rao, D., Webb, J. S., Holmström, C., Case, R., Low, A., Steinberg, P. and Kjelleberg, S. (2007) 'Low Densities Of Epiphytic Bacteria From The Marine Alga Ulva Australis Inhibit Settlement Of Fouling Organisms', *Applied and Environmental Microbiology*, 73(24), pp. 7844–7852. doi: 10.1128/AEM.01543-07.

Read, B., Kegel, J., Klute, M., Kuo, A., Lefebvre, S., Maumus, F., Mayer, C., Miller, J., Monier, A., Salamov, A., *et al.* (2013) 'Pan genome of the phytoplankton *Emiliania* underpins its global distribution', *Nature*, 499(7457), pp. 209–13. doi: 10.1038/nature12221.

Reape, T. J., Molony, E. M. and McCabe, P. F. (2008) 'Programmed cell death in plants: distinguishing between different modes', *Journal of Experimental Botany*, 59(3), pp. 435–444. doi: 10.1093/jxb/erm258.

Rosenwasser, S., Mausz, M. a, Schatz, D., Sheyn, U., Malitsky, S., Aharoni, A., Weinstock, E., Tzfadia, O., Ben-Dor, S., Feldmesser, E., *et al.* (2014) 'Rewiring host lipid metabolism by large viruses determines the fate of *Emiliania huxleyi*, a bloom-forming alga in the ocean', *The Plant cell*, 26(June), pp. 2689–2707. doi: 10.1105/tpc.114.125641.

Russell, A. B., Peterson, S. B. and Mougous, J. D. (2014) 'Type VI Secretion System effectors: poisons with a purpose', *Nature Reviews Microbiology*. doi: 10.1038/nrmicro3185.

Rutkowski, D. T., Kaufman, R. J., Stirling, P. C., al., et, Harding, H. P., al., et, Kaufman, R. J., Harding, H. P., al., et, Bertolotti, A., *et al.* (2004) 'A trip to the ER: Coping with stress', *Trends in cell biology*. doi: 10.1016/J.TCB.2003.11.001.

Sapp, M., Schwaderer, A. S., Wiltshire, K. H., Hoppe, H. G., Gerdts, G. and Wichels, A. (2007) 'Species-specific bacterial communities in the phycosphere of microalgae?', *Microbial Ecology*, 53(4), pp. 683–699. doi: 10.1007/s00248-006-9162-5.

Schatz, D., Shemi, A., Rosenwasser, S., Sabanay, H., Wolf, S. G., Ben-Dor, S. and Vardi, A. (2014) 'Hijacking of an autophagy-like process is critical for the life cycle of a DNA virus infecting oceanic algal blooms', *New Phytologist*, 204(4), pp. 854–863. doi: 10.1111/nph.13008.

Schulein, R., Guye, P., Rhomberg, T. A., Schmid, M. C., Schröder, G., Vergunst, A. C., Carena, I. and Dehio, C. (2005) 'A bipartite signal mediates the transfer of Type IV Secretion Substrates of *Bartonella henselae* into human cells', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0406796102.

Segev, E., Tellez, A., Vlamakis, H. and Kolter, R. (2015) 'Morphological heterogeneity and attachment of *Phaeobacter inhibens*', *PLoS ONE*, 10(11), pp. 1–13. doi: 10.1371/journal.pone.0141300.

Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N., Paulson, J. N., Chai, L., Clardy, J., *et al.* (2016) 'Dynamic metabolic exchange governs a marine algal-bacterial interaction', *eLife*, 5, p. e17473. doi: 10.7554/eLife.17473.

Segovia, M., Haramaty, L., Berges, J. a and Falkowski, P. G. (2003) 'Cell death in the unicellular Chlorophyte *Dunaliella tertiolecta*. a hypothesis on the evolution of apoptosis in higher plants and metazoans', *Plant physiology*, 132(1), pp. 99–105. doi: 10.1104/pp.102.017129.

Seyedsayamdost, M. R., Case, R. J., Kolter, R. and Clardy, J. (2011) 'The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*', *Nature chemistry*, 3(4), pp. 331–5. doi: 10.1038/nchem.1002.

Seymour, J. R., Amin, S. A., Raina, J.-B. and Stocker, R. (2017) 'Zooming in on the phycosphere: The ecological interface for phytoplankton–bacteria relationships', *Nature Microbiology*, 2(May), p. 17065. doi: 10.1038/nmicrobiol.2017.65.

Shao, F. (2003) 'Cleavage of *Arabidopsis* PBS1 by a bacterial Type III Effector', *Science*. doi: 10.1126/science.1085671.

Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B. and Tsujimoto, Y. (2004) 'Role of Bcl-2 family proteins in a non-apoptotic programmed cell

death dependent on autophagy genes.', *Nature cell biology*, 6(12), pp. 1221–1228. doi: 10.1038/ncb1192.
Si, M., Zhao, C., Burkinshaw, B., Zhang, B., Wei, D., Wang, Y., Dong, T. G. and Shen, X. (2017) 'Manganese scavenging and oxidative stress response mediated by Type VI Secretion system in *Burkholderia thailandensis*', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1614902114.
Signorini, S. R. and McClain, C. R. (2009) 'Environmental factors controlling the Barents Sea spring-summer phytoplankton blooms', *Geophysical Research Letters*, 36(10), pp. 1–5. doi: 10.1029/2009GL037695.
Sikora, A. E. (2013) 'Proteins secreted via the Type II Secretion System: smart strategies of *Vibrio cholerae* to maintain fitness in different ecological niches', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1003126.
Silhavy, T. J., Kahne, D. and Walker, S. (2010) 'The bacterial cell envelope', *Cold Spring Harbor perspectives in biology*, 2(5), p. a000414. doi: 10.1101/cshperspect.a000414.
Slightom, R. N. and Buchan, A. (2009) 'Surface colonization by marine roseobacters: integrating genotype and phenotype', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01508-09.
Smriga, S., Fernandez, V. I., Mitchell, J. G. and Stocker, R. (2016) 'Chemotaxis toward phytoplankton drives organic matter partitioning among marine bacteria', *Proceedings of the National Academy of Sciences of the United States of America*, 113(6), pp. 1576–81. doi: 10.1073/pnas.1512307113.
Stennicke, H. and Salvesen, G. (1999) 'Catalytic properties of the caspases', *Cell Death and Differentiation*, (6), pp. 1054–1059.
Stocker, R. (2012) 'Marine microbes see a sea of gradients', *Science*, 338(6107), pp. 628–633. doi: 10.1126/science.1208929.
Stocker, R., Seymour, J. R., Samadani, A., Hunt, D. E. and Polz, M. F. (2008) 'Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches', *Proceedings of the National Academy of Sciences of the United States of America*, 105(11), pp. 4209–4214. doi: 10.1073/pnas.0709765105.
Strom, S., Wolfe, G., Slajer, A., Lambert, S. and Clough, J. (2003) 'Chemical defense in the microplankton II: Inhibition of protist feeding by β-dimethylsulfoniopropionate (DMSP)', *Limnology and oceanography*. American Society of Limnology and Oceanography, 48(1), pp. 230–237.
Suchéras-Marx, B. and Henderiks, J. (2014) 'Testing the impact of calcareous nannofossil size evolution on pelagic carbonate burial over the past 17 million years', *Global and Planetary Change*. Elsevier B.V., 123, pp. 94–109. doi: 10.1016/j.gloplacha.2014.10.015.
Sunda, W., Kieber, D. J., Kiene, R. P. and Huntsman, S. (2002) 'An antioxidant function for DMSP and DMS in marine algae', *Nature*, 418(6895), pp. 317–20. doi: 10.1038/nature00851.
Sundström, J. F., Vaculova, A., Smertenko, A. P., Savenkov, E. I., Golovko, A., Minina, E., Tiwari, B. S., Rodriguez-Nieto, S., Zamyatnin, A. a, Välineva, T., *et al.* (2009) 'Tudor staphylococcal nuclease is an evolutionarily conserved component of the Programmed Cell Death degradome', *Nature cell biology*. Nature Publishing Group, 11(11), pp. 1347–1354. doi: 10.1038/ncb1979.

Suttle, C. A., López-Bueno, A., Pearce, D. A., Alcamí, A., Suttle, C. A., Ignacio-Espinoza, J. C., Solonenko, S. A., Sullivan, M. B., Fuhrman, J. A., Mann, N. H., *et al.* (2005) 'Viruses in the sea', *Nature*. doi: 10.1038/nature04160.

Thornton, D. C. O. (2014) 'Dissolved Organic Matter (DOM) release by phytoplankton in the contemporary and future ocean', *European Journal of Phycology*. © 2014 British Phycological Society, 49(1), pp. 20–46. doi: 10.1080/09670262.2013.875596.

Tyrrell, T. and Merico, A. (2004) '*Emiliania huxleyi*: bloom observations and the conditions that induce them'. doi: 10.1007/978-3-662-06278-4_4.

Tyrrell, T. and Young, J. R. (2009) 'Coccolithophores', *Encyclopedia of Ocean Sciences*, (c), pp. 606–614. doi: 10.1016/B978-012374473-9.00662-7.

Tzfira, T., Vaidya, M. and Citovsky, V. (2004) 'Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*', *Nature*. doi: 10.1038/nature02857.

Vardi, A., Haramaty, L., Van Mooy, B., Fredricks, H. F., Kimmance, S., Larsen, A. and Bidle, K. D. (2012) 'Host-virus dynamics and subcellular controls of cell fate in a natural coccolithophore population', *Proceedings of the National Academy of Sciences of the United States of America*, 109(47), pp. 19327–32. doi: 10.1073/pnas.1208895109.

Vardi, A., Mooy, B. A. S. Van, Fredricks, H. F., Popendorf, K. J., Ossolinski, J. E., Haramaty, L. and Bidle, K. D. (2009) 'Viral glycosphingolipids induce lytic infection and cell death in marine phytoplankton', *AAAS*, 326(5954), pp. 861–865.

Vartapetian, A., Tuzhikov, A., Chichkova, N., Taliansky, M. and Wolpert, T. (2011) 'A plant alternative to animal caspases: Subtilisin-like proteases', *Cell death and differentiation*. Nature Publishing Group, 18(8), pp. 1289–1297. doi: 10.1038/cdd.2011.49.

Vergunst, A. C. (2000) 'VirB/D4-Dependent Protein Translocation From *Agrobacterium* Into Plant Cells', *Science*, 290(5493), pp. 979–982. doi: 10.1126/science.290.5493.979.

Voth, D. E., Broederdorf, L. J. and Graham, J. G. (2012) 'Bacterial Type IV Secretion Systems: versatile virulence machines', 7(2), pp. 241–257. doi: 10.2217/fmb.11.150.Bacterial.

Wagner-Döbler, I., Ballhausen, B., Berger, M., Brinkhoff, T., Buchholz, I., Bunk, B., Cypionka, H., Daniel, R., Drepper, T., Gerdts, G., *et al.* (2010) 'The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: A hitchhiker's guide to life in the sea', *The ISME journal*, 4(1), pp. 61–77. doi: 10.1038/ismej.2009.94.

Wagner-Döbler, I. and Biebl, H. (2006) 'Environmental biology of the marine roseobacter lineage.', *Annual review of microbiology*, 60, pp. 255–280. doi: 10.1146/annurev.micro.60.080805.142115.

Wallden, K., Rivera-Calzada, A. and Waksman, G. (2010) 'Type IV Secretion Systems: Versatility and diversity in function', *Cellular Microbiology*, 12(9), pp. 1203–1212. doi: 10.1111/j.1462-5822.2010.01499.x.

Wang, H., Tomasch, J., Michael, V., Bhuju, S., Jarek, M., Petersen, J. and Wagner-Döbler, I. (2015) 'Identification of genetic modules mediating the Jekyll and Hyde interaction of *Dinoroseobacter shibae* with the dinoflagellate *Prorocentrum minimum*', *Frontiers in Microbiology*, 6(NOV), pp. 1–8. doi: 10.3389/fmicb.2015.01262.

Wang, R. and Seyedsayamdost, M. R. (2017) 'Roseochelin B, an algaecidal natural product synthesized by the roseobacter *Phaeobacter inhibens* in response to algal sinapic acid', *Organic Letters*, p. acs.orglett.7b02424. doi: 10.1021/acs.orglett.7b02424.

Wetmore, K. M., Price, M. N., Waters, R. J., Lamson, J. S., He, J., Hoover, C. A., Blow, M. J., Bristow, J., Butland, G., Arkin, A. P., *et al.* (2015) 'Rapid quantification of mutant fitness in

diverse bacteria by sequencing randomly bar-coded transposons', *mBio*, 6(3), pp. 1–15. doi: 10.1128/mBio.00306-15.
Whitman, W., Coleman, D. C. and Wiebe, W. J. (1998) 'Prokaryotes: The unseen majority', *Proceedings of the…*, 95(June), pp. 6578–6583.
Wilhelm, S. W. and Suttle, C. A. (1999) 'Viruses and nutrient cycles in the sea', *BioScience*, 49(10), pp. 781–788. doi: 10.2307/1313569.
Wilson, W. H., Schroeder, D. C., Allen, M. J., Holden, M. T. G., Parkhill, J., Barrell, B. G., Churcher, C., Hamlin, N., Mungall, K., Quail, M. A., *et al.* (2015) 'Complete genome sequence and lytic phase transcription profile of a coccolithovirus', *Science*, 309, pp. 1090–1092.
Winter, A., Henderiks, J., Beaufort, L., Rickaby, R. E. M. and Brown, C. W. (2014) 'Poleward expansion of the coccolithophore *Emiliania huxleyi*', *Journal of Plankton Research*, 36(2), pp. 316–325. doi: 10.1093/plankt/fbt110.
Woltering, E. J. (2004) 'Death proteases come alive', *Trends in Plant Science*, 9(10), pp. 469–472. doi: 10.1016/j.tplants.2004.08.001.
Worden, A. Z., Cuvelier, M. L. and Bartlett, D. H. (2006) 'In-depth analysis of marine microbial community genomics', *Trends in Microbiology*. doi: 10.1016/j.tim.2006.06.008.
Wyllie, A. H. and Golstein, P. (2001) 'More than one way to go', *PNAS*, 98(26), pp. 11–13. doi: 10.1073/pnas.98.1.11.
Xu, Q. and Zhang, L. (2009) 'Plant caspase-like proteases in plant programmed cell death', *Plant signaling & behavior*, 4(9), pp. 902–904. doi: 10.4161/psb.4.9.9531.
Yao, N., Tada, Y., Sakamoto, M., Nakayashiki, H., Park, P., Tosa, Y. and Mayama, S. (2002) 'Mitochondrial oxidative burst involved in apoptotic response in oats', *Plant Journal*, 30(5), pp. 567–579. doi: 10.1046/j.1365-313X.2002.01314.x.
Young, J., Geisen, M., Cros, L., Kleijne, A., Sprengel, C., Probert, I. and Østergaard, J. (2003) 'A guide to extant coccolithophore taxonomy', *Journal of Nannoplankton Research*, p. 125.
Yu, L., Lenardo, M. J. and Baehrecke, E. H. (2004) 'Authophagy and caspases: A new cell death program', *Cell Cycle*, 3(9–September), pp. 1124–1126. doi: 10.4161/cc.3.11.1203.
Zechner, E. L., Lang, S. and Schildbach, J. F. (2012) 'Assembly and mechanisms of bacterial Type IV Secretion machines', *Philosophical Transactions of the Royal Society B: Biological Sciences*. doi: 10.1098/rstb.2011.0207.
Zhang, D. C., Li, H. R., Xin, Y. H., Liu, H. C., Chi, Z. M., Zhou, P. J. and Yu, Y. (2008) '*Phaeobacter arcticus* sp. nov., a psychrophilic bacterium isolated from the arctic', *International Journal of Systematic and Evolutionary Microbiology*, 58(6), pp. 1384–1387. doi: 10.1099/ijs.0.65708-0.
Zhang, Y., Higashide, W., Dai, S., Sherman, D. M. and Zhou, D. (2005) 'Recognition and ubiquitination of *Salmonella* Type III Effector SopA by a ubiquitin E3 ligase, HsRMA1', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M506309200.
Zubkov, M. V., Fuchs, B. M., Archer, S. D., Kiene, R. P., Amann, R. and Burkill, P. H. (2001) 'Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea', *Environmental Microbiology*, 3(5), pp. 304–311. doi: 10.1046/j.1462-2920.2001.00196.x.
Zupan, J., Hackworth, C. A., Aguilar, J., Ward, D. and Zambryski, P. (2007) 'VirB1* Promotes T-pilus Formation In The Vir-type IV Secretion System Of Agrobacterium Tumefaciens', *Journal of Bacteriology*. doi: 10.1128/JB.00480-07.

Chapter 2. A small volume bioassay to assess bacterial-phytoplankton co-culture using Pulse-Amplitude-Modulated fluorometry

2.1. Introduction

Phytoplankton physiology has traditionally been studied in mesoscale experiments ranging from 20 mL in conical flasks to 5 L in carboys (Moore *et al.*, 1995; Scarratt *et al.*, 2006; Bidle *et al.*, 2007; Chen *et al.*, 2011). This experimental scale typically requires subsampling for experimental monitoring, as sacrificing replicate samples for ever time point creates an unmanageable experimental setup. Miniaturizing the experimental setup will reduce or eliminate the limitations associated with larger volume studies (e.g., subsampling, pseudo-replication, etc.), while simultaneously maximizing the number of independent replicates.

A microtiter plate format has been developed for algal bioassays using a 1 mL culture volume for experimentally manipulating algal cultures in variable conditions. This small experimental volume allows for the number of replicates to be increased, increases experimental reproducibility, and allows for increased experimental replication within the same diurnal incubator (Seyedsayamdost *et al.*, 2011). This microtiter plate format is easily adapted for testing a variety of experimental questions, such as: does a bacterium have a symbiotic, neutral, or pathogenic interaction with its algal host? or Is the addition of a compound stimulating or toxic to an alga? These and other questions can be addressed in a rapid medium-throughput manner using this new miniaturized format (Blaise *et al.*, 2005; Skjelbred *et al.*, 2012; Nagai *et al.*, 2013).

The 48-well microtiter plate allows each 1 mL well to be an independent experimental setup that is sacrificially sampled at a single time-point. Various parameters can be assessed using this 1 mL volume including, but not limited to chlorophyll fluorescence parameters using Pulse-Amplitude-Modulated (PAM) fluorometry (Schreiber, 1998). PAM fluorometry is a rapid and non-invasive technique that can be used to monitor algal photosynthetic efficiency and PSII health from a small culture volume (Beer *et al.*, 2000; Jones *et al.*, 2000). In addition to PAM fluorometry, this setup can be used to assess a variety of other parameters including, but not limited to: microscopic visualization, enumeration of bacteria, and enumeration of algae.

This chapter presents the major methods used throughout this thesis. A detailed procedure of setting up a microtiter plate assay to assess algal health when an alga is grown in co-culture with a bacterium is presented. An example of how to test algal strains for long-term viability in the miniaturized microtiter plate assay and an example co-culture experiment are then presented. Finally, the method is discussed in detail and the potential importance of this medium-throughput assay is examined.

2.2. Procedure

The microtiter plates used for this assay hold a final volume of 1 mL per well. When preforming co-culture experiments using two organisms (i.e., one alga and one bacteria) it is important to mix the two organisms in a 50:50 ratio, to ensure well suspended and evenly mixed populations. For this reason, you should first calculate the volume of algae and bacteria needed to fill as many wells and plates as are required for your experimental needs. For example, when preforming an experiment sampling every 2 days for 10 days, with 3 replicates of control and co-culture each sampling day (see sample experiment) you will need a different amount of algal and bacterial stock solutions than would be required for a 20-day experiment requiring 6 replicates each day. To plan your experiments correctly follow the procedure and calculations below.

2.2.1. Calculations for experimental setup

Step 2.2.1-1 Calculate the volume of algal and/or bacterial cultures needed for algal and bacterial controls throughout the entire experiment by using Equation 1. This example is assuming 3 experimental replicates (see Equation 1 and 2: '*3 wells*'); alter this value as needed.

Equation 1:

$$V_{control} = (0.5 \text{ mL control culture/well}) \times (3 \textit{ wells}/\text{control}) \times (\boldsymbol{y} \text{ controls/d}) \times (\boldsymbol{z} \text{ d})$$

Where ***y*** equals the number of controls and ***z*** is the number of sampling days (d) required.

Step 2.2.1-2 Calculate the volume of algal and/or bacterial cultures required for co-cultures for the experiment using Equation 2.

Equation 2:

$$V_{co\text{-}culture} = (0.5 \text{ mL co-culture/well}) \times (3 \textit{ wells}/\text{co-culture}) \times (\boldsymbol{w} \text{ co-cultures/d}) \times (\boldsymbol{z} \text{ d})$$

Where ***w*** equals the number of co-cultures needed and ***z*** equals the number of sampling days (d) required for your experiment.

Step 2.2.1-3Use Equation 1 and Equation 2 to calculate the final volume of mid-exponential

algal culture required for the experiment using Equation 3.

Equation 3:

$\mathbf{V_A}$ = volume of mid-exponential algal culture required

$= V_{control} + V_{co\text{-}culture} + 10$ mL

Step 2.2.1-4Use Equation 1 and Equation 2 to calculate the final volume of 10^5 cfu/mL bacterial culture (or desired inoculation concentration) required for the experiment using Equation 4.

Equation 4:

$\mathbf{V_B}$ = volume of bacterial culture

$= V_{control} + V_{co\text{-}culture} + 10$ mL

NOTE: The extra 10 mL is meant 0 d evaluations (e.g., PAM, flow cytometry, microscopy, etc.).

2.2.2. Growing algal cells for experimental setup

Step 2.2.2-1 Isolate or obtain an actively growing axenic algal culture. For the purpose of this thesis axenic calcifying *Emiliania huxleyi* culture CCMP3266, hereafter referred to as *E. huxleyi*, was obtained from the National Centre for Marine Algae and Microbiota (East Boothbay, ME, USA). These cultures were routinely maintained in L1-Si medium at 18 °C in a diurnal incubator (8:16 h dark-light cycle) and 41.51 +/- 11.15 µmol/m^2/s (Guillard *et al.*, 1993) for five days prior to all algal experiments. Under these conditions *E. huxleyi* reaches mid-exponential phase (10^5-10^6 cells/mL), in five days. Each alga has a unique growth curve depending on culturing conditions. Mid-exponential phase for all strains and culture conditions should be determined by performing an algal growth curve and enumerating algal cells/mL using flow cytometry.

Step 2.2.2-2 After a fresh parent culture reaches mid-exponential phase (~10^5 cells/mL), re-culture the alga at a 1:9 dilution in L1-Si, ensure the final volume of algae is equal to the V_A calculated (Equation 3). This will be your algal stock bottle.

NOTE: It is important to ensure these cultures are axenic. Test all algal media and algal stock bottle(s) for contamination by plating onto a general marine bacterial medium (e.g., ½ marine agar (½MA) [18.7 g Difco Marine Broth 2216 and 9 g NaCl supplemented with 15 g Difco agar in 1 L deionized water] is used throughout this work).

2.2.3. Preparing bacterial cells for inoculation

Step 2.2.3-1 For the purpose of this work marine bacterium *Phaeobacter inhibens* DSM17395 was grown on ½MA at 30 ºC and then an isolated colony was transferred into 5 mL ½ marine

broth (½MB) [18.7 g Difco Marine Broth 2216 and 9 g NaCl, in 1 L deionized water] and grown shaking at 160 rpm, at either 18 ºC or 21.5 ºC for 30 hr or 24 hr, respectively so that the bacterium reached stationary phase (which was identified to be roughly 10^8 cfu/mL for this bacterium and these growth conditions).

Step 2.2.3-2 Plan the experiment so that the bacteria have reached the correct density (10^8 cfu/mL) at the same time that the algal cells have reached mid-exponential phase (~10^5 algal cells/mL).

Step 2.2.3-3 Once the bacterial cells have grown, use a pipette to wash any biofilm attached to the test tube down into the medium. Pipette 1 mL of well-mixed bacterial culture into a sterile 1.5 mL microtube. Centrifuge for 1 min at 14000 x g.

Step 2.2.3-4Remove and dispose of the supernatant (bacterial media) without disrupting the pellet (bacterial cells). Add 1 mL of sterile algal media (L1-Si) to the microtube (with pellet), and vortex to suspend pellet in algal media.

Step 2.2.3-5 Wash cells by centrifuging cells again and repeating Step 2.2.3-4.

NOTE: It is critical to wash the bacterial cells with algal media to remove all bacterial media, excreted proteins, and small molecules from the cells prior to inoculation as this might alter the nutrient composition of the algal media or introduce bioactive molecules to the screen.

Step 2.2.3-6 Serially dilute the washed bacterial cells in algal media, to a final concentration that is 100 fold more concentrated than the desired final bacterial concentration (cfu/mL) and save this microtube for Step 2.2.4-2.

2.2.4. Preparing bacteria for experimental setup

Step 2.2.4-1Prepare 4 sterile autoclaved glass conical flasks and label them: 1) '*algal control flask*', 2) 'diluted bacterial stock flask', 3) '*bacterial control flask*', and 4) '*co-culture flask*'.

Step 2.2.4-2 Dilute the bacterial suspension (from Step 2.2.3-6) 1:99 in sterile algal media to a final volume = V_B (Step 2.2.1-4). This is your 'diluted bacterial stock flask'.

Step 2.2.4-3 Pipette $V_{control}$ (Step 2.2.1-1) from the 'diluted bacterial stock flask' and put it in the '*bacterial control flask*'.

Step 2.2.4-4 Pipette $V_{control}$ of sterile algal medium into the '*bacterial control flask*' (this is a 1:1 dilution). Swirl flask to mix cells and set aside (for Step 2.2.3-3).

Step 2.2.4-5 Pipette $V_{co\text{-}culture}$ from the 'diluted bacterial stock flask' into the '*co-culture flask*', set aside (for Step 2.2.3-3).

2.2.1. Preparing algal controls

Step 2.2.1-1 Gently mix the mid-exponential algal culture (from Step 2.2.2-2) with a wide-mouth pipette tip until cells appear well mixed.
Step 2.2.1-2 Pipette $V_{control}$ from the algal stock bottle to the '*algal control flask*'. Then return the algal stock bottle to the diurnal incubator.
Step 2.2.1-3 Pipette $V_{control}$ of sterile algal medium to the '*algal control flask*' (this is a 1:1 dilution). Swirl to mix flask and place in the diurnal incubator, until needed for Step 2.2.3-4.

2.2.2. Preparing experimental co-culture

Step 2.2.2-1 Gently pipette $V_{co\text{-}culture}$ from the *algal stock bottle* into the bacterial '*co-culture flask*'. Return the '*co-culture flask*' to the diurnal incubator until needed for Step 2.2.3-3.
NOTE: The concentration of bacteria in the bacterial control should equal the bacterial concentration in the experimental co-culture. Similarly, the concentration of algae in the algal control should equal the concentration of algae in the experimental co-culture.

2.2.3. Setting up microtiter plates

Step 2.2.3-1 Divide a sterile 48-well microtiter plate as per (Figure 2.1). Label above outer wells containing either sterile diluent or non-photosynthetic samples.
Step 2.2.3-2 Pipette 1 mL 1X phosphate buffer solution (PBS) (pH 7.4) into the appropriate wells (Figure 2.1).
Step 2.2.3-3 Pipette 1 mL of '*bacterial control flask*' into wells labelled bacterial control (Figure 2.1). Swirl the '*bacterial control flask*' before pipetting to avoid bacterial settling.
Step 2.2.3-4 Pipette 1 mL of '*algal control culture*' into wells labelled algal control using a wide mouth pipette tip (Figure 2.1). Swirl the algal flask as regularly as the bacterial flask. If the alga tends to sink or float, swirl as regularly as is needed to maintain a visually uniform culture.
Step 2.2.3-5 Using a wide mouth pipette tip, pipette 1 mL from '*co-culture flask*' into the appropriate wells (Figure 2.1). Swirl the co-culture flask as regularly as the algal control flask.
Step 2.2.3-6 Seal each plate with parafilm and place in a diurnal incubator at the desired temperature and diurnal light cycle (18˚C with a 16:8 h light: dark cycle is used throughout this work). Position plates in the same direction to ensure consistent light exposure. Leave the microtiter plates in the diurnal incubator until ready to take PAM readings (Section 2.2.4).

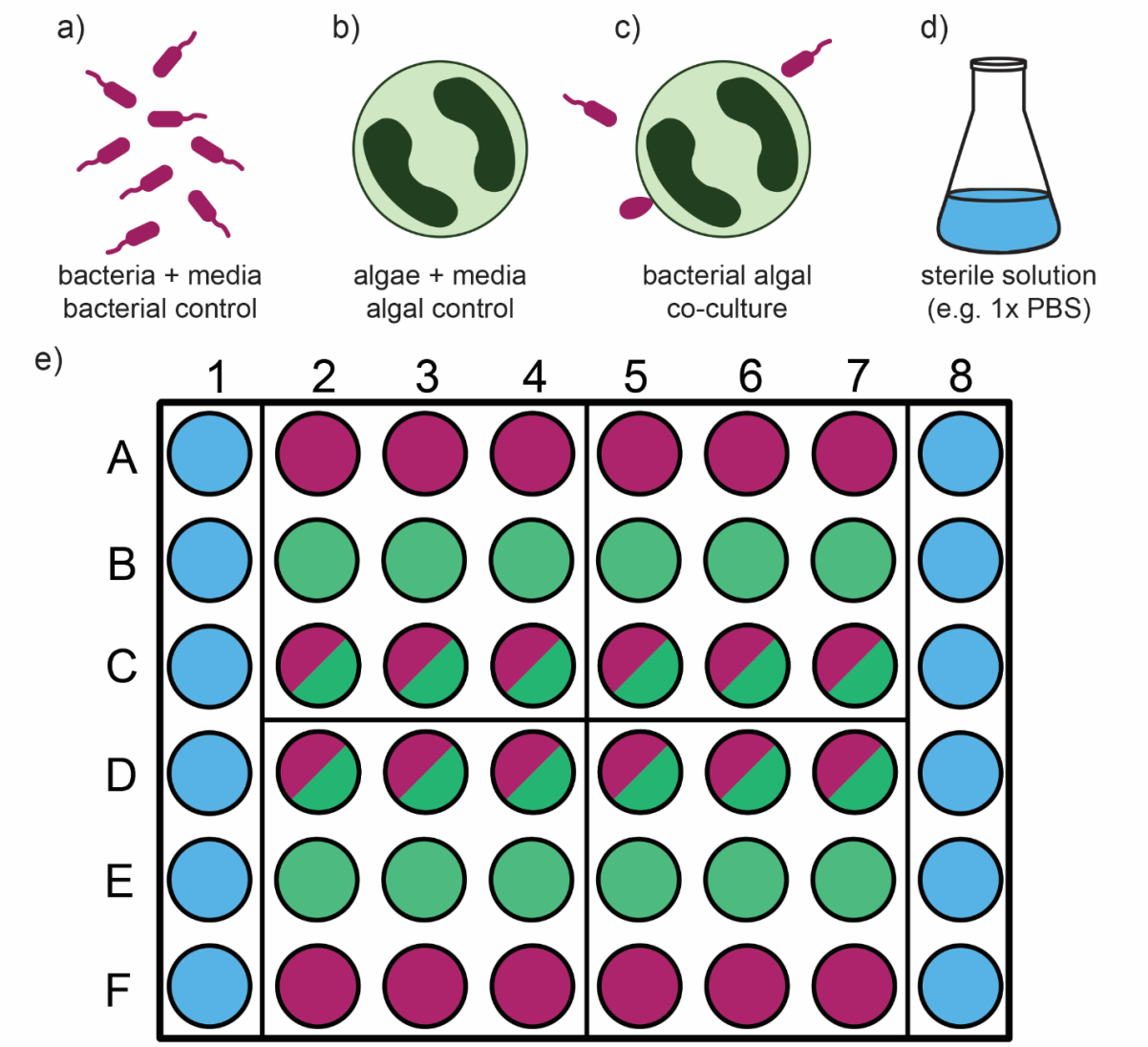

Figure 2.1. **Schematic of experimental setup in a 48-well microtiter plate.** Schematic representation of experimental setup: (a) Bacterial control (purple), (b) algal control (green), (c) algal-bacterial co-culture (purple/green), (d) sterile 1xPBS (blue). Schematic representation of microtiter plate setup (e) wells to be filled as follows: columns 1 and 8 filled with 1xPBS (or other sterile solution); rows A and F, wells 2-7 (purple) are filled with 1 mL bacterial control; rows B and E wells 2-7 (green) are filled with 1 mL algal control; rows C and D, wells 2-7 (purple/green) are filled with 1 mL co-culture. The plate is divided into 4 quadrants, with triplicate wells in each quadrant, to allow for triplicate sampling on sequential days (e.g., on days 2, 4, 6, and 8). It is advisable to randomize samples within each quadrant samples using a random number generator.

Step 2.2.3-7 Pipette five replicate 20 µl drops from the PBS, algal media, algal stock flask(s), and *algal control flask* onto ½MA and incubate plates at 30 ºC for 72 h to test for contamination.
Step 2.2.3-8 Take PAM fluorometry readings using the remaining sample from the algal control and co-culture flasks for the experimental 0 d (see Step 2.2.4-1).

2.2.4. Taking PAM fluorometry readings

Step 2.2.4-1 Zero the PAM with sterile algal media (e.g., L1-Si) in a clean cuvette before taking readings (MacIntyre *et al.*, 2005).
Step 2.2.4-2 Pipette 300 µL from '*algal control flask*' or '*co-culture flask*' into a clean cuvette containing 2.7 mL of the same sterile algal medium used in the experiment (e.g., L1-Si). Mix the sample and diluent gently.
Step 2.2.4-3 Clean cuvette and place it into PAM machine, cover with the cap and dark-adapt for 3 min. Dark adaptation times vary depending on the species of algae and must be determined for the specific alga used in the experiment. Avoid lengthy dark adaptation times (>20 min) by taking PAM readings during the middle of the dark cycle (Maxwell *et al.*, 2000).
Step 2.2.4-4 After dark adaption, hit the F_0 button. If fluorescence readings are over 3900, dilute sample 1:1 in algal medium. Dark-adapt for an additional 3 min and re-read. Continue to dilute the sample 1:1 in algal medium until the F_0 and F_m readings are below 3900.
NOTE: Account for these dilutions when recording final fluorescence. For example: if the sample is diluted 1:9 during the initial transfer from the well into the cuvette, then an algal fluorescence reading of 500 should be multiplied by the inverse of the dilution factor (in this case 10) and the actual fluorescence of the tube is recorded as 5000.
Step 2.2.4-5 After setting F_0, take a saturating pulse (SAT-Pulse) reading every 1 min 30 s by hitting the SAT button to take F_m readings. The time interval between readings may be adjusted depending on algal strain. Discard sample.
Step 2.2.4-6 Repeat Step 2.2.4-1-5 for the remaining samples.
NOTE: When sampling from a microtiter plate follow the same procedure, but remove the 300µL sample from a well-mixed microtiter plate well.

2.2.5. Additional parameters of interest

2.2.5.1. *Determine bacterial concentration (cfu/mL):*

To determine bacterial concentration, perform a serial dilution of the bacterial control and/or co-culture wells in sterile algal medium. First, pipette 900 µL sterile algal medium into sterile eppindorf tubes, then transfer 100 µL from a well-mixed microtiter plate into the first dilution tube (dilution factor: 10^{1-}) (Figure 2.2a). Vortex the tube (2 m) and transfer 100 µL from the 10^{1-} dilution into the next dilution (10^{2-}), and so on until 10^{7-}. Then vortex tubes again before immediately pipetting five 20 µl drops from each dilution (starting with the most dilute) onto ½MA (Figure 2.2b). Incubate the plates at 30 °C for 2 d and count the cfu per drop to determine the bacterial cfu/mL (Figure 2.2c) (Herigstad *et al.*, 2001). After incubation, count all dilutions where all five drops have between 2-20 cfu per drop (this range was chosen because the count is high enough to be statistically accurate and low enough to avoid nutrient competition among the developing colonies (Herigstad *et al.*, 2001)). Then average the five replicate drops for each dilution within the countable range (2-20 cfu/drop) and calculate cfu/mL using Equation 5. In this example the 10^{7-} dilution is too few to count (less than 2-20 cfu per drop), so for this example choose the 10^{6-} dilution, which has an average of 9.2 cfu/drop (Figure 2.2c).

Equation 5:

$$\text{cfu/mL} = \frac{[(\text{average cfu/X µL drop}) \times (1000\ \text{µL/mL})]}{\text{dilution factor}}$$

$$\text{cfu/mL} = \frac{[(9.2\ \text{cfu/20 µL drop}) \times (1000\ \text{µL/mL})]}{10^{6-}}$$

$$\text{cfu/mL} = 4.60 \times 10^{7}\ \text{cfu/mL}$$

2.2.5.2. *Determine algal cell concentration*

Fix algal control and co-cultures with a 0.15% final concentration of glutaraldehyde. Incubate for 10 min in the dark, then flash freeze in liquid nitrogen, and store at -80 °C. Process all samples on a flow cytometer (e.g., FACSCalibur) to count algal cells.

2.2.5.3. *Observe algal cell morphology*

Observe algal controls and bacterial-algal co-cultures using microscopy (e.g., epifluorescence, or similar) to assess bacterial induced changes to algal morphology.

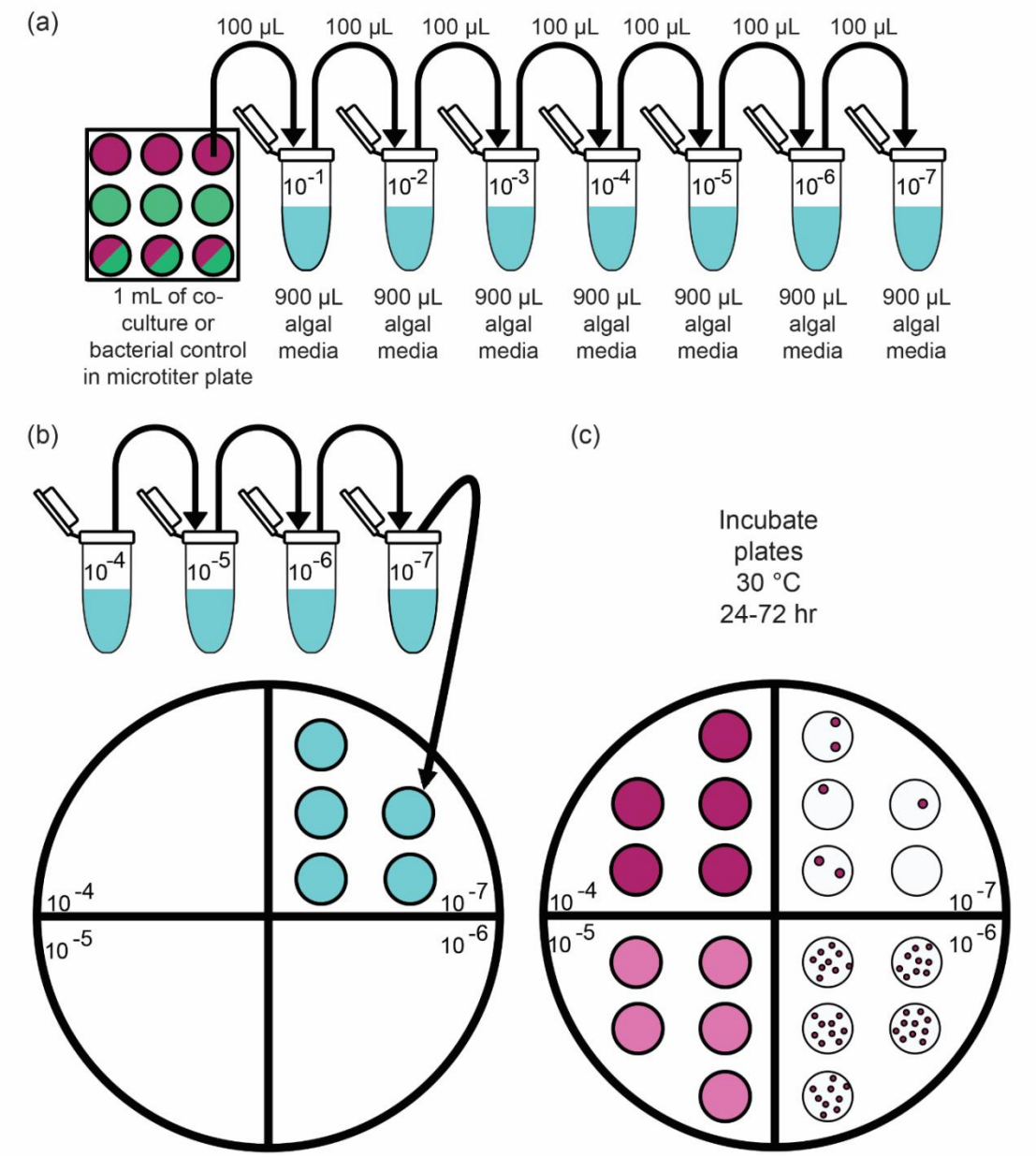

Figure 2.2. **Schematic representation of drop-plate experiment setup and resulting cfu/drop.** Schematic representation of experimental setup: (a) After mixing bacterial control well from mitrotiter plate (purple), remove 100 µL and pipette it into 900 µL sterile algal media (or similar). Preform a serial dilution being careful to vortex each tube well before subsequent dilutions. (b) Beginning with the most dilute dilution tube (10^{7-}) vortex the tube and carefully pipette five drops of the dilution onto a dry ½ MA plate. (c) Incubate the plates and count colony forming units per drop, then calculate cfu/mL.

2.3. Representative Results

The first example of representative results is a standard growth curve of the ubiquitous calcifying coccolithophore species *Emiliania huxleyi*, grown in the microtiter plate format for 60 d (Figure 2.3). The second experiment presented here is a 10-day co-culture of the calcifying microalga *E. huxleyi* and a marine bacterium *P. inhibens* (Figure 2.4). Both experiments are performed in the microtiter plate format and monitored using PAM fluorometry.

2.3.1. Long-term viability in the microtiter plate format

Figure 2.3 depicts how algal cultures should be tested to ensure long-term viability in microtiter plate format. This growth curve of axenic *E. huxleyi* cultures, shows that the alga retains elevated PSII maximum quantum efficiency (>0.5) for 60 d in the microtiter plate format (Figure 2.3a). The minimal dark fluorescence graph demonstrates that the algal cultures maintain high fluorescence until d 40, after which fluorescence begins to slowly diminish until 60 d (Figure 2.3b). Other algal strain used in this thesis (*E. huxleyi* non-calcifying diploid CCMP2090 and *E. huxleyi* flagellated haploid CCMP3268) are assessed in the same way (Appendix B-1).

2.3.2. Co-culture in microtiter plate format

Figure 2.4 depicts how the algal fluorescence (F_0 and F_m) and potential quantum yield (F_v/F_m) are influenced by the bacterium throughout the 10-day co-culture experiment (Figure 2.4). In this example the bacteria appear to be negatively influencing the algal host, causing prematurely diminished algal fluorescence (F_0 and F_m) and potential quantum yield (F_v/F_m) (Figure 2.4). The standard error bars are derived from triplicate microtiter wells, which are independent replicate experiments. The consistently small standard error demonstrates the robustness and reproducibility of the microtiter plate format for algal bioassays.

2.4. Discussion

PAM fluorometry is a quick and efficient method to determine algal fluorescence (a proxy for chlorophyll content) and potential quantum yield (F_v/F_m) (an estimate of photosynthetic health) of algal cultures (Maxwell *et al.*, 2000; Schreiber, 2004; da Silva *et al.*, 2007). This microtiter plate assay allows for a robust and highly reproducible medium-throughput assay of algal cultures, without the need for subsampling, making this a useful method for any lab interesting in rapidly assessing algal cultures under a variety of conditions.

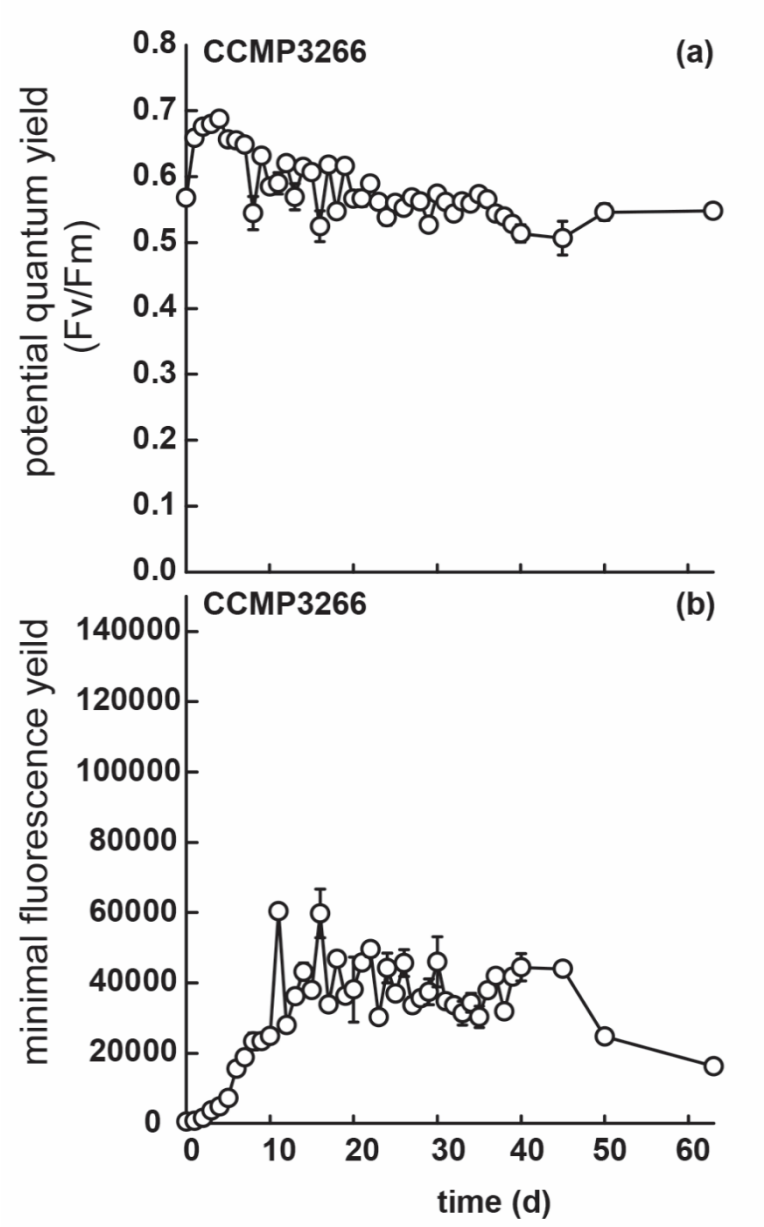

Figure 2.3. **Representative PAM fluorometry graphs of a 60 d growth curve of axenic *Emiliania huxleyi* CCMP3266.** Readings for the (a) potential quantum yield (F_v/F_m) and (b) initial algal fluorescence (F_0) for *E. huxleyi* are shown as white circles. Potential quantum yield (F_v/F_m) is a dimensionless expression of photosynthetic health, which is calculated as (F_m-F_0)/ F_m. Error bars represent the standard error between triplicate wells.

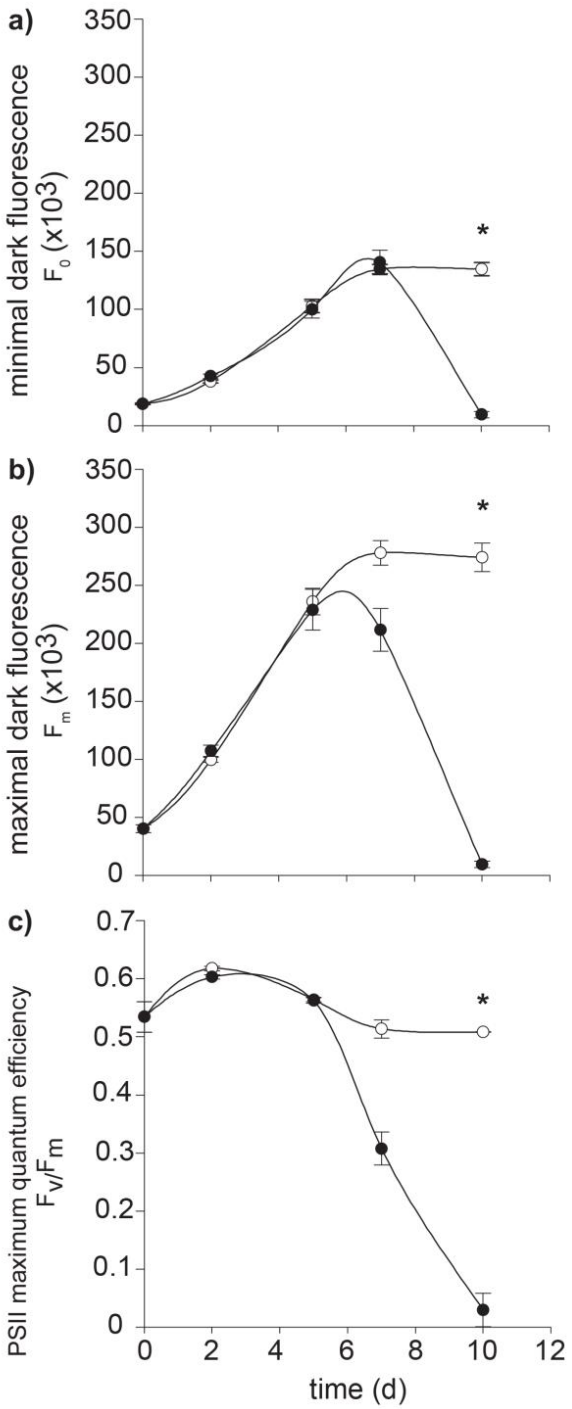

Figure 2.4. **Representative PAM fluorometry graphs of a 10 d co-culturing experiment of *Emiliania huxleyi* CCMP3266 with *Phaeobacter inhibens*** The initial algal fluorescence (F_0) (a), maximum algal fluorescence (F_m) (b), and potential quantum yield (F_v/F_m) (c) are graphed for control algal (open circles) and alga co-cultured with a bacterium (black circles). Potential quantum yield (F_v/F_m) is a dimensionless expression of photosynthetic health, which is calculated as $(F_m-F_0)/F_m$. Error bars represent the standard error between triplicate wells. Statistical significance (*) was determined using a one-way ANOVA and Tukey HSD test.

2.4.1. Algal growth in a miniaturized format

The miniaturization of algal cultures to a 1 mL culture volume in a microtiter plate allows for the replication within an experiment to be increased. It is important to ensure the alga is healthy throughout the experiment. To test this always perform a growth curve using the microtiter plate format to ensure the nutritional requirements of the alga are met (Figure 2.3). The other algal strains used in this thesis (Chapter 3) were also tested for long-term viability in the microtiter plate format (Appendix B-1). Additionally, it may be important to optimize the diurnal cycle (light and dark periods) and growth temperature. Proper optimization for a given alga can allow for maintaining healthy algal cultures at peak fluorescence for 26 d and detectable potential quantum yield for 60 d (Figure 2.3).

2.4.2. Minimizing evaporative effects

It is important to minimize the evaporative effects of liquid based assays as an 'edge effect' is commonly observed where there is greater evaporation in wells at the edge of the microtiter plate than in wells located towards the middle of the plate. While evaporation has been observed at the edge of plates, the rate of evaporation does not limit the experimental duration as healthy algal cultures; with peak fluorescence at 26 d has been maintained in this format (Figure 2.3). To minimize any potential 'edge effect' 1X PBS (pH 7.4), is aliquoted into wells along all four edges (columns 1 and 8, rows A and F, Figure 2.1).

2.4.3. Dark adaptation of algal samples for PAM fluorometry

Before conducting PAM readings it is important to dark-adapt the algal samples so that the PSII reaction centers are fully open and the light-induced transthylakoidal pH gradient is fully dissipated, thus giving true F_o and F_m values from which to calculate F_v/F_m. Sampling the alga from the assay for PAM measurements in the middle of the dark phase of the diurnal cycle (i.e., for a 16:8 h light: dark cycle, a two hour sampling session would be performed from T(dark)=3-5 h) makes dark adaptation time shorter (3-5 min) compared with the middle of the light cycle (T(light)=7-9 h) when dark adaptation is longer (>20 min) (Maxwell *et al.*, 2000). The alga's dark adaption time will also vary depending on the algal species, growth conditions and the light conditions of its natural habitat range.

2.4.4. Future Applications

This small volume bioassay provides a rapid screening method for microalgae by combining a microtiter plate format with PAM fluorometry. Examples of future applications are various, and could include using alternate methods such as Imaging PAM fluorometry, which provides insight into cell-cell variation of PSII health within a population as it assesses individual cells (Vieira *et al.*, 2013). The bioassay can also be combined with microscopy and flow cytometry as previously discussed. Another combination with the potential to provide further insight is cell staining for flow cytometry and microscopy to elucidate morphological and/or physiological variations within subpopulations of the algal culture. Some of these future directions are employed in later chapters of this thesis (Chapter 3, Chapter 4).

2.5. Acknowledgements

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2.6. Works Cited

Beer, S., Larsson, C., Poryan, O. and Axelsson, L. (2000) 'Photosynthetic rates of *Ulva* (Chlorophyta) measured by Pulse Amplitude Modulated fluorometry', *European journal of phycology*, 35(1), pp. 69–74. doi: 10.1080/09670260010001735641.

Bidle, K. D., Haramaty, L., Barcelos E Ramos, J. and Falkowski, P. (2007) 'Viral Activation And Recruitment Of Metacaspases In The Unicellular Coccolithophore, *Emiliania Huxleyi*', *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), pp. 6049–6054. doi: 10.1073/pnas.0701240104.

Blaise, C. and Vasseur, P. (2005) 'Algal microplate toxicity test', in Blaise, C. and Férard, J.-F. (eds) *Small-scale Freshwater Toxicity Investigations, Vol. 1: Toxicity Test Methods*. Dordrecht, Netherlands: Springer, p. 137−179.

Chen, M., Tang, H., Ma, H., Holland, T. C., Ng, K. Y. S. and Salley, S. O. (2011) 'Effect Of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*', *Bioresource technology*. Elsevier Ltd, 102(2), pp. 1649–55. doi: 10.1016/j.biortech.2010.09.062.

Guillard, R. R. L. and Hargraves, P. E. (1993) '*Stichochrysis immobilis* is a diatom, not a chrysophyte', *Phycologia*, 32(3), pp. 234–236. doi: 10.2216/i0031-8884-32-3-234.1.

Herigstad, B., Hamilton, M. and Heersink, J. (2001) 'How To optimize the drop plate method for enumerating bacteria', *Journal of microbiological methods*, 44(2), pp. 121–9.

Jones, R. J., Ward, S., Amri, A. Y. and Hoegh-Guldber, O. (2000) 'Changes in quantum efficiency of Photosystem II of symbiotic dinoflagellates of corals after heat stress, and of bleached corals sampled after the 1998 Great Barrier Reef mass bleaching event', *Marine and freshwater research* , 51(1), pp. 63–71. doi: 10.1071/MF99100.

MacIntyre, H. L. and Cullen, J. J. (2005) 'Using cultures to investigate the physiological ecology of microalgae', in Anderson, R. (ed.) *Algal Culturing Techniques*. 1st Editio. Burlington, MA: Elsevier Academic Press, pp. 287–326.

Maxwell, K. and Johnson, G. N. (2000) 'Chlorophyll fluorescence-a practical guide', *Journal of experimental botany*, 51(345), pp. 659–668. doi: 10.1093/jexbot/51.345.659.

Moore, L. R., Goericke, R. and Chisholm, S. W. (1995) 'Comparative Physiology Of Synechococcus And Prochlorococcus: Influence Of Light And Temperature On Growth, Pigments, Fluorescence And Absorptive', *Marine Ecology Progress Series*, 116, pp. 259–275. doi: 10.3354/meps116259.

Nagai, T., Taya, K., Annoh, H. and Ishihara, S. (2013) 'Application Of A Fluorometric Microplate Algal Toxicity Assay For Riverine Periphytic Algal Species', *Ecotoxicology and Environmental Safety*, 94, pp. 37–44. doi: 10.1016/j.ecoenv.2013.04.020.

Scarratt, M. G., Marchetti, A., Hale, M. S., Rivkin, R. B., Michaud, S., Matthews, P., Levasseur, M., Sherry, N., Merzouk, A., Li, W. K. W. W., *et al.* (2006) 'Assessing microbial responses to iron enrichment in the subarctic northeast pacific: do microcosms reproduce the *in situ* condition?', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 53(20–22), pp. 2182–2200. doi: 10.1016/j.dsr2.2006.05.035.

Schreiber, U. (1998) 'Chlorophyll fluorescence: new instruments for special applications', *Photosynthesis: mechanisms and effects*, V, pp. 4253–4258.

Schreiber, U. (2004) 'Pulse-Amplitude-Modulation (PAM) fluorometry and saturation pulse method: an overview', in Papageorgiou, G. C. and Govindjee (eds) *Chlorophyll a Fluorescence: A Signature of Photosynthesis*. Netherlands: Springer, pp. 279–319. doi: 10.1007/978-1-4020-3218-9.

Seyedsayamdost, M. R., Case, R. J., Kolter, R. and Clardy, J. (2011) 'The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*', *Nature chemistry*, 3(4), pp. 331–5. doi: 10.1038/nchem.1002.

da Silva, J. M., da Silva, A. B. and Pádua, M. (2007) 'Modulated chlorophyll *a* fluorescence: A tool for teaching photosynthesis', *Journal of Biological Education*, 41(4), pp. 178–183. doi: 10.1080/00219266.2007.9656094.

Skjelbred, B., Edvardsen, B. and Andersen, T. (2012) 'A high-throughput method for measuring growth and loss rates in microalgal cultures', *Journal of applied phycology*, 24, pp. 1589–1599. doi: 10.1007/s10811-012-9819-z.

Vieira, S., Ribeiro, L., Jesus, B., Cartaxana, P. and da Silva, J. M. (2013) 'Photosynthesis Assessment In Microphytobenthos Using Conventional And Imaging Pulse Amplitude Modulation Fluorometry.', *Photochemistry and photobiology*, 89(1), pp. 97–102. doi: 10.1111/j.1751-1097.2012.01224.x.

Chapter 3. Roseobacter *Phaeobacter inhibens* is a selective pathogen of its algal host *Emiliania huxleyi*

3.1. Introduction

On a microscopic scale, the marine environment is a heterogeneous mixture of nutrient 'hotspots' formed by plankton and marine snow (Azam, 1998). Marine microbes take advantage of this aquatic array of nutrient gradients and 'hotspots' by preferentially occupying specific niches (Hunt *et al.*, 2008; Stocker, 2012). For example, the concentrations of algal metabolites are highest within the algal phycosphere (Bell *et al.*, 1972), so microbes capable of associating directly with phytoplankton benefit from a continuous stream of algal metabolites issuing from their algal hosts (Sapp *et al.*, 2007; Geng *et al.*, 2010). In this way, algal-associated bacteria might experience a nutrient benefit compared to the non-associated marine consortia.

Specialized bacteria have fine-tuned their ability to sense and respond to host exudates as a way of engaging in symbiotic or pathogenic relationships with their host (Miller *et al.*, 2006; Seymour *et al.*, 2010). Bacterial bioactive molecules have been implicated in bacterial-algal interactions at the cell-cell interface (Wagner-Döbler *et al.*, 2006). Bacteria can engage in symbiotic relationships with their algal host by producing small molecules that are beneficial or even required by the alga, such as vitamins (Bolch *et al.*, 2011). Other bacteria use bioactives in pathogenesis to coordinate virulence or kill their host (Case *et al.*, 2011; Fernandes *et al.*, 2011).

Coccolithophores, such as the wide spread bloom-forming *Emiliania huxleyi*, are calcifying microalgae that incorporate dissolved bicarbonate and calcium into delicate calcite scales called coccoliths. The *E. huxleyi* species complex, is made up of three distinct cell types: diploid calcifying, diploid non-calcifying, and haploid flagellated cells (Klaveness, 1972a; Frada *et al.*, 2012; von Dassow *et al.*, 2014). *E. huxleyi* blooms are comprised of complex mixtures of all three cell types, but the fast growing calcifying cells make up the majority of the bloom (Baumann *et al.*, 2008; Frada *et al.*, 2012). It is not presently understood how *E. huxleyi* alternates between haploid and diploid cell types, but each cell type is chloroplast-bearing and capable of asexual proliferation (Klaveness, 1972a). The fusion or syngamy of two haploid cells to generate a calcifying diploid cell was postulated based on the paleo-record (Young and Henriksen, 2003), but such events are rarely identified in laboratory cultures. The reverse

transition from diploid calcifying to motile haploid cells has been documented, supporting the suggestion that the alga's life cycle involves both asexual reproduction and meiosis of diploid cells generating haploid cells (von Dassow *et al.*, 2014). Interestingly, the viral pathogen or *E. huxleyi* viruses (EhVs), increases the generation of haploid cells in cultures either by inducing meiosis of calcifying cells or mitosis of a stable sub-population of haploid cells (Frada *et al.*, 2008). During pathogenesis the virus prompts both diploid cell types (calcifying and non-calcifying) to produce viral glycosphingolipids (vGLS), which induce algal programmed cell death (PCD) of diploid *E. huxleyi* cells (Bidle *et al.*, 2007), while the haploid cells escape viral lysis (Frada *et al.*, 2008).

Although its viral predators and grazers have been studied (Wolfe *et al.*, 1994; Wilson, Tarran and Zubkov, 2002), the association of bacteria with *E. huxleyi* is only recently being explored (Mayers *et al.*, 2016; Segev *et al.*, 2016). To gain insights into such bacterial-algal relationships, we investigated how *E. huxleyi* interacts with the marine alphaproteobacteria: *Phaeobacter inhibens* strain DSM17395, hereafter called *P. inhibens*. *P. inhibens* produces a number of novel bioactives, including the antibiotic tropodithetic acid (TDA) (Thole *et al.*, 2012) and potent algicides called roseobacticides (Seyedsayamdost *et al.*, 2011). These bioactives might allow *P. inhibens* to live a duplicitous lifestyle as both a beneficial symbiont and a pathogenic one. For example, TDA has been implicated in *P. inhibens'* chemical defense of various hosts (D'Alvise *et al.*, 2012), while various roseobacticides might facilitate pathogenesis, as they can directly cause lysis of specific non-calcifying diploid strains (Seyedsayamdost *et al.*, 2011). *P. inhibens* is also a pathogen of one representative strain of calcifying *E. huxleyi* (Segev *et al.*, 2016), however the way in which this bacterial pathogen might alter the ratios of the three algal cell types within a complex population has not yet been tested. This research aims to elucidate the interaction between *P. inhibens* and each of the *E. huxleyi* cell types and clarify the role roseobacticides play in killing this microalga.

3.2. Methods

3.2.1. Algal and bacterial strains

All axenic *E. huxleyi* strains were obtained, maintained, and grown as described in Chapter 2. Axenic *E. huxleyi* strains used include: calcifying CCMP3266, haploid CCMP3268, and non-calcifying diploid strains: CCMP370, CCMP372, CCMP374, CCMP379, and CCMP2090. Most

of the strains were all isolated from unique geographical locations, however CCMP3268 was single cell isolated from CCMP3266 after isolation from the Tasman Sea over 10 years ago (von Dassow *et al.*, 2014) (Table 3.1).

The wild type (WT) *P. inhibens* DSM17395 (Frank *et al.*, 2014), hereafter called *P. inhibens*, was maintained at 30 °C on ½ MA. Colonies were checked for purity and uniform appearance (dark brown colony morphology) then several isolated colonies were transferred into ½MB and grown at 18 °C, 30 h, 160 rpm, prior to co-cultivation.

3.2.2. Bacterial and algal co-cultivation

Bacterial-algal co-cultivation was performed as previously described (Chapter 2). Briefly, *E. huxleyi* and *P. inhibens* were mixed 1:1 (volume:volume) with 10^2 cfu/mL bacteria and 10^5 cells/mL algae, then 1 mL of this co-culture was aliquoted into 48-well plates (Chapter 2).

3.2.3. *P. inhibens* transposon mutants co-cultured with *E. huxleyi*

Four transposon mutants of *P. inhibens*, each interrupting required steps of the roseobacticide biosynthesis pathway, were obtained from the Joint Genome Institute (JGI) (Wetmore *et al.*, 2015; Wang *et al.*, 2016). The mutants were maintained on ½ MA amended with 200 μg/mL kanamycin (Sigma-Aldrich), where they all presented with white colony morphology, allowing for easy differentiation from WT *P. inhibens*. Mutants screened include those interrupting two genes in the *tda* gene cluster: *tdaB* (AFO93379) and *paaZ2* (AFO93362), which interrupt both TDA biosynthesis and roseobacticide biosynthesis; and two genes in the *paa* gene cluster: *paaA* (AFO90142) and *patB* (AFO89832), which interrupt only roseobacticide biosynthesis (Wetmore *et al.*, 2015; Wang *et al.*, 2016). Lack of roseobacticide production for all mutants was confirmed by detecting roseobacticides (430 nm) via spectrometry (Seyedsayamdost *et al.*, 2011).

Successful interruption of the targeted genes by the insertion of the 1,570 base pair transposon was confirmed by sequencing over the mutation site (Wetmore *et al.*, 2015). Prior to extracting DNA, mutants were grown both on ½ MA and for 24 h in ½MB and extracted together to ensure plasmid expression in all mutants. Genomic DNA was extracted using GeneJet Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's protocol. Gene-specific PCR amplifications were performed using Phusion High-Fidelity DNA polymerase (Thermo Scientific) according to the manufacturer's protocol. PCR products were either column- or gel-purified using QIAquick PCR Purification Kit or MinElute Gel Extraction

Kit (QIAGEN), respectively. Amplicon sequencing was performed using the Sanger dideoxy method (Applied Biosystems 3730 Genetic Analyzer, Life Technologies).

Co-cultivation of CCMP3266 and CCMP3268 with the four transposon mutants of *P. inhibens* were performed as stated above; all mutants were added to co-cultures at target cell concentration of 10^2 cfu/mL. Control and co-cultures were amended with kanamycin to a final concentration of 100 µg/mL (Wetmore *et al.*, 2015). Various concentrations (including 0, 10, 50, 100, 200 µg/mL) of kanamycin were tested on control cultures of *E. huxleyi*, and it was determined that 100 µg/mL kanamycin did not adversely affect algal cultures (Appendix C-1). Mutants were monitored on ½ MA with and without 200 µg/mL kanamycin throughout the co-cultivation with algae to ensure no reversion to WT *P. inhibens* morphology.

3.2.4. Roseobacticide B and algal co-cultivation

Freeze-dried roseobacticide B was obtained from Seyedsayamdost *et al.*, and dissolved in methanol. The compound was added to senescent algal cultures (9 d) of CCMP3266 and CCMP3268 at a final concentration of 0.019 µM, 0.19 µM, and 1.9 µM roseobacticide B. Methanol solvent added was under 2% of the final volume. Samples were well mixed and then microtiter plates were returned to the diurnal incubator for 24 h prior to measuring PAM fluorescence.

3.2.5. Microscopy

Brightfield images were obtained using an Axio Scope.A1, equipped with an Optronics digital camera and PictureFrame software v2.3 (Zeiss). Epifluorescence images were obtained using an Axio Imager.M2, equipped with a monochrome camera (AxioCam 506). Unfixed *E. huxleyi* control and co-culture aliquots were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) (Life Technologies) according to manufacturer's instructions (30 °C, 20 min) then immediately pelleted by centrifugation (5,000 × g, room temperature, 2 min). Cells were gently washed twice in sterile L1-Si media and analyzed immediately on the epifluorescence microscope. Three images were acquired simultaneously and then overlaid using Zen 2 Blue Edition software, 1) differential interference contrast (DIC) channel was overlaid with 2) algal chlorophyll auto-fluorescence (red: excitation = 610-650 nm; emission = 670-720 nm) and 3) DNA-DAPI complex fluorescence (blue: excitation = 350-400 nm; emission = 417-477 nm).

3.2.6. Fluorescence measurements

A pulse-amplitude-modulation (PAM) fluorometer was used to measure chlorophyll fluorescence (as previously described Chapter 2). Data were subsequently processed using SigmaPlot 12.0. Statistical significance determined via a one-way ANOVA and Tukey HSD test.

3.2.7. Flow cytometry and bacterial counts

E. huxleyi control and co-culture populations were fixed for flow cytometry by incubating cells in the dark for 10 min with 0.15% glutaraldehyde (Sigma-Aldrich), flash-freezing in liquid nitrogen, and storing frozen aliquots at -80 °C. Flow cytometry was performed using a FACSCalibur (Becton Dickinson) and an excitation laser of 488 nm. Samples were run for 30 sec counting all particles. Algal cell enumeration flow cytometry data were processed using FlowJo 9.2, as follows. Doublet discrimination was first done using forward scatter height (FSC-H) versus forward scatter width (FSC-W), and cells containing the same height but double the width were considered doublets or clumps of algal cells and were removed (Donnenberg *et al.*, 2015). Algal cells were then counted using both chlorophyll auto-fluorescence (emission: 667 nm) and forward scatter area (FSC-A), which corresponds to cell size.

The bacterial population density grown alone and in co-culture was enumerated by the drop-plate method (described in Chapter 2, Figure 2.2). Statistical significance was determined using a one-way ANOVA and Tukey HSD test.

3.3. Results and Discussion

E. huxleyi blooms are frequently associated with a diverse microbial consortia (Green *et al.*, 2015), commonly dominated by members of the roseobacter clade, such as *P. inhibens*, especially toward the end of bloom (Green *et al.*, 1996; Segev *et al.*, 2016). Even so, specific interactions between members of the microbial consortia and the various *E. huxleyi* cell types (diploid calcifying, diploid non-calcifying, and haploid flagellated) remain largely unexamined. The interactions between various EhVs and the different cell types of *E. huxleyi* have been studied (Bidle *et al.*, 2007), leading to the proposal of differential viral infection depending on algal cell type (Frada *et al.*, 2008). To determine if there was a different bacterial-algal interaction based on the origin or cell type of the host, we co-cultured various axenic (calcifying and non-calcifying) *E. huxleyi* strains with the model marine roseobacter *P. inhibens* (Table 3.1)

Table 3.1. ***Emiliania huxleyi*** **strains and their susceptibility to** ***Phaeobacter inhibens*** **pathogenesis.**

CCMP strain code	RCC strain code	Isolation location	Axenic	Cell type*	Killed by *P. inhibens*†
CCMP370	RCC1255	Oslo Fjord, Norway, North Atlantic	Yes	N	No
CCMP372		Sargasso Sea, North Atlantic	Yes	N	No
CCMP374	RCC1259	Gulf of Maine, North Atlantic	Yes	N	No
CCMP379		English Channel, North Atlantic	Yes	N	No
CCMP2090		Coast of South America, South Pacific	Yes‡	N	No
CCMP3266	RCC1216	Tasman Sea, South Pacific	YesX	C	Yes
CCMP3268	RCC1217	clonal isolate from RCC1216	YesX	S	Yes

*Calcifying diploid (C), non-calcifying diploid (N), and scale-bearing haploid (S)
†Co-culture experiments were observed over 30 d without precipitous algal death
‡The axenic form of the polymicrobial non-calcifying diploid strain CCMP1516
XCCMP cultures are the only commercially available axenic versions

3.3.1. *P. inhibens* is a selective pathogen

P. inhibens was previously shown to have a unique interaction with *E. huxleyi,* whereby algal ageing resulted in the release of the chemical cue *p*-coumaric acid (*p*CA), which triggered the bacteria to produce potent algicides, called roseobacticides (Seyedsayamdost *et al.*, 2011). Purified roseobacticides can kill at least one non-calcifying diploid strain of *E. huxleyi* (CCMP372) (Seyedsayamdost *et al.*, 2011). However, only the axenic calcifying strain CCMP3266 and the haploid strain CCMP3268 were killed by *P. inhibens* when grown in co-culture. Whereas, all tested non-calcifying diploid strains, regardless of geographic origin, survived (including the strain CCMP372, which was previously shown to be killed by purified roseobacticides (Seyedsayamdost *et al.*, 2011)). These findings might suggest that the algicidal activity of *P. inhibens* against the microalga host *E. huxleyi* is potentially dependent on algal cell type (Table 3.1). Alternatively, this differential killing might be related to the presence of different variable genes in sensitive strains compared to resistant non-calcifying strains (Read *et al.*, 2013). Regardless, this finding differentiates the bacterial pathogen from the viral pathogen,

which instead kills both diploid strains, while the haploid cell type escapes EhV-induced lysis (Frada *et al.*, 2008).

3.3.2. Non-calcifying diploid resistance to pathogenesis

There are a few possible explanations as to why non-calcifying diploid *E. huxleyi* cells are widely resistant to *P. inhibens* pathogenesis. If survival is due to an algal escape from the pathogen, it is likely to be different from the proposed reasons for haploid escape from EhVs. These viruses are thought to be incapable of recognizing and infecting the haploid cell type (Frada *et al.*, 2008), while EhVs effectively co-opt the metabolic sphingolipid pathway of both diploid *E. huxleyi's* strains, subsequently inducing PCD (Bidle *et al.*, 2007). It was postulated that the haploid scaly coverings or perhaps variation/loss of viral receptor molecules might infer this viral resistance of the haploid cell type (Frada *et al.*, 2008). Supporting this theory, slightly elevated temperature confers temperature-induced resistance to EhVs by altering the outer sphingolipids of representative calcifying and non-calcifying diploid *E. huxleyi* strains, thereby impeding viral recognition and infection of target cells (Kendrick *et al.*, 2014).

In terms of the bacterial pathogen, attached bacteria are identifiable on all three cell types (Figure 3.1), suggesting that the bacteria attach to the non-calcifying algae successfully. Alternatively, genetic differences might play an important role in the escape of non-calcifying strains. *E. huxleyi* is a vast species complex with a pan genome, meaning *E. huxleyi* strains share a set of core genes and each have additional variable genes, which enhances *E. huxleyi*'s pronounced intraspecies variability (Read *et al.*, 2013). So, it is possible that genetic differences between the sensitive and resistant algal strains might be responsible for this differential interaction. Additionally, non-calcifying diploid cells are generated from cultures of calcifying diploid cells (Zhang *et al.*, 2016), reportedly due to a series of mutations resulting in, among other differences, a malformed coccolith-forming vesicle (van der Wal *et al.*, 1983). Given the way non-calcifying diploid strains arise in the population (Zhang *et al.*, 2016), it is possible that these non-calcifying strains might also have genetic differences from their calcifying parent strains, which might also confer resistance.

3.3.3. Targeted pathogenesis of *P. inhibens* against specific cell types of *E. huxleyi*

To further investigate the differential pathogenesis of *P. inhibens* when grown in co-culture with *E. huxleyi*, axenic representative strains of each of the three cell types were used for more

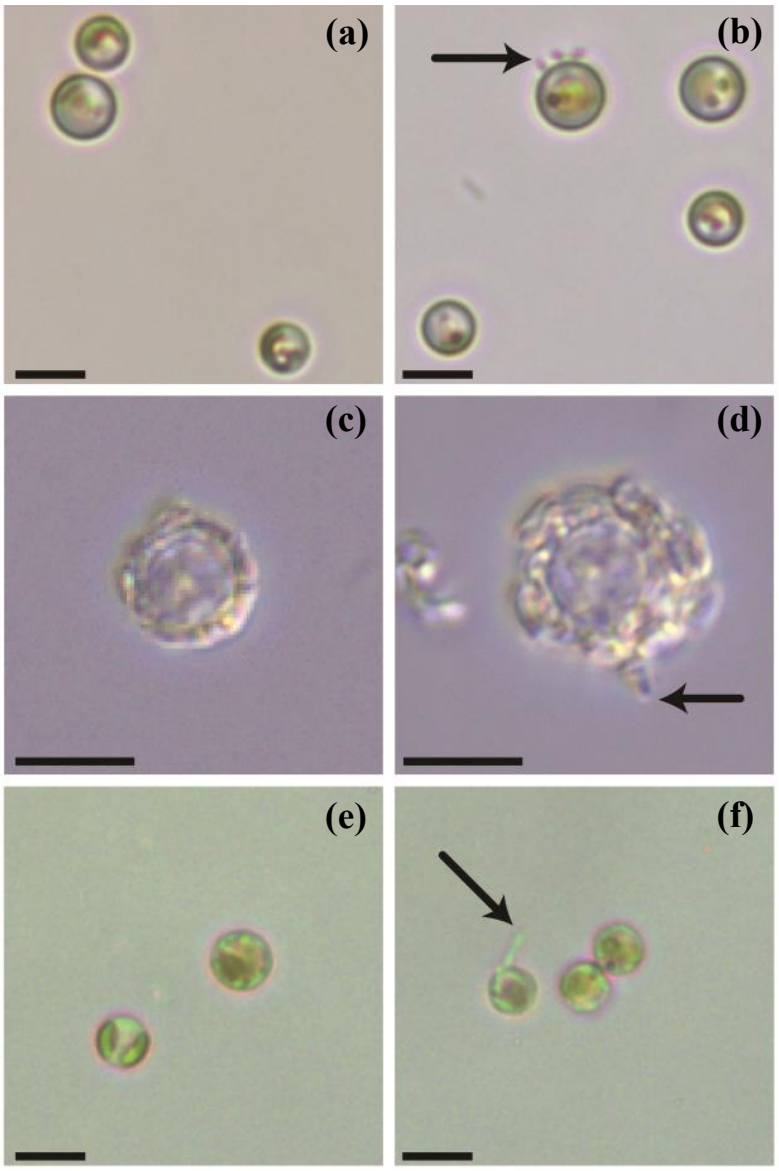

Figure 3.1 **Brightfield light microscopy of *Phaeobacter inhibens* attached to non-calcifying diploid (CCMP2090), diploid calcifying (CCMP3266), and haploid (CCMP3268) *Emiliania huxleyi* strains**. Brightfield light microscopy of *E. huxleyi*: (a) control CCMP2090, (b) CCMP2090 co-culture with *P. inhibens*, (c) control CCMP3266, (d) CCMP3266 co-culture with *P. inhibens*, (e) control CCMP3268, (f) CCMP3268 co-culture with *P. inhibens*. *P. inhibens* cells attached to algal host are indicated by black arrows.

in-depth analysis. The algal strains selected were: 1) calcifying CCMP3266 (the only known axenic calcifying strain). 2) haploid CCMP3268 (which was single cell isolated from the newly isolated calcifying diploid RCC1216 culture (von Dassow *et al.*, 2014), and 3) non-calcifying CCMP2090. Throughout the experiment *P. inhibens* were easily identifiable attached to each of the representative cell types (Figure 3.1). Photosystem II (PSII) health of the algal population was monitored throughout the experiment using PAM fluorometry (Figure 3.2), thereby detecting declining PSII maximum quantum efficiency (F_v/F_m), which can be the result of cellular stress and/or losses of functional PSII centers per cell (Parkhill *et al.*, 2001).

When grown alone, all three algal cell types (CCMP2090, 3266, and 3268) demonstrated stable PSII maximum quantum efficiency ($F_v/F_m > 0.5$) throughout the experiment (Figure 3.2a-c). The non-calcifying strain maintained chlorophyll health and functional PSII systems when grown alone or in co-culture with *P. inhibens* (Figure 3.2a). However, when grown in co-culture with *P. inhibens,* both calcifying and non-calcifying haploid strains (CCMP3266 and 3268, respectively), experienced an accelerated premature decline in photosystem health (8-10 d) (Figure 3.2b,c). The rapid decline of PSII efficiency is intriguing as it has not been observed in senescent *E. huxleyi* cells (Franklin *et al.*, 2012), but is a common feature of EhV-induced death of *E. huxleyi* (Bidle *et al.*, 2007). It is tempting to suggest that this similar *E. huxleyi* physiology (rapid loss of PSII efficiency) during algal death might imply that perhaps *E. huxleyi* cells in co-culture with the viral or bacterial pathogen might undergo a similar death process. However, investigating this question further would first require elucidating the mechanism of *E. huxleyi* death as a result of *P. inhibens* pathogenesis.

3.3.4. Population dynamics of the algae and bacterium in co-culture

Algal population dynamics when grown axenically and in co-culture with *P. inhibens* were monitored using flow cytometry (Figure 3.3a-c), highlighting the differential pathogenesis of the bacterial symbiont. Bacterial population dynamics when grown alone and in co-culture were monitored using colony forming units (cfu), demonstrating a population-wide benefit to being grown in co-culture with an algal host, regardless of host cell type (Figure 3.3d-f). Under the experimental conditions used (algal medium: L1-Si at 18 °C), *P. inhibens* was able to initially grow to a maximum cell density of 10^5 cfu/mL without a host (Figure 3.3d-f), followed by declining cell numbers (6-14 d). However, its growth was greatly enhanced by the presence of *E. huxleyi*, where its population density reached 10^7 cfu/mL after 14 d in co-culture (Figure 3.3d-f).

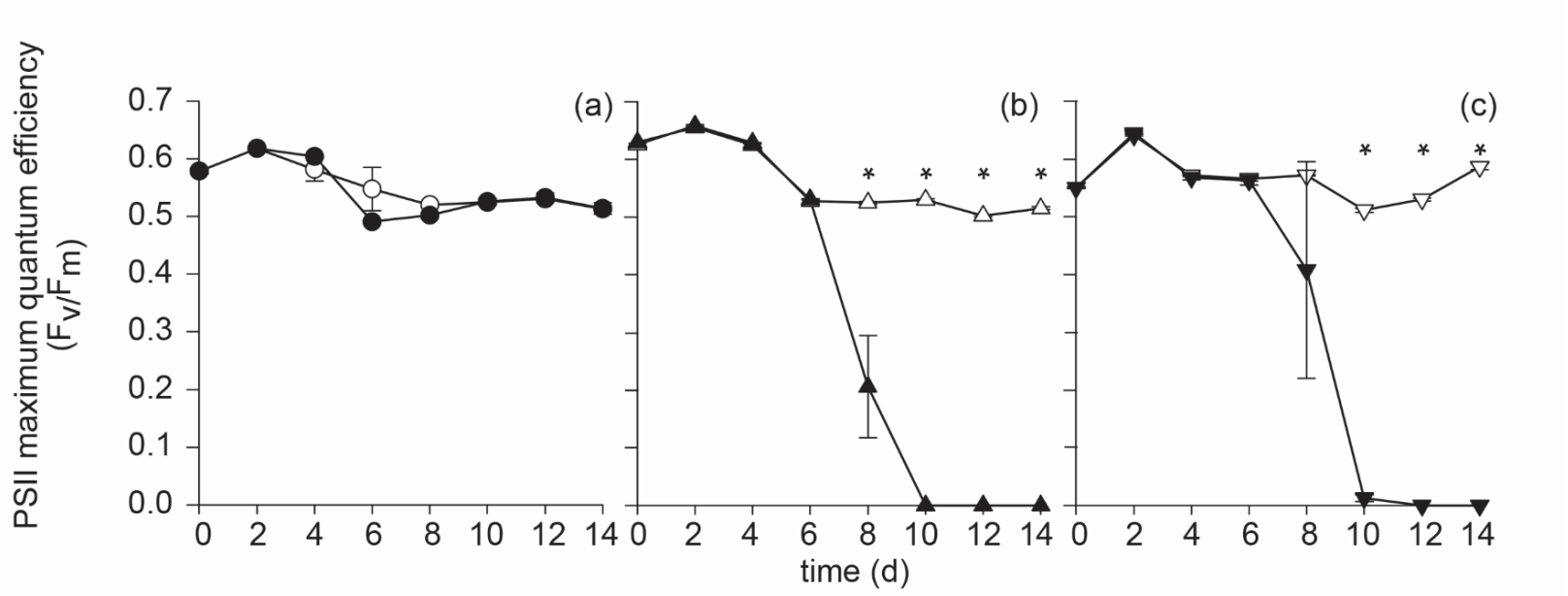

Figure 3.2. ***Phaeobacter inhibens*** **affects the photosynthetic health of** ***Emiliania huxleyi*** **cell types: non-calcifying diploid CCMP2090, diploid calcifying CCMP3266, and haploid CCMP3268**. The maximum quantum efficiency (F_v/F_m) is shown for axenic controls (white) and co-culture experiments (black) for all three tested cell types: (a) CCMP2090 (circles), (b) CCMP3266 (triangles) and (c) CCMP3268 (inverted triangles). Triplicate wells were run, error bars represent ±1 standard error. An asterisk (*) at a time point indicates that the co-culture (black) is significantly different from the cooresponding control (white).

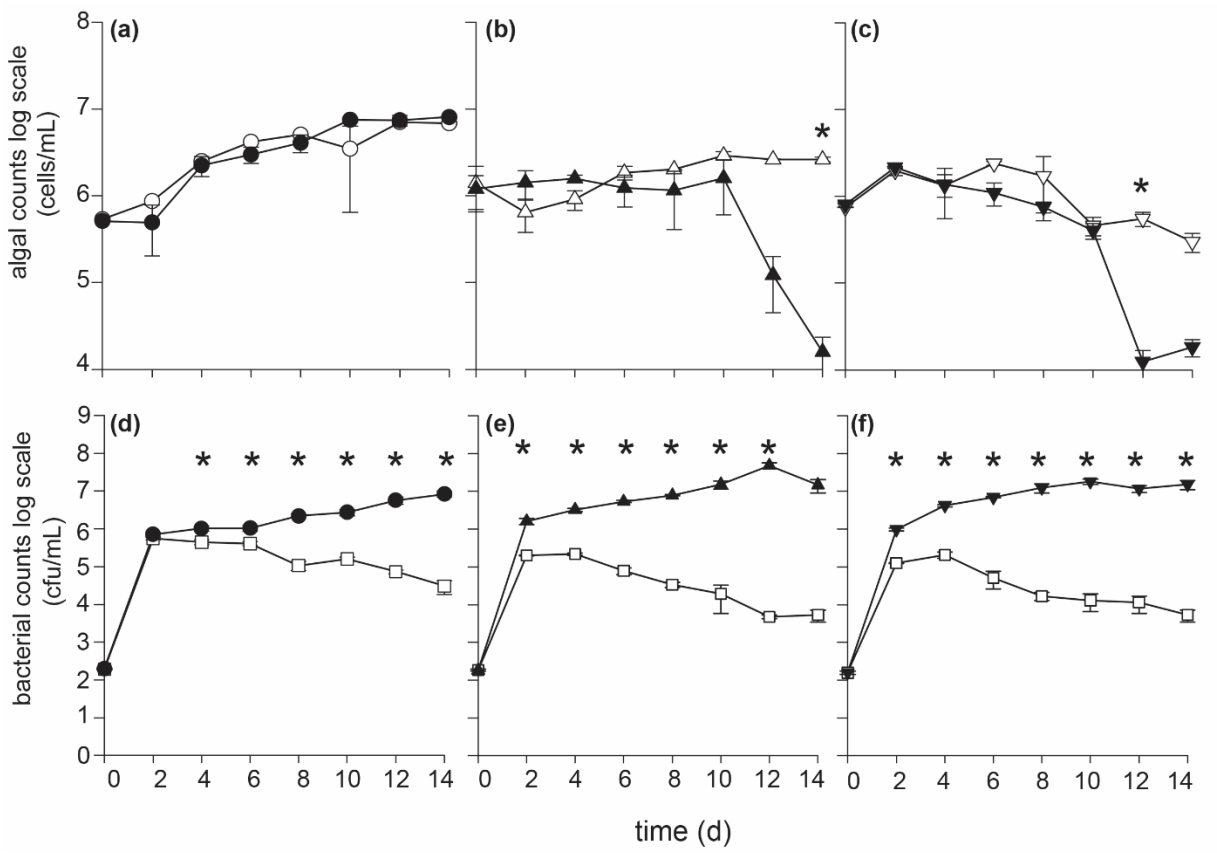

Figure 3.3. **Co-culturing experiment of *Phaeobacter inhibens* with three *Emiliania huxleyi* cell types: non-calcifying diploid CCMP2090, diploid calcifying CCMP3266, and haploid CCMP3268**. Algal cell counts (cells/mL) were performed using flow cytometry for (a) CCMP2090 (circles), (b) CCMP3266 (triangles), and (c) CCMP3268 (upside down triangles) for both axenic control (white) and grown in co-culture with *P. inhibens* (black). Bacterial counts (cfu/mL) were performed on *P. inhibens* cultured in algal medium (white squares) and co-cultured with the algae: (d) CCMP2090 (black circles) (e) CCMP3266 (black triangles), and (f) CCMP3268 (black upside-down triangles). Triplicate wells were run, error bars represent ±1 standard error. An asterisk (*) at a time point indicates that it is significantly different to the control.

Without a host *P. inhibens* peak cell density was rapidly achieved (2-4 d), whereas with a host peak bacterial density occurs after death of the algal host (10-14 d) (Figure 3.3). Prolonged monitoring of *P. inhibens* grown without a host revealed that all bacteria died by 25 d. This increased persistence and higher population density of *P. inhibens* when *E. huxleyi* is present is likely the result of nutrient rich algal exudates (Borchard *et al.*, 2012). Phytoplankton constantly leak sugars and oxygen from photosynthesis, as well as other nutrients such as dimethylsulfopropionate (DMSP) and amino acids, which are chemoattractants for roseobacters (Miller and Belas, 2004; Miller, Hnilicka, *et al.*, 2004). Additionally, DMSP-degrading microbes, like *P. inhibens*, benefit directly from being able to efficiently assimilate the sulfur from DMSP directly into bacterial amino acids (González *et al.*, 2000).

P. inhibens does not influence algal cell numbers for the non-calcifying diploid strain CCMP2090 (Figure 3.3a). *P. inhibens* grown in co-culture with CCMP2090 maintained high cell counts (10^8 cfu/mL) (Figure 3.3d) as long as there was a healthy algal host, until at least 30 d. Similarly, related roseobacter pathogen, *Ruegeria* sp. R11, kills both calcifying and haploid cell types, while not affecting non-calcifying diploid CCMP2090 (Mayers *et al.*, 2016).

CCMP3266 grown alone has exponential growth, until the culture reaches a maximum cell density (10 d), which is typical of the growth dynamics of axenic CCMP3266 within the microtiter format (Chapter 2). Conversely, when grown with *P. inhibens,* chlorophyll containing algal cells prematurely decline (14 d) compared to the axenic control (Figure 3.3b). A similar trend is observed for the haploid CCMP3268 (Figure 3.3c). Grown alone, the culture reaches a maximum cell density (6 d), then the algae enters senescence, typified by gradual losses of cell numbers and chlorophyll a fluorescence (Franklin *et al.*, 2012). Following gradual senescence, control cultures remain alive in stationary phase for the remainder of the experiment; but when grown in co-culture with *P. inhibens* the haploid *E. huxleyi* strain demonstrates premature losses of cells/mL (12 d; Figure 3.3c). This indicates that the pathogen first induces loss of algal photosynthetic health (8 d) (Figure 3.2b,c), followed by a rapid decline in the number of chlorophyll containing algal cells (12-14 d) (Figure 3.3b,c).

The benefit to *P. inhibens* persisted until long after the death of the sensitive algal host (d 12-14). In fact, *P. inhibens* grown with CCMP3266 and CCMP3268 maintained high cell counts until at least 30 d, when both co-cultures had 10^6 and 10^7 cfu/mL, respectively, despite lacking a healthy algal host. This would seem to indicate that as algal health and densities of calcifying

CCMP3266 and haploid CCMP3268 rapidly decline, the bacteria in co-culture derives a population-wide benefit, presumably from increased nutrients released by algal cell lysis (Kolb *et al.*, 2013). The benefit to *P. inhibens* in killing its host makes it an opportunistic pathogen rather than a parasite, as parasites can reduce the health of their host but benefit from prolonging the host's life, while *P. inhibens* causes CCMP3266 and CCMP3268 to die prematurely.

3.3.5. Haploid cells form a dynamic sub-population of a diploid calcifying cultures

Initial control experiments were performed to verify the ploidy of our model strains CCMP2090 (diploid 2N), CCMP3266 (diploid 2N), and CCMP3268 (haploid 1N) (Paasche *et al.*, 1970; Green *et al.*, 1996; von Dassow *et al.*, 2009). While, ploidy analysis is typically performed after nuclear extraction, this method has not yet been successful on cultures of calcifying *E. huxleyi* (von Dassow *et al.*, 2009). For this reason, ploidy analysis of calcifying strains is routinely performed using stained whole algal cells and then sorting based on the relative intensity of the dsDNA-complex after intercalation with the fluorescent stain (dsDNA-SYBR emission: 520 nm) (Green *et al.*, 1996; von Dassow *et al.*, 2009) (Figure 3.4).

Ploidy comparisons of all three cell types were performed using whole cell flow cytometry analysis of early-log SYBR-I stained *E. huxleyi* cultures (Green *et al.*, 1996; Medlin *et al.*, 1996; Laguna *et al.*, 2001). The high nucleic acid peaks, representing diploid (2N) cells (CCMP2090 and CCMP3266) appear to have identical nucleic acid content (Figure 3.4). Expectedly, the haploid population of CCMP3268 has one prominent low nucleic acid peak (1N), the slightly higher peak representing dividing haploid cells with double nucleic acid (2N) (Figure 3.4a).

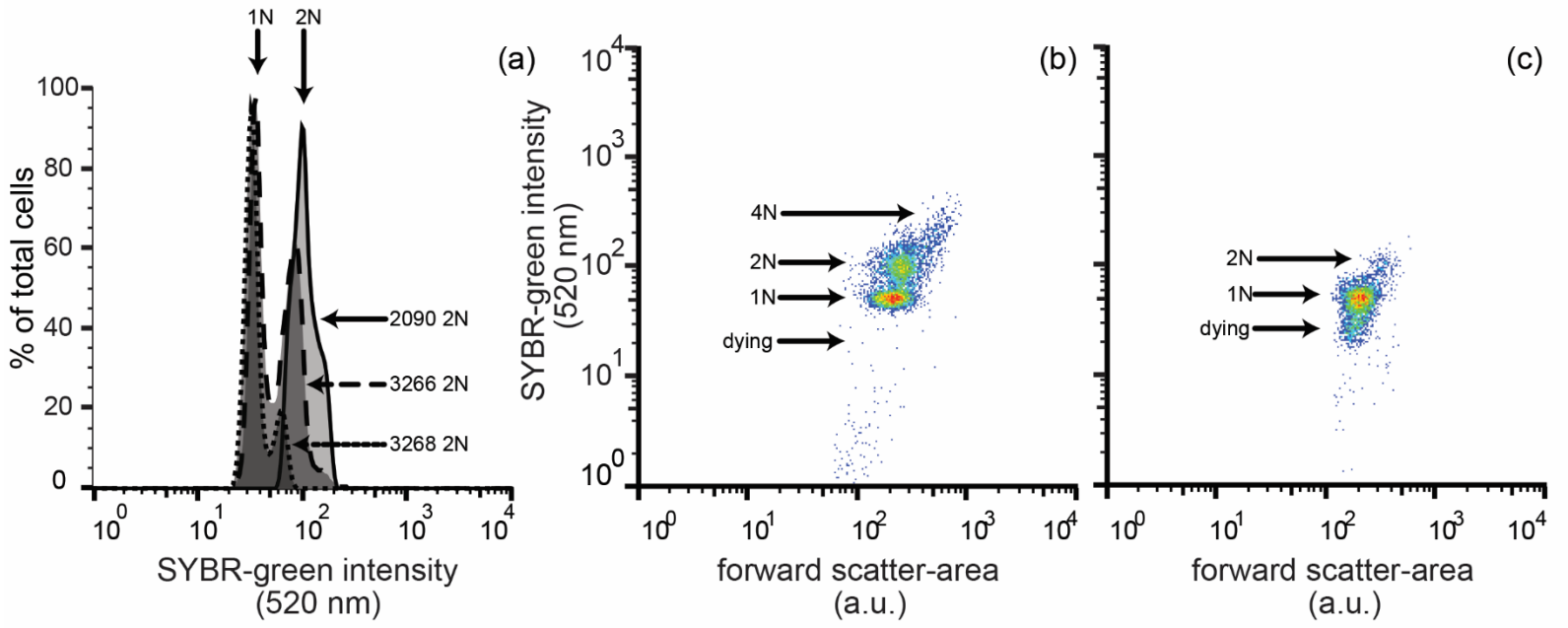

Figure 3.4. **Comparing the haploid-diploid population structure of *Emiliania huxleyi* CCMP2090, CCMP3266, CCMP3268.** The non-calcifying diploid (CCMP2090), calcifying diploid (CCMP3266), and haploid (CCMP3268) strains were stained with SYBR green to determine if cultures contained a mix of haploid (1N) and diploid (2N) cells. (a) The non-calcifying diploid culture (solid line) is predominantly 2N cells, the calcifying diploid culture (long dash line) contains two peaks corresponding to 2N and 1N (haploid + dividing 2N) cell populations. The haploid culture has a dominant 1N peak and a secondary 2N (dotted line). For clarity individual populations for (b) CCMP3266 and (c) CCMP3268 are also depicted as non-edited density dot plots, where each dot represents a stained algal cell. Cells are displayed with SYBR-DNA complex intensity (520nm) on the y-axis and algal particle size (forward scatter in arbitrary units (a.u.)) on the x-axis.

Predictably, the mono-culture of haploid cells had nucleic acid that was roughly half of that identified in the mono-culture of non-calcifying diploid non-calcifying cells (Green *et al.*, 1996). Unexpectedly, this analysis revealed that CCMP3266 is a diploid calcifying strain (2N) that also produces a strong 1N peak, which was suspected of including both dividing calcifying cells and possibly a dynamic sub-population of haploid non-calcifying cells (1N) (Figure 3.4b). Microscopic observation of CCMP3266 grown in co-culture with *P. inhibens* confirmed the presence of flagellated haploid cells in the CCMP3266 culture (Figure 3.5).

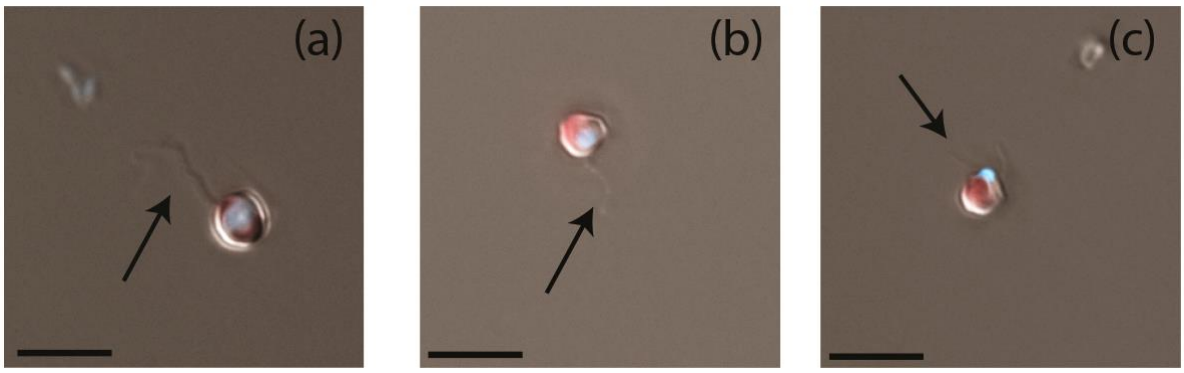

Figure 3.5. **Flagellated algal cells identified within diploid calcifying CCMP3266 grown in co-culture with *Phaeobacter inhibens* (4 d).** DIC images of CCMP3266 co-cultured with *P. inhibens*. Three separate cells (a-c) are shown overlaid with two fluorescent channels: 1) chlorophyll auto-fluorescence (red: excitation 610-650 nm; emission 670-720 nm) and 2) DNA-DAPI complex fluorescence (blue: excitation 350-400 nm; emission 417-477 nm). Flagella indicated by black arrows.

To better understand how the bacterial pathogen might influence this dynamic population of calcifying diploid cells and the sub-population of haploid flagellated cells, we tracked the two cell types in cultures of CCMP3266 alone and in co-culture with *P. inhibens* (Figure 3.6). When CCMP3266 is grown alone, haploid cells initially represented a high proportion of the population (Figure 3.6a). Overtime, this proportion naturally became diploid (2N) dominated (8-14 d), as would be expected of a calcifying *E. huxleyi* culture. However, in the co-culture, there appears to be a more rapid switch away from haploid cells (2-6 d, Figure 3.6b), which might suggest that *P. inhibens* is able to change the population structure of *E. huxleyi*. Haploid cells are vulnerable to *P. inhibens*, but the timing of death is comparable to CCMP3266, suggesting that *P. inhibens* is not prematurely killing the haploid population, but that the bacterium might be decreasing the proportion of haploid cells in this dynamic population by either limiting mitosis of haploid cells or meiosis of calcifying diploid cells.

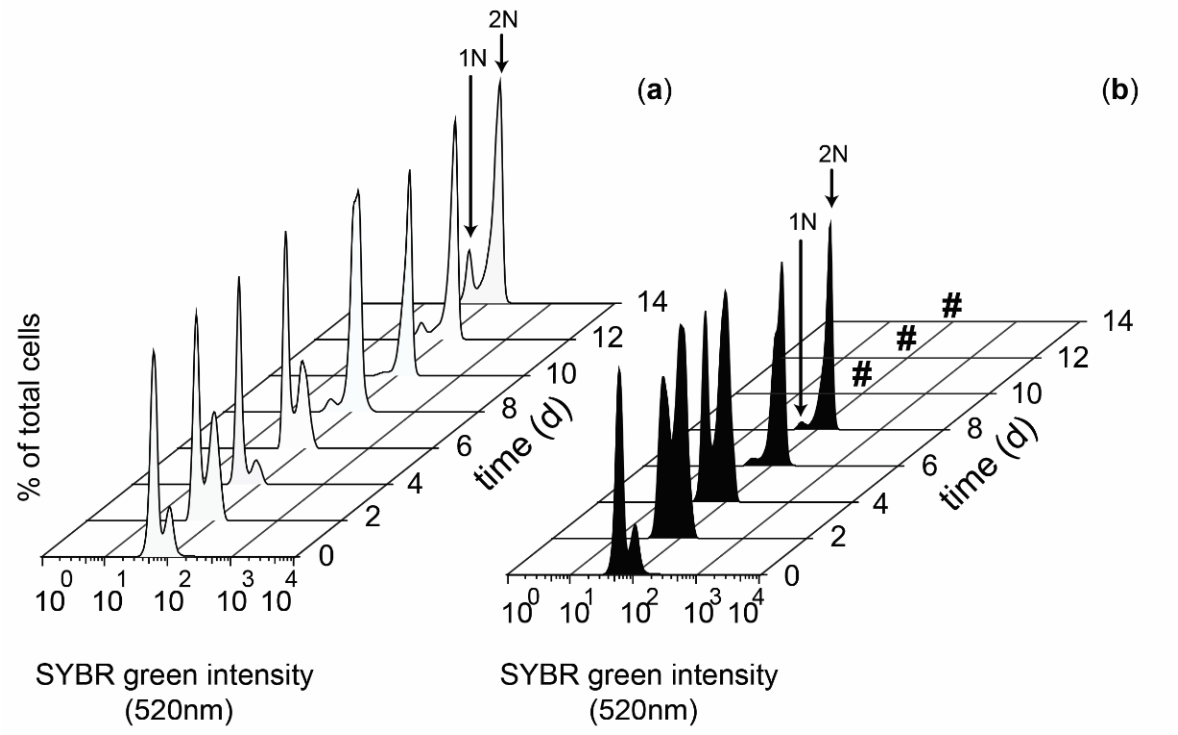

Figure 3.6. **The influence of *Phaeobacter inhibens* on haploid and diploid population dynamics in diploid calcifying *Emiliania huxleyi* strain CCMP3266.** The relative proportion of 1N and 2N cells was determined by flow cytometry of SYBR-DNA complex intensity (520 nm) for the (a) CCMP3266 axenic culture (white) and (b) the CCMP3266-*P. inhibens* co-culture (black). The histograms show relative proportion of haploid (1N) compared to diploid (2N) cells. # denotes where algal cells are no longer chlorophyll-containing or of the expected size of *E. huxleyi.*

This finding might imply that bacterial interaction with calcifying diploid and haploid cells might alter the proportions of haploid cells that are present within calcifying cultures. Dynamic calcifying cultures (with identifiable haploid cells) have been reported before in studies tracking the viral pathogen's interaction with calcifying cultures (Frada *et al.*, 2008). However, the cues to signal this transition are not yet understood. When previously identified, the authors suggested that either 1) the pathogen was stimulating meiosis of calcifying cells, resulting in a higher proportion of haploid cells in the culture or 2) the viral pathogen was targeting the more competitive calcifying cell, thereby allowing the sub-population of haploid cells to become comparatively more competitive (Frada *et al.*, 2008). In the case of the bacterial pathogen, it appears to do the opposite, possibly prematurely limiting the proportion of haploid cells relative to calcifying diploid cells. Mechanisms of inducing or halting meiosis of calcifying cells are not

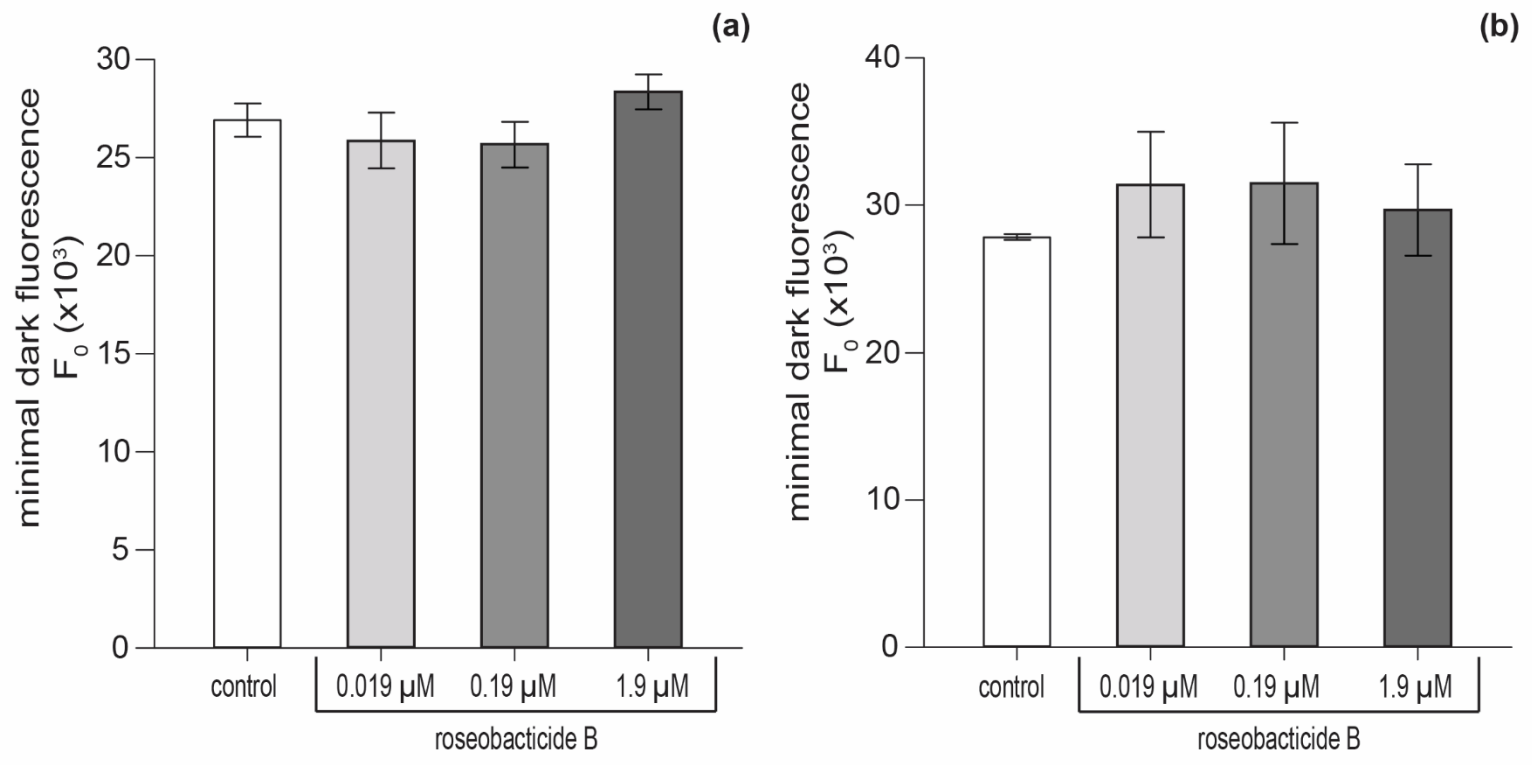

Figure 3.7. **Effect of various concentrations of roseobacticide B on senescent diploid calcifying CCMP3266 and haploid non-calcifying CCMP3268 *Emiliania huxleyi* strains.** Minimal algal fluorescence of the population (F_0) is shown for (a) senescent calcifying CCMP3266 cells and (b) senescent non-calcifying haploid CCMP3268 cells. Both cell types were incubated for 24 hours with methanol (white bar) and with 0.019 µM (lightest grey), 0.19 µM (grey), and 1.9 µM (darkest grey) roseobacticide B, dissolved in methanol. Triplicate wells were run, error bars represent ±1 standard error.

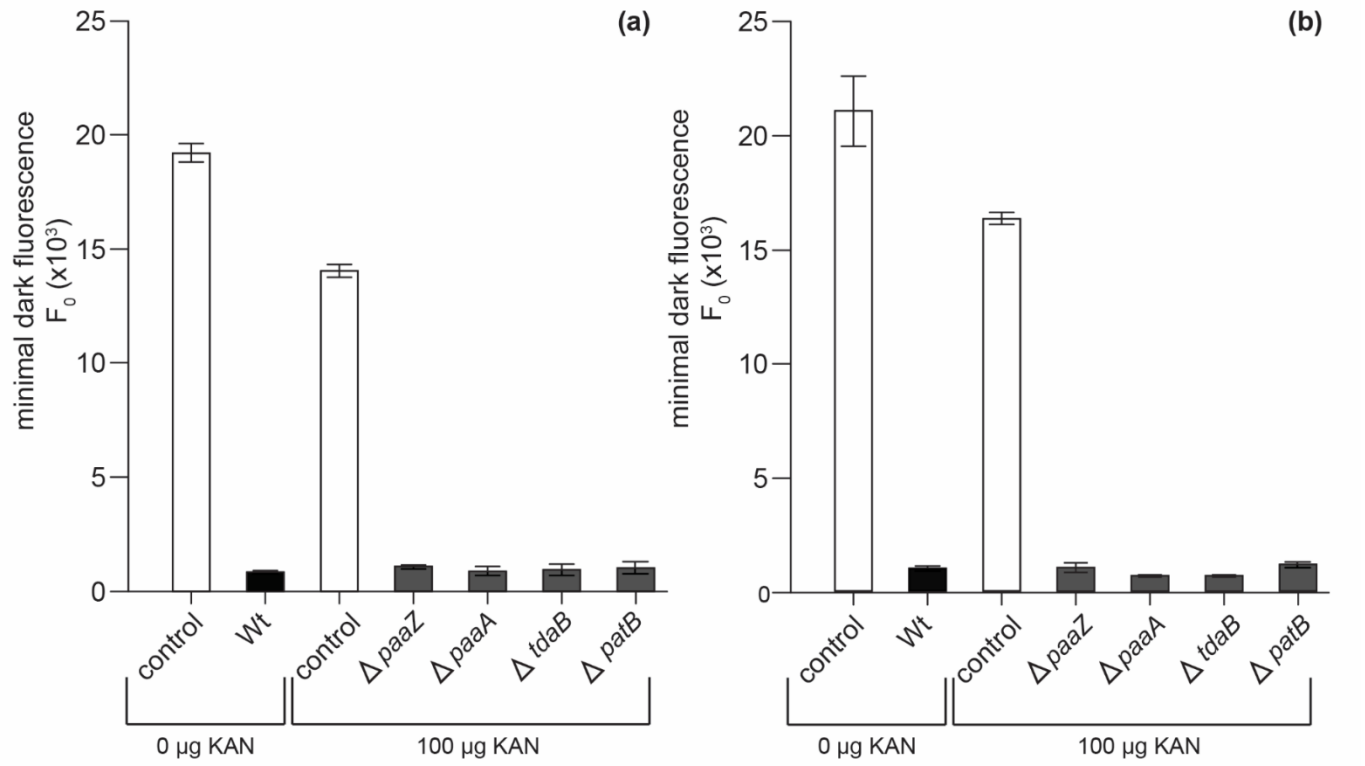

Figure 3.8. **Effect of transposon mutants *Phaeobacter inhibens* missing parts of the roseobacticide synthesis pathway on the diploid calcifying CCMP3266 and haploid CCMP3268 *Emiliania huxleyi* strains.**Minimal algal fluorescence of the population (F_0) is shown for (a) calcifying cell type CCMP3266 and (b) non-calcifying haploid CCMP3268 (10 d). Triplicate wells were run, error bars represent ±1 standard error.

well understood and more research is required to understand how the bacterial pathogen might be arresting meiosis of calcifying cells or mitosis of haploid cells. However, halting cell cycle progression is an effective strategy used by bacterial pathogens to control eukaryotic host cell proliferation and can directly trigger mitotic arrest and host cell death (Coburn *et al.*, 2000; Kurokawa *et al.*, 2010), so it is possible this phenomenon is due to *P. inhibens*' pathogenic strategy.

3.3.6. Roseobacticides implicated in killing diploid calcifying and haploid flagellated *E. huxleyi*

The rapid decline in maximum quantum yield and loss of chlorophyll observed throughout CCMP3266 and CCMP3268 cultures at 8-10 d (Figure 3.7) is consistent with the physiological response of *E. huxleyi* to roseobacticides, which can directly cause lysis of non-calcifying algal cells (Seyedsayamdost *et al.*, 2011). To test whether roseobacticides might be the causative agent of algal death, roseobacticide B was added to senescent calcifying diploid and haploid *E. huxleyi* on day nine (when the bacterium causes death of these algae) at half maximal lethal concentration (LC_{50}) of 0.19 μM determined for CCMP372 (Seyedsayamdost *et al.*, 2011), as well as tenfold lower and higher. Unexpectedly, no death was observed in either of the tested sensitive cell types (CCMP3266 or CCMP3268) (Figure 3.7). However, the bacterium attaches to the alga (Figure 3.1), so we postulated it might be possible that bacterial attachment might be necessary to transfer the roseobacticides at their lethal concentration to the alga.

To address this question, several *P. inhibens* transposon mutants with verified insertion mutations in essential genes required for roseobacticide synthesis were obtained (Wetmore *et al.*, 2015; Wang *et al.*, 2016). Then they were co-cultured with either CCMP3266 or CCMP3268 to determine if pathogen induced death was delayed or abolished. Death occurred in all co-cultures of the mutants with both CCMP3266 and CCMP3268 at the same time and in the same manner as when co-cultured with *P. inhibens* WT (Figure 3.8). This finding demonstrates that roseobacticides are not the only causative agent of death in the co-culture between *P. inhibens* and the tested calcifying and haploid strains of *E. huxleyi*. In fact, this finding is suggestive that there is some other, currently unknown, mechanism of *P. inhibens* pathogenesis.

3.4. Conclusion

A better understanding of how the two sensitive *E. huxleyi* strains die during this interaction is required to shed light on how the non-calcifying diploid strains appear to escape pathogenesis. Interestingly, the same non-calcifying strain shown here to be resistant to *P. inhibens* pathogenesis (CCMP2090), is killed by EhVs, which induce programmed cell death (PCD) with autophagy of the algal host (Schatz *et al.*, 2014). Autophagy, or the genetically programed lysosomal degradation of cellular constituents (Kroemer *et al.*, 2009), is a vital part of the host immune response and can infer protection against pathogens by ensuring the rapid degradation of bacterial virulence factors (Cemma *et al.*, 2012). It is interesting to speculate that the autophagy response of CCMP2090 might also protect against bacterial virulence factors by rapidly degrading them. However, understanding the complexities of how the sensitive *E. huxleyi* strains die when confronted with *P. inhibens* is required to better understand this bacterial-algal interaction. The goal of this chapter was to elucidate the interaction of *P. inhibens* with all three cell types of *E. huxleyi* and to determine the role roseobacticides play in *P. inhibens* pathogenesis. The remainder of this thesis will focus in on mechanistically describing the pathogenic interaction between *P. inhibens* and the dominant calcifying cell type of *E. huxleyi* (CCMP3266).

3.5. Works Cited

Azam, F. (1998) 'Microbial control of oceanic carbon flux: the plot thickens', *Science*, (280), pp. 694–696.

Baumann, K., Boeckel, B. and Čepek, M. (2008) 'Spatial distribution of living coccolithophores along an east- west transect in the subtropical South Atlantic', *Journal of Nannoplankton Research*, 30(1), pp. 9–21.

Bell, W. and Mitchell, R. (1972) 'Chemotactic and growth responses of marine bacteria to algal extracellular products', *Marine Biological Laboratory*, 143(2), pp. 265–277.

Bidle, K. D., Haramaty, L., Barcelos E Ramos, J. and Falkowski, P. (2007) 'Viral activation and recruitment of metacaspases in the unicellular coccolithophore, *Emiliania huxleyi*', *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), pp. 6049–6054. doi: 10.1073/pnas.0701240104.

Bolch, C. J. S., Subramanian, T. A. and Green, D. H. (2011) 'The toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae) requires marine bacteria for growth', *Journal of Phycology*. doi: 10.1111/j.1529-8817.2011.01043.x.

Borchard, C. and Engel, A. (2012) 'Organic matter exudation by *Emiliania huxleyi* under simulated future ocean conditions', *Biogeosciences*, 9(8), pp. 3405–3423. doi: 10.5194/bg-9-3405-2012.

Case, R. J., Longford, S. R., Campbell, A. H., Low, A., Tujula, N., Steinberg, P. D. and Kjelleberg, S. (2011) 'Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga', *Environmental Microbiology*, 13(2), pp. 529–537. doi: 10.1111/j.1462-2920.2010.02356.x.

Cemma, M. and Brumell, J. H. (2012) 'Interactions of pathogenic bacteria with autophagy systems', *Current biology : CB*. Elsevier, 22(13), pp. R540-5. doi: 10.1016/j.cub.2012.06.001.

Coburn, J. and Leong, J. M. (2000) 'Microbiology. arresting features of bacterial toxins', *Science (New York, N.Y.)*. doi: 10.1126/science.290.5490.287.

D'Alvise, P. W., Lillebø, S., Prol-Garcia, M. J., Wergeland, H. I., Nielsen, K. F., Bergh, Ø. and Gram, L. (2012) '*Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents *Vibriosis* in cod larvae', *PLoS ONE*, 7(8). doi: 10.1371/journal.pone.0043996.

von Dassow, P., John, U., Ogata, H., Probert, I., Bendif, E. M., Kegel, J. U., Audic, S., Wincker, P., Da Silva, C., Claverie, J.-M., *et al.* (2014) 'Life-cycle modification in open oceans accounts for genome variability in a cosmopolitan phytoplankton', *The ISME Journal*, 9(6), pp. 1–13. doi: 10.1038/ismej.2014.221.

von Dassow, P., Ogata, H., Probert, I., Wincker, P., Da Silva, C., Audic, S., Claverie, J.-M. and de Vargas, C. (2009) 'Transcriptome analysis of functional differentiation between haploid and diploid cells of *Emiliania huxleyi*, a globally significant photosynthetic calcifying cell', *Genome biology*, 10(10), p. R114. doi: 10.1186/gb-2009-10-10-r114.

Donnenberg, V. S. and Donnenberg, A. D. (2015) 'Coping with artifact in the analysis of flow cytometric data', *Methods*. Elsevier Inc., 82, pp. 3–11. doi: 10.1016/j.ymeth.2015.03.012.

Fernandes, N., Case, R. J., Longford, S. R., Seyedsayamdost, M. R., Steinberg, P. D., Kjelleberg, S. and Thomas, T. (2011) 'Genomes and virulence factors of novel

bacterial pathogens causing bleaching disease in the marine red alga *Delisea pulchra*', *PloS one*, 6(12), p. e27387. doi: 10.1371/journal.pone.0027387.
Frada, M. J., Bidle, K. D., Probert, I. and de Vargas, C. (2012) '*In situ* survey of life cycle phases of the coccolithophore *Emiliania huxleyi* (Haptophyta)', *Environmental Microbiology*, 14(6), pp. 1558–1569. doi: 10.1111/j.1462-2920.2012.02745.x.
Frada, M., Probert, I., Allen, M. J., Wilson, W. H. and de Vargas, C. (2008) 'The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection', *Proceedings of the National Academy of Sciences of the United States of America*, 105(41), pp. 15944–15949. doi: 10.1073/pnas.0807707105.
Frank, O., Pradella, S., Rohde, M., Scheuner, C., Klenk, H.-P., Göker, M. and Petersen, J. (2014) 'Complete genome sequence of the *Phaeobacter gallaeciensis* type strain CIP 105210T (= DSM 26640T = BS107T)', *Standards in Genomic Sciences*. doi: 10.4056/sigs.5179110.
Franklin, D. J., Airs, R. L., Fernandes, D. J., Bell, T. G., Bongaerts, R. J., Berges, J. A. and Malin, G. (2012) 'Identification of senescence and death in *Emiliania huxleyi* and *Thalassiosira pseudonana*: cell staining, chlorophyll alterations, and dimethylsulfoniopropionate (DMSP) metabolism', *Limnology and oceanography*, 57(1), pp. 305–317. doi: 10.4319/lo.2012.57.1.0305.
Geng, H. and Belas, R. (2010) 'Molecular mechanisms underlying roseobacter-phytoplankton symbioses', *Current opinion in biotechnology*. Elsevier Ltd, 21(3), pp. 332–8. doi: 10.1016/j.copbio.2010.03.013.
González, J. M., Simó, R., Massana, R., Covert, J. S., Casamayor, E. O., Pedrós-Alió, C. and Moran, M. a (2000) 'Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom', *Applied and environmental microbiology*, 66(10), pp. 4237–46. doi: 10.1128/AEM.66.10.4237-4246.2000.
Green, D. H., Echavarri-Bravo, V., Brennan, D. and Hart, M. C. (2015) 'Bacterial diversity associated with the coccolithophorid algae *Emiliania huxleyi* and *Coccolithus pelagicus F. braarudii*', *BioMed Research International*, 2015. doi: 10.1155/2015/194540.
Green, J. C., Course, P. A. and Tarran, G. A. (1996) 'The life-cycle of *Emiliania huxleyi*: a brief review and a study of relative ploidy levels analysed by flow cytometry', *Journal of Marine Systems*, 9(1–2), pp. 33–44. doi: 10.1016/0924-7963(96)00014-0.
Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J. and Polz, M. F. (2008) 'Resource partitioning and sympatric differentiation among closely related bacterioplankton', *Science*, 320(5879), pp. 1081–1085. doi: 10.1126/science.1157890.
Kendrick, B. J., DiTullio, G. R., Cyronak, T. J., Fulton, J. M., Van Mooy, B. a. S. and Bidle, K. D. (2014) 'Temperature-induced viral resistance in *Emiliania huxleyi* (Prymnesiophyceae)', *PLoS ONE*, 9(11), p. e112134. doi: 10.1371/journal.pone.0112134.
Klaveness, D. (1972) '*Coccolithus huxleyi* (Lohm.) Kamptn II. the flagellate cell, aberrant cell types, vegetative propagation and life cycles', *British Phycological Journal*, 7(3), pp. 309–318. doi: 10.1080/00071617200650321.

Kolb, A. and Strom, S. (2013) 'An inducible antipredatory defense in haploid cells of the marine microalga *Emiliania huxleyi* (Prymnesiophyceae)', *Limnology and Oceanography*, 58(3), pp. 932–944. doi: 10.4319/lo.2013.58.3.0932.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E., Baehrecke, E., Blagosklonny, M., El-Deiry, W., Golstein, P., Green, D., *et al.* (2009) 'Classification of cell death: recommendations of the nomenclature committee on cell death 2009', *Cell death and differentiation*, 16(1), pp. 3–11. doi: 10.1038/cdd.2008.150.

Kurokawa, M. and Kornbluth, S. (2010) 'Stalling in mitosis and releasing the apoptotic brake', *The EMBO Journal*, 29, pp. 2255–2257. doi: 10.1038/emboj.2010.150.

Laguna, R., Romo, J., Read, B. a and Wahlund, M. (2001) 'Induction of phase variation events in the life cycle of the marine coccolithophorid *Emiliania huxleyi*', 67(9), pp. 3824–3831. doi: 10.1128/AEM.67.9.3824.

Mayers, T., Bramucci, A., Yakimovich, K. and Case, R. (2016) 'A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*', *Frontiers in Microbiology*, 7(JUN). doi: 10.3389/fmicb.2016.00892.

Medlin, L. K., Barker, G. L. A., Campbell, L., Green, J. C., Hayes, P. K., Marie, D., Wrieden, S. and Vaulot, D. (1996) 'Genetic characterisation of *Emiliania huxleyi* (Haptophyta)', *Journal of Marine Systems*. doi: 10.1016/0924-7963(96)00013-9.

Miller, T. R. and Belas, R. (2004) 'Dimethylsulfoniopropionate metabolism by *Pfiesteria* -associated roseobacter spp .', *Applied and environmental microbiology*, 70(6), pp. 3383–3391. doi: 10.1128/AEM.70.6.3383.

Miller, T. R. and Belas, R. (2006) 'Motility is involved in *Silicibacter* sp. TM1040 interaction with dinoflagellates', *Environmental Microbiology*, 8(9), pp. 1648–1659. doi: 10.1111/j.1462-2920.2006.01071.x.

Miller, T. R., Hnilicka, K., Dziedzic, A., Desplats, P. and Belas, R. (2004) 'Chemotaxis of *Silicibacter* sp. strain TM1040 toward dinoflagellate products', *Society*, 70(8), pp. 4692–4701. doi: 10.1128/AEM.70.8.4692.

Paasche, E. and Klaveness, D. (1970) 'A physiological comparison of coccolith-forming and naked cells of *Coccolithus huxleyi', Arch. Mikrobiol.*, 73(2), pp. 143–152. doi: 10.1007/BF00410317.

Parkhill, J. P., Maillet, G. and Cullen, J. J. (2001) 'Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress', *Journal of Phycology*. doi: 10.1046/j.1529-8817.2001.037004517.x.

Read, B., Kegel, J., Klute, M., Kuo, A., Lefebvre, S., Maumus, F., Mayer, C., Miller, J., Monier, A., Salamov, A., *et al.* (2013) 'Pan genome of the phytoplankton *Emiliania* underpins its global distribution', *Nature*, 499(7457), pp. 209–13. doi: 10.1038/nature12221.

Sapp, M., Schwaderer, A. S., Wiltshire, K. H., Hoppe, H. G., Gerdts, G. and Wichels, A. (2007) 'Species-specific bacterial communities in the phycosphere of microalgae?', *Microbial Ecology*, 53(4), pp. 683–699. doi: 10.1007/s00248-006-9162-5.

Schatz, D., Shemi, A., Rosenwasser, S., Sabanay, H., Wolf, S. G., Ben-Dor, S. and Vardi, A. (2014) 'Hijacking of an autophagy-like process is critical for the life cycle of a DNA virus infecting oceanic algal blooms', *New Phytologist*, 204(4), pp. 854–863. doi: 10.1111/nph.13008.

Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N., Paulson, J. N., Chai, L., Clardy, J., *et al.* (2016) 'Dynamic metabolic

exchange governs a marine algal-bacterial interaction', *eLife*, 5, p. e17473. doi: 10.7554/eLife.17473.
Seyedsayamdost, M. R., Case, R. J., Kolter, R. and Clardy, J. (2011) 'The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*', *Nature chemistry*, 3(4), pp. 331–5. doi: 10.1038/nchem.1002.
Seymour, J. R., Simo, R., Ahmed, T. and Stocker, R. (2010) 'Chemoattraction to dimethylsulfoniopropionate throughout the marine microbial food web', *Science*, 329(July), pp. 342–346.
Stocker, R. (2012) 'Marine microbes see a sea of gradients', *Science*, 338(6107), pp. 628–633. doi: 10.1126/science.1208929.
Thole, S., Kalhoefer, D., Voget, S., Berger, M., Engelhardt, T., Liesegang, H., Wollherr, A., Kjelleberg, S., Daniel, R., Simon, M., *et al.* (2012) '*Phaeobacter gallaeciensis* genomes from globally opposite locations reveal high similarity of adaptation to surface life', *The ISME Journal*. Nature Publishing Group, 6(12), pp. 2229–2244. doi: 10.1038/ismej.2012.62.
Wagner-Döbler, I. and Biebl, H. (2006) 'Environmental biology of the marine *roseobacter* lineage', *Annual review of microbiology*, 60, pp. 255–280. doi: 10.1146/annurev.micro.60.080805.142115.
van der Wal, P., de Jong, E. W., Westbroek, P., de Bruijn, W. C. and Mulder-Stapel, A. A. (1983) 'Ultrastructural polysaccharide localization in calcifying and naked cells of the coccolithophorid *Emiliania huxleyi*', *Protoplasma*, 118(2), pp. 157–168. doi: 10.1007/BF01293073.
Wang, R., Gallant, É. and Seyedsayamdost, M. R. (2016) 'Investigation of the genetics and biochemistry of roseobacticide production in the roseobacter clade bacterium *Phaeobacter inhibens*', *mBio*, 7(2), pp. 1–10. doi: 10.1128/mBio.02118-15.
Wetmore, K. M., Price, M. N., Waters, R. J., Lamson, J. S., He, J., Hoover, C. A., Blow, M. J., Bristow, J., Butland, G., Arkin, A. P., *et al.* (2015) 'Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons', *mBio*, 6(3), pp. 1–15. doi: 10.1128/mBio.00306-15.
Wilson, W. H., Tarran, G. and Zubkov, M. V (2002) 'Virus dynamics in a coccolithophore-dominated bloom in the North Sea', *Deep Sea Research Part II: Topical Studies in Oceanography*, 49(15), pp. 2951–2963. doi: 10.1016/S0967-0645(02)00065-6.
Wolfe, G. V, Sherr, E. B. and Sherr, B. F. (1994) 'Release and consumption of dmsp from *Emiliania-huxleyi* during grazing by *Oxyrrhis marina'*, *Marine Ecology-Progress Series*. doi: 10.3354/meps111111.
Young, J. and Henriksen, K. (2003) 'Biomineralization within vesicles : the calcite of coccoliths', *Biomineralisaton. Reviews in Mineralogy and Geochemistry*, pp. 189–215. doi: 10.2113/0540189.
Zhang, X., Gamarra, J., Castro, S., Carrasco, E., Hernandez, A., Mock, T., Hadaegh, A. R. and Read, B. A. (2016) 'Characterization of the small rna transcriptome of the marine Coccolithophorid, *Emiliania huxleyi*', *PLoS ONE*. doi: 10.1371/journal.pone.0154279.

Chapter 4. *Phaeobacter inhibens* induces algal apoptosis-like-Programmed Cell Death in calcifying *Emiliania huxleyi*

4.1. Introduction

Coccolithophores are well known for their precipitation of dissolved bicarbonate to produce characteristic ornate calcite disks or coccoliths (Poulton *et al.*, 2010). They are globally important bloom-forming algae, frequently forming blooms that cover 100,000-250,000 km^2 stretches of the upper ocean (Holligan *et al.*, 1993; Brown *et al.*, 1994). Coccolithophore blooms are primarily made up of the ubiquitous species complex of *Emiliania huxleyi* (Read *et al.*, 2013), which is the most dominant and smallest coccolithophore in modern oceans (Monteiro *et al.*, 2016). These *E. huxleyi* populations are dominated by coccolith-bearing cells, which reproduce rapidly to densities of over 10^5 cells/mL (Baumann *et al.*, 2008). *E. huxleyi* bloom dynamics (peak density and subsequent crash) are determined by the rates of reproduction and losses from the population. These losses occur via gradual senescence—death after peak exponential growth (Franklin *et al.*, 2012)—or interactions with predators and pathogens. Indeed, the duration of coccolithophore blooms can be dramatically shortened by predation from microzooplankton (Harvey *et al.*, 2015) and attack by viruses (Wilson, Tarran and Zubkov, 2002). The latter can trigger premature collapse of *E. huxleyi* blooms by inducting algal programmed cell death (PCD) (Bidle *et al.*, 2007, 2012; Schatz *et al.*, 2014). Such a role for PCD in bloom collapse is not unique to *E. huxleyi* and has been observed in a number of other unicellular phytoplankton such as cyanobacteria (Berman-Frank *et al.*, 2004), diatoms (Berges *et al.*, 1998), dinoflagellates (Franklin *et al.*, 2004), and green algae (Segovia *et al.*, 2003).

PCD is the potentially interruptible process by which an independent cell responds to internal or external signals by genetically initiating and biochemically orchestrating its own deconstruction. Apoptotic-PCD (or apoptosis) was initially defined as having: 1) a strict reliance on the biochemical activity of highly specific proteases called caspases (i.e., cysteine aspartic proteases that cleave proteins after aspartic acid residues) and 2) conserved cellular morphologies during death (cell shrinkage, chromatin condensation, nuclear blebbing, apoptotic bodies, etc. (Lawen, 2003; Kroemer *et al.*, 2009)). Furthermore, the required dependence on

caspase activity means that apoptosis can be abolished, or interrupted, by biochemically inhibiting the responsible caspases (Chang *et al.*, 2000; Kroemer *et al.*, 2009).

Caspases have not been identified in non-metazoans, which is why apoptosis was initially assumed to be a strictly metazoan process (Chowdhury *et al.*, 2008). However, the identification of caspase-like cleavage of peptides in plants and unicellular phytoplankton that lack caspases (Chowdhury *et al.*, 2008), led to the recognition of an alternate death process called: apoptosis-like-PCD (AL-PCD). AL-PCD is now used to describe PCD (with apoptosis morphologies), but lacking the hallmark caspase activity (Danon *et al.*, 2000). Instead, AL-PCD can rely on either metacaspase or caspase-like protease activities. A few caspase-like molecules have been identified in plants, for example vacuolar processing enzyme (YVADase, caspase-1-like (Hatsugai *et al.*, 2015)), proteasomes (DEVDase, caspase-3-like (Hatsugai *et al.*, 2009)), and saspases (IETDase, caspase-8-like (Coffeen *et al.*, 2004; Bosch *et al.*, 2007)), along with others discussed elsewhere (Reape and McCabe, 2008; Xu *et al.*, 2009; Vartapetian *et al.*, 2011). Which explains why some 'caspase-specific' probes, such as those used in the current and previous studies (Bidle *et al.*, 2007; Mayers *et al.*, 2016), are not in fact specific only for caspases.

AL-PCD has been implicated as a critical cell death pathway in a variety of unicellular phytoplankton and a range of activation mechanisms for AL-PCD have been documented. AL-PCD can occur in response to various environmental stresses such as nutrient starvation (Bidle *et al.*, 2008), oxidative stress (Bidle *et al.*, 2011), or prolonged darkness (Segovia *et al.*, 2003). A similar PCD process was also shown to occur in *E. huxleyi* population in response to infection by lytic *E. huxleyi* viruses (EhVs) (Bratbak *et al.*, 1993; Vardi *et al.*, 2009). During this the process the virus upregulates algal directed synthesis of bioactive viral glycosphingolipids (vGSLs), which accumulate and subsequently induce increased generation of algal reactive oxygen species (ROS) and activate host-directed PCD (Bidle *et al.*, 2007; Rosenwasser *et al.*, 2014; Schatz *et al.*, 2014).

Members of the prolific marine roseobacter clade can sense algal exudates and are closely associated with algal blooms in the open ocean (González *et al.*, 2000; Zubkov *et al.*, 2001; Segev *et al.*, 2016). The roseobacter *Phaeobacter inhibens*, for example, has been identified within blooming populations of *E. huxleyi* (Green *et al.*, 2015) and was recently shown to be a pathogen of the only known axenic calcifying *E. huxleyi* culture CCMP3266 (Chapter 3). Additionally, *P. inhibens* produces several algicidal bioactives, like roseochelins (Wang *et al.*,

2017), which have been postulated to facilitate pathogenic interactions with calcifying *E. huxleyi*. However, the mechanism of *E. huxleyi* death in response to the roseobacter pathogen *P. inhibens* has not been elucidated. This work aims to investigate the mechanism of calcifying *E. huxleyi* death during this pathogenic interaction. Given previous findings that EhVs producing a bioactive vGSL that triggers *E. huxleyi* PCD pathways, we propose a similar mechanism of pathogenesis might take place in this bacterial-algal interaction. It is tempting to suggest, for instance, that *P. inhibens* bioactives might initiate activation of algal caspase-like molecules, which then propagate algal death via AL-PCD.

4.2. Methods

4.2.1. Bacterial and algal strains

The axenic diploid coccolith-bearing *Emiliania huxleyi* strain CCMP3266, hereafter called *E. huxleyi*, was grown in L1-Si and incubated statically at 18 °C in a diurnal incubator (8:16 hr dark-light cycle) (Chapter 2).

The roseobacter strain used in this study was *Phaeobacter inhibens* strain DSM17395 (Ruiz-Ponte *et al.*, 1999; Martens *et al.*, 2006), hereafter called *P. inhibens*. *P. inhibens* colonies were transferred to 25 mL ½MB and grown to early stationary phase at 18 °C in a shaking incubator (160 rpm, 30 hr), as previously described (Chapter 3).

4.2.2. Algal and bacterial co-cultivation

E. huxleyi and *P. inhibens* were co-cultured with 2.6 x 10^5 cells/mL and 1.2 x 10^2 cfu/mL, respectively, at 0 d. Briefly, 1 mL aliquots of control algal and bacterial monocultures, as well as experimental co-cultures were aliquoted into triplicate microtiter plate wells, as previously described (Chapter 2). The resulting microtiter plates were incubated statically at 18 °C in a diurnal incubator and sacrificial sampling was used throughout the experiment.

4.2.3. Pulse-Amplitude-Modulated (PAM) Fluorometry

Algal maximum quantum yield (F_v/F_m) is a useful measure of the photosynthetic efficiency of the algal Photosystem II (PSII)—chlorophyll *a* complex to excite and subsequently pass electrons to a functional electron transport chain (Schreiber, 1998). All samples were taken at the mid-point of the dark cycle and diluted in L1-Si media to within the detection range of the PAM fluorometer, as previously described (Chapter 2).

4.2.4. Quantification of reactive oxygen species (ROS)

Intracellular algal ROS were measured using the membrane permeable 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen) fluorescent ROS probe (Molecular Probes Inc.). The probe diffuses passively into live cells, is hydrolyzed by intracellular esterases to 2',7'-di-chlorohydrofluorescin (DCFDA), and remains trapped within the cell. The DCFDA is rapidly oxidized to the fluorescent compound dichlorofluorescein (DCF) and DCF staining per algal cell is enumerated using flow cytometry (detailed below). Fresh 1 mM stock solutions were made in dimethyl sulfoxide (DMSO) before use. Staining conditions used in previous studies indicated that a final concentration of 5 mM CM-H2DCFDA followed by a dark incubation for 1 h was effective for staining *E. huxleyi* cells (Evans *et al.*, 2006; Vardi *et al.*, 2012). The same conditions were determined sufficient for the *E. huxleyi*. Flow cytometry was performed using a FACSCalibur equipped with a 488 nm excitation laser (Becton Dickinson). Samples were analyzed based on DCF fluorescence per algal (green: emission 520 nm) and algal cell size (FSC-A).

Algal ROS quantification flow cytometry data were processed using FlowJo 9.2, as follows. Flow cytometry dot plots portray every counted cell in a different quadrant of the plot based on that specific cells size (x axis) and DCF fluorescence signal (y axis), with control *E. huxleyi* cells (black dots) overlaid on top of co-culture cells (orange dots). Algal populations were gated into quadrants (q) based on the algal cell forward scatter and DCF fluorescence of a non-stained 5 d old axenic *E. huxleyi* culture. Using this method >95% of non-stained algal cells were constrained in q 1, which can be defined as an algal cell without DCF fluorescence. q 2 and q 3 contained algal cells with decreased size (proportion <10% of the total), and q 4 is defined as algal cells of the correct cell size with increased DCF staining (or ROS activity) per algal cell.

4.2.5. Quantification of IETDase activity

In vitro caspase-like IETDase (Ile-Glu-Thr-Asp) activity in *E. huxleyi* cells was measured as previously described (Bidle *et al.*, 2007). Briefly, triplicate 950 uL aliquots from control and co-culture wells were pelleted by centrifugation (14,000 x g, 4 °C, 10 min), immediately flash-frozen in liquid nitrogen, and stored at -80 °C until processed. Cells were suspended, then a subsample was used for protein extraction (extraction buffer: 20 mM Tris pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$) and Bradford protein assay (BioRad). The remainder of the sample was

centrifuged (16,000 x g; room temperature; 2 min), resuspended in caspase activity buffer, and sonicated, before pelleting of cellular debris (16,000 x g; room temperature; 2 min). The supernatant was then incubated with IETD-AFC (Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin) according to the manufactures instructions (Caspase-8 Activity Kit, EMB Millipore). Extracts were incubated for 4 h at 25 °C and fluorescence (excitation 400 nm, emission 505 nm) was measured using a Synergy H1 microplate reader (BioTek). *In vitro* caspase activity was successfully abolished (>95%) with the irreversible caspase inhibitor z-VAD-fmk at a 20 µM final concentration (z-Val-Ala-Asp-fluoromethyl-ketone; Calbiochem), as previously described (Bidle *et al.*, 2007). To depict relative increase in active enzymes capable of IETDase cleavage, the ratio of IETD cleavage activity in pathogen infected co-culture extracts was normalized to IETD cleavage activity in *E. huxleyi* controls.

4.2.6. Algal and bacterial cell enumeration

Aliquots from *E. huxleyi* control monocultures and co-cultures with *P. inhibens* were fixed for flow cytometry, as described in Chapter 3. Algal cells were fixed using a cross-linking agent to ensure that intracellular algal DNA was retained within algal cell membranes, regardless of nuclear membrane fractionation (Wlodkowic *et al.*, 2009; Darzynkiewicz *et al.*, 2010). Algal cells without an intact cellular membrane (typical of late-stage programed cell death) appeared as debris. Flow cytometry was performed using a FACSCalibur equipped with a 488 nm excitation laser. Samples were first run using chlorophyll auto-fluorescence for detection then rerun after staining with SYBR-I (as described in Chapter 3). Data were processed as described (Chapter 3).

P. inhibens population density from control (without *E. huxleyi*) and co-cultivation experiments were enumerated by drop-plate counting and enumerating counting colony forming units (cfu), as previously described (described in Chapter 2, Figure 2.2).

4.2.7. Inhibition of caspase-like protease activity

Algal control monocultures and co-cultures with bacteria were grown for 6 d in the microtiter plate format; then all wells were mixed carefully with a wide mouth pipette to suspend algal cells. Active caspase-like molecules were inhibited in the algal controls or co-cultures using the cell permeable and irreversible pan-caspase inhibitor: z-VAD(OMe)-fmk (z-Val-Ala-Asp-(OMe)-fluoromethyl-ketone). The inhibitor was added *in vivo* on 6 d at a final concentration of 20µM v-VAD(OMe)-fmk.

4.2.8. Microscopy

During microtiter plate sampling wells were subsampled for brightfield and epifluorescence microscopy. Brightfield images were obtained using Zeiss Axio Scope.A1, equipped with an Optronics digital camera and PictureFrame Software Ver 2.3. Epifluorescence images were obtained using a Zeiss Axio Imager.M2 microscope, equipped with a monochrome camera (AxioCam 506 mono). Active algal caspase-like proteases were visualized using epifluorescence microscopy after *in vivo* staining of cells with the cell permeable, irreversibly binding, fluorescent pan-caspase marker (FITC-VAD-fmk, Millipore). The pan-caspase marker binds to active caspase and caspase-like proteases (Pozarowski *et al.*, 2003), having both the characteristic cysteine—histidine dyad and an available Val-Ala-Asp (VAD) binding site. This method has been used effectively to label activated caspase-like molecules in *E. huxleyi* (Bidle *et al.*, 2007; Mayers *et al.*, 2016). Epifluorescence microscopy was also used to image algal chlorophyll auto-florescence and to visualize loss of condensed double-stranded DNA (dsDNA) bundle when stained with DAPI (4'; 6-Diamidino-2-phenylindole dihydrochloride), which stains dsDNA (Jiménez *et al.*, 2009) (DAPI-dsDNA complex: Ex = 364 nm, Em = 454 nm) (Life Technologies).

Unfixed *E. huxleyi* control and co-culture aliquots were stained with pan-caspase marker (FITC-VAD-fmk) and DAPI according to manufacturer's instructions (30 °C, 20 min), then immediately pelleted by centrifugation (5,000 x g, room temperature, 2 min). Cells were gently washed twice in filter sterile L1-Si medium and analyzed immediately on the epifluorescence microscope. Images were acquired simultaneously for four different channels and overlaid using Zen 2 Blue Edition software. Channel 1) differential interference contrast (DIC) channel was overlaid each of the following fluorescent channels individually: 2) chlorophyll auto-fluorescence (red: excitation 610-650 nm; emission 670-720 nm), 3) localization of active caspase-like proteases using specific pan-caspase marker: FITC-VAD-FMK (green: excitation 450-490 nm; emission 515-586 nm, green), 4) Nuclear integrity based on dsDNA-DAPI complex fluorescence (blue: excitation 350-400 nm; emission 417-477 nm). Image processing was performed using Zeiss software and all four channels for all images were optimized in the same way and the colored channels were overlaid with the same DIC image.

4.3. Results

We examined the interaction occurring between marine pathogen *P. inhibens* and calcifying microalga *E. huxleyi*. To do this, we grew these two organisms alone and together in prolonged co-culture. During this co-culture it was established that the bacterial pathogen induced an accelerated loss of functional PSII systems (8 to 12 d, Figure 4.1a). Next, we investigated the mechanism of algal death by first assessing ROS concentrations in the algal population.

4.3.1. *P. inhibens* enhanced ROS generation in *E. huxleyi* co-cultures

Briefly, the non-fluorescent cell permeable indicator of intracellular ROS (CM-H2DCFDA) was added to algal cultures, rapidly hydrolyzed by intracellular algal esterases, and retained in the cytoplasm where it reacts with algal ROS, resulting in intracellular fluorescent DCF. Therefore, more algal esterases and ROS in a given algal cell is directly correlated to higher DCF fluorescence per algal cell. Algal cells shifting from q1 (baseline DCF fluorescence) into q 4 (elevated DCF fluorescence) are demonstrating an increase in DCF fluorescence or ROS per algal cell.

Algal control populations are not free of ROS staining (q1; Figure 4.1b-g), even though they maintained a high maximum potential yield (>0.6) for the duration of the experiment (Figure 4.1a). The proportion of control algal cells with intracellular ROS activity increases on day 6, but never surpasses 38.4% of the population (Figure 4.1c, q1). The co-cultures, on the other hand, routinely have both higher proportions of cells stained positive for ROS and more DCF fluorescence per individual algal cell, as indicated by the vertical shift in orange dots (Figure 4.1; q4). As the photosynthetic health of the algal population begins to decline (10 d), the proportion of algal cells in co-culture containing DCF fluorescence increases dramatically to 74.9% (Figure 4.1a,e). At that time, only 18.9% of the remaining algal cells in co-culture have baseline levels of DCF fluorescence, and by day 12 that number drops to 13.7% of the remaining population (Figure 4.1e,f).

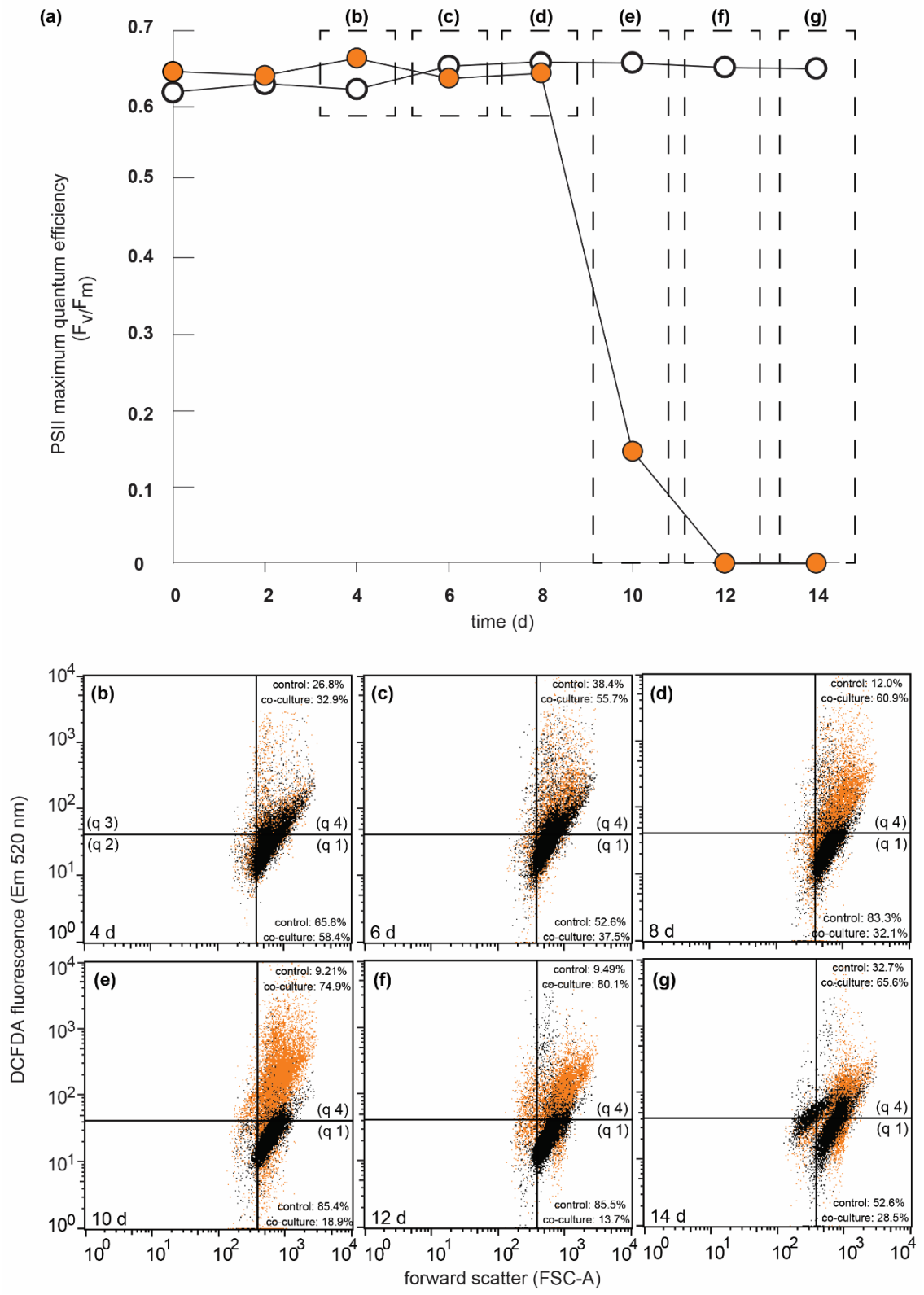
(a)
(b)
(c)
(d)
(e)
(f)
(g)
PSII maximum quantum efficiency (Fv/Fm)
time (d)
DCFDA fluorescence (Em 520 nm)
forward scatter (FSC-A)
control: 26.8%
co-culture: 32.9%
control: 65.8%
co-culture: 58.4%
4 d
control: 38.4%
co-culture: 55.7%
control: 52.6%
co-culture: 37.5%
6 d
control: 12.0%
co-culture: 60.9%
control: 83.3%
co-culture: 32.1%
8 d
control: 9.21%
co-culture: 74.9%
control: 85.4%
co-culture: 18.9%
10 d
control: 9.49%
co-culture: 80.1%
control: 85.5%
co-culture: 13.7%
12 d
control: 32.7%
co-culture: 65.6%
control: 52.6%
co-culture: 28.5%
14 d
(q 1)
(q 2)
(q 3)
(q 4)

Figure 4.1. **Elevated Reactive Oxygen Species (ROS) per algal cell grown in co-culture with *Phaeobacter inhibens*.** Control cultures of *E. huxleyi* and co-cultures of *E. huxleyi* grown with *P. inhibens* were assessed for (a) PSII maximum quantum efficiency levels and then immediately stained with General Oxidative Stress Indicator (CM-H2DCFDA) to detect algal ROS. Samples were then immediately assessed via flow cytometry (excitation 488 nm, emission 520 nm) and cell size (FSC-A). Data for control *E. huxleyi* cells (black dot plots) overlaid above co-culture of *E. huxleyi* and *P. inhibens* algal cells (orange dot plots): (b) 4 d, (c) 6 d, (d) 8 d, (e) 10 d, (f) 12 d, and (g) 14 d. The density of the dots is proportional to the density of the detections events. Flow cytometry events were counted and gated based on algal control cell size (FSC-A) and non-ROS stained control algal cells. The vertical and horizontal quadrants are based on >95% of non-ROS-stained algal cells being in quadrant 1 (q 1). Cells to the left of the vertical line show loss of algal cell size (FSC-A) and are therefore considered dead regardless of ROS staining (q2 and q3 total < 10% of cells counted). The proportions control or co-culture *E. huxleyi* cells present in either q1 (healthy) or q4 (elevated ROS) are labeled in the lower or upper corner of the dot plots, respectively.

4.3.2. Caspase-like activities in co-cultures

Next, we assessed algal control and co-culture populations for a relative increase of predicted caspase-like activities (i.e., IETDase) in the algal population due to co-culture with *P. inhibens*. The relative increase in algal IETDase identified in co-cultures is not significant between 0 and 8 d. However, as the PSII of algal hosts crashed (10 d, Figure 4.1a) there was a simultaneous four-fold increase of IETDase activity in the co-culture relative to the axenic control (Figure 4.2). Concurrently, there was an increase in morphological and physiological features of AL-PCD throughout the algal population. Throughout the experiment, algal monocultures retained a tight packet of dsDNA, visible chlorophyll autofluorescence, and had no visible FITC-VAD-fmk labeling (Figure 4.3a-d). Algal cells grown in co-culture, on the other hand, began displaying apoptotic-like morphologies, such as nuclear blebbing-like, as early as day 8 (Figure 4.4). Nuclear blebbing was followed by signs of active caspase-like molecules localized to the algal chloroplasts (9 d) (Figure 4.5). By day 10 *E. huxleyi* cells grown with *P. inhibens* showed signs of having one or more AL-PCD morphologies (Figure 4.3). Occasionally, cells with visible autofluorescence and FITC-VAD-fmk labeling were still identifiable on day 10 (Figure 4.3, white arrows). Complete loss of algal chlorophyll autofluorescence often coincides with loss of a condensed nucleus, indicating that chloroplast DNA is likely part of the extra-nuclear DNA observed filling the cells (Figure 4.3). As death progressed autofluorescence disappeared, and

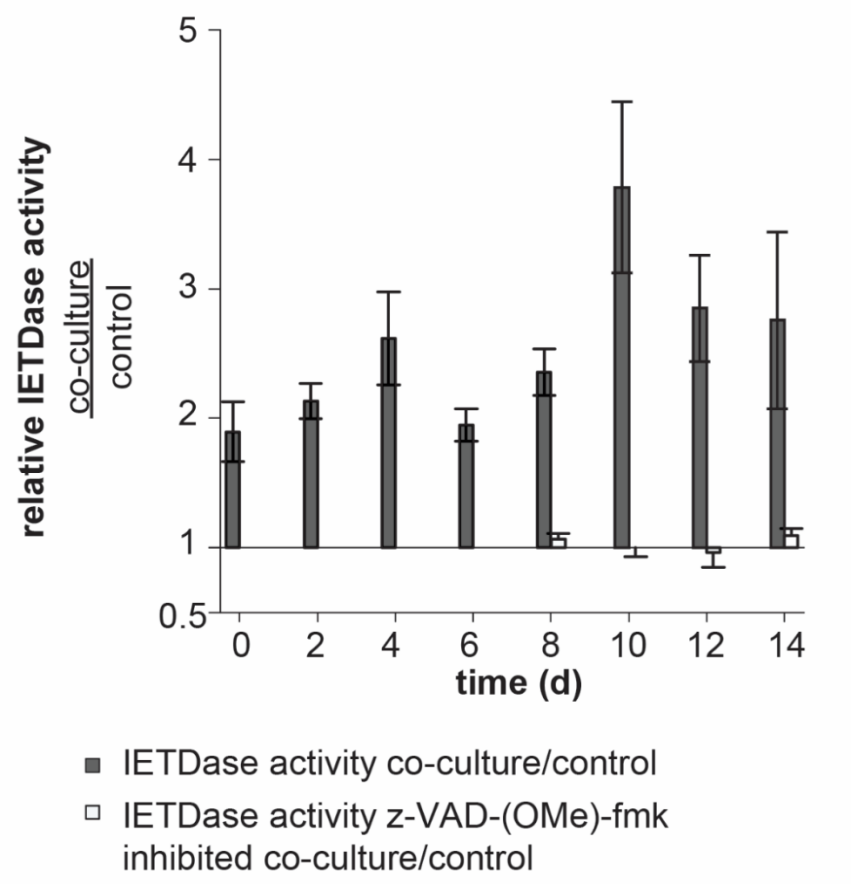

Figure 4.2. **Relative increase in IETDase activity in *Emiliania huxleyi* grown in co-culture with *Phaeobacter inhibens***. *In vitro* caspase-like IETDase (Ile-Glu-Thr-Asp) activity in *E. huxleyi* control and co-cultures was measured using Caspase-8 Activity Kit (EMB Millipore). The ratio of IETD cleavage in pathogen infected co-culture extracts was normalized to IETD cleavage activity in algal control to depict relative increase in algal enzymes capable of IETDase cleavage. Non-inhibited co-culture and control IETDase (black bars) were measured throughout the experiment, displayed as a ratio to show relative increase. After adding the pan-caspase inhibitor to control and co-cultures on 6 d, inhibited samples were also assessed for IETDase (grey bars). Error bars = ± SE of counts for triplicate wells.

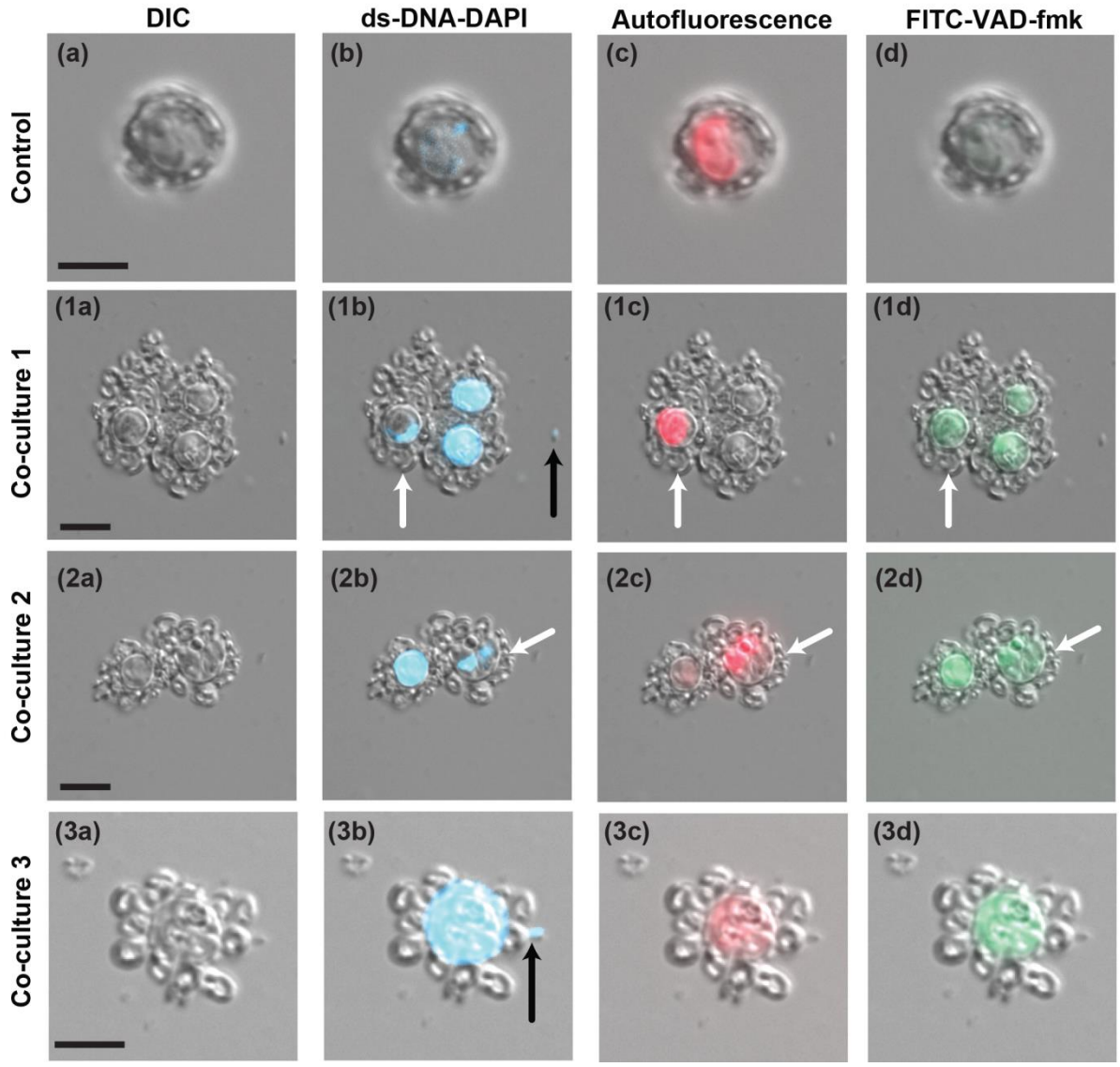

Figure 4.3. **Visualization of morphological indicators of AL-PCD in *Emiliania huxleyi* cells when grown in co-culture with *Phaeobacter inhibens*.** 1) DIC images of algal control (10 d) (a) and three different co-cultures (1a-3a). Simultaneous images of DIC and three indivitually overlaid fluorescent channels (10 d): 2) dsDNA-DAPI complex (DNA localization) (blue: excitation 350-400 nm; emission 417-477 nm) of algal control (b) and co-cultures (1b-3b). 3) autofluorescence (red: excitation 610-650 nm; emission 670-720 nm) of algal control (c) and co-cultures (1c-3c), 4) pan-caspase marker (v-VAD-FMK) highlighting active caspase-like proteases (green: excitation 450-490 nm; emission 515-586 nm) of algal control (d) and co-cultures (1d-3d). Scale bar is 5 µm. White arrows pointing out algal cells in the early stages of death (indicated by tight packet of dsDNA and healthy chloroplast autofluorescence (red). DAPI stained *P. inhibens* cell attached to algal host indicated by black arrows.

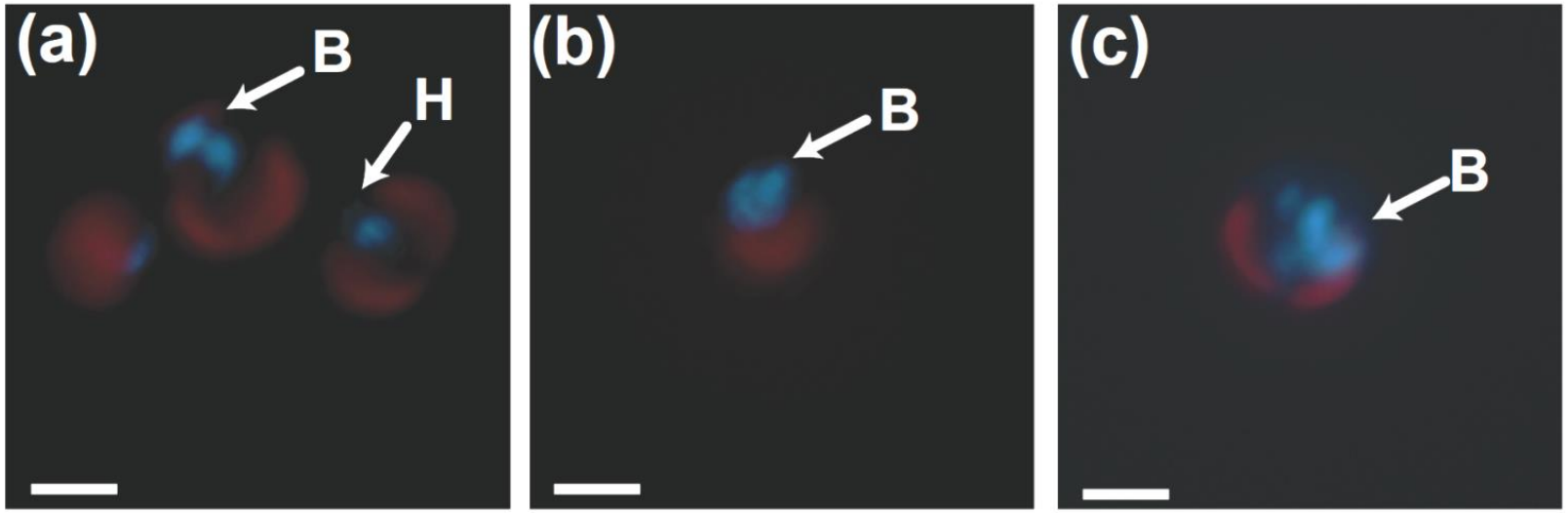

Figure 4.4. ***Phaeobacter inhibens*** **induces nuclear blebbing in** ***Emiliania huxleyi.*** Epifluorescence microscopy of co-culture (8 d): overlaid with chlorophyll auto-fluorescence (red: emission 670-720 nm) and dsDNA—DAPI stain complex fluorescence (blue: emission 417-477 nm). *E. huxleyi* cells where nucluear membrane appears to be blebbing are indicated by B and condensed packet of nuclear materia, typical of healthy cells is indicated by H. Scale bar is 5 μm.

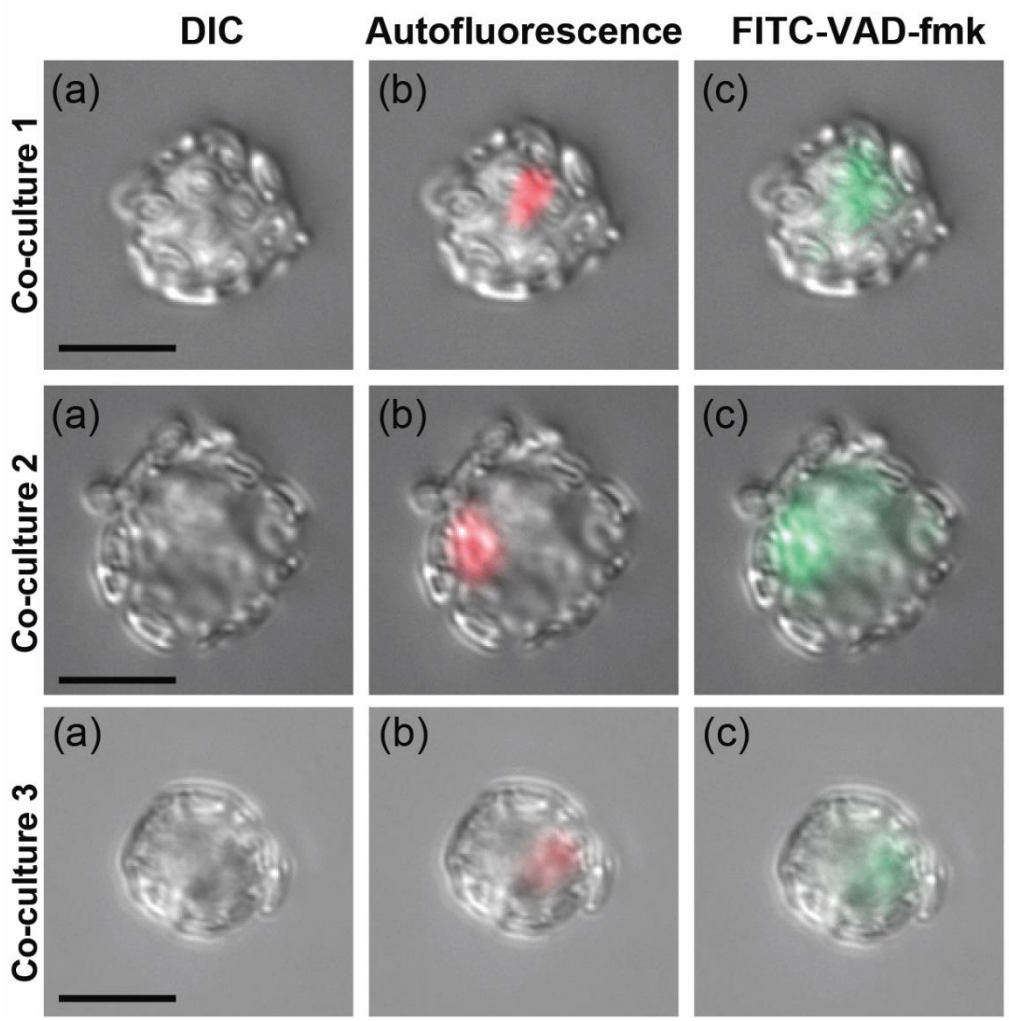

Figure 4.5. **Early localization of active caspase-like molecules within algal chloroplasts (9 d).** Three *E. huxleyi* cells grown in co-culture with *P. inhibens* (co-culture 1-3), shown. Each cell has three simultaneous images taken: (a) DIC alone, (b) DIC overlayed with chlorophyll autofluorescence (red: excitation 610-650 nm; emission 670-720 nm), and (c) DIC overlayed with (green: excitation 450-490 nm; emission 515-586 nm), to show fluorescence of active caspase-like proteases stained with specific pan-caspase marker: v-VAD-fmk (green). Scale bar is 5 μm.

both extra-nuclear DNA and FITC-VAD-fmk labeling routinely filled entire algal cells (Figure 4.3, 1d-3d).

4.3.3. Inhibition of caspase-like activity restores PSII efficiency

The pathogenic interaction was assessed by tracking algal PSII maximum quantum efficiency (F_v/F_m) throughout the experiment (Figure 4.6). While grown in monoculture, *E. huxleyi* displayed high photosynthetic efficiency throughout the 14 d experiment (maximum quantum yield: >0.55; Figure 4.6a). During the first six days of growth with *P. inhibens, E. huxleyi* photosystem efficiency was not statistically different and healthy algal cells with autofluorescence and attached bacteria were readily identified (Figure 4.6b,c, white arrows). However, *E. huxleyi* grown in co-culture exhibited slightly lower maximum quantum efficiency and as the co-culture progressed, the PSII damage became irreparably damaged (10 d; Figure 4.6a).To establish bacterial induction of algal AL-PCD we next attempted to biochemically inhibit the caspase-like molecules responsible for propagating AL-PCD. When the pan-caspase inhibitor was added on 6 d to control algal cultures, there was no statistically significant effect on algal PSII efficiency (Figure 4.6a, inverted triangles). However, pan-caspase inhibition of co-cultures completely abolished *P. inhibens* induced damage to the photosystem efficiency of algal host *E. huxleyi* (Figure 4.6a). Importantly, the irreversible pan-caspase inhibitor that abolished algal death also abolished IETDase activities (Figure 4.2).

4.3.4. Pan-caspase inhibition abolishes *P. inhibens*-induced AL-PCD

E. huxleyi cell density in control monocultures and co-cultures with *P. inhibens* was exponential (0-6 d), followed by a period of gradual algal senescence typified by limited algal cell losses after peak exponential growth (6 to 9 d; Figure 4.7a). The algal-bacterial co-cultures displayed a slightly accelerated exponential peak and then remain stable until day 9. As the algal controls and co-cultures enter late-senescence (9 d), the co-cultures rapidly undergo a population-wide algal cell death event, irreversibly losing over 90% of the algal cells from the population in <24 hr (9-10 d; Figure 4.7a). The timing of this massive cell death event occurs in conjunction with the loss of functional PSII systems (Figure 4.6a). While the *E. huxleyi* control cultures rapidly rebound after the period of limited losses, the algal cells grown in co-culture continue to irreversibly decline. Membrane integrity is maintained in the few algal cells

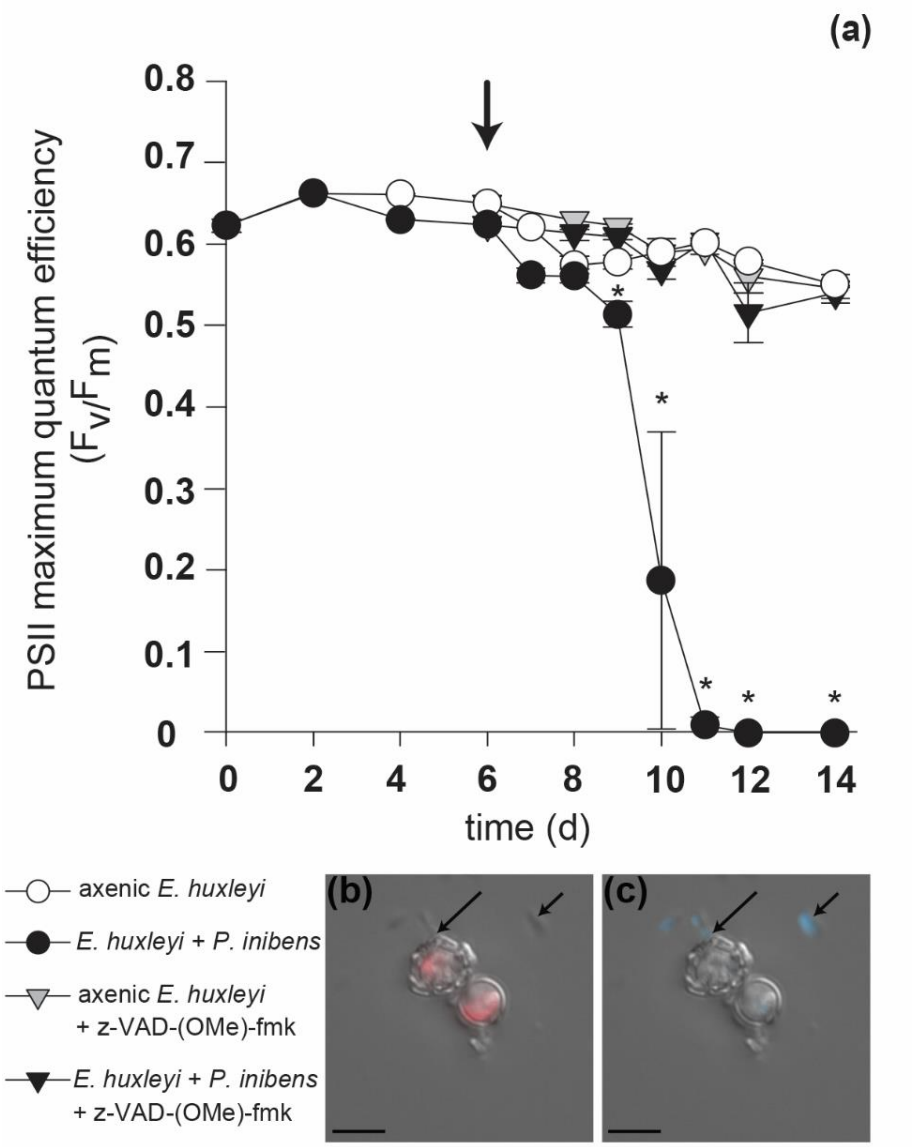

Figure 4.6. ***Emiliania huxleyi* Photosystem II (PSII) maximum quantum efficiency when grown alone or in co-culture with *Phaeobacter inhibens***. (a) Algal PSII maximum quantum efficiency (Fv/Fm) of axenic *E. huxleyi* (white circles) and co-culture with *P. inhibens* (back circles). Cell permeable pan-caspase inhibitor added in vivo on day 6 at 20 µM z-VAD-(OMe)-fmk (black arrow), from that point on pan-caspase inhibited control *E. huxleyi* (light grey inverted triangles) and co- co-culture with *P. inhibens* (black inverted triangles) were also measured. Error bars = ± SE of counts for triplicate wells. An asterix (*) indicates that algal co-culture Fv/Fm is statistically different from the Fv/Fm of the non-inhibited algal control. Statistics done using SigmaPlot 12 with a Student's T-test, p value <0.001. Epifluorescence microscopy of co-culture on 6 d: differential interference contrast (DIC) image overlaid with (b) chlorophyll auto-fluorescence (red: emission 670-720 nm) and (c) dsDNA—DAPI stain complex fluorescence (blue: emission 417-477 nm). *P. inhibens* cells indicated by black arrows. Scale bar is 5 µm.

remaining in the co-culture, until after the loss of all functional PSII systems (11 to 14 d, Figure 4.6a). Again, pan-caspase inhibition on day 6 did not alter algal control population dynamics, but once again abolished *E. huxleyi* cell losses from the inhibited co-culture (10 to 14 d; Figure 4.7a).

4.3.5. Population density of *P. inhibens* with and without an algal host

Bacterial monocultures grown in sterile seawater L1-Si algal media, rapidly reached 10^5 cfu/mL and then gradually declined throughout the experiment (Figure 4.7b). As early as day 2, *P. inhibens* grown in co-culture with *E. huxleyi* demonstrated a statistically significant increase in overall bacterial load compared to the bacteria grown alone (Figure 4.7b). As the algal hosts die (9 to 10 d; Figure 4.7a), the bacterial population of the co-culture experiences an impressive 1.9 fold jump in *P. inhibens* cfu/mL (Figure 4.7b). This extension of exponential growth of the pathogen coincides with the massive death event of the algal hosts (10 d, Figure 4.7a). The pan-caspase inhibitor, added at d 6 was not detrimental to the pathogen, which displayed a similar population size in the uninhibited co-culture until 8 d. As algae in the uninhibited co-culture died (10 d), the bacteria experienced an increase in population density. Interestingly, the bacterial population in the pan-caspase inhibited co-culture, where no algal death occurred (Figure 4.7a), remained in stationary phase for the rest of the experiment (Figure 4.7b).

4.3.6. DNA loss per algal cell during late-stage AL-PCD

We next attempted to evaluate the DNA content per algal cell for the algal population using flow cytometry. On day 8 the DNA content per algal cell in controls (black) and co-cultures (blue) is roughly equivalent (Figure 4.8a). The initial decline in algal cells/mL in co-culture occurs when the majority of algal cells die on d 10 (Figure 4.7a). After this point, the remaining algal cells in co-culture have fractional dsDNA content per cell (blue) compared to control algal cells (black) (Figure 4.8b,c,e,f). This dramatic loss of DNA content per cell is depicted first by the vertical shift downward of algal cells in co-culture (blue dots) compared to control (black dots) (10-12 d, Figure 4.8b,c). These data are subsequently depicted as histograms on a logarithmic scale to show the shift left (toward lower dsDNA content) in co-culture cells (blue) compared to the dsDNA content of control cells (black) (Figure 4.8f). As *E. huxleyi* cells begin to display reduced dsDNA content per cell (12 d), the algal cells from co-cultures all lack a

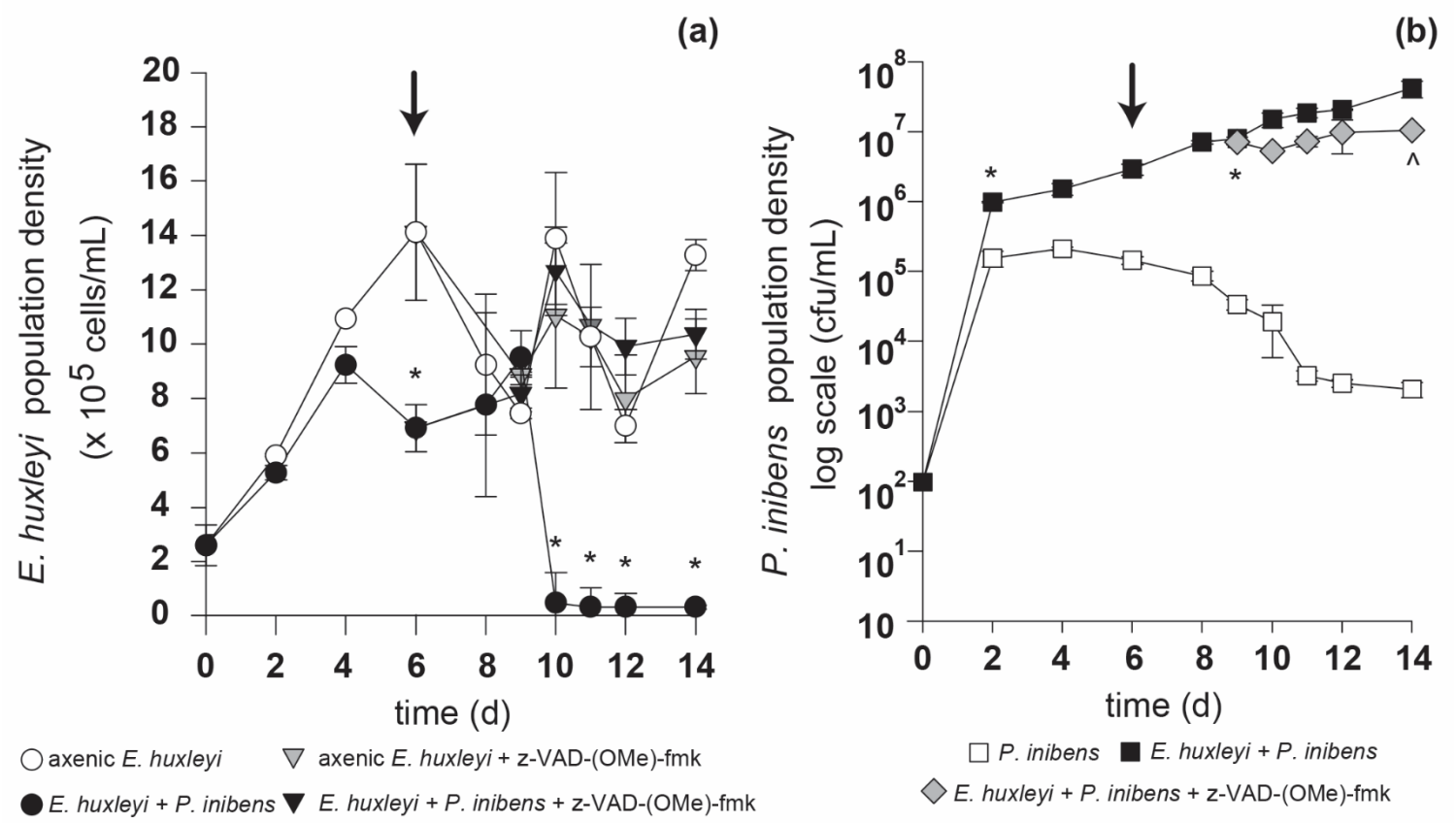

Figure 4.7. **Bacterial and algal population dynamics when grown alone or in co-culture**. Cell permeable pan-caspase inhibitor (z-VAD-(OMe)-fmk) added 6 d (black arrow). (a) Algal cell numbers of control *E. huxleyi* (white circles), co-culture with *P. inhibens* (back circles), and pan-caspase inhibited control *E. huxleyi* (light grey inverted triangles) and pan-caspase inhibited co-culture with *P. inhibens* (black inverted triangles). An asterix (*) indicates that algal co-culture cells/mL is statistically different from the cells/mL of the non-inhibited algal control. (b) *P. inhibens* cfu/mL counts in control (L1-Si media, white squares) and co-culture (grown with *E. huxleyi*, black squares), and pan-caspase inhibited *E. huxleyi* co-culture with *P. inhibens* (grew diamonds). Error bars = ± SE of counts for triplicate wells. After 2 d the cfu/mL in non-inhibited co-culture (black squares) and inhibited co-culture (grey diamonds) are statistically different (*) from the cfu/mL of the *P. inhibens* control (white squares). (^) indicates that the cfu/mL in the inhibited co-culture is statistically different from the cfu/mL of the non-inhibited co-culture (14 d). Error bars = ± SE of counts for duplicate runs. Statistics done using SigmaPlot 12 with a Student's T-test, p value <0.001.

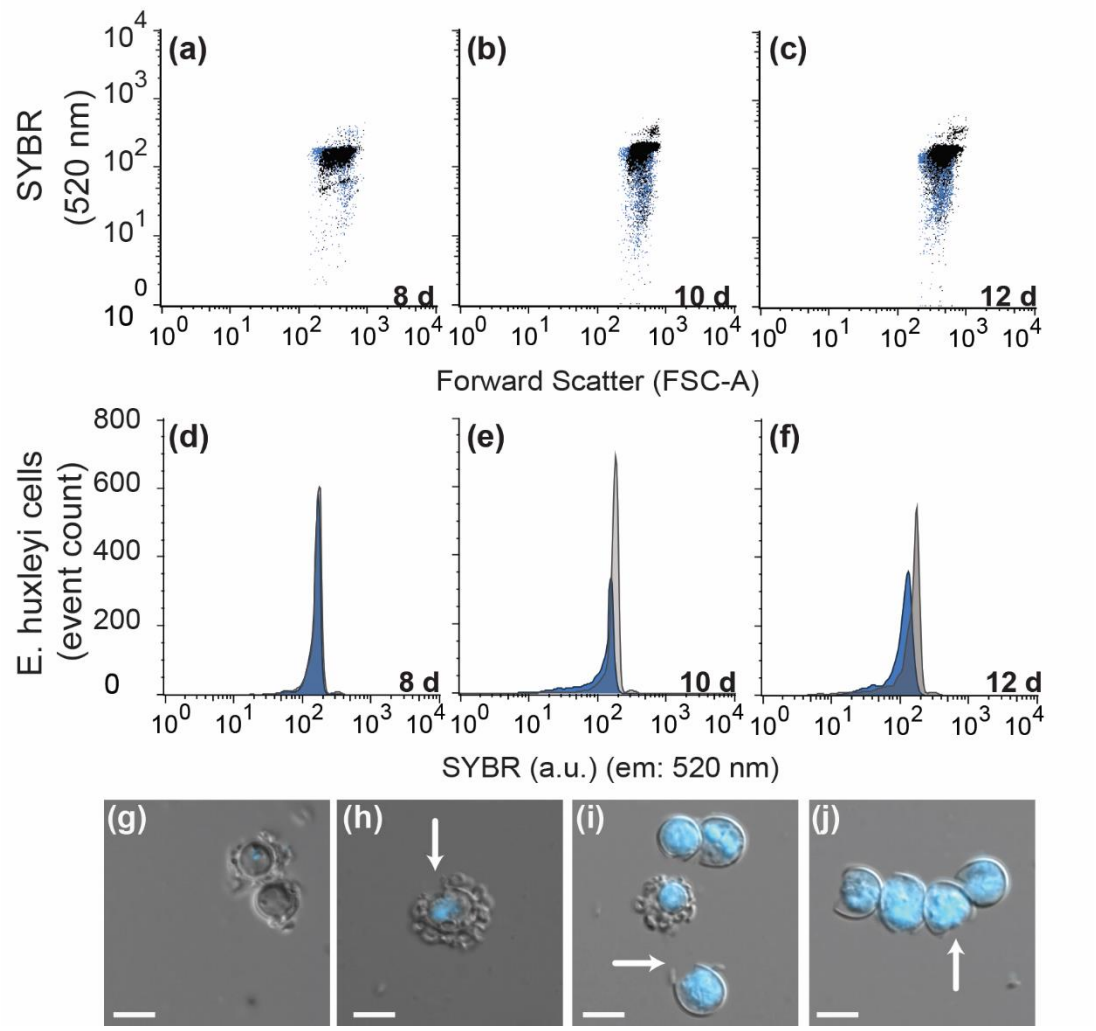

Figure 4.8. **Loss of DNA content per *Emiliania huxleyi* cell during prolonged co-culture with *Phaeobacter inhibens.*** Control cultures of *E. huxleyi* and co-cultures of *E. huxleyi* grown with *P. inhibens* were assessed for DNA content after standing with DNA specific SYBR green stain (520 nm) and cell size (FSC-A) using flow cytometry and shown on the logarithmic scale. Data for axenic *E. huxleyi* cells are represented by black dot plots, while co-culture of *E. huxleyi* and *P. inhibens* algal cells are represented in blue dot plots: (a) 8 d, (b) 10 d, and (c) 12 d. The density of the dots is proportional to the density of the detections events. Histograms of cell number (y-axis) and SYBR stained DNA content per counted cell on a logarithmic scale (x-axis) for co-cultures (blue) compared to controls (grey): (d) 8 d, (e) 10 d, and (f) 12 d. Images show day 12 control (g) and co-culture (h-j) images on day 12, show DIC overlaid with dsDNA-DAPI stain (blue: excitation 350-400 nm; emission 417-477 nm).

condensed nucleus (Figure 4.8h-j). At this point, calcified cells (and coccolith-free diploid cells) both begin showing signs of visibly broken membranes (Figure 4.8h-j, white arrows).

4.4. Discussion

The current work has established that bacterial pathogen *P. inhibens* dynamically interacts with its calcifying microalga host. During the interaction the alga experiences increased generation of ROS, subsequent loss of PSII function, and elevation of caspase-like activities. However, inhibition of caspase-like molecules rescues the algal population from bacterial induced death, thereby biochemically confirming that the *E. huxleyi* population dies via AL-PCD. Finally, during late-stage AL-PCD we assessed the algal population for loss of DNA per algal cell, as a way of confirming a state of algal death (Kroemer *et al.*, 2009).

4.4.1. *P. inhibens* induces AL-PCD in *E. huxleyi*

It is important to remember here that AL-PCD mediated cell death (like apoptosis-PCD) is a stochastic process, not a synchronous process (Kroemer *et al.*, 2009), so at a given time varying proportions of the unicellular population are at various stages of the AL-PCD process. Furthermore, the progression from lacking AL-PCD markers to expressing one or more AL-PCD markers and subsequently succumbing to cell death is very rapid (<24 hr) (Figure 4.9). The current study biochemically and physiologically characterizes *P. inhibens* induced AL-PCD in *E. huxleyi* (Figure 4.9). Biochemical inhibition of caspase-like molecules abolished pathogen induced death, demonstrating that caspase-like activity is required for algal death. Rescuing viral infected cultures from death using pan-caspase inhibition has not been successfully in haptophytes (Ray *et al.*, 2014), including *E. huxleyi* (Bidle *et al.*, 2007). However, the same pan-caspase inhibitor (z-VAD(OMe)-fmk) has successfully abolished AL-PCD in other phytoplankton populations including cyanobacteria (Berman-Frank *et al.*, 2004), green algae (Segovia *et al.*, 2003), and diatoms (Bidle *et al.*, 2008). The fact that the pan-caspase inhibitor completely abolished bacterial induced *E. huxleyi* death in this study confirms that the pathogen induced AL-PCD is dependent on algal caspase-like molecules (9-10 d).

As the pathogen induces the massive algal death event, the bacterial population appears to re-enter exponential growth, with a 1.9 fold increase in cfu/mL (9-10 d; Figure 4.7). This population-wide increase can be explained by the pathogen benefiting from the death of their calcifying algal hosts. The pathogen population in the pan-caspase inhibited populations, where

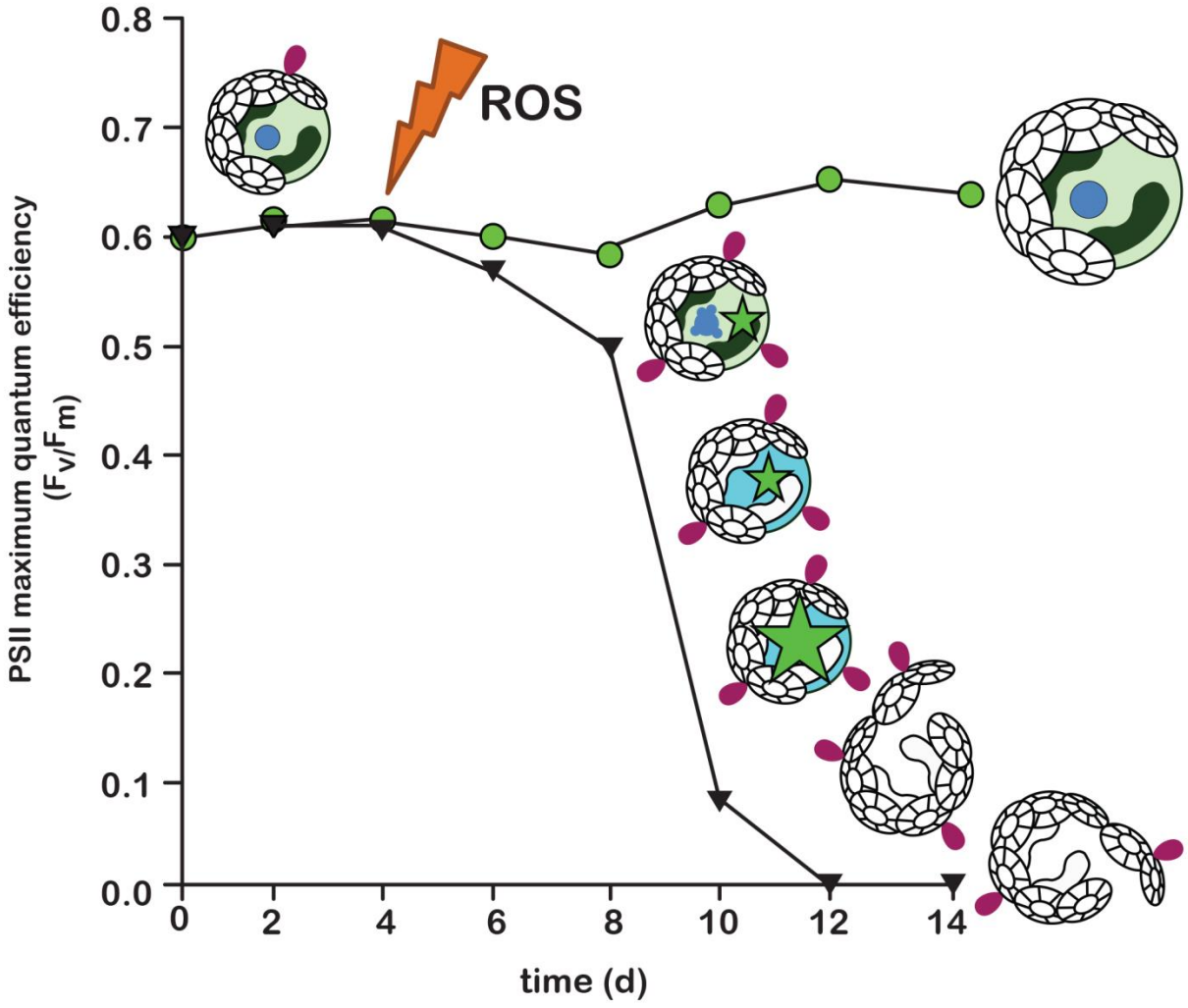

Figure 4.9. **Schematic diagram demonstrating the progression of*Emiliania huxleyi* AL-PCD morphologies and physiologies during co-culture with *Phaeobacter inhibens*.** Control cultures of *E. huxleyi* (green) remain healthy. Co-cultures of *E. huxleyi* grown with *P. inhibens* (attached pink) display AL-PCD morphologies: spike in ROS (d 6), nuclear blebbing and active caspase-like molecules (green star) localized to the chloroplasts (9 d), loss of chlorophyll autofluorescence (9 d), then caspase-like activities are visible filling the cell and tight packet of dsDNA is lost, while coccolithosphere and/or cell membrane retains structure (10 d).

the algal hosts remain healthy do not display this increase in population size (Figure 4.7b). This supports previous suggestions that pathogen-induced algal AL-PCD may alter the way algal metabolites flow through the marine ecosystem, possibly accelerating the transfer of DOM to heterotrophic microbes (Berman-Frank *et al.*, 2004; Thornton, 2014).

4.4.2. ROS generation in control *E. huxleyi* cultures

ROS generation in viral infected *E. huxleyi* cultures appears to be a critical (Evans *et al.*, 2007; Sheyn *et al.*, 2016) or even required step in the death process (Schatz *et al.*, 2014). When the intracellular generation of ROS exceeds the organism's elimination capacity, the ROS become toxic to the cell (Evans *et al.*, 2006). Given the importance of ROS during viral infection of *E. huxleyi* we wanted to track ROS generation in control and co-cultures. Algal senescence of control cultures is preceded by elevated ROS (6-9 d) (Figure 4.1), but not inhibited by the pan-caspase inhibition (Figure 4.7), which is suggestive of autophagy (Kroemer *et al.*, 2009). In fact, ROS induced autophagy-like processes, which then degrade the ROS generating chloroplasts has been identified before for plants and other algal species (Perez-Perez *et al.*, 2012). Importantly, this period of algal senescence of control cultures is directly mirrored in the algal populations grown in co-culture and with the pan-caspase inhibitor (Figure 4.7a). These findings are suggestive that *E. huxleyi* may rely more on a ROS dependent or autophagy-like process to regulate algal senescence of cultures rather than AL-PCD. Additionally, autophagy inhibitors have successfully abolished viral induced autophagy of tested *E. huxleyi* strains (Schatz *et al.*, 2014), so it would be interesting to see if the same inhibitors might delay or abolish *E. huxleyi* senescence of control cultures.

4.4.3. Potential interplay between ROS and AL-PCD

Chloroplasts are particularly sensitive to ROS damage, because when electrons escape the photosynthetic electron transport chain, they quickly encounter O_2 in the chloroplasts, generating superoxide radicals ($O_2^{\bullet-}$) (Chen *et al.*, 2004). Buildup of algal ROS precedes loss of maximum quantum efficiency in viral infected *E. huxleyi* cultures (Sheyn *et al.*, 2016), just as it does in *P. inhibens*-*E. huxleyi* co-cultures (Figure 4.1). However, once caspase-like molecules, localized to the chloroplasts, begin to be activated (9 d, Figure 4.5), the progression to population-wide death is rapid (<24 hr) (10 d, Figure 4.7). This timing fits with previous reports that activation of caspase-like molecules is the point of no return for AL-PCD, because only 24 h after the peak

activation of caspase-like molecules the majority of the algal population dies. The timing of these events is suggestive of potential chloroplast involvement in the AL-PCD of *E. huxleyi*, particularly because active caspase-like molecules appeared in the chloroplasts before IETDase activity spiked (Figure 4.2).

It is tempting to suggest that some type of dynamic interplay might be occurring between ROS and caspase-like molecules in *E. huxleyi* co-cultures. Interestingly, in plant systems, ROS generated from the chloroplasts can escape the chloroplasts and become deadly signaling molecules directly activating caspase-like molecules, thereby amplifying AL-PCD (De Jong *et al.*, 2002; Ambastha *et al.*, 2015). Or conversely, activation of caspase-like molecules can act as a positive feedback to increase release of ROS from chloroplasts (De Jong *et al.*, 2002). Given the findings presented here, it is not possible to decipher between these possibilities: 1) ROS generated from the chloroplasts activate caspase-like molecules inducing AL-PCD or 2) caspase-like molecules act as a positive feedback increasing generation and release of ROS.

4.4.4. *E. huxleyi* loss of DNA content per cell

One overlap between metazoan apoptotic morphologies and those identified in algal AL-PCD is the rapid progression from condensed nucleus to subsequent loss of DNA content per cell. In unicellular algae this phenomenon was first noted in chlorophytes *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii* (Segovia *et al.*, 2003; Moharikar *et al.*, 2006; Jiménez *et al.*, 2009). During *C. reinhardtii* AL-PCD, nuclear blebbing quickly progresses to DNA disintegrating and diffusing out of the nucleus filling the whole cell (Moharikar *et al.*, 2006), which is reminiscent of what occurs throughout *E. huxleyi* co-cultures (10 d, Figure 4.3). Additionally, rapid degradation and recycling of cellular DNA can quickly result in deficit in DNA content per cell (Bethke, 1999; Darzynkiewicz *et al.*, 2010; Mayers *et al.*, 2016), due to the degradation of intracellular DNA, as seen in *E. huxleyi* (Figure 4.8).

4.4.5. Lack of apoptosis bodies during AL-PCD

As previously discussed, not all classical apoptosis morphologies are mirrored during the process of AL-PCD. Late-stage membrane blebbing into apoptotic bodies is considered one of the hallmark morphologies of apoptosis (Kerr *et al.*, 1972), but is not a common feature of plants undergoing AL-PCD (Fath *et al.*, 2000; Reape, Molony, *et al.*, 2008). The lack of membrane blebbing of calcifying *E. huxleyi* is likely due to the presence of several layers of rigid coccoliths

surrounding the cell. A similar phenomenon was seen in during late-stage *D. tertiolecta* AL-PCD, and was explained by noting that *D. tertiolecta*'s rigid outer cell envelope likely maintains membrane integrity into late-stage AL-PCD (Krishnamurthy *et al.*, 2000; Segovia *et al.*, 2003).

4.4.6. How viral and bacterial pathogenesis of *E. huxleyi* differ

Pathogens have a plethora of strategies allowing them to manipulate and control the fate of a host cell by hijacking the cellular process of PCD (Gao *et al.*, 2000; Zhou *et al.*, 2015; Mukhtar *et al.*, 2016). For instance, viruses can delay eukaryotic apoptosis, rerouting the cell to the slower process of autophagy (Müller *et al.*, 2001; Blaho, 2004). Autophagy is a major degradation and recycling system requiring the packaging of cellular constituents into autophagy lysosome vesicles that are degraded intracellularly (Van Doorn *et al.*, 2005), which benefits viruses by indirectly supporting the production and release of virions (Blaho, 2004). Viral rerouting of AL-PCD to autophagy might explain why the timing of viral-induced collapse of *E. huxleyi* cultures tends to occur at a steady rate over three to five days (Bidle *et al.*, 2007; Schatz *et al.*, 2014), much longer than *P. inhibens* killing. Additionally, while biochemical inhibition abolishes bacterial induction of AL-PCD, viral killing of *E. huxleyi* is delayed, rather than abolished, by the same pan-caspase inhibitor (Bidle *et al.*, 2007). This is suggestive that viral lysis is not strictly dependent on caspase-like molecules as is *P. inhibens* pathogenesis of *E. huxleyi*.

Finally, recent studies of a non-calcifying strain of *E. huxleyi* (CCMP2090) infected with a virus (EhV201), describe viral induction of autophagy-PCD (abolished by autophagy inhibitors) (Schatz *et al.*, 2014). Interestingly, the same non-calcifying *E. huxleyi* strain (CCMP2090) was recently shown to be resistant to bacterial infection by the widespread roseobacter pathogens *Ruegeria* R11 and *P. inhibens* (Mayers *et al.*, 2016; Chapter 3). Autophagy has also been found to have a protective role in cells, by rapidly degrading virulence factors, such as those released by pathogenic bacteria (Cemma *et al.*, 2012). This suggests that the ability of non-calcifying diploid *E. huxleyi* to undergo autophagy might protect them against certain bacterial pathogens (Mayers *et al.*, 2016; Chapter 3).

Interestingly, genome sequencing of *P. inhibens* revealed the presence of a chromosomally encoded T4SS (Thole *et al.*, 2012). Bacterial T4SS are versatile virulence machines, some classes of which transport effector molecules into target host cells to modulate host processes (Christie *et al.*, 2014). In fact, there are examples of intracellular and extracellular bacteria manipulating host PCD processes by translocating T3SS or T4SS effectors that induce mammalian apoptotic-PCD

(Schulein *et al.*, 2006; Banga *et al.*, 2007; Niu *et al.*, 2010). So, it is tempting to suggest that might be the mechanism of inducing algal AL-PCD identified in this study.

4.4.7. Conclusion

Emiliania huxleyi bloom collapse is frequently attributed to various intracellular coccolithoviruses, which infect blooming populations of *E. huxleyi* in the open ocean *(Frada et al*., 2012). However, the associated bacterial consortia are frequently overlooked, even though marine pathogens, such as those from the roseobacter clade, can track algal populations and sense and respond to algal exudates (Green *et al*., 2015; Segev *et al*., 2016). The demonstration of bacteria inducing rapid AL-PCD in algal populations, is a reminder that bacterial pathogens may play a key role in determining how algal metabolites flow through the marine ecosystem into the microbial loop. As ocean temperatures continue to rise, viral pathogens might become less of a threat to the longevity of *E. huxleyi* blooms, as viral resistance is enhanced with increasing temperatures (Kendrick *et al*., 2014). Conversely, roseobacter pathogens have been recently found to have enhanced pathogenesis of *E. huxleyi* at higher temperatures (Mayers *et al*., 2016), indicating that certain marine pathogens might begin to occupy larger pathogenic niches in light of increasing ocean temperatures.

4.5. Works Cited

Ambastha, V., Tripathy, B. C. and Tiwari, B. S. (2015) 'Programmed Cell Death in plants: a chloroplastic connection', *Plant Signaling & Behavior*, 10(2), p. e989752. doi: 10.4161/15592324.2014.989752.

Banga, S., Gao, P., Shen, X., Fiscus, V., Zong, W., Chen, L. and Luo, Z. (2007) '*Legionella pneumophila* Inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family', *PNAS (Proceedings of the National Academy of Sciences of the United States of America)*, 104(12), pp. 5121–5126.

Baumann, K., Boeckel, B. and Čepek, M. (2008) 'Spatial distribution of living coccolithophores along an East-West transect in the subtropical South Atlantic', *Journal of Nannoplankton Research*, 30(1), pp. 9–21.

Berges, J. A. and Falkowski, P. G. (1998) 'Physiological stress and cell death in marine phytoplankton: induction of proteases in response to nitrogen or light limitation', *Limnology and Oceanography*, 43(1), pp. 129–135. doi: 10.4319/lo.1998.43.1.0129.

Berman-Frank, I., Bidle, K. D., Haramaty, L. and Falkowski, P. G. (2004) 'The demise of the marine cyanobacterium, *Trichodesmium* Spp., Via An Autocatalyzed Cell Death Pathway', *Limnology and Oceanography*, 49(4), pp. 997–1005. doi: 10.4319/lo.2004.49.4.0997.

Bethke, P. C. (1999) 'Hormonally regulated programmed cell death in barley aleurone cells', *the Plant Cell Online*, 11(6), pp. 1033–1046. doi: 10.1105/tpc.11.6.1033.

Bidle, K. D. and Bender, S. J. (2008) 'Iron starvation and culture age activate metacaspases and Programmed Cell Death in the marine diatom *Thalassiosira pseudonana*', *Eukaryotic Cell*, 7(2), pp. 223–236. doi: 10.1128/EC.00296-07.

Bidle, K. D., Haramaty, L., Barcelos E Ramos, J. and Falkowski, P. (2007) 'Viral activation and recruitment of metacaspases in the unicellular coccolithophore, *Emiliania huxleyi*', *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), pp. 6049–6054. doi: 10.1073/pnas.0701240104.

Bidle, K. D. and Kwityn, C. J. (2012) 'Assessing the role of caspase activity and metacaspase expression on viral susceptibility of the coccolithophore, *Emiliania huxleyi* (Haptophyta).', *Journal of Phycology*, 48(5), pp. 1079–1089. doi: 10.1111/j.1529-8817.2012.01209.x.

Bidle, K. D. and Vardi, A. (2011) 'A Chemical arms race at sea mediates algal host-virus interactions', *Current opinion in microbiology*. Elsevier Ltd, 14(4), pp. 449–57. doi: 10.1016/j.mib.2011.07.013.

Blaho, J. (2004) 'Virus infection and apoptosis (Issue II) an introduction: cheating death or death as a fact of life?', *International reviews of immunology*, 23(1–2), pp. 1–6. doi: 10.1080/0883018049026551.

Bosch, M. and Franklin-Tong, V. E. (2007) 'Temporal and spatial activation of caspase-like enzymes induced by self-incompatibility in papaver pollen', *Proceedings of the National Academy of Sciences of the United States of America*, 104(46), pp. 18327–18332. doi: 10.1073/pnas.0705826104.

Bratbak, G., Egge, J. K. and Heldal, M. (1993) 'Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms', *Marine Ecology Progress Series*, 93(1–2), pp. 39–48. doi: 10.3354/meps093039.

Brown, C. W. and Yoder, J. a. (1994) 'Coccolithophorid blooms in the global ocean', 99(93), pp. 7467–7482. doi: 10.1029/93JC02156.

Cemma, M. and Brumell, J. H. (2012) 'Interactions of pathogenic bacteria with autophagy systems', *Current biology : CB*. Elsevier, 22(13), pp. R540-5. doi: 10.1016/j.cub.2012.06.001.

Chang, H. Y. and Yang, X. (2000) 'Proteases for cell suicide: Functions and regulation of caspases', *Microbiology and molecular biology reviews : MMBR*, 64(4), pp. 821–46. doi: 10.1128/MMBR.64.4.821-846.2000.

Chen, S. and Dickman, M. B. (2004) 'Bcl-2 family members localize to tobacco chloroplasts and inhibit Programmed Cell Death Induced by chloroplast-targeted herbicides', *Journal of Experimental Botany*, 55(408), pp. 2617–2623. doi: 10.1093/jxb/erh275.

Chowdhury, I., Tharakan, B. and Bhat, G. K. (2008) 'Caspases-An update', *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 151(1), pp. 10–27. doi: 10.1016/j.cbpb.2008.05.010.

Christie, P. J., Whitaker, N. and González-Rivera, C. (2014) 'Mechanism and structure of the bacterial Type IV Secretion Systems', *Biochimica et Biophysica Acta - Molecular Cell Research*. doi: 10.1016/j.bbamcr.2013.12.019.

Coffeen, W. C. and Wolpert, T. J. (2004) 'Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with Programmed Cell Death in *Avena sativa*', *The Plant Cell Online*, 16(4), pp. 857–873. doi: 10.1105/tpc.017947.

Danon, A., Delorme, V., Mailhac, N. and Gallois, P. (2000) 'Plant Programmed Cell Death: A common way to die', *Plant Physiology and Biochemistry*, 38(9), pp. 647–655. doi: 10.1016/S0981-9428(00)01178-5.

Darzynkiewicz, Z., Halicka, H. D. and Zhao, H. (2010) 'Analysis of cellular DNA content by flow and laser scanning cytometry', *Advances in Experimental Medicine and Biology*, 675, pp. 137–147. doi: 10.1007/978-1-4419-6199-0_9.

Van Doorn, W. G. and Woltering, E. J. (2005) 'Many ways to exit? Cell death categories in plants', *Trends in Plant Science*, 10(3), pp. 117–122. doi: 10.1016/j.tplants.2005.01.006.

Evans, C., Kadner, S. V., Darroch, L. J., Wilson, W. H., Liss, P. S. and Malin, G. (2007) 'The relative significance of viral lysis and microzooplankton grazing as pathways of Dimethylsulfoniopropionate (DMSP) cleavage: An *Emiliania huxleyi* culture study', *Limnology and Oceanography*, 52(3), pp. 1036–1045. doi: 10.4319/lo.2007.52.3.1036.

Evans, C., Malin, G., Mills, G. P. and Wilson, W. H. (2006) 'Viral infection of *Emiliania huxleyi* (Prymnesiophyceae) leads to elevated production of reactive oxygen species', *Journal of Phycology*, 42(5), pp. 1040–1047. doi: 10.1111/j.1529-8817.2006.00256.x.

Fath, A., Bethke, P., Lonsdale, J., Meza-Romero, R. and Jones, R. (2000) 'Programmed Cell Death in *Cereal aleurone*', *Plant Molecular Biology*, 44(3), pp. 255–266. doi: 10.1023/A:1026584207243.

Frada, M. J., Bidle, K. D., Probert, I. and de Vargas, C. (2012) '*In situ* Survey of life cycle phases of the coccolithophore *Emiliania huxleyi* (Haptophyta)', *Environmental Microbiology*, 14(6), pp. 1558–1569. doi: 10.1111/j.1462-2920.2012.02745.x.

Franklin, D. J., Airs, R. L., Fernandes, D. J., Bell, T. G., Bongaerts, R. J., Berges, J. A. and Malin, G. (2012) 'Identification of senescence and death in *Emiliania huxleyi* and *Thalassiosira pseudonana*: cell staining, chlorophyll alterations, and Dimethylsulfoniopropionate (DMSP) Metabolism', *Limnology and oceanography*, 57(1), pp. 305–317. doi: 10.4319/lo.2012.57.1.0305.

Franklin, D. J. and Berges, J. a (2004) 'Mortality in cultures of the Dinoflagellate *Amphidinium carterae* during culture senescence and darkness.', *Proceedings. Biological sciences / The Royal Society*, 271(1553), pp. 2099–107. doi: 10.1098/rspb.2004.2810.

Gao, L. Y. and Abu Kwaik, Y. (2000) 'Hijacking of apoptotic pathways by bacterial pathogens', *Microbes and Infection*, 2(14), pp. 1705–1719. doi: 10.1016/S1286-4579(00)01326-5.

González, J. M., Simó, R., Massana, R., Covert, J. S., Casamayor, E. O., Pedrós-Alió, C. and Moran, M. a (2000) 'Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom.', *Applied and environmental microbiology*, 66(10), pp. 4237–46. doi: 10.1128/AEM.66.10.4237-4246.2000.

Green, D. H., Echavarri-Bravo, V., Brennan, D. and Hart, M. C. (2015) 'Bacterial diversity associated with the coccolithophorid algae *Emiliania huxleyi* and *Coccolithus pelagicus F. braarudii*', *BioMed Research International*, 2015. doi: 10.1155/2015/194540.

Harvey, E. L., Bidle, K. D. and Johnson, M. D. (2015) 'Consequences of strain variability and calcification in *Emiliania huxleyi* on microzooplankton grazing', *Journal of Plankton Research*, 0(0), pp. 1–12. doi: 10.1093/plankt/fbv081.

Hatsugai, N., Iwasaki, S., Tamura, K., Kondo, M., Fuji, K., Ogasawara, K., Nishimura, M. and Hara-Nishimura, I. (2009) 'A novel membrane fusion-mediated plant immunity against bacterial pathogens', *Genes and Development*. doi: 10.1101/gad.1825209.

Hatsugai, N., Yamada, K., Goto-Yamada, S. and Hara-Nishimura, I. (2015) 'Vacuolar Processing enzyme in plant progammed cell death', *Frontiers in Plant Science*, 6(April), pp. 1–11. doi: 10.3389/fpls.2015.00234.

Holligan, P. M., Fernandez, E., Aiken, J., Balch, W. M., Boyd, P., Burkill, P., Finch, M., Groom, S., Malin, G., Muller, K., *et al.* (1993) 'A biogeochemical study of the coccolithopore, *Emiliania huxleyi*, in the North Atlantic', 7(4), pp. 879–900.

Jiménez, C., Capasso, J. M., Edelstein, C. L., Rivard, C. J., Lucia, S., Breusegem, S., Berl, T. and Segovia, M. (2009) 'Different ways to die: Cell death modes of the unicellular Chlorophyte *Dunaliella viridis* exposed to various environmental stresses are mediated by the caspase-like activity DEVDase', *Journal of Experimental Botany*, 60(3), pp. 815–828. doi: 10.1093/jxb/ern330.

De Jong, A. J., Yakimova, E. T., Kapchina, V. M. and Woltering, E. J. (2002) 'A critical role for ethylene in hydrogen peroxide release during programmed cell death in tomato suspension cells', *Planta*, 214(4), pp. 537–545. doi: 10.1007/s004250100654.

Kendrick, B. J., DiTullio, G. R., Cyronak, T. J., Fulton, J. M., Van Mooy, B. a. S. and Bidle, K. D. (2014) 'Temperature-induced viral resistance in *Emiliania huxleyi* (Prymnesiophyceae)', *PLoS ONE*, 9(11), p. e112134. doi: 10.1371/journal.pone.0112134.

Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) 'Apoptosis: A basic biological phenomenon with wide- ranging implications in tissue kinetics', *Journal of Internal Medicine*, 258(6), pp. 479–517. doi: 10.1111/j.1365-2796.2005.01570.x.

Krishnamurthy, K. V., Krishnaraj, R., Chozhavendan, R. and Samuel Christopher, F. (2000) 'The programme of cell death in plants and animals: A comparison', *Current Science*, 79(9), pp. 1169–1181.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E., Baehrecke, E., Blagosklonny, M., El-Deiry, W., Golstein, P., Green, D., *et al.* (2009) 'Classification of cell death: recommendations of the nomenclature committee on cell death 2009', *Cell death and differentiation*, 16(1), pp. 3–11. doi: 10.1038/cdd.2008.150.

Lawen, A. (2003) 'Apoptosis: An introduction', *BioEssays*, 25(9), pp. 888–896. doi: 10.1002/bies.10329.
Martens, T., Heidorn, T., Pukal, R., Simon, M., Tindall, B. J. and Brinkhoff, T. (2006) 'Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al*. 1998 As *Phaeobacter gallaeciensis* gen. nov., comb. nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria algicola* Lafay *et al.* 1995) Uchino *et al.* 19', *International Journal of Systematic and Evolutionary Microbiology*, 56(6), pp. 1293–1304. doi: 10.1099/ijs.0.63724-0.
Mayers, T., Bramucci, A., Yakimovich, K. and Case, R. (2016) 'A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*', *Frontiers in Microbiology*, 7(JUN). doi: 10.3389/fmicb.2016.00892.
Moharikar, S., D'Souza, J. S., Kulkarni, A. B. and Rao, B. J. (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(2), pp. 423–433. doi: 10.1111/j.1529-8817.2006.00207.x.
Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P., Rickaby, R. E. M., Poulton, A. J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., Gibbs, S., *et al.* (2016) 'Why marine phytoplankton calcify', *Science Advances*, 2(7), pp. e1501822–e1501822. doi: 10.1126/sciadv.1501822.
Mukhtar, M. S., McCormack, M. E., Argueso, C. T. and Pajerowska-Mukhtar, K. M. (2016) 'Pathogen tactics to manipulate plant cell death', *Current Biology*. Elsevier Ltd, 26(13), pp. R608–R619. doi: 10.1016/j.cub.2016.02.051.
Müller, A. and Rudel, T. (2001) 'Modification of host cell apoptosis by viral and bacterial pathogens.', *International journal of medical microbiology : IJMM*, 291(3), pp. 197–207. doi: 10.1078/1438-4221-00125.
Niu, H., Kozjak-pavlovic, V., Rudel, T. and Rikihisa, Y. (2010) '*Anaplasma phagocytophilum* Ats-1 is imported into host cell mitochondria and interferes with apoptosis induction', 6(2). doi: 10.1371/journal.ppat.1000774.
Perez-Perez, M. E., Lemaire, S. D. and Crespo, J. L. (2012) 'Reactive oxygen species and autophagy in plants and algae', *Plant Physiology*, 160(1), pp. 156–164. doi: 10.1104/pp.112.199992.
Poulton, A. J., Charalampopoulou, A., Young, J. R., Tarran, G. a., Lucas, M. I. and Quartly, G. D. (2010) 'Coccolithophore dynamics in non-bloom conditions during late summer in the central iceland basin (July-August 2007)', 55(4), pp. 1601–1613. doi: 10.4319/lo.2010.55.4.1601.
Pozarowski, P., Huang, X., Halicka, D. H., Lee, B., Johnson, G. and Darzynkiewicz, Z. (2003) 'Interactions of fluorochrome-labeled caspase inhibitors with apoptotic cells: a caution in data interpretation', *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 55(1), pp. 50–60. doi: 10.1002/cyto.a.10074.
Ray, J. L., Haramaty, L., Thyrhaug, R., Fredricks, H. F., Van Mooy, B. A. S., Larsen, A., Bidle, K. D. and Sandaa, R. A. (2014) 'Virus infection of *Haptolina ericina* and *Phaeocystis pouchetii* implicates evolutionary conservation of programmed cell death induction in marine Haptophyte-virus interactions', *Journal of Plankton Research*, 36(4), pp. 943–955. doi: 10.1093/plankt/fbu029.
Read, B., Kegel, J., Klute, M., Kuo, A., Lefebvre, S., Maumus, F., Mayer, C., Miller, J., Monier, A., Salamov, A., *et al.* (2013) 'Pan genome of the phytoplankton *Emiliania* underpins its global distribution', *Nature*, 499(7457), pp. 209–13. doi: 10.1038/nature12221.

Reape, T. J. and McCabe, P. F. (2008) 'Apoptotic-like programmed cell death in plants', *New Phytologist*, 180(1), pp. 13–26. doi: 10.1111/j.1469-8137.2008.02549.x.
Reape, T. J., Molony, E. M. and McCabe, P. F. (2008) 'Programmed cell death in plants: distinguishing between different modes', *Journal of Experimental Botany*, 59(3), pp. 435–444. doi: 10.1093/jxb/erm258.
Rosenwasser, S., Mausz, M. a, Schatz, D., Sheyn, U., Malitsky, S., Aharoni, A., Weinstock, E., Tzfadia, O., Ben-Dor, S., Feldmesser, E., *et al.* (2014) 'Rewiring host lipid metabolism by large viruses determines the fate of *Emiliania huxleyi*, a bloom-forming alga in the ocean', *The Plant cell*, 26(June), pp. 2689–2707. doi: 10.1105/tpc.114.125641.
Ruiz-Ponte, C., Samain, J. J. F., Sánchez, J. L. J. and Nicolas, J. J. L. (1999) 'The benefit of a roseobacter species on the survival of scallop larvae.', *Marine biotechnology*, 1(1), pp. 52–59. doi: 10.1007/PL00011751.
Schatz, D., Shemi, A., Rosenwasser, S., Sabanay, H., Wolf, S. G., Ben-Dor, S. and Vardi, A. (2014) 'Hijacking of an autophagy-like process is critical for the life cycle of a DNA virus infecting oceanic algal blooms', *New Phytologist*, 204(4), pp. 854–863. doi: 10.1111/nph.13008.
Schreiber, U. (1998) 'Chlorophyll fluorescence: new instruments for special applications', *Photosynthesis: mechanisms and effects*, V, pp. 4253–4258.
Schulein, R., Guye, P., Schmid, M. C., Scheidegger, F., Dehio, M., Chennakesava, C. S., Biedermann, B. and Dehio, C. (2006) 'A translocated bacterial protein protects vascular endothelial cells from apoptosis', 2(11). doi: 10.1371/journal.ppat.0020115.
Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N., Paulson, J. N., Chai, L., Clardy, J., *et al.* (2016) 'Dynamic metabolic exchange governs a marine algal-bacterial interaction', *eLife*, 5, p. e17473. doi: 10.7554/eLife.17473.
Segovia, M., Haramaty, L., Berges, J. a and Falkowski, P. G. (2003) 'Cell death in the unicellular Chlorophyte *Dunaliella tertiolecta*. a hypothesis on the evolution of apoptosis in higher plants and metazoans.', *Plant physiology*, 132(1), pp. 99–105. doi: 10.1104/pp.102.017129.
Sheyn, U., Rosenwasser, S., Ben-Dor, S., Porat, Z. and Vardi, A. (2016) 'Modulation of host ROS metabolism is essential for viral infection of a bloom-forming coccolithophore in the ocean', *The ISME journal*. Nature Publishing Group, 10(7), pp. 1–13. doi: 10.1038/ismej.2015.228.
Thole, S., Kalhoefer, D., Voget, S., Berger, M., Engelhardt, T., Liesegang, H., Wollherr, A., Kjelleberg, S., Daniel, R., Simon, M., *et al.* (2012) '*Phaeobacter gallaeciensis* genomes from globally opposite locations reveal high similarity of adaptation to surface life', *The ISME Journal*. Nature Publishing Group, 6(12), pp. 2229–2244. doi: 10.1038/ismej.2012.62.
Thornton, D. C. O. (2014) 'Dissolved Organic Matter (DOM) release by phytoplankton in the contemporary and future ocean', *European Journal of Phycology*. © 2014 British Phycological Society, 49(1), pp. 20–46. doi: 10.1080/09670262.2013.875596.
Vardi, A., Haramaty, L., Van Mooy, B., Fredricks, H. F., Kimmance, S., Larsen, A. and Bidle, K. D. (2012) 'Host-virus dynamics and subcellular controls of cell fate in a natural coccolithophore population', *Proceedings of the National Academy of Sciences of the United States of America*, 109(47), pp. 19327–32. doi: 10.1073/pnas.1208895109.
Vardi, A., Mooy, B. A. S. Van, Fredricks, H. F., Popendorf, K. J., Ossolinski, J. E., Haramaty, L. and Bidle, K. D. (2009) 'Viral glycosphingolipids induce lytic infection and cell death in marine phytoplankton', *AAAS*, 326(5954), pp. 861–865.

Vartapetian, A., Tuzhikov, A., Chichkova, N., Taliansky, M. and Wolpert, T. (2011) 'A plant alternative to animal caspases: subtilisin-like proteases', *Cell death and differentiation*. Nature Publishing Group, 18(8), pp. 1289–1297. doi: 10.1038/cdd.2011.49.

Wang, R. and Seyedsayamdost, M. R. (2017) 'Roseochelin B, an algaecidal natural product synthesized by the roseobacter *Phaeobacter inhibens* in response to algal sinapic acid', *Organic Letters*, p. acs.orglett.7b02424. doi: 10.1021/acs.orglett.7b02424.

Wilson, W. H., Tarran, G. and Zubkov, M. V (2002) 'Virus dynamics in a coccolithophore-dominated bloom in the North Sea', *Deep Sea Research Part II: Topical Studies in Oceanography*, 49(15), pp. 2951–2963. doi: 10.1016/S0967-0645(02)00065-6.

Wlodkowic, D., Skommer, J. and Darzynkiewicz, Z. (2009) 'Flow cytometry-based apoptosis detection', 559(0), pp. 1–14. doi: 10.1007/978-1-60327-017-5.

Xu, Q. and Zhang, L. (2009) 'Plant caspase-like proteases in plant programmed cell death', *Plant signaling & behavior*, 4(9), pp. 902–904. doi: 10.4161/psb.4.9.9531.

Zhou, Y. and Zhu, Y. (2015) 'Diversity of bacterial manipulation of the host ubiquitin pathways', *Cellular Microbiology*. doi: 10.1111/cmi.12384.

Zubkov, M. V., Fuchs, B. M., Archer, S. D., Kiene, R. P., Amann, R. and Burkill, P. H. (2001) 'Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea', *Environmental Microbiology*, 3(5), pp. 304–311. doi: 10.1046/j.1462-2920.2001.00196.x.

Zupan, J., Hackworth, C. A., Aguilar, J., Ward, D. and Zambryski, P. (2007) 'VirB1* promotes T-pilus formation in the Vir-type IV Secretion System Of *Agrobacterium tumefaciens*', *Journal of Bacteriology*. doi: 10.1128/JB.00480-07

Chapter 5. *Phaeobacter inhibens* Type IV Secretion System facilitates pathogenesis of calcifying *Emiliania huxleyi*

5.1. Introduction

Calcifying phytoplankton, such as coccolithophores, are important drivers of the marine carbon cycle. They fix dissolved inorganic carbon into organic carbon and precipitate dissolved bicarbonate into delicate calcite disks or coccoliths. Coccolithophore blooms are easy to track in the upper ocean because their calcite coccoliths scatter light characteristically, highlighting vast million kilometer patches of coccolith rich algal blooms (Holligan *et al.*, 1993; Tyrrell *et al.*, 2009). Coccolithophore blooms—dominated by coccolith-bearing cells—last several weeks before suddenly crashing in what is called a bloom collapse (Baumann *et al.*, 2008). During bloom collapse the dominant blooming cells (calcified diploid cells) die rapidly and sink into the deep ocean, resulting in the burial of biogenic carbonates into marine sediments (Suchéras-Marx *et al.*, 2014). Although a portion of this calcite undergoes dissolution while sinking, the majority of coccolithophore calcite sinks in sticky clumps, marine snow (Ziveri *et al.*, 2000; Engel *et al.*, 2004), or zooplankton feces, which sink more rapidly and have lower dissolution rates (Schaechter *et al.*, 2007; Ziveri *et al.*, 2007). In fact, deep sea sediments are dominated by coccolith nannofossils, which make up roughly half of the buried biogenic carbonate, the other half being largely provided by planktonic foraminifera (Suchéras-Marx *et al.*, 2014).

The dominant coccolithophore in modern oceans is the species complex *Emiliania huxleyi* (Baumann *et al.*, 2008). *E. huxleyi* is also the only coccolithophore species that regularly overproduces and sheds coccoliths (Paasche, 2002). These coccoliths are produced within an internal vesicle and then are excreted to the outer surface of the cell to form the coccosphere around the cell (Klaveness, 1972b). Healthy calcified *E. huxleyi* cells have a coccosphere made up of approximately 12-15 interlocking coccoliths (Poulton *et al.*, 2011). The reason for microalgal calcification is still unclear, but it has been postulated that their calcite coccoliths might play an important protective role for coccolithophores, possibly impeding microzooplankton grazing, viral lysis, and/or bacterial pathogenesis (Monteiro *et al.*, 2016). However, contrary to this suggestion, the calcified *E. huxleyi* cell type appear to be more susceptible to viral pathogens than the scale-bearing haploid cells are (Frada *et al.*, 2008). As for

grazers, there are indications that microzooplankton may be negatively influenced by consuming calcified *E. huxleyi* due to their high particulate carbon (calcite coccoliths) to organic carbon (sugars like glucose) ratios (Harvey *et al.*, 2015). But nonetheless, calcified *E. huxleyi* remain highly grazed, perhaps even preferentially consumed (Evans *et al.*, 2008), by heterotrophic microzooplankton (Olson *et al.*, 2002). Finally, recent reports of the roseobacter pathogen *Phaeobacter inhibens* killing the calcifying diploid cell type, while the non-calcifying diploid cell type survives (Chapter 3), implies that bacterial pathogens are also able to overcome the physical calcite barrier. However, the mechanism employed by these bacterial pathogens to kill their calcified host, is currently unknown.

The central aim of this chapter is to investigate genetic features that facilitate the pathogenic interaction between *P. inhibens* and the calcified microalga *E. huxleyi* CCMP3266, hereafter called *E. huxleyi*. *P. inhibens* readily attaches to and interacts directly with the microalgal coccolithophore *E. huxleyi* during co-culture (Segev *et al.*, 2016). Additionally, a few bacterial produced molecules have been implicated as potentially important factors in bacterial pathogenesis of *E. huxleyi* (Seyedsayamdost *et al.*, 2011; Segev *et al.*, 2016; Wang *et al.*, 2017), but it remains unclear how these molecules might be transferred from the bacteria into the calcifying alga. Some bioactive molecules passively diffuse across cellular membranes (Zazímalová *et al.*, 2010), but in an aquatic environment, such a diffuse mechanism would likely result in rapid dilution of the molecule to an ineffective concentrations.

Specialized pathogens overcome diffusive effects by employing secretion systems, such as the type III, type IV, and type VI secretion systems (T3SS, T4SS, and T6SS respectively), which actively export bioactive effectors from the bacterial cell directly into target cells (Christie, 2001; Russell *et al.*, 2014; Buttner, 2016). These cross-kingdom transport systems have been implicated in playing a key role in bacterial-algal interactions (Persson *et al.*, 2009), but their importance in roseobacter pathogenesis has not yet been experimentally demonstrated. Distinct among secretion systems is the versatile T4SS, which can transfer an array of effectors (protein, DNA, or protein-DNA complexes) into the extracellular milieu (DNA release/uptake T4SS), directly into bacterial cells (conjugal T4SS), or directly into eukaryotic cells (effector T4SS) (Christie, 2001).

Conjugal T4SS are the most widespread subclass of T4SS; they mediate horizontal gene transfer between bacteria of the same species (or even across genera), increasing genomic

plasticity and contributing to the evolution of infectious pathogens by rapidly spreading antibiotic resistance and virulence genes (Wallden *et al.*, 2010). However, conjugal T4SS have only very rarely been linked directly to bacterial virulence (Seubert *et al.*, 2003). DNA release/uptake T4SS mediate the transfer of naked DNA into the extracellular environment (rather than a donor cell) and facilitate the uptake of DNA from the environment by naturally competent bacteria (Wallden *et al.*, 2010). Finally, effector T4SSs deliver a variety of bacterial proteins and protein-DNA complexes directly into target eukaryotic cells, thereby directly contributing to bacterial pathogenicity (Christie *et al.*, 2005; Voth *et al.*, 2012).

Effector T4SSs are so diverse that they are further subdivided into two subgroups (T4SSA and T4SSB) based on structural organization of the T4SS genes and the number of required genes. Conventional effector T4SSs resemble the archetypal VirB/VirD4 system of the plant pathogen *Agrobacterium tumefaciens*: subgroup T4SSA (Voth *et al.*, 2012). Typically, pathogens sharing this system are composed of subunits similar in both composition and number, to those first identified in *A. tumefaciens* (Voth *et al.*, 2012). Several pathogens rely on a T4SSA for pathogenesis. For instance, *Helicobacter pylori* requires its T4SSA cag system to infect gastric epithelial cells to cause gastritis (Fischer, 2011) and *Bordetella pertussis* requires its T4SSA Ptl system to cause whooping cough (Locht *et al.*, 2011). Intracellular pathogens like *Legionella pneumophila* and *Coxiella burnetii*, on the other hand, utilize the subclass T4SSB, which is more structurally similar to the conjugal transfer system of the self-transmissible IncI plasmid (Voth *et al.*, 2012).

Roughly half of the genome sequenced roseobacters have at least one copy of T4SS genes (Moran *et al.*, 2007; Persson *et al.*, 2009) and roughly a quarter possess multiple (2-3) copies of T4SS genes (Slightom *et al.*, 2009). Interestingly, while T4SS genes are widespread throughout the roseobacter clade, they are not ubiquitous, even within the *Phaeobacter* genus (Newton *et al.*, 2010). In fact, a genetic comparison of two closely related *P. inhibens* strains with only 3% difference in average nucleotide identity, revealed that one strain had a chromosomally encoded T4SS (DSM17395), while the other strain lacks T4SS genes (DSM24588) (Thole *et al.*, 2012). However, the particular class of *P. inhibens* T4SSs (conjugation vs effector), has not been experimentally confirmed. Experimentally demonstrating what type of T4SS genes are present is important as there are several subclasses of T4SS (conjugal T4SS, DNA uptake/release, and effector T4SS) all with at least a few homologous components (Cascales *et al.*, 2003). For

example, it was recently reported that the *Dinoroseobacter shibae* requires a 191 kb plasmid, which houses a functional T4SS, in order to kill its dinoflagellate host *Prorocentrum minimum* (Wang *et al.*, 2015). This was initially suggestive of T4SS mediated pathogenesis of a eukaryote (Wang *et al.*, 2015), which is suggestive of an effector T4SS apparatus. However, a more recent publication showed the cross-genera transfer of this killer plasmid from *D. shibae* into *P. inhibens*, indicating that this plasmid encoded T4SS is likely a conjugal T4SS rather than an effector T4SS (Patzelt *et al.*, 2016).

Given the importance of some classes of T4SS in translocating virulence factors into eukaryotic cells, we hypothesized that an effector type T4SS might enable *P. inhibens* DSM17395 to kill calcifying *E. huxleyi* populations, bypassing their protective coccoliths to deliver effectors into the host cytoplasm. This study expands on previous findings in Chapter 4, which demonstrate that the extracellular pathogen *P. inhibens* DSM17395 directly modulates host cell PCD processes in order to trigger algal AL-PCD.

5.2. Methods

5.2.1. Bacterial and algal strains

Phaeobacter inhibens DSM17395 (isolated from Spanish coastal waters (Ruiz-Ponte *et al.*, 1999; Martens *et al.*, 2006)) and DSM24588 (isolated from the surface of the green macroalga *Ulva australis*, near Sydney, Australia (Rao *et al.*, 2006)), were both maintained as previously described (Chapter 3). Liquid cultures were grown in ½MB at 18 °C, 160 rpm, 30 h, for algal-bacterial co-culture experiments. *P. inhibens* DSM17395 transposon mutants were obtained from collaborators at the Joint Genome Institute (JGI), and successful interruption of the targeted genes was verified (Table 5.1), as previously described (Wetmore *et al.*, 2015) and (Chapter 3). Mutants were maintained and grown as previously described (Chapter 3).

Diploid calcifying *E. huxleyi* was obtained, maintained, and grown as described (Chapter 2).

5.2.2. Co-cultivation experiments

Early-log 5 d *E. huxleyi* was co-cultured with 10^2 cfu/mL *P. inhibens* (DSM17395 or DSM24588) at 0 d. Triplicate 1 mL experiments for each time point (*E. huxleyi* and *P. inhibens* inoculated together) and controls (*E. huxleyi* or *P. inhibens* alone) were aliquoted into of 48-well microtiter plates, as previously described (Chapter 2).

Table 5.1. ***P. inhibens*** **gene names and accession numbers of T4SS VirB/VirD mutants**obtained from (Wetmore *et al.*, 2015) and used in the current study.

DSM17395 gene	Accession number	Gene name	Mutant
PGA1_c22820	WP_014880577	Lytic transglycosylase 1	Δ*LT-1*
PGA1c22850	WP_014880580	*virB4*	Δ*virB4*
PGA1c22870	WP_014880582	Lytic transglycosylase 2	Δ*LT-2*
PGA1c22880	WP_014880583	*virB5*	Δ*virB5*
PGA1c22900	WP_014880585	*virB9*	Δ*virB9*
PGA1c22960	WP_014880591	Hypothetical	ΔPGA1c22960
PGA1c22970	WP_014880592	*virD4*	Δ*virD4*
PGA1c22980	WP_014880593	*virD2*	Δ*virD2*
PGA1c23030	WP_014880598	*ard*C antirestriction	Δ*ardC* antirestriction

5.2.3. Pulse-Amplitude-Modulated Fluorometry

The Photosystem II (PSII) maximum quantum efficiency of the algal populations was monitored throughout experiments, using pulse-amplitude-modulation (PAM) fluorometry as previously described (Chapters 2, 3).

5.2.4. Flow cytometry and bacterial counts

Flow cytometry for algal cell/mL counts were enumerated using flow cytometry (as described in (Chapter 3)) and bacterial population density was enumerated using the drop-plate method (as described in Chapter 2, Figure 2.2).

5.2.5. Epifluorescence microscopy

Epifluorescence images were obtained using an Axio Imager.M2 equipped with a monochrome camera (AxioCam 506). Epifluorescence microscopy was used to visualize chlorophyll autoflorescence (red emission = 670-720 nm) and DNA stained with SYBR-I (Life Technologies) (green emission = 520 nm) within bacterial and algal cells. Unfixed *E. huxleyi* control and co-culture aliquots were stained with SYBR-I according to manufacturer's instructions then gently pelleted by centrifugation (5,000 × g, room temperature, 2 min) and washed in sterile L1-Si. Simultaneous acquisition of images overlaid using Zen 2 software v2.

5.2.6. Virulence assay of *P. inhibens* T4SS transposon mutants

E. huxleyi was inoculated with nine transposon mutants of *P. inhibens* separately. These co-cultures were performed as described except that controls (*E. huxleyi* or *P. inhibens* mutants grown alone) were amended with kanamycin (100 μg/mL) to maintain selection for the transposon (Appendix C-1).

5.3. Results and Discussion

Effector T4SSs are widespread in pathogenic bacteria and facilitate the transfer of proteins and protein-DNA complexes into eukaryotic cells (Fronzes *et al.*, 2009). Although the genome encoded *P. inhibens* DSM17395 T4SS has not yet been functionally characterized, we hypothesized that if this system is an effector translocator, it might facilitate pathogenesis through delivery of virulence factors. Conversely, if this T4SS is a conjugal transfer apparatus (as in *D. shibae* (Patzelt *et al.*, 2016)), it could play a role in spreading a plasmid encoding virulence factors throughout the population, but would likely not be directly involved in pathogenesis. Because of the high sequence similarity between the structural proteins of all subclasses of T4SSs (with various functions), the genomic context of the genes encoding this apparatus is essential to predict its function. To determine the type of T4SS encoded by DSM17395, the amino acid sequences of the proteins encoded by genes in the vicinity of the *P. inhibens* DSM17395 *virB/virD* operons were compared to those of the prototypical *A. tumefaciens* VirB/D4 system (Figure 5.1).

5.3.1. Architecture of VirB/D4 T4SS in *P. inhibens*

Effector T4SS apparatuses generally have high sequence similarity to most or all of the components of the archetypical effector translocator T4SS first identified in the plant pathogen *Agrobacterium tumefaciens,* which mediates formation of tumors in plant cells (Alvarez-Martinez *et al.*, 2009). Indeed, *P. inhibens* DSM17395 possesses a VirB/D4 type T4SS system, sharing extensive sequence similarity and operon structure to the prototypical *A. tumefaciens* VirB/D4 type T4SS (Figure 5.1). *A. tumefaciens* T4SS is comprised of a core set of 12 genes *virB1-virB11* and *virD4* (Fronzes *et al.*, 2009). The same nomenclature was used here to annotate *P. inhibens* DSM17395 T4SS proteins based on their amino-acid similarity to other characterized VirB/D4 proteins (Figure 5.1).

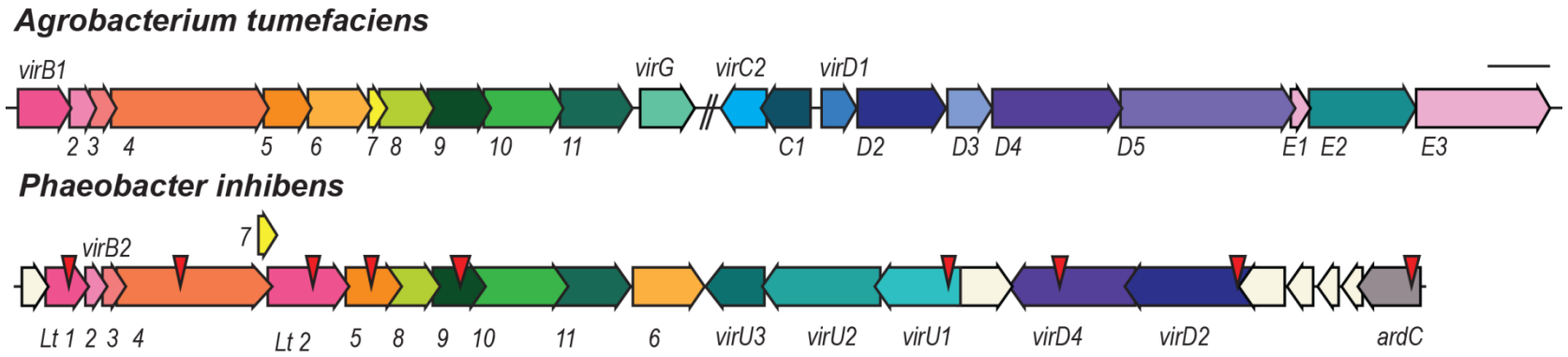

Figure 5.1. **Genetic architecture of Type IV Secretion System (T4SS) of *Phaeobacter inhibens* DSM17395 (CP002976) and *Agrobacterium tumerifaciens* LBA4213 (Ach5) plasmid Ti (CP007228).** The prototypical T4SSA gene arrangement of *Agrobacterium tumefaciens* was used to demonstrate the orientation and arrangement of homologous *virB* and *virD* genes identified within *P. inhibens* chromosome. Homologues were identified using BLASTP against *Rhizobium/Agrobacterium* species with characterized T4SSA systems. Homologues were defined for each gene separately, using a combination of factors including gene location, sequence length, % coverage, e-value, and % protein identity (Table 5.2). Homologous genes are color coded and annotated. The location of the 1,570 bp insertion from transposon mutagenesis (Wetmore *et al.*, 2015) indicated as red triangles. Scale bar denotes the length of 900 bp.

Table 5.2. ***P. inhibens*** **DSM17395 (CP002976) Type IV Secretion System (T4SS) gene accession numbers, current NCBI annotation, predicted function based on protein % similarity, and protien length (aa).** This table accompanies Figure 5.1.

Operon	*P. inhibens* DSM17395	NCBI annotation	Predicted function based on protein % similarity	aa
virB	WP_014880576	hypothetical	hypothetical	108
	WP_014880577	lytic transglycosylase	lytic transglycosylase 1[a]	192
	WP_014880578	VirB2	VirB2[b]	96
	WP_014880579	VirB3	VirB3[b]	92
	WP_014880580	VirB4	VirB4[c]	788
	WP_014880581	hypothetical	VirB7[d]	52
	WP_014880582	hypothetical	lytic transglycosylase 2[a]	382
	WP_014880583	TraF	VirB5	259
	WP_014880584	VirB8	VirB8[a]	216
	WP_014880585	TrbG	VirB9[a]	231
	WP_014880586	conjugal transfer protein	VirB10[c]	437
	WP_014880587	VirB11	VirB11[c]	331
	WP_014880589	hypothetical	VirB6[b]	332
virU	WP_081497208	hypothetical	VirU3	159
	WP_014880590	hypothetical[#]	VirU2	587
	WP_014880591	hypothetical[*]	VirU1	414
	WP_0144041585	hypothetical	hypothetical	243

Operon	*P. inhibens* DSM17395	NCBI annotation	Predicted function based on protein % similarity	aa
virD	WP_014880592	VirD4	VirD4	630
	WP_014880593	VirD2	VirD2	602
	WP_014880594	hypothetical	hypothetical	196
	WP_014880595	hypothetical	hypothetical	93
	WP_014880596	hypothetical	hypothetical	74
	WP_014880597	hypothetical	hypothetical	67
	WP_014880598	ArdC antirestriction	ArdC antirestriction	292

#hypothetical protein containing an MPN deubiuitinating (DUB) activity domain
*hypothetical protein containing an UBCc Ubiquitin-conjugating enzyme E2 catalytic domain and ThiF Ubiquitin-activating enzyme E1 domain
[a]Predicted functions are based on protein homology (% cover >30%; % identity >30%; e-value $<10^{11-}$) to *Agrobacterium tumefaciens* strain Kerr 14
[b]Predicted functions are based on protein homology (% cover >86%; % identity >52%; e-value $<10^{35-}$) to *Rhizobium* sp. NT-26
[c]Predicted functions are based on protein homology (% cover >52%; % identity >26%; e-value $<5^{29-}$) to *Agrobacterium tumefaciens* strain LBA4213 (Ach5) plasmid Ti (CP007228)
[d]Predicted functions are based on protein homology (% cover >60%; % identity >35; functional domain) to *Rhizobium* sp. NT-26

There are interesting differences between the *vir* gene organization of the VirB/D4 type T4SS in the marine roseobacter *P. inhibens* DSM17395 and the T4SS from the soil bacterium and plant pathogen *A. tumefaciens*. It is important to note that the *virB/D4* genes of *A. tumefaciens* are encoded on the Ti-plasmid of the bacteria, while in *P. inhibens* DSM17395 the *virB/D4* genes are chromosomally encoded (Thole *et al.*, 2012). Second, this comparison revealed that *P. inhibens* DSM17395 has two extra genes in the *virB* operon that are not present in the *A. tumefaciens* model T4SSA (Figure 5.1). The first extra gene is the hypothetical open-reading frame (ORF) at the 5'-end of the *virB* operon, which function is unclear, given its location and lack of sequence similarity to other *virB* genes. Secondly, there are two genes with similarity to lytic transglycosylases, which is suggestive of a functional similarity to *A. tumefaciens* VirB1. One is at the same position as the canonical *virB1* of *A. tumefaciens* and the other is inserted after *virB7*. The presence of an extra lytic transglycosylase gene might imply *P. inhibens* uses two the lytic transglycosylases in a manner similar to *A. tumefaciens'* periplasmic VirB1 protein (Christie *et al.*, 2014). Apart from being critical to both the assembly and biogenesis T4SS, this lytic transglycosylase is also required for local lysis of the peptidoglycan to allow insertion of bulky T4SS proteins into the membrane (Zupan *et al.*, 2007). Additionally, VirB1 is cleaved during T4SS construction and part of the protein is translocated out the T4SS channel before the pilus is extended; this translocated fragment is critical to pilus extension and organization (Zupan *et al.*, 2007).

Three ATPases (VirB4, VirB11, and VirD4), associated with the inner membrane and extending into the cytoplasm, power the T4SS and are essential for secretion of substrates by *A. tumefaciens* (Fronzes *et al.*, 2009). Proteins highly similar to these three ATPases were also identified in the *P. inhibens* DSM17395 VirB/D4 T4SS (Figure 5.1). The central secretion channel, spanning the two bacterial membranes, is made up of proteins VirB6, VirB7, VirB8, VirB9, and VirB10 (Wallden *et al.*, 2010). Usually a T4SS has a main pilus consisting of a major (VirB2) and minor (VirB5) subunit, which extends from the secretion channel into the extracellular space (Fronzes *et al.*, 2009). These genes (*virB2* and *virB5*) are present in *P. inhibens* DSM17395 in positions similar to their homologs in the *A. tumefaciens virB* operon. Based on their sequence similarity and similar gene order to *A. tumefaciens* homologs, genes annotated as *traF*, *trbG*, and hypothetical conjugal transfer protein in the *P. inhibens* DSM17395 genome should be renamed *virB5*, *virB9*, and *virB10*, respectively (Figure 5.1).

5.3.2. Predicted *virU* operon in *P. inhibens* T4SS

There is an extra operon containing a set of four putative ORFs of unknown function between the *virB* and *virD* operons of *P. inhibens* DSM17395 (Figure 5.1). Unexpectedly, two of the proteins within this operon (WP_0144880590 and WP_0144880591) do have sequence similarity to deubiquitinating (DUB) active domains and ubiquitin activating enzyme containing both E1 and E2 catalytic domains, respectively. Although it is unlikely that these genes would play a role in either biogenesis or structure of the T4SS, it is possible that one or more of these hypothetical genes encode for a T4SS effector protein that is translocated through the VirB/D4 T4SS. In fact, *virD5*, *virE2*, and *virE3* are all genes within the *A. tumefaciens virD* operon that encode for effector proteins that are translocated through the effector T4SS to infect plant cells (Figure 5.1) (Zechner *et al.*, 2012). Additionally, ubiquitination does not occur in prokaryotes, so detection of these protein domains suggests that they might be effectors that mimic eukaryotic ubiquitin, ubiquitin-like, or DUB-like proteins (Angot *et al.*, 2007). Given the arrangement of these genes and their relationship to ubiquitin proteins, we propose renaming the genes in this operon *virU1, virU2*, and *virU3* (Figure 5.1). The fourth ORF in this region is a truncated hypothetical gene that is in the reverse orientation to the other three *virU* genes, and was therefore not re-annotated.

5.3.3. *P. inhibens* strains differ in their interaction with *E. huxleyi*

Given the importance of T4SSs in translocating effectors and virulence factors into eukaryotic cells, it was hypothesized that the predicted T4SS identified in *P. inhibens* DSM17395 genome plays a role in bacterial interactions with calcifying *E. huxleyi* cells. Wild-type *P. inhibens* DSM17395 (predicted to contain a T4SS) and *P. inhibens* DSM24588 (lacking T4SS genes) were both grown in prolonged (20 d) co-culture with the calcifying microalga *E. huxleyi*. As expected, there was a distinct advantage to bacteria grown with an algal host, compared to those grown without an algal host (Appendix D-1). After 20 days in co-culture with *E. huxleyi*, *P. inhibens* DSM17395 and DSM24588 reached stationary phase densities of 10^8 and 10^7 cfu/mL, respectively (Appendix D-1).

However, for *E. huxleyi*, its interaction with the two *Phaeobacter* strains is distinct. Algal control cultures maintained high PSII maximum quantum efficiency (>0.5) throughout the experiment (Figure 5.2). However, *P. inhibens* DSM17395 induced a rapid population-wide decline of *E. huxleyi* PSII quantum efficiency between 8 d and 10 d (Figure 5.2a). In contrast, *P.*

inhibens strain DSM24588 (lacking T4SS) did not significantly influence algal PSII quantum efficiency (Figure 5.2b). Algal dark-adapted state constant fluorescent yield (F_0) was used to evaluate changes in overall algal fluorescence of the algal populations. Control *E. huxleyi* cultures F_0 increased throughout the experiment until (10 d), then experienced gradual decline until the end of the experiment (20 d) (Figure 5.2c,d). *P. inhibens* DSM24588 had no statistical influence on F_0 compared to the axenic control (Figure 5.2d). However, when grown with *P. inhibens* DSM17395, *E. huxleyi* experienced a rapid population-wide loss of algal F_0 (Figure 5.2c). In the case of both the controls and co-culture with *P. inhibens* DSM24588, the gradual loss of F_0 can be attributed primarily to a loss of cells/mL (Appendix D-2), rather than a gradual loss of chlorophyll *a* autofluorescence per cell (Appendix D-3). However, in the co-culture of *E. huxleyi* and *P. inhibens* DSM17395, *E. huxleyi* cells have declining chlorophyll per cell and *E. huxleyi* cells/mL (12 d) (Appendix D-2, D-3). These results demonstrate that while *P. inhibens* DSM17395 is a pathogen of calcified *E. huxleyi* and that the closely related *P. inhibens* DSM24588 is non-pathogenic and does not negatively influence either algal PSII maximum quantum efficiency or algal fluorescence. This difference in pathogenesis is suggestive that the algicides, roseobacticides and roseochelins, which are produced by both pathogenic (DSM17395) and non-pathogenic (DSM24588) strains (Wang *et al.*, 2017), are not the algicides responsible for killing the host, nor are they likely secreted via the T4SS system. Instead, it is likely that the critical bacterial virulence factor enabling *P. inhibens* DSM17395 to kill *E. huxleyi* might be identifiable in the genes that are unique present in the pathogen *P. inhibens* DSM17395 but absent from non-pathogenic *P. inhibens* DSM24588.

5.3.4. T4SS-mediated *P. inhibens* virulence

Given the high degree of genetic similarity between the two *P. inhibens* strains (Thole *et al.*, 2012) tested in co-culture with *E. huxleyi*, it was predicted that the T4SS might be key difference that enhances *P. inhibens* DSM17395 pathogenesis of the calcified eukaryotic host. To demonstrate that T4SS is required for virulence of *E. huxleyi*, mutants in nine of the genes within the *virB* and *virD* operons of the VirB/D4 T4SS system were obtained from the random transposon mutant library of *P. inhibens* DSM17395 (Wetmore *et al.*, 2015). The location of each insert was verified by PCR and sequencing (Figure 5.1). The co-culture experiments with the respective mutants and calcifying *E. huxleyi* revealed that virulence was completely attenuated in seven of the tested *virB* and *virD* mutants (Figure 5.3a-g). The list of genes that

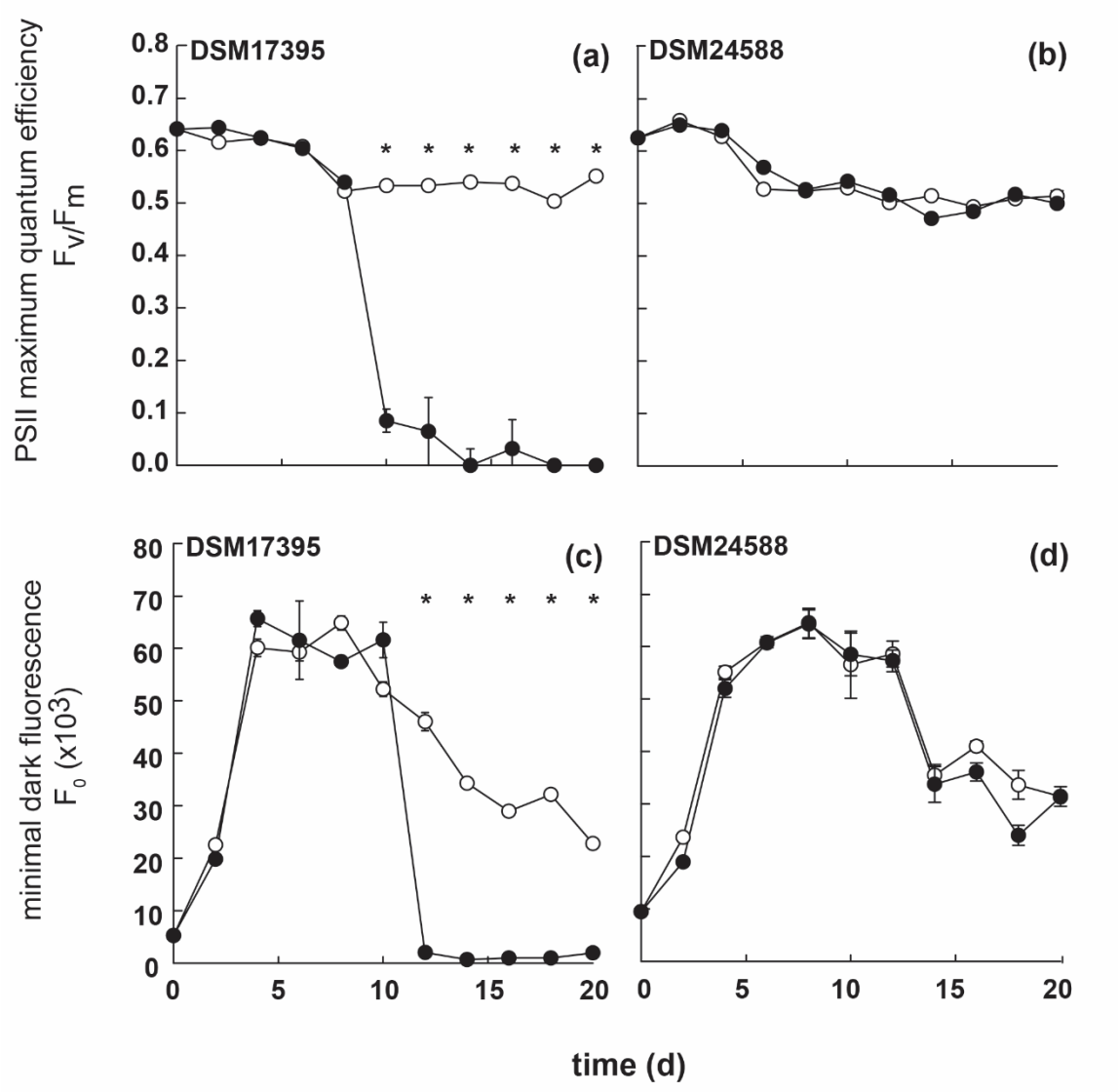

Figure 5.2. **Differential pathogenesis of *Phaeobacter inhibens* strains DSM17395 and DSM24588 on *Emiliania huxleyi* CCMP3266.** Potential quantum yield (F_v/F_m) of axenic *E. huxleyi* (control) (white circles) and co-cultures (black circles): (a) co-culture with *P. inhibens* strain DSM17395 and (b) co-culture with *P. inhibens* DSM24588 (black circles). A dark adapted state constant fluorescent yield (F_0) of (c) co-culture with DSM17395 and (d) DSM24588. Error bars = ± SE. An asterix (*) indicates that the quantum potential yield of the control and co-culture are statistically different. SigmaPlot 12 was used to perform a one-way ANOVA followed by a Tukey test, p value <0.05.

attenuated virulence includes six homologs of genes required for the *A. tumefaciens* VirB/D4 T4SS system, as well as one hypothetical gene (Figure 5.1).

A. tumefaciens requires *virB2-virB11* and *virD4* in order to translocate proteins and ssT-DNA into recipient plant cells (Fullner, 1998). These genes are involved in both the assembly of effector T4SSA and the eventual function of the apparatus (Christie *et al.*, 2014). The construction of the T4SSA pilus is a complex sequence of events orchestrated by several proteins, most importantly VirB1 (Zupan *et al.*, 2007). In *A. tumefaciens*, deletion of the gene encoding lytic transglycosylase VirB1 abolishes pilus biogenesis and decreases secretion (but does not abolish virulence) (Fronzes *et al.*, 2009). It is therefore interesting that a mutation in either LT-1 or LT-2 resulted in a complete attenuation of virulence of *P. inhibens* (Figure 5.3a, c). It is possible these transposon mutations resulted in downstream effects that compromised other aspects of T4SS assembly and/or biogenesis, but it is nonetheless suggestive that both lytic transglycosylases may be required for *P. inhibens* T4SS function.

Other mutations resulting in an avirulent phenotype include two of the three ATPases, encoded by *virB4, virB11* and *virD4* (Figure 5.3b,g). In *A. tumefaciens*, the deletion of any one of the three ATPases resulted in the arrest of the substrate prior to being transferred into the channel proteins VirB6 and VirB8 (Atmakuri *et al.*, 2004). During translocation from the bacterial periplasm to the outer membrane the substrate eventually contacts VirB9 and VirB2 (Fronzes *et al.*, 2009), highlighting the likely importance of VirB9 in substrate transfer to the host cell. VirB5 is also essential for substrate transfer and possibly releasing the effector into the target cell's membrane (Fronzes *et al.*, 2009). Mutations in the genes encoding two of these periplasm transfer proteins in *P. inhibens*, *virB5* and *virB9,* also resulted in avirulent phenotypes (Figure 5.3d,e). Additionally, VirB5 plays an important role in adhesion of the T4SS pilus to plant cells (Zechner *et al.*, 2012). The mutation in *virB5* did not decrease bacterial attachment to glass surfaces (Appendix D-4), but this protein could be required for transfer of substrates from the pilus into the host membrane, as is true for the transfer of the nucleoprotein complex by *A. tumefaciens* (Lacroix *et al.*, 2011). Although it is unclear whether these mutations might interrupt downstream genes or the biogenesis of the pilus unit, these findings do demonstrate that overall, the genes of the *virB* operon and *virD4* appear to be critical for T4SS assembly and/or function, and consequently *P. inhibens* virulence against *E. huxleyi*. While, *A. tumefaciens* requires *virB2-virB11* and *virD4* to infect plant cells (Fullner, 1998; Vergunst, 2000), the current findings

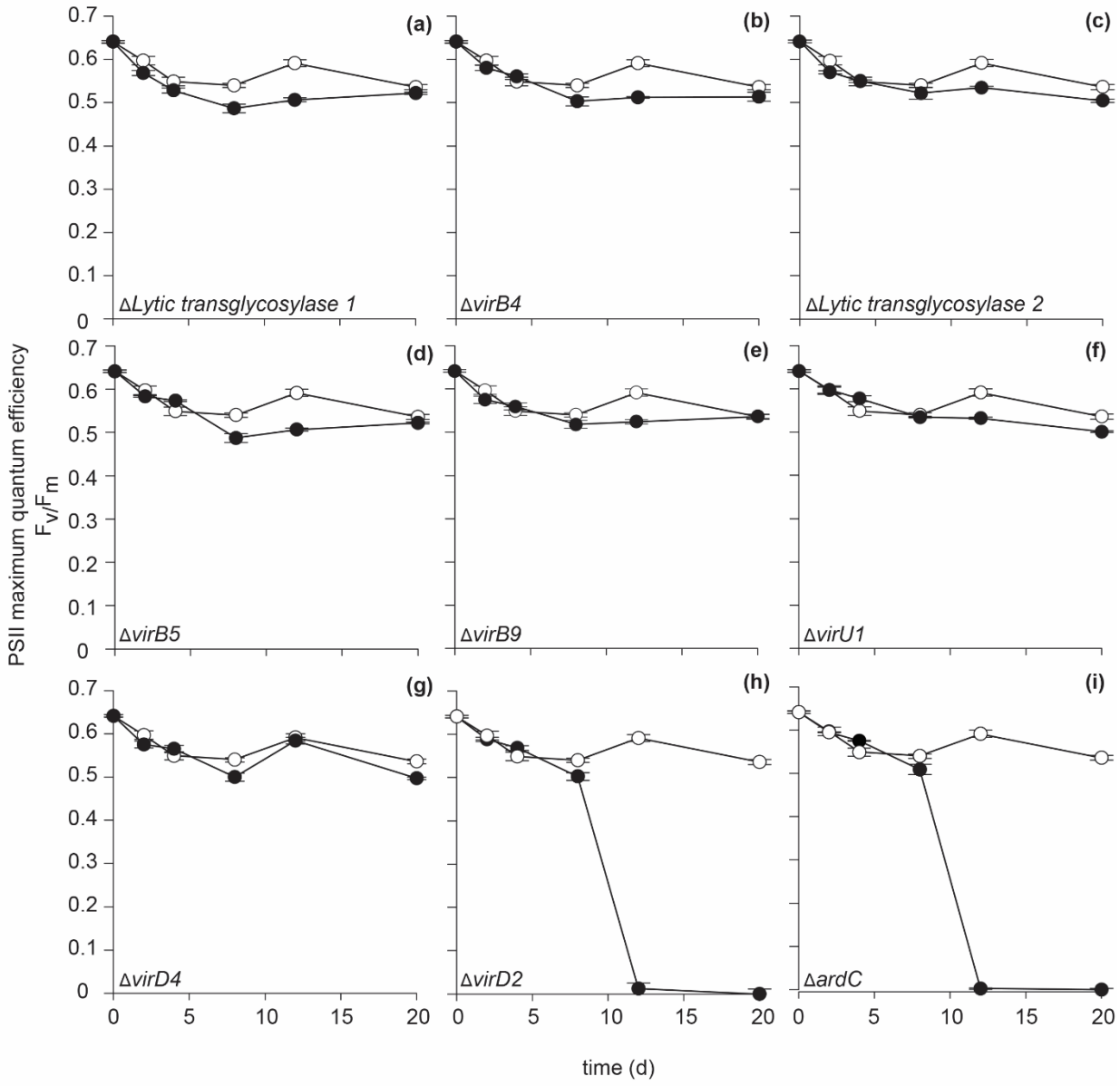

Figure 5.3. **Attenuated virulence of *Phaeobacter inhibens* DSM17395.** Potential quantum yield (Fv/Fm) of axenic *E. huxleyi* control cultures (white circles) and co-cultures (black circles): (a) co-culture with *P. inhibens* strain DSM17395 and (b) co-culture with *P. inhibens* DSM24588 (black circles). And dark adapted state constant fluorescent yield (F_0) of (c) co-culture with *P. inhibens* strain DSM17395 and (d) co-culture with *P. inhibens* DSM24588. Error bars = ± SE. An asterix (*) indicates that the quantum potential yield of the control and co-culture are statistically different. Statistics done using SigmaPlot 12 and testing a one-way ANOVA followed by a Tukey test, p value <0.05.

suggest that *P. inhibens* requires two lytic transglycosylases, as well as *virB4*,*virB5*,*virB9*, and *virD4*, to kill calcified *E. huxleyi* (Figure 5.3).

5.3.5. Genes not required for virulence

Only two mutants in this region, both in the *virD* operon (*virD2* and anti-restriction *ardC*), did not display attenuated virulence (Figure 5.3h,i). In the model *A. tumefaciens* system, the protein VirD2 associates with the ssT-DNA in the cytosol and is then translocated to the ATPase VirD4, which recruits the ssT-DNA/VirD2 complex into the T4SS channel (Wallden *et al.*, 2010). Once in the host cell VirD2 and VirE2 protect the T-DNA and translocate it into the host nucleus (Păcurar *et al.*, 2011). Importantly, mutations in *A. tumefaciens virD2* diminish but do not attenuate virulence (Bravo-Angel *et al.*, 1998), because although infection of plant cells relies on VirD2 to protect the bacterial ssT-DNA from plant endonucleases, other proteins, like VirE2, have a similar function (Păcurar *et al.*, 2011). However, in the *P. inhibens* DSM17395 T4SS, it appears that mutations in these two genes (*virD2*) does not result in attenuation or delay of killing, which suggests that in this case the effector is likely a bacterial protein rather than a protein/DNA complex as it is in the *A. tumefaciens* system. Similarly, anti-restriction genes belonging to the Ard family aid in protecting bacterial T-DNA from host endonuclease degradation by efficiently inhibiting endonucleases (Rastorguev *et al.*, 2003). However, while Ard genes are critical to DNA transfers and T4SSB type systems, they are also redundant and typically not required for T4SSA protein transfers (Belogurov *et al.*, 2000), so it is perhaps not unexpected that this mutation does not attenuate or delay virulence of *P. inhibens.*

5.3.6. Candidate T4SS effectors: *virU* genes

The novel *virU* operon inserted between *virB* and *virD* operons is of particular interest, as a mutation in its first gene (*virU1*) attenuated *P. inhibens* virulence of its algal host (Figure 5.1, Figure 5.3). This attenuation of virulence implies that *virU1* or downstream genes that might be affected by the insertion mutation (*virU2* or *virU3*) might play an important role in T4SS-mediated pathogenesis. The first indication that genes in this hypothetical *virU* operon are possible candidate T4SSA effectors is that *virU1* or *virU2* contain domains related to ubiquitination functions. This suggests they have a role in modulating the algal host's protein ubiquitination pathway. While ubiquitination is absent in prokaryotes, it is a key eukaryotic host

target for bacterial pathogens, which have evolved a diverse arsenal of T3SS or T4SS effectors that mimic ubiquitination-related functions (Singer *et al.*, 2013).

The protein ubiquitin pathway is a highly regulated mechanism of rapidly earmarking and subsequently degrading unwanted proteins (Welchman *et al.*, 2005). This highly selective protein recycling system is exclusive to eukaryotes and is used to control the abundance of key regulatory proteins and enzymes (Ingvardsen *et al.*, 2001). The degradation of such regulatory proteins can act as a cellular switch, abruptly eliminating or activating particularly cellular processes by removing critical transcription factors, cell growth modulators, and cell cycle proteins (Shabek *et al.*, 2010). Secondarily, this pathway ensures the rapid removal of abnormal or damaged proteins (Ingvardsen *et al.*, 2001). The tagging of unwanted proteins is initiated by the ubiquitin-activating enzyme known as enzyme-1 (E1), which transfers the ubiquitin protein (a polypeptide of 76 aa) to the ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) complex, and covalently attaches ubiquitin to the target substrate protein (Welchman *et al.*, 2005). This process allows eukaryotes to label proteins with a tail of ubiquitin proteins, which is sometimes extended by the combined action of E2 and E3 enzymes, resulting in polyubiquitination (>4 ubiquitin attached in a chain) (Thrower, 2000). Polyubiquitination is the signal for the protein to be degraded by the 26S proteasome (Thrower, 2000). Substrate recognition is a critical aspect of this process that is not yet entirely understood, but ubiquitination usually occurs on a lysine residue of the target protein (Ribet *et al.*, 2010). Additionally, it is thought that E2/E3 complexes recognize a specific degradation signal on the substrates, but only a few of these signals are known. Finally, eukaryote control of ubiquitination relies on having deubiquitinating enzymes (DUBs), which are a family of protease that reverse the process of protein ubiquitination by removing ubiquitin's from the substrate (Ingvardsen *et al.*, 2001).

Bacterial pathogens can exploit this eukaryotic post translational modification system by mimicking host ubiquitin proteins, thereby initiating the polyubiquitination of host proteins resulting in their degradation (Welchman *et al.*, 2005). Several examples of bacterial T3SS or T4SS effectors that directly interfere with host ubiquitin/proteasome pathway have recently been identified (Zhou *et al.*, 2015). For example, during infection of cells the intracellular pathogen *Salmonella enterica* serovar Typhimurim, utilizes T3SS to secrete at least three ubiquitin-related enzymes ubiquitin-activating E1, ubiquitin-conjugating E2, and E3 ubiquitin ligase (Zhang *et al.*,

2006). Plant pathogen *Pseudomonas syringae* injects a suite of T3SS effectors including E3 ubiquitin ligases into host plant cells to cause speck disease, where the bacterial E3 proteins recruit plant E2 enzymes and catalyze auto-ubiquitination (Abramovitch *et al.*, 2006). Similarly, *Legionella pneumophila* relies on its T4SSB to inject E3 ubiquitin ligases, which cooperate with host E2 enzymes to induce polyubiquitination of host proteins (Kubori *et al.*, 2008). Although the protein substrates targeted by E2 and E3 proteins are not always known (Ingvardsen *et al.*, 2001), these proteins tend to target substrates that are integral to host cellular processes, including transcription factors, key metabolic enzymes, and various programmed cell death (PCD) regulators (Kim *et al.*, 2003). In fact, there is mounting evidence that the ubiquitin/proteasome pathway might play an important role in apoptosis and autophagy PCD (Jesenberger *et al.*, 2002; Kim *et al.*, 2003).

Although it remains unclear how the algal ubiquitin/proteasome pathway might be connected with algal apoptosis-like PCD (AL-PCD), it is tempting to speculate that one of the predicted hypothetical *P. inhibens* effector proteins (related to DUB or ubiquitin-activating enzymes) might initiate *E. huxleyi* AL-PCD (Chapter 4). An intricate strategy of plant and mammalian pathogens is to secrete T4SS effectors that induce either AL-PCD (Iakimova *et al.*, 2013) or apoptosis (Amieva *et al.*, 2008), respectively. In fact, a mammalian pathogen produces E2 proteins (ubiquitin-activating enzyme) that have been shown to directly activate pro-caspase-8 (an initiator caspase with IETDase (Ile-Glu-Thr-Asp) activity in metazoans), thereby inducing apoptosis (Demeret *et al.*, 2003). This finding is interesting as the same caspase-like activity (IETDase) was recently identified spiking as *E. huxleyi* cultures died via AL-PCD (Chapter 4). This speaks to the possible interaction between the candidate VirU effector(s) identified here and algal caspase-like molecules with IETDase activities, which spiked directly before population-wide induction of AL-PCD (Chapter 4). However, it is also possible that another or several, as of yet unknown T4SS effector proteins or small molecules might also activate the algal caspase-like molecules that induce AL-PCD (Chapter 4).

5.3.7. *P. inhibens* DSM17395 enhances coccoliths cover in *E. huxleyi*

On day eight of the co-cultures between *E. huxleyi* and either *P. inhibens* DSM17395 or DSM24588, a striking phenotypic variation in *E. huxleyi* occurs when grown with *P. inhibens* DSM17395 (Figure 5.4). *E. huxleyi* cells appeared to have heavier calcification (Figure 5.4d-f), compared to the algae grown alone (Figure 5.4a-c) or with *P. inhibens* DSM24588 (Figure 5.4g-

i). To determine if this increase in coccolith cover was a population-wide phenomenon, side scatter area (SSC-A) properties of cells were assessed using flow cytometry, where shifts to the right (high scatter) correspond to increased roughness due to an increase in the number of coccoliths on each cell's surface (Figure 5.4a-d). *E. huxleyi* co-cultures with *P. inhibens* DSM17395 had an increased side scatter compared to the control cultures (Figure 5.5a,b). *E. huxleyi* co-cultured with non-pathogenic *P. inhibens* DSM24588 appeared more similar to the control, in terms of both algal cell size and scattering properties (Figure 5.5c,d). To further investigate this trend of increasing coccolith-cover throughout the population, the change in forward scatter area (FSC-A) of control and co-cultures were compared over time. Initially, algal cells both grown alone and in co-culture had similar size distributions (Figure 5.5e,g). However, on 8 d there is a noticeable increase (shift right) in the size and scattering properties of the algal cells grown with the pathogen *P. inhibens* DSM17395, compared to those grown alone (Figure 5.5f), or grown with the non-pathogenic strain DSM24588 (Figure 5.5h). This shift to the right demonstrates a population wide increase in cell roughness or coccolith cover on *E. huxleyi* cells grown with *P. inhibens* DSM17395.

Healthy *E. huxleyi* typically bear one layer of interlocking coccoliths (Poulton *et al.*, 2011), but the degree of calcification can vary between strains. However, nutrient limitation has been associated with an increased degree of calcification of *E. huxleyi* cells (Henriksen *et al.*, 2003). Researchers have since reasoned that cells entering stationary phase are unable to divide as frequently, but continue to produce coccoliths, leading to increased calcification during stationary phase (Müller *et al.*, 2008; Gibbs *et al.*, 2013). Alternatively, there is a suggestion that nutrient limitation (specifically of nitrogen and phosphorus) might enhance coccolith-cover as a means of accelerating algal sinking to more nutrient rich waters (Paasche, 1998). Conversely, periods of low irradiance (Fritz *et al.*, 1996), bloom termination (Fritz, 1999), and viral infection (Wilson, Tarran, Schroeder, *et al.*, 2002) are frequently associated with increased rates of coccolith detachment from calcifying *E. huxleyi* cells (Chow *et al.*, 2015).

This stimulation of coccoliths appears to be specific to specific roseobacters, like *P. inhibens* DSM17395, as it was not observed when the same algal strain was co-culture with the non-pathogenic *P. inhibens* DSM24588 or the roseobacter *Ruegeria* R11 (Chapter 5, Mayers *et al.*, 2016), which also rapidly kills the calcifying *E. huxleyi*. This is suggestive that instead of being solely nutrient limitation driven, this phenomenon may be in part due to bacterial induced

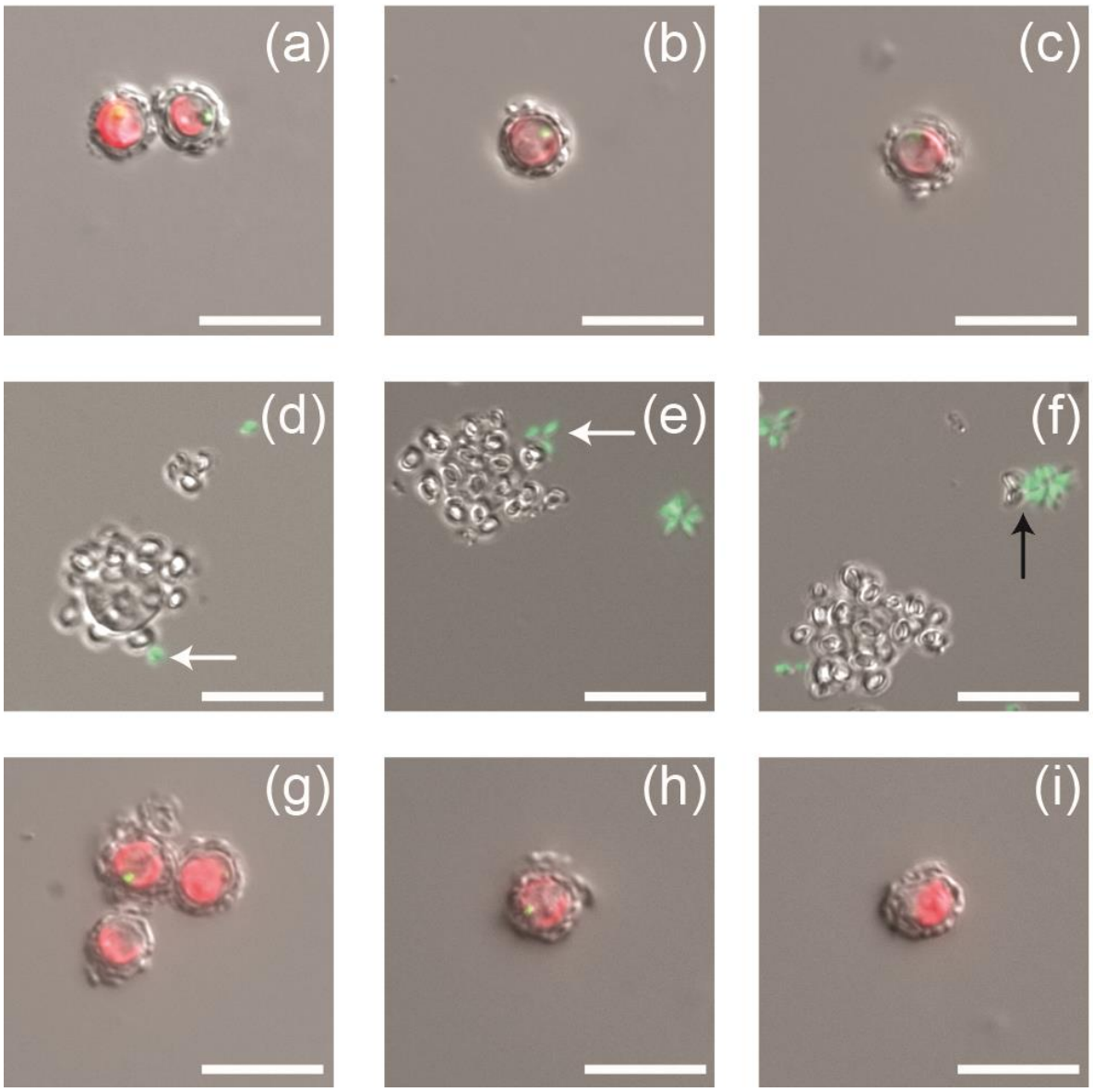

Figure 5.4. **Epifluorescence microscopy of increasing coccolithosphere per algal cell in calcifying *Emiliania huxleyi* in co-culture with *Phaeobacter inhibens* DSM17395.** Epifluorescence microscopy on day 8 of axenic control *E. huxleyi* cultures (a-c), co-cultures with *P. inhibens* DSM17395 (d-f), and co-cultures with P. inhibens DSM24588 (g-i). Merged images show differential interference contrast (DIC) overlaid with two fluorescent channels: 1) chlorophyll auto-fluorescence (red emission 670-720 nm) and 2) DNA-SYBR green complex fluorescence (green emission 520 nm). SYBR green stained P. inhibens cells attached to algal host (white arrow) and attached to free coccoliths in rosettes (black arrow).

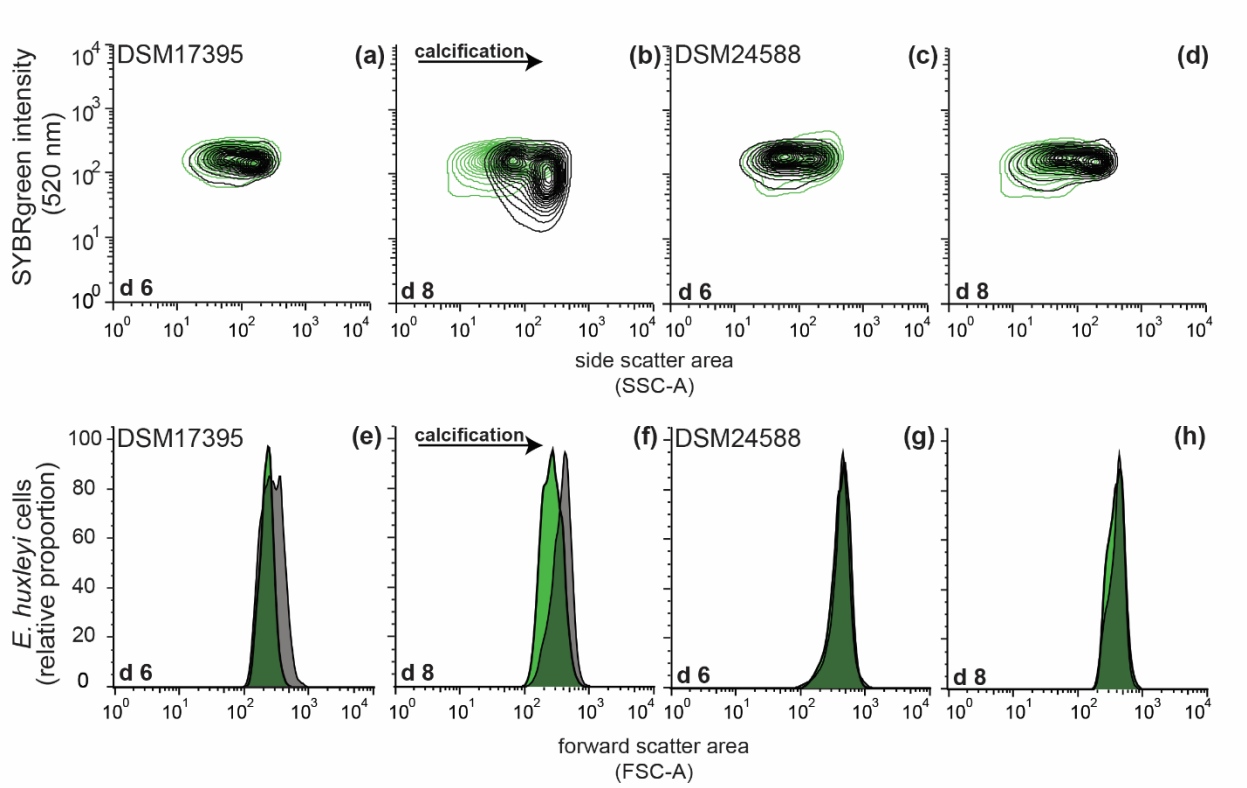

Figure 5.5. **Increasing coccolithosphere and algal cell size of calcifying *Emiliania huxleyi* (CCMP3266) in co-culture with *Phaeobacter inhibens* DSM17395 compared to those grown in co-culture with DSM24588.** Algal populations were assessed for algal SYBR green-DNA complex fluorescence (green: excitation 497 nm; emission 520 nm) and cell scattering properties (side scatter area) using flow cytometry. Contour diagrams of control cultures of *E. huxleyi* (green contour lines) and co-cultures (black contour lines) of co-cultures with *P. inhibens* DSM17395 on 6 d (a), 8 d (b) and with *P. inhibens* DSM24588 on 6 d (c), d 8 (d). The density of the contour lines is proportional to the density of detection events. The same samples were also assessed for cell size (forward scatter area) and depicted in a histogram to show the relative proportion of algal cells of a given cell size. Co-culture with *P. inhibens* DSM17395 on 6 d (e), 8 d (f) and with *P. inhibens* DSM24588 on 6 d (g), d 8 (h). A shift right to higher forward scatter area indicates an increase in calcification.

exchanges with the algal host. Bacterial driven increased calcification of an algal host might be beneficial to the host (and by extension to the pathogen). The possible biological benefits of increasing coccoliths per cell remain uncertain but include increasing light scattering (Tyrrell *et al.*, 1999, 2009), shielding from increased irradiance, and accelerating sinking within an algal population (Eppley *et al.*, 1967; Bach *et al.*, 2012). Another possible effect of increasing algal coccolith-cover is that it skews the inorganic to organic carbon content of the algal cells, and although this has already been shown not to deter grazing it might negatively affect microzooplankton grazers of heavily calcified cells (Harvey *et al.*, 2015).

5.4. Conclusion

T4SS genes are widespread in the roseobacter clade, but this is the first time roseobacter effector translocator T4SS has been directly demonstrated to be required for pathogenesis towards an algal host. This finding is suggestive that other roseobacters with secretion systems capable of interacting with eukaryotic cells (T3SS, T4SSA, and T6SS) might also translocate effectors during pathogenic interactions. In this way, pathogens would overcome the likely diffusive effects of releasing virulence effectors into the extracellular environment, and these attached pathogens would be more likely to directly benefit from killing their algal host. Additionally, this work suggests a candidate effector protein that might play a role in subverting the algal ubiquitin/proteasome pathway to direct the host to AL-PCD. These findings are suggestive that bacteria, particularly chemosensory bacteria like roseobacters, might be able to seek out, attach to, and kill algae in the natural environment.

5.5. Works Cited

Abramovitch, R. B., Janjusevic, R., Stebbins, C. E. and Martin, G. B. (2006) 'Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0507892103.

Alvarez-Martinez, C. E. and Christie, P. J. (2009) 'Biological diversity of prokaryotic Type IV Secretion Systems', *Microbiol Mol Biol Rev*, 73(4), pp. 775–808. doi: 10.1128/MMBR.00023-09.

Amieva, M. R. and El-Omar, E. M. (2008) 'Host-bacterial interactions in *Helicobacter pylori* infection', *Gastroenterology*. doi: 10.1053/j.gastro.2007.11.009.

Angot, A., Vergunst, A., Genin, S. and Peeters, N. (2007) 'Exploitation of Eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial Type III and Type IV Secretion Systems', *PLoS Pathogens*, 3(1), pp. 0001–0013. doi: 10.1371/journal.ppat.0030003.

Atmakuri, K., Cascales, E. and Christie, P. J. (2004) 'Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial Type IV Secretion', *Molecular Microbiology*, 54(5), pp. 1199–1211. doi: 10.1111/j.1365-2958.2004.04345.x.

Bach, L. T., Riebesell, U., Sett, S., Febiri, S., Rzepka, P. and Schulz, K. G. (2012) 'An Approach For Particle Sinking Velocity Measurements In The 3-400um Size Range And Considerations On The Effect Of Temperature On Sinking Rates', *Marine Biology*, 159(8), pp. 1853–1864. doi: 10.1007/s00227-012-1945-2.

Baumann, K., Boeckel, B. and Čepek, M. (2008) 'Spatial Distribution of living coccolithophores along an East-West transect in the subtropical South Atlantic', *Journal of Nannoplankton Research*, 30(1), pp. 9–21.

Belogurov, A. A., Delver, E. P., Agafonova, O. V, Belogurova, N. G., Lee, L. Y. and Kado, C. I. (2000) 'Antirestriction Protein Ard (Type C) encoded by IncW plasmid PSa has a high similarity to the "protein transport" domain of TraC1 primase of promiscuous plasmid RP4', *J Mol Biol*. doi: 10.1006/jmbi.1999.3493.

Bravo-Angel, A. M., Hohn, B. and Tinland, B. (1998) 'The omega sequence of VirD2 is important but not essential for efficient transfer of T-DNA by *Agrobacterium tumefaciens*', *Molecular plant-microbe interactions : MPMI*. doi: 10.1094/MPMI.1998.11.1.57.

Buttner, D. (2016) 'Behind the lines-actions of bacterial Type III effector proteins in plant cells', *FEMS Microbiology Reviews*, 40(6), pp. 894–937. doi: 10.1093/femsre/fuw026.

Cascales, E. and Christie, P. J. (2003) 'The versatile bacterial Type IV Secretion Systems', *Nature Reviews Microbiology*. doi: 10.1038/nrmicro753.

Chow, J. S., Lee, C. and Engel, A. (2015) 'The Influence Of Extracellular Polysaccharides, Growth Rate, And Free Coccoliths On The Coagulation Efficiency Of *Emiliania Huxleyi*', *Marine Chemistry*. Elsevier B.V., 175, pp. 5–17. doi: 10.1016/j.marchem.2015.04.010.

Christie, P. J. (2001) 'Type IV Secretion: Intercellular Transfer Of Macromolecules By Systems Ancestrally Related To Conjugation Machines', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2001.02302.x.

Christie, P. J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S. J. and Cascales, E. (2005) 'Biogenesis, Architecture, And Function Of Bacterial Type IV Secretion Systems', *Annual review of microbiology*.

Christie, P. J., Whitaker, N. and González-Rivera, C. (2014) 'Mechanism And Structure Of The Bacterial Type IV Secretion Systems', *Biochimica et Biophysica Acta - Molecular Cell Research*. doi: 10.1016/j.bbamcr.2013.12.019.

Demeret, C., Garcia-Carranca, A. and Thierry, F. (2003) 'Transcription-independent Triggering Of The Extrinsic Pathway Of Apoptosis By Human Papillomavirus 18 E2 Protein', *Oncogene*, 22(2), pp. 168–175. doi: 10.1038/sj.onc.1206108.

Engel, A., Delille, B., Jacquet, S., Riebesell, U., Rochelle-Newall, E., Terbrüggen, A. and Zondervan, I. (2004) 'Transparent Exopolymer Particles And Dissolved Organic Carbon Production By *Emiliania huxleyi* Exposed To Different CO_2 Concentrations: A Mesocosm Experiment', *Aquatic Microbial Ecology*, 34(1), pp. 93–104. doi: 10.3354/ame034093.

Eppley, R. W., Holmes, R. W. and Strickland, J. D. H. (1967) 'Sinking Rates Of Marine Phytoplankton Measured With A Fluorometer', *Journal of Experimental Marine Biology and Ecology*, 1(2), pp. 191–208. doi: 10.1016/0022-0981(67)90014-7.

Evans, C. and Wilson, W. H. (2008) 'Preferential Grazing Of *Oxyrrhis Marina* On Virus Infected *Emiliania huxleyi*', *Limnology and Oceanography*, 53(5), pp. 2035–2040. doi: 10.4319/lo.2008.53.5.2035.

Fischer, W. (2011) 'Assembly And Molecular Mode Of Action Of The *Helicobacter pylori* Cag Type IV Secretion Apparatus', *FEBS Journal*. doi: 10.1111/j.1742-4658.2011.08036.x.

Frada, M., Probert, I., Allen, M. J., Wilson, W. H. and de Vargas, C. (2008) 'The "Cheshire Cat" escape Strategy Of The Coccolithophore *Emiliania Huxleyi* In Response To Viral Infection.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(41), pp. 15944–15949. doi: 10.1073/pnas.0807707105.

Fritz, J. J. (1999) 'Carbon Fixation And Coccolith Detachment In The Coccolithophore *Emiliania Huxleyi* In Nitrate-limited Cyclostats', *Marine Biology*, 133(3), pp. 509–518. doi: 10.1007/s002270050491.

Fritz, J. J. and Balch, W. M. (1996) 'A Light-limited Continuous Culture Study Of *Emiliania huxleyi*: Determination Of Coccolith Detachment And Its Relevance To Cell Sinking', *Journal of Experimental Marine Biology and Ecology*. doi: 10.1016/S0022-0981(96)02633.

Fronzes, R., Christie, P. J. and Waksman, G. (2009) 'The Structural Biology Of Type IV Secretion Systems.', *Nature reviews. Microbiology*, 7(10), pp. 703–14. doi: 10.1038/nrmicro2218.

Fullner, K. J. (1998) 'Role Of Agrobacterium VirB Genes In Transfer Of T Complexes And RSF1010', *Journal of Bacteriology*.

Gibbs, S. J., Poulton, A. J., Bown, P. R., Daniels, C. J., Hopkins, J., Young, J. R., Jones, H. L., Thiemann, G. J., O'Dea, S. A. and Newsam, C. (2013) 'Species-specific Growth Response Of Coccolithophores To Palaeocene–Eocene Environmental Change', *Nature Geoscience*. doi: 10.1038/ngeo1719.

Harvey, E. L., Bidle, K. D. and Johnson, M. D. (2015) 'Consequences Of strain variability and calcification in *Emiliania huxleyi* on microzooplankton grazing', *Journal of Plankton Research*, 0(0), pp. 1–12. doi: 10.1093/plankt/fbv081.

Henriksen, K., Stipp, S. L. S., Young, J. R. and Bown, P. R. (2003) 'Tailoring Calcite: Nanoscale AFM of coccolith biocrystals', *American Mineralogist*, 88(11–12 PART 2), pp. 2040–2044.

Holligan, P. M., Fernandez, E., Aiken, J., Balch, W. M., Boyd, P., Burkill, P., Finch, M., Groom, S., Malin, G., Muller, K., *et al.* (1993) 'A Biogeochemical study of the coccolithopore, *Emiliania huxleyi*, in the North Atlantic', 7(4), pp. 879–900.

Iakimova, E. T., Sobiczewski, P., Michalczuk, L., Wegrzynowicz-Lesiak, E., Mikiciński, A. and Woltering, E. J. (2013) 'Morphological and biochemical characterization of *Erwinia amylovora*-induced hypersensitive cell death in apple leaves', *Plant Physiology and Biochemistry*, 63, pp. 292–305. doi: 10.1016/j.plaphy.2012.12.006.

Ingvardsen, C. and Veierskov, B. (2001) 'Ubiquitin- and proteasome-dependent proteolysis in plants', *Physiologia Plantarum*. doi: 10.1034/j.1399-3054.2001.1120401.x.
Jesenberger, V., Jentsch, S., V, J. and S, J. (2002) 'Deadly Encounter: Ubiquitin Meets Apoptosis.', *Nature reviews. Molecular cell biology*. doi: 10.1038/nrm731.
Kim, M., Ahn, J. W., Jin, U. H., Choi, D., Paek, K. H. and Pai, H. S. (2003) 'Activation of the programmed cell death pathway by inhibition of proteasome function in plants', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M210539200.
Klaveness, D. (1972) '*Coccolitus Huxleyi* (Lohmann) Kamptner I. Morphological investigations on the vegetative cell and the process of coccolith formation', *Protistologica*, 8(3), pp. 335–346.
Kubori, T., Hyakutake, A. and Nagai, H. (2008) '*Legionella* translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2008.06124.x.
Lacroix, B. and Citovsky, V. (2011) 'Extracellular VirB5 enhances T-DNA transfer from *Agrobacterium* to the host plant', *PLoS ONE*. doi: 10.1371/journal.pone.0025578.
Locht, C., Coutte, L. and Mielcarek, N. (2011) 'The ins and outs of *Pertussis* toxin', *Febs Journal*. doi: 10.1111/j.1742-4658.2011.08237.x.
Martens, T., Heidorn, T., Pukal, R., Simon, M., Tindall, B. J. and Brinkhoff, T. (2006) 'Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al*. 1998 As *Phaeobacter gallaeciensis* gen. nov., comb. nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria algicola* Lafay *et al.* 1995) Uchino *et al.* 19', *International Journal of Systematic and Evolutionary Microbiology*, 56(6), pp. 1293–1304. doi: 10.1099/ijs.0.63724-0.
Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P., Rickaby, R. E. M., Poulton, A. J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., Gibbs, S., *et al.* (2016) 'Why marine phytoplankton calcify', *Science Advances*, 2(7), pp. e1501822–e1501822. doi: 10.1126/sciadv.1501822.
Moran, M. A., Belas, R., Schell, M. A., González, J. M., Sun, F., Sun, S., Binder, B. J., Edmonds, J., Ye, W., Orcutt, B., *et al.* (2007) 'Ecological genomics of marine roseobacters', *Applied and Environmental Microbiology*, 73(14), pp. 4559–4569. doi: 10.1128/AEM.02580-06.
Müller, M. N., Antia, A. N. and LaRoche, J. (2008) 'Influence of cell cycle phase on calcification in the coccolithophore *Emiliania huxleyi*', *Limnology and Oceanography*. doi: 10.4319/lo.2008.53.2.0506.
Newton, R. J., Griffin, L. E., Bowles, K. M., Meile, C., Gifford, S., Givens, C. E., Howard, E. C., King, E., Oakley, C. A., Reisch, C. R., *et al.* (2010) 'Genome Characteristics Of A Generalist Marine Bacterial Lineage.', *The ISME journal*. Nature Publishing Group, 4(6), pp. 784–98. doi: 10.1038/ismej.2009.150.
Olson, M. B. and Strom, S. L. (2002) 'Phytoplankton growth, microzooplankton herbivory and community structure in the southeast Bering Sea: Insight into the formation and temporal persistence of an *Emiliania huxleyi* bloom', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 49(26), pp. 5969–5990. doi: 10.1016/S0967-0645(02)00329-6.
Paasche, E. (1998) 'Roles Of Nitrogen And Phosphorus In Coccolith Formation In *Emiliania Huxleyi* (Prymnesiophyceae)', *European Journal of Phycology*, 33(1), pp. 33–42. doi: 10.1017/S0967026297001480.
Paasche, E. (2002) 'A review of the coccolithophorid *Emiliania huxleyi*', *Phycologia*, 40(6), pp. 503–529.

Păcurar, D. I., Thordal-Christensen, H., Păcurar, M. L., Pamfil, D., Botez, M. L. and Bellini, C. (2011) '*Agrobacterium tumefaciens*: From crown gall tumors to genetic transformation', *Physiological and Molecular Plant Pathology*, 76(2), pp. 76–81. doi: 10.1016/j.pmpp.2011.06.004.

Patzelt, D., Michael, V., Päuker, O., Ebert, M., Tielen, P., Jahn, D., Tomasch, J., Petersen, J. and Wagner-Döbler, I. (2016) 'Gene flow across genus barriers - conjugation of *Dinoroseobacter shibae*'s 191-kb killer plasmid into *Phaeobacter inhibens* and AHL-mediated expression of Type IV Secretion Systems', *Frontiers in Microbiology*, 7(MAY), pp. 1–12. doi: 10.3389/fmicb.2016.00742.

Persson, O. P., Pinhassi, J., Riemann, L., Marklund, B. I., Rhen, M., Normark, S., González, J. M. and Hagström, Å. (2009) 'High abundance of virulence gene homologues in marine bacteria', *Environmental Microbiology*, pp. 1348–1357. doi: 10.1111/j.1462-2920.2008.01861.x.

Poulton, A. J., Young, J. R., Bates, N. R. and Balch, W. M. (2011) 'Biometry of detached *Emiliania huxleyi* along the Patagonian Shelf', *Marine Ecology Progress Series*, 443, pp. 1–17. doi: 10.3354/meps09445.

Rao, D., Webb, J. S. and Kjelleberg, S. (2006) 'Microbial colonization and competition on the marine alga *Ulva australis*', *Applied and Environmental Microbiology*, 72(8), pp. 5547–5555. doi: 10.1128/AEM.00449-06.

Rastorguev, S. M. and Zavilgelsky, G. B. (2003) 'Role of "antirestriction" motif in functional activity of antirestriction protein ArdA PKM101 (IncN)', *Russian Journal of Genetics*. doi: 10.1023/A:1022444212370.

Ribet, D. and Cossart, P. (2010) 'Pathogen-mediated posttranslational modifications: A re-emerging field', *Cell*. doi: 10.1016/j.cell.2010.11.019.

Ruiz-Ponte, C., Samain, J. J. F., Sánchez, J. L. J. and Nicolas, J. J. L. (1999) 'The benefit of a roseobacter species on the survival of scallop larvae', *Marine biotechnology*, 1(1), pp. 52–59. doi: 10.1007/PL00011751.

Russell, A. B., Peterson, S. B. and Mougous, J. D. (2014) 'Type VI Secretion System effectors: poisons with a purpose', *Nature Reviews Microbiology*. doi: 10.1038/nrmicro3185.

Schaechter, M., Ingraham, J. L. and Neidhardt, F. C. (2007) 'Eukaryotic Microbes', in *Microbe - Das Original mit Übersetzungshilfen*.

Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N., Paulson, J. N., Chai, L., Clardy, J., *et al.* (2016) 'Dynamic metabolic exchange governs a marine algal-bacterial interaction', *eLife*, 5, p. e17473. doi: 10.7554/eLife.17473.

Seubert, A., Hiestand, R., De La Cruz, F. and Dehio, C. (2003) 'A bacterial conjugation machinery recruited for pathogenesis', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2003.03650.x.

Seyedsayamdost, M. R., Case, R. J., Kolter, R. and Clardy, J. (2011) 'The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*', *Nature chemistry*, 3(4), pp. 331–5. doi: 10.1038/nchem.1002.

Shabek, N. and Ciechanover, A. (2010) 'Degradation of ubiquitin: The fate of the cellular reaper', *Cell Cycle*, 9(3), pp. 523–530. doi: 10.4161/cc.9.3.11152.

Singer, A. U., Schulze, S., Skarina, T., Xu, X., Cui, H., Eschen-Lippold, L., Egler, M., Srikumar, T., Raught, B., Lee, J., *et al.* (2013) 'A pathogen Type III effector with a novel E3 ubiquitin ligase architecture', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1003121.

Slightom, R. N. and Buchan, A. (2009) 'Surface colonization by marine roseobacters: integrating genotype and phenotype', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01508-09.
Suchéras-Marx, B. and Henderiks, J. (2014) 'Testing the impact of calcareous nannofossil size evolution on pelagic carbonate burial over the past 17 million years', *Global and Planetary Change*. Elsevier B.V., 123, pp. 94–109. doi: 10.1016/j.gloplacha.2014.10.015.
Thole, S., Kalhoefer, D., Voget, S., Berger, M., Engelhardt, T., Liesegang, H., Wollherr, A., Kjelleberg, S., Daniel, R., Simon, M., *et al.* (2012) '*Phaeobacter gallaeciensis* genomes from globally opposite locations reveal high similarity of adaptation to surface life', *The ISME Journal*. Nature Publishing Group, 6(12), pp. 2229–2244. doi: 10.1038/ismej.2012.62.
Thrower, J. S. (2000) 'Recognition of the polyubiquitin proteolytic signal', *The EMBO Journal*. doi: 10.1093/emboj/19.1.94.
Tyrrell, T., Holligan, P. M. and Mobley, C. D. (1999) 'Optical impacts of oceanic coccolithophore blooms', *Journal of Geophysical Research*, 104(C2), p. 3223. doi: 10.1029/1998JC900052.
Tyrrell, T. and Young, J. R. (2009) 'Coccolithophores', *Encyclopedia of Ocean Sciences*, (c), pp. 606–614. doi: 10.1016/B978-012374473-9.00662-7.
Vergunst, A. C. (2000) 'VirB/D4- dependent protein translocation from *Agrobacterium* into plant cells', *Science*, 290(5493), pp. 979–982. doi: 10.1126/science.290.5493.979.
Voth, D. E., Broederdorf, L. J. and Graham, J. G. (2012) 'Bacterial Type IV Secretion Systems: versatile virulence machines', 7(2), pp. 241–257. doi: 10.2217/fmb.11.150.Bacterial.
Wallden, K., Rivera-Calzada, A. and Waksman, G. (2010) 'Type IV Secretion Systems: Versatility and diversity in function', *Cellular Microbiology*, 12(9), pp. 1203–1212. doi: 10.1111/j.1462-5822.2010.01499.x.
Wang, H., Tomasch, J., Michael, V., Bhuju, S., Jarek, M., Petersen, J. and Wagner-Döbler, I. (2015) 'Identification of genetic modules mediating the Jekyll and Hyde interaction of *Dinoroseobacter shibae* with the dinoflagellate *Prorocentrum minimum*', *Frontiers in Microbiology*, 6(NOV), pp. 1–8. doi: 10.3389/fmicb.2015.01262.
Wang, R. and Seyedsayamdost, M. R. (2017) 'Roseochelin B, an algaecidal natural product synthesized by the roseobacter *Phaeobacter inhibens* in response to algal sinapic acid', *Organic Letters*, p. acs.orglett.7b02424. doi: 10.1021/acs.orglett.7b02424.
Welchman, R. L., Gordon, C. and Mayer, R. J. (2005) 'Ubiquitin And ubiquitin-like proteins as multifunctional signals', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm1700.
Wetmore, K. M., Price, M. N., Waters, R. J., Lamson, J. S., He, J., Hoover, C. A., Blow, M. J., Bristow, J., Butland, G., Arkin, A. P., *et al.* (2015) 'Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons', *mBio*, 6(3), pp. 1–15. doi: 10.1128/mBio.00306-15.
Wilson, W. H., Tarran, G., Schroeder, D., Cox, M., Oke, J. and Malin, G. (2002) 'Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel', *Journal of the Marine Biological Association of the United Kingdom*, 82, pp. 369–377.
Zazímalová, E., Murphy, A. S., Yang, H., Hoyerová, K. and Hosek, P. (2010) 'Auxin transporters--why so many?', *Cold Spring Harbor perspectives in biology*. doi: 10.1101/cshperspect.a001552.
Zechner, E. L., Lang, S. and Schildbach, J. F. (2012) 'Assembly and mechanisms of bacterial Type IV Secretion machines', *Philosophical Transactions of the Royal Society B: Biological Sciences*. doi: 10.1098/rstb.2011.0207.

Zhang, Y., Higashide, W. M., McCormick, B. A., Chen, J. and Zhou, D. (2006) 'The inflammation-associated *Salmonella* SopA Is A HECT-like E3 ubiquitin ligase', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2006.05407.x.

Zhou, Y. and Zhu, Y. (2015) 'Diversity of bacterial manipulation of the host ubiquitin pathways', *Cellular Microbiology*. doi: 10.1111/cmi.12384.

Ziveri, P., de Bernardi, B., Baumann, K. H., Stoll, H. M. and Mortyn, P. G. (2007) 'Sinking of coccolith carbonate and potential contribution to organic carbon ballasting in the deep ocean', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 54(5–7), pp. 659–675. doi: 10.1016/j.dsr2.2007.01.006.

Ziveri, P., Broerse, A. T. C., vanHinte, J. E., Westbroek, P. and Honjo, S. (2000) 'The fate of coccoliths at 48 degrees N 21 degrees W, Northeastern Atlantic', *Deep - Sea Research Part II - Topical Studies in Oceanography*.

Zupan, J., Hackworth, C. A., Aguilar, J., Ward, D. and Zambryski, P. (2007) 'VirB1* Promotes T-pilus Formation In The Vir-type IV Secretion System Of Agrobacterium Tumefaciens', *Journal of Bacteriology*. doi: 10.1128/JB.00480-07.

Chapter 6. General Conclusion

6.1. Brief summary

The research in this thesis aims to mechanistically describe the pathogenic interaction between the roseobacter *Phaeobacter inhibens* and the ubiquitous coccolithophore *Emiliania huxleyi*. In Chapter 2, I established and tested a novel miniaturized bioassay to assess phytoplankton physiology when grown alone or with a bacterial symbiont. This bioassay was then used in the subsequent chapters. Chapter 3 established that *P. inhibens* DSM17395 demonstrates a specific pathogenesis, killing both the calcifying diploid and the flagellated haploid cells of *E. huxleyi*, but not several tested diploid non-calcifying cells (Figure 6.1). In that chapter, I also experimentally demonstrated that the novel algicidal bioactive roseobacticides produced by *P. inhibens* do not kill the tested calcifying or haploid strains of *E. huxleyi* (CCMP3266 and CCMP3268, respectively). Chapter 4 investigated the death mechanism the calcifying *E. huxleyi* strain CCMP3266 undergoes when challenged with the bacterial pathogen *P. inhibens* DSM17395 (Figure 6.1). In that chapter, I demonstrated for the first time that *P. inhibens* induces activation of caspase-like molecules and apoptosis-like programmed cell death (AL-PCD) in the calcifying *E. huxleyi* (Figure 6.1). Finally, Chapter 5 established that *P. inhibens* DSM17395 pathogenesis requires a functional effector translocator Type VI Secretion System (effector T4SS) to kill calcifying *E. huxleyi* cells (Figure 6.1). Chapter 5 also proposed a possible mechanism for the candidate T4SS effectors to induce AL-PCD in *E. huxleyi* cells.

"An essential goal for the future is to conclusively demonstrate that the phenomenon of phytoplankton death due to bacteria does indeed occur in the ocean…This will require a mechanistic understanding of how algicidal bacteria kill their phytoplankton prey…"

Mayali and Azam 2004

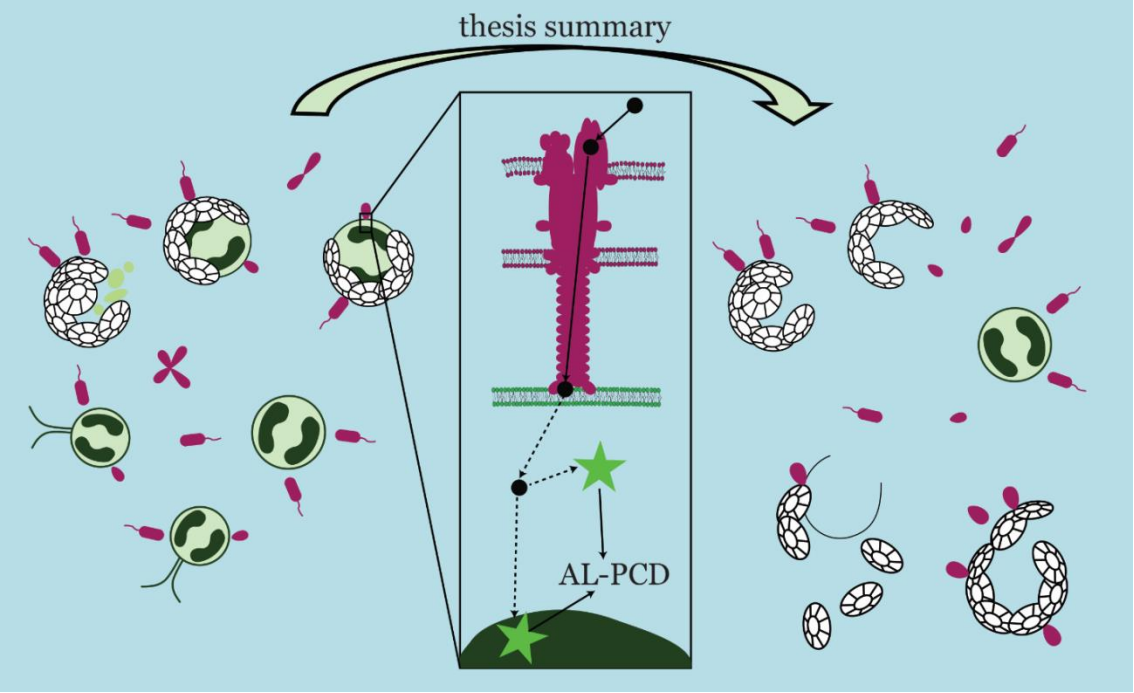

Figure 6.1. **Pictorial representation of the key findings of this thesis**. *Phaeobacter inhibens* DSM17395 (pink) is a selective pathogen of calcified diploid and flagellated haploid *Emiliania huxleyi* cells, but not non-calcifying diploid cells (Chapter 3). Further study of the dominant calcifying strain identified that *P. inhibens* induced the activation of algal caspase-like molecules (green star), which led to AL-PCD of the calcifying algal (Chapter 4). Finally (inset), Chapter 5 identified that pathogenic *P. inhibens* requires a functional T4SSA to translocated T4SSA effector(s) (black circle) in order to successfully kill the calcifying alga.

6.2. Potential scope of findings

6.2.1. *E. huxleyi* bloom composition and various strains

After discovering that *P. inhibens* DSM17395 has a highly specific pathogenic interaction, killing one representative strain of calcifying and haploid strain of *E. huxleyi* (Figure 6.1) (Chapter 3), the remainder of my work focused on only the representative axenic calcifying cell type of *E. huxleyi* strain CMP3266 (Chapters 4 and 5). I chose to focus on just the calcifying cell type because coccolithophore blooms (defined as marine waters having $>10^6$ cells/mL coccolithophores (Tyrrell *et al.*, 2004)) are typically dominated by the fast growing diploid coccolith-bearing *E. huxleyi* cells (Baumann *et al.*, 2008). In fact, calcifying *E. huxleyi* cells frequently make up the majority of the algal cells (80-90%) in a *E. huxleyi* bloom (Baumann *et al.*, 2008; Frada *et al.*, 2012). The remaining *E. huxleyi* cells are bald diploid cells; which are actually a mixture of calcifying cells that have shed coccoliths and non-calcifying diploid cells

(Frada *et al.*, 2012). Flagellated haploid cells account for between 1% and 10% of the cells within an *E. huxleyi* bloom (Baumann *et al.*, 2008; Frada *et al.*, 2012). Haploid *E. huxleyi* cells tend to occupy predominantly surface waters (Baumann *et al.*, 2008), whereas diploid calcifying cells are thought to accelerate sinking by overproducing coccoliths (Monteiro *et al.*, 2016). For these reasons, and because of the ecological importance of calcifying cells in terms of the carbon cycle (Ziveri *et al.*, 2000, 2007), Chapters 4 and 5 focused on the interaction between *P. inhibens* and the calcifying cell type of *E. huxleyi*.

6.2.2. Calcifying bloom-forming *E. huxleyi* cells and AL-PCD

The overarching goal of this thesis was to mechanistically elucidate the microbial interaction between a roseobacter and its calcifying algal host. This goal meant that it was imperative that the chosen calcifying algal strain was capable of growing axenically (without bacteria). Several marine algae require bacteria to survive in culture (Bolch *et al.*, 2011), but fortunately calcifying *E. huxleyi* strain CCMP3266 can remain physiologically and morphologically stable in prolonged culture without bacteria (Chapter 2). This limitation makes it important to consider how widespread the findings of potential roseobacter induced AL-PCD might be in a mixed bloom scenario with various strains and morphotypes of *E. huxleyi*.

E. huxleyi is a species complex with a pan genome containing a set of core genes and a set of auxiliary genes (that appear to be unique or shared by only a limited number of strains) (Read *et al.*, 2013). Evaluating the core genome of *E. huxleyi* is one way of assessing how widespread the genes required for AL-PCD and/or autophagy might be throughout the *E. huxleyi* species complex. In terms of AL-PCD, the genes required for the process are not always known, but metacaspases (which have been associated with some caspase-like activities (Bidle *et al.*, 2012)) are abundant in *E. huxleyi*. *E. huxleyi* CCMP1516 (the polymicrobial parent culture of CCMP2090), has nine metacaspase genes in its genome (Choi *et al.*, 2013). However, these genes appear to be less uniformly distributed as only three of these metacaspases are identifiable in the core genome of *E. huxleyi* (Read *et al.*, 2013). Presently, it remains unclear if *E. huxleyi* relies predominantly on metacaspases or on other caspase-like proteases, discussed in Chapter 4, to produce the caspase-like cleavage that is associated with AL-PCD. In the future, it would be beneficial if metacaspase activity was identified and/or inhibited by metacaspase-specific probes, of which there are a few (Watanabe *et al.*, 2005), instead of continuing to rely on caspase-specific probes in these studies (Bidle *et al.*, 2007).

In addition to these nine metacaspase genes (Choi *et al.*, 2013), *E. huxleyi* CCMP1516 has 13 conserved autophagy related genes (ATG) and three ubiquitin-like regulators of apoptosis (Dep. Energy JGI, 2014). Autophagy genes appear to be widespread throughout *E. huxleyi* strains (Read *et al.*, 2013); for instance, the calcifying strain used in the current study (CCMP3266) has a complete set of all 13 ATG genes, as well as an additional eight related autophagy genes that are required for autophagosome biogenesis (Shemi *et al.*, 2015). The presence of genes for both PCD pathways (autophagy and apoptosis-like) in the *E. huxleyi* genome highlights the possibility that these pathways are tightly intertwined in *E. huxleyi*, the way they are in higher plants and mammals (Jäättelä, 2017). This finding poses an interesting question regarding the likelihood that autophagy and AL-PCD might be occurring throughout mixed populations of *E. huxleyi*. This initial genetic comparison is suggestive that perhaps autophagy is more likely to occur than AL-PCD, which might require more than three of the nine metacaspases to initiate or complete the death process. However, AL-PCD appears to rely on promiscuous caspase-like proteases in addition to metacaspases, which might suggest that AL-PCD could be more widespread throughout *E. huxleyi* populations than expected. In short, more research is certainly needed to evaluate how likely it is that mixed *E. huxleyi* populations might succumb to either autophagy or AL-PCD.

6.2.3. Other identified roseobacter pathogens

When considering the scope of the findings presented here, it is also important to evaluate how common it might be for roseobacters to be able to kill calcifying *E. huxleyi*. To date, six roseobacters have been tested in co-culture with calcifying *E. huxleyi* cells and five of them are capable of prematurely killing this microalga (Chapter 3, Appendix E-1, Mayers *et al.*, 2016). Of these five roseobacter pathogens, three of them induce rapid (<24 hr) population-wide death of *E. huxleyi* cultures at 18 ºC: *P. inhibens* DSM17395, *Leisingera* ANG-1, and *Ruegeria* R11 (Chapter 5, Appendix E-1, Mayers *et al.*, 2016). Interestingly, although this thesis has shown that the T4SS is required for *P. inhibens* pathogenesis of the calcifying microalga *E. huxleyi*, this secretion system is only present in two of the known roseobacter pathogens of *E. huxleyi*: *P. inhibens* DSM17395 and *Silicibacter* sp. TM1040 (Slightom *et al.*, 2009) (Chapter 5, Appendix E-1). In contrast, T4SS genes are generally lacking from the *Leisingera* genus, which instead have a functional T6SS (Collins *et al.*, 2015). This finding is perplexing, as it means that these two pathogens of *E. huxleyi* appear to both kill the alga in a similar rapid manner (<24 h), but

likely have distinct mechanisms of pathogenesis. While *P. inhibens* induces T4SS-mediated death, *Leisingera* ANG-1 might rely on either T6SS, twin-arginine transport system (Tat), Sec (Collins *et al.*, 2011), or use an entirely unrelated mechanism to kill the calcifying alga. Alternatively, two of the other known bacterial pathogens of *E. huxleyi*, both from the *Silicibacter* genus (*Silicibacter* sp. TM1040 and *S. pomeroyi* DSM15171 (=DSS3)), induce a distinctly different type of algal death. These *Silicibacter* pathogens both kill *E. huxleyi* at much slower rates than *P. inhibens* DSM17395 does (Appendix E-1). While *Silicibacter* sp. TM1040 contains a functional, but uncharacterized T4SS, *S. pomeroyi* DSM15171 does not (Slightom *et al.*, 2009). It is possible that these other roseobacter pathogens kill *E. huxleyi* without inducing any PCD process in the alga. This thesis has provided the framework for rapidly assessing the algal death process by identifying the AL-PCD markers established in Chapter 4 (e.g., caspase-like activity, ROS, pan-caspase inhibition, etc.), thus providing the framework to rapidly identify the algal mode of death. Alternatively, it would be interesting to use biochemical inhibition of autophagy to identify if any of the slower *E. huxleyi* death processes (perhaps induced by either of the *Silicibacter* pathogens) is an autophagy-PCD process, rather than the typically more rapid AL-PCD process.

In conclusion, these findings have demonstrated that there are several roseobacters capable of killing calcifying *E. huxleyi*, using a variety of potential pathogenic strategies to do so. This demonstrates that not only are roseobacter pathogens of *E. huxleyi* readily identifiable throughout the clade, but also that there appears to be several distinct successful mechanisms of killing this microalga. This diverse array of successful pathogenic attacks on *E. huxleyi* highlights just how much more there is to learn about bacterial pathogens of *E. huxleyi* in order to begin to appreciate their importance.

6.3. Potential EhV-infected versus *P. inhibens*-infected *E. huxleyi* populations

There is still much that we do not understand about the potential for algicidal bacteria to successfully kill phytoplankton in the marine ecosystem. To date, bacterial pathogens have not been linked to killing phytoplankton in the ocean, let alone to decimating natural algal populations. However, several bacteria have been isolated from algal bloom environments and later found to produce algicides that kill algae (Mayali *et al.*, 2004). In some instances, these bioactive algicides are secreted into the extracellular milieu (where it is possible to suggest that they might interact with several algal species) (Mayali *et al.*, 2004; Demuez *et al.*, 2015).

Conversely, others rely on a contact-dependent mechanism, which might result in selective killing of specific strains or even cell types, as demonstrated in this work (Chapter 3 and 5).

Five roseobacter pathogens, with range distributions that overlap with *E. huxleyi* distributions have now been shown to kill populations of this microalga in the laboratory setting (Chapter 5, Appendix E-1, Mayers, *et al.*, 2016). The next step, as Mayali and Azam stated in 2004, is to demonstrate that bacterial pathogens kill algal hosts in the ocean. Achieving this goal will require more extensive sampling efforts that are aimed at field-based identification of both the eukaryotic consortia and the associated bacterial consortia. This is the direction the field must move toward in order to demonstrate that bacterial pathogens can kill phytoplankton in the ocean.

6.3.1. EhV-infected *E. huxleyi* populations

Emiliania huxleyi populations recurrently bloom in marine waters (Houdan *et al.*, 2006), with some preference for colder more oligotrophic oceans (Baumann *et al.*, 2008). In brief, EhVs infect *E. huxleyi*, inducing either a PCD process or autophagy (Bidle *et al.*, 2007; Schatz *et al.*, 2014), and lyse both diploid strains of *E. huxleyi* (Bratbak *et al.*, 1993; Brussaard *et al.*, 1996). This infection process has been tracked in mesocosms experiments and shown to skew the algal population from diploid dominated to only haploid cells remaining (Vardi *et al.*, 2012) (Figure 6.2). Traditionally, viral directed lysis of algae is predicted to increase the release of algal DOM, thereby stimulating the microbial loop (Azam, 1998). However, if viruses activate algal autophagy pathways, instead of killing via viral lysis alone, the process of autophagy could potentially decrease the release of algal nutrients into the DOM pool (Bidle, 2015). Furthermore, it is presently unknown how increasing marine temperatures might influence potential viral lysis of *E. huxleyi* and release of DOM into the microbial loop.

Over the last century, ocean temperatures increased by roughly 0.67 ºC (IPCC, 2007). It has been predicted that by the end of the 21st century, ocean waters will have globally warmed by anywhere from 1.1-6.4 ºC (IPCC, 2007). Importantly, if ocean temperatures continue to rise, the threat viral pathogens have on *E. huxleyi* might begin to decline. An increase in temperature from 18 ºC to 21 ºC causes resistance to viral infection in a representative non-calcifying diploid *E. huxleyi* strain (Kendrick *et al.*, 2014). This finding means that viral pathogenesis of *E. huxleyi* blooms might be on the decline in some warmer oceans. In fact, loss of EhV signatures during *E. huxleyi* dominated blooms is already occurring in warmer low-latitude oceans, while the viral

signatures remain in high-latitude coastal waters (von Dassow *et al.*, 2014). The diminished pathogenicity of EhVs, coupled with their shrinking distribution, might have major implications for the longevity of future *E. huxleyi* blooms. Alternatively, the potential loss of EhVs as a major pathogen might imply that other pathogens, like the roseobacter pathogens discussed here (Chapter 4, Appendix E-1), could become increasingly important.

6.3.2. *P. inhibens*-infected *E. huxleyi* populations

Ruegeria R11 and *P. inhibens* both kill calcifying diploid and haploid non-calcifying *E. huxleyi* cells, while the non-calcifying diploid CCMP2090 escapes (Chapter 3, Mayers *et al.* 2016). This type of pathogenic interaction might shift natural populations in a way that favors the non-calcifying diploid strains, which appear to be widely resistant to bacterial pathogens (Chapter 3, Figure 6.2). However, it is important to note here, that haploid cells represent a small fraction of a natural bloom, and have several advantages over calcifying cells, making escape from bacterial pathogens possible in the marine environment. For instance, haploid *E. huxleyi* are motile cells with far lower DMSP concentrations than calcified cells (Brady Olson *et al.*, 2017), which might provide some means of escape from motile roseobacters chemo-taxing toward waters with the highest DMSP concentrations (Miller, Hnilicka, *et al.*, 2004). Additionally, some *E. huxleyi* strains can ingest bacteria (Rokitta *et al.*, 2011), so in the marine environment it is possible this ability might act to protect from occasional bacterial pathogens, as well.

It is interesting to speculate about the potential difference in the amount of dissolved inorganic carbon (DIC) burial that might occur during viral-induced death versus *P. inhibens*-induced death of calcifying *E. huxleyi*. Viral-induced death of calcifying cells is associated with the shedding of coccoliths throughout the algal population (Bidle *et al.*, 2007; Frada *et al.*, 2008). In contrast, *P. inhibens* enhances coccolith-cover of calcifying cells (Chapter 5), which might further increase the amount of DIC that is buried when cells die (Figure 6.2). Deposition of coccolithophore calcite to the sea floor is notably enhanced in highly calcified algae, while free coccoliths shed from cells are less likely to reach the sediments (Broerse *et al.*, 2000). Together these findings are suggestive that, when compared to the EhV-infection, loss of coccoliths, and subsequent lysis of algae, bacterial induced algal AL-PCD might enhance the long-term burial of DIC (Figure 6.2). These findings highlight the importance of studying these interactions, and the ways they might alter sedimentation rates in the future.

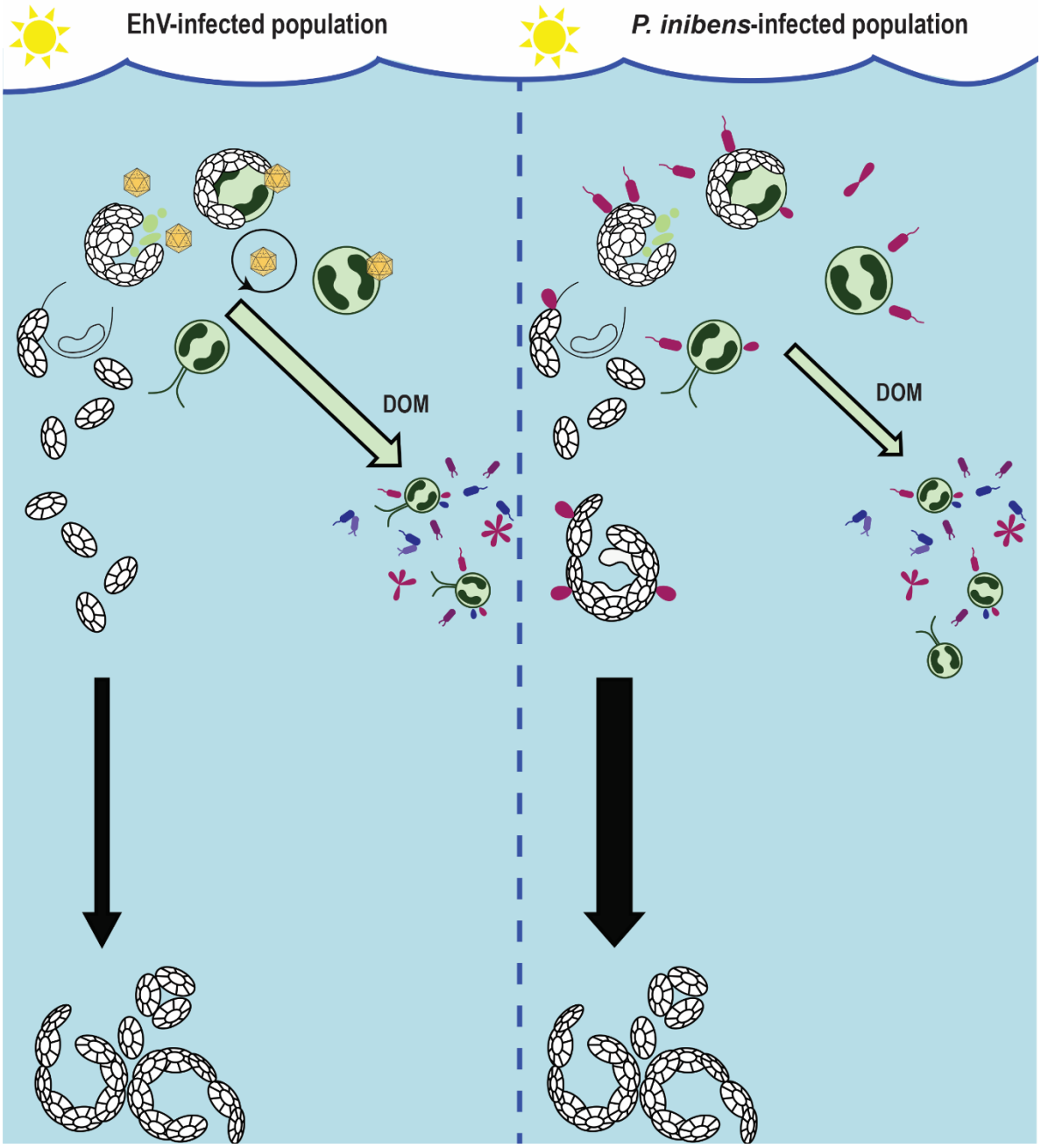

Figure 6.2. **Pictorial representation of possible outcome of EhV-infected *E. huxleyi* populations (left) and *P. inhibens*-infected *E. huxleyi* populations (right)**. EhV infection, associated with viral lysis and redistribution of DOM into the upper ocean would likely increase passage of nutrients to the microbial loop. EhV infection is also associated with increase coccolith-shedding, which might mean less burial of DIC than might be expected from *P. inhibens*-infected *E. huxleyi* populations (right). *P. inhibens*-infected algae have enhanced coccolith-cover (Chapter 5), which might further enhance DIC burial (Chapter 4).

6.4. An updated view of *P. inhibens—E. huxleyi* interactions

In the introduction of this thesis, I presented a model demonstrating the current understanding of *P. inhibens—E. huxleyi* interactions (Figure 1.4). Throughout my thesis I expanded on this model and elucidated some of the complex exchanges occurring between *P. inhibens* and its microalgal host (Figure 6.3). Now I will present an updated view of the interactions occurring between *P. inhibens* and the three cell types of *E. huxleyi* (calcifying diploid, motile haploid, and non-calcifying diploid), and highlight areas requiring further investigation.

6.4.1. Calcifying *E. huxleyi* cells undergo *P. inhibens* induced AL-PCD

P. inhibens was initially chosen for this research because it is known to make a variety of bioactive and even algicidal molecules (i.e. roseobacticides, roseochelins, and the plant hormone indole-3-acetic acid (IAA)), which have all been postulated to enhance pathogenic interactions with *E. huxleyi* (Seyedsayamdost *et al.*, 2011; Segev *et al.*, 2016; Wang *et al.*, 2017). Given that both the pathogenic and non-pathogenic *P. inhibens* strains used in Chapter 5 are capable of producing detectable amounts of algicidal roseochelins (Wang *et al.*, 2017), we reasoned that these molecules are not killing calcifying *E. huxleyi*, nor are they likely secreted via the T4SS (Chapter 5). However, the suggestion that IAA might be the potential killing molecule is more difficult to rule out.

Future work should evaluate whether the non-pathogenic strain *P. inhibens* DSM24588 is able to produce IAA, as a way of further clarifying if IAA is indeed the bioactive killing *E. huxleyi*. One way of investigating this still further would be to determine if the seven avirulent *P. inhibens* mutants in the VirB/D operons of the T4SS produce IAA in response to exogenous tryptophan. If the seven avirulent mutants produce and release as much IAA in response to tryptophan as the wild type *P. inhibens* DSM17395 does, then it is likely that either: 1) IAA is not the molecule responsible for killing *E. huxleyi* or 2) there are multiple ways to release IAA from the bacterial cell. However, it has already been established in plant systems that protonated IAA passively diffuse across cellular membranes (Zazímalová *et al.*, 2010), suggesting that this molecule is likely not a T4SS effector, which would rule it out as the killing molecule.

Narrowing down a possible T4SS effector protein is a difficult task, particularly as some pathogens can excrete a plethora of different T4SS effectors. For instance, the pathogen

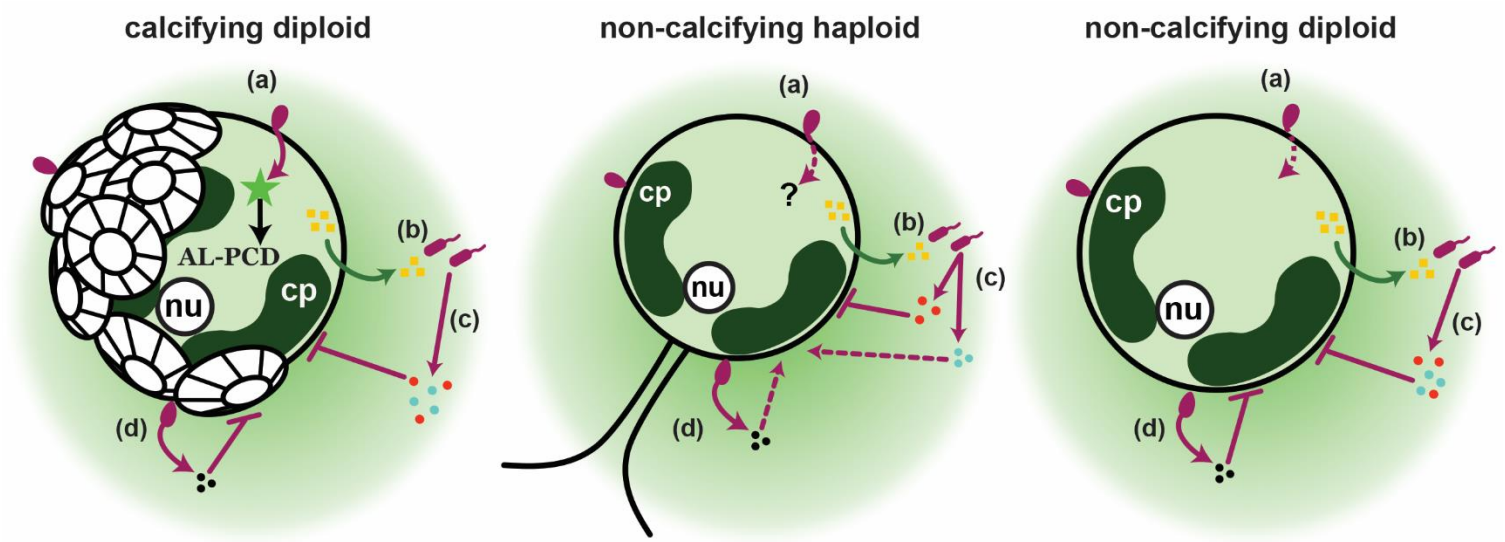

Figure 6.3.**Updated model demonstrating the current understanding of *P. inhibens*—*E. huxleyi* interactions.** Califying diploid CCMP3266: (a) *P. inhibens* injects T4SS effector(s) which induces the activation of caspase-like molecules (green star), inducing AL-PCD of the calcifying strain. (b) *P. inhibens* responds to *p*CA to produces algicidal roseobacticides (red) and roseochelins (blue), neither of which kill CCMP3266.
Haploid flagellated CCMP3268: (a) *P. inhibens* might inject T4SS effector(s) or the bacteria might release a variety of known bioactives that could also induce death, including: RTX toxins, roseochelins (blue), or IAA (black). (b) *P. inhibens* responds to *p*CA to produces algicidal roseobacticides (red), which do not kill CCMP3268.
Non-calcifying diploid CCMP2090: does not die when co-cultured with *P. inhibens*, (a) the bacteria might inject T4SS effector(s) that are either degraded in autophagy vesicles or perhaps unable to induce AL-PCD. Or the alga might impeded bacterial T4SS assembly and/or effectors. (b) *P. inhibens* responds to *p*CA to produces algicidal roseobacticides (red) and produces (c) roseochelins (blue), neither of which kill CCMP2090. (d) IAA is also produced and does not kill the host (black).

Legionella pneumophila excretes over 300 unique T4SS effectors into its eukaryotic host (Rolando *et al.*, 2014). However, by considering all the avirulent T4SS mutants, I presented a candidate effector that might interact with algal AL-PCD pathways to trigger algal population collapse of the calcifying *E. huxleyi* strain CCMP3266 (Chapter 5). Alternatively, AL-PCD might be induced by another (as of yet undetermined) T4SS effector or possibility a complex combination of several T4SS effectors. To confirm that the candidate effector presented in Chapter 5 is one of the T4SS effectors of interest in this interaction, future work should attempt to demonstrate its involvement by generating deletion mutations in each of the genes in the *virU* operon, which might demonstrate a loss of pathogenesis without disrupting the function of the T4SS. Additionally, T4SS secreted proteins are commonly associated with a positively charged C-terminal translocation sequence tag (Vergunst, 2000; Vergunst *et al.*, 2005), which has helped researchers accurately identify candidate T4SS secreted proteins. So, identifying a similar sequence tag on the candidate effectors from the *virU* operon might further strengthen the argument for these as potential effectors. Finally, as we expect this molecule to be a T4SS secreted protein, it should be identifiable by comparing the exoproteomes of the wildtype (which has already been successful for this strain (Durighello *et al.*, 2014; Wienhausen *et al.*, 2017)) and the various avirulent mutant strains (Chapter 5). Next, it would be interesting to use the findings from Chapters 4 and 5 to investigate the interactions occurring between *P. inhibens* and the other sensitive cell type: the haploid flagellated *E. huxleyi*.

6.4.2. Haploid *E. huxleyi* cells are rapidly killed by *P. inhibens*

P. inhibens might utilize T4SS to kill CCMP3268 in a manner similar to the mechanism employed by *P. inhibens* to kill the diploid calcifying CCMP3226 (Chapters 4 and 5). It would be interesting to further investigate the *P. inhibens* DSM17395 T4SS mutants utilized in Chapter 5, to understand if these mutants are able to kill haploid flagellated *E. huxleyi* strain CCMP3268. If that is the case, then the pathogen has two distinct mechanisms for killing these two cell types of *E. huxleyi*. In this case it might be that some other, as of yet undetermined, bioactive molecule either diffuses through the bacterial membrane or is secreted out one of the other secretion systems such as T1SS or T2SS (Thole *et al.*, 2012). I will therefore briefly suggest possible non-T4SS mediated mechanisms of killing the haploid cell type, including: production of repeats-in-toxins (RTX), boactive roseochelins, or hypothetically IAA.

6.4.2.1. *P. inhibens releases RTX-toxins*

Indirect pathogenesis relies on secretion of proteins from the bacterial pathogen into the extracellular environment, where the transported proteins initially interact with the cellular wall or outer membrane of the target cell. T1SS are periplasm-spanning secretion channels, dedicated to the transport of polypeptide cargo, such as gene-encoded antimicrobial peptides or larger proteins with RTX motifs (Chang *et al.*, 2014). T1SS effectors function to increase bacterial biofilm formation or binding to target plant cells (Perez-Mendoza *et al.*, 2011). T1SS effectors can also function to rapidly degrade the plant cell wall, as in the example of *Dickeya dadantii*, which uses T1SS to secrete RTX-proteases, causing soft-rot (Chang *et al.*, 2014).

P. inhibens DSM17395 is known to secrete several bioactive small molecules, some of which have already been implicated in possible pathogenesis. For instance, 60% of the exoproteome of *P. inhibens* DSM17395 was found to be made of a novel RTX-toxin, which is related to a hemolysin-type calcium binding protein (Durighello *et al.*, 2014). Another roseobacter, *Ruegeria* R11, kills both calcifying and haploid *E. huxleyi* slowly (14 d and 12 d, respectively) at 18 ºC (Mayers *et al.*, 2016). However, when temperatures were increased to 25 ºC, the pathogen killed both cell types more rapidly (4 d) (Mayers *et al.*, 2016). At temperatures above 24 ºC, *Ruegeria* R11 excretes over 207 proteins into the extracellular milieu, including two potentially virulent RTX-like proteins (Gardiner *et al.*, 2017). It is therefore tempting to suggest that *Ruegeria* R11 kills *E. huxleyi* in a contact-independent manner, which makes sense, given that it lacks both the T4SS and the T6SS (Collins *et al.*, 2015). However, more research is needed to confirm that either of these roseobacters might utilize T1SS to translocate RTX-toxins that kill haploid *E. huxleyi.*

6.4.2.2. *Bioactive roseobacticides, roseochelins, and/or IAA*

This work demonstrates that roseobacticides are not the effectors causing death of calcified or haploid flagellated *E. huxleyi* strains (CCMP3266 and CCMP3268, respectively) (Chapter 3). However, roseochelins and/or IAA might be involved in killing the haploid strain (CCMP3268) in a potentially non-T4SS dependent manner. In short, more research is required to ascertain how haploid CCMP3268 is being killed by the pathogen *P. inhibens*.

6.4.3. How autophagy might protect CCMP2090 from *P. inhibens* pathogenesis

Although this work has focused on *E. huxleyi* strains sensitive to *P. inhibens*, it is very interesting that several tested non-calcifying strains escaped pathogenesis (Chapter 3, Figure 7-

3). When considering why CCMP2090 and other non-calcifying strains escape this bacterial pathogen, it is important to recall that they are genetically different from the tested calcifying and haploid strains (CCMP3266 and CCMP3268, respectively) used in this study. It would therefore be interesting to isolate non-calcifying diploid cells originating from a CCMP3266 culture and identify if they are sensitive or resistant to *P. inhibens*. This work (Chapters 2,3,4,5) never identified a persister population of non-calcifying diploid cells from the CCMP3266 co-cultures with *P. inhibens*. This suggests that either 1) non-calcifying diploid cells are not commonly generated from CCMP3266 or 2) non-calcifying cells are generated, but are sensitive to the bacterial pathogen and die along with the calcifying cells.

When comparing CCMP2090 to the calcifying sensitive strain, it is necessary to consider the potential genetic differences that might influence this bacterial-algal interaction. For instance, viral hijacking of host alga's autophagy pathway has already been identified for non-calcifying diploid *E. huxleyi* strain CCMP2090 when infected by EhVs (Schatz *et al.*, 2014). Autophagy has a protective role in plants, since lysosomes can degrade bacterial virulence factors (Cemma *et al.*, 2012), which might be an algal defense mechanism of interest in regard to microalga-bacterial interactions. Additionally, autophagy can directly regulate eukaryotic ubiquitination processes, which might be an alternative way CCMP2090 might escape *P. inhibens* induced apoptosis. Conversely, it is possible that this non-calcifying algal strain lacks some required metacaspase or caspase-like genes that are integral to the process of apoptosis, thereby inferring resistance to the roseobacter pathogen. The same diploid non-calcifying *E. huxleyi* cell type (CCMP2090) that was recently shown to be resistant to roseobacter pathogens *Ruegeria* R11 and *P. inhibens* (Chapter 3) (Mayers *et al.*, 2016), was also recently shown to die via autophagy when infected with the *E. huxleyi* virus (EhV201) (Schatz *et al.*, 2014). This might suggest that the autophagy response of CCMP2090 could play a protective role by degrading possible bacterial virulence factors.

6.5. Conclusion

This thesis presents the first evidence of bacterial induction of AL-PCD in a marine microalga. This cross-kingdom induction of AL-PCD in a calcifying microalga population has interesting implications for future studies of bacterial-algal pathogenesis in the marine ecosystem. If marine roseobacters have the widespread ability to promote rapid death of calcifying *E. huxleyi* cells, these bacteria might play an important role in increasing potential

draw down and burial of DIC during death events. Improving this potential still further is the finding that certain roseobacter strains enhance the calcification of *E. huxleyi* cells. This thesis also presents the first evidence of bacterial T4SS-directed pathogenesis of calcifying *E. huxleyi*. This complex interaction, involving a potential T4SS effector that might interact directly with the algal proteasome degradation pathway and/or AL-PCD, highlights how much there is left to learn about algal-bacterial interactions in the marine ecosystems. Marine bacteria are not the silent machines they were once assumed to be, they are complex microorganisms, capable of interacting with their algal hosts in multifarious ways that are only now beginning to be understood.

6.6. Works Cited

Azam, F. (1998) 'Microbial control of oceanic carbon flux: the plot thickens', *Science*, (280), pp. 694–696.

Baumann, K., Boeckel, B. and Čepek, M. (2008) 'Spatial distribution of living coccolithophores along an East-West transect in the subtropical South Atlantic', *Journal of Nannoplankton Research*, 30(1), pp. 9–21.

Bidle, K. D. (2015) 'The molecular ecophysiology of Programmed Cell Death in marine phytoplankton', *Annual Review of Marine Science*, 7, p. 24.1–24.35. doi: 10.1146/annurev-marine-010213-135014.

Bidle, K. D., Haramaty, L., Barcelos E Ramos, J. and Falkowski, P. (2007) 'Viral activation and recruitment of metacaspases in the unicellular coccolithophore, *Emiliania huxleyi*', *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), pp. 6049–6054. doi: 10.1073/pnas.0701240104.

Bidle, K. D. and Kwityn, C. J. (2012) 'Assessing The role of caspase activity and metacaspase expression on viral susceptibility of the coccolithophore, *Emiliania huxleyi* (Haptophyta).', *Journal of Phycology*, 48(5), pp. 1079–1089. doi: 10.1111/j.1529-8817.2012.01209.x.

Bolch, C. J. S., Subramanian, T. A. and Green, D. H. (2011) 'The toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae) requires marine bacteria for growth', *Journal of Phycology*. doi: 10.1111/j.1529-8817.2011.01043.x.

Brady Olson, M., Wuori, T. A., Love, B. A. and Strom, S. L. (2017) 'Ocean acidification effects on haploid and diploid *Emiliania huxleyi* strains: Why changes in cell size matter', *Journal of Experimental Marine Biology and Ecology*. Elsevier B.V., 488, pp. 72–82. doi: 10.1016/j.jembe.2016.12.008.

Bratbak, G., Egge, J. K. and Heldal, M. (1993) 'Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms', *Marine Ecology Progress Series*, 93(1–2), pp. 39–48. doi: 10.3354/meps093039.

Broerse, A. T. ., Ziveri, P., van Hinte, J. E. and Honjo, S. (2000) 'Coccolithophore export production, species composition, and coccolith-$CaCO_3$ fluxes in the NE Atlantic (34°N21°W And 48°N21°W)', *Deep Sea Research Part II: Topical Studies in Oceanography*. doi: 10.1016/S0967-0645(00)00010-2.

Brussaard, C. P. D., Kempers, R. S., Kop, A. J., Riegman, R. and Heldal, M. (1996) 'Virus-like Particles in a summer bloom of *Emiliania huxleyi* in the North Sea', *Aquatic Microbial Ecology*, 10, pp. 105–113.

Cemma, M. and Brumell, J. H. (2012) 'Interactions Of pathogenic bacteria with autophagy systems', *Current biology : CB*. Elsevier, 22(13), pp. R540-5. doi: 10.1016/j.cub.2012.06.001.

Chang, J. H., Desveaux, D. and Creason, A. L. (2014) 'The ABCs and 123s of bacterial secretion systems in plant pathogenesis', *Annual Review of Phytopathology*. doi: 10.1146/annurev-phyto-011014-015624.

Choi, C. J. and Berges, J. a (2013) 'New types of metacaspases in phytoplankton reveal diverse origins of cell death proteases.', *Cell death & disease*. Nature Publishing Group, 4(2), p. e490. doi: 10.1038/cddis.2013.21.

Collins, A. J., Fullmer, M. S., Gogarten, J. P. and Nyholm, S. V. (2015) 'Comparative genomics of roseobacter clade bacteria isolated from the accessory nidamental gland of *Euprymna scolopes*', *Frontiers in Microbiology*, 6(FEB), pp. 1–14. doi: 10.3389/fmicb.2015.00123.

Collins, A. J. and Nyholm, S. V. (2011) 'Draft genome of *Phaeobacter gallaeciensis* ANG1, a dominant member of the accessory nidamental gland of *Euprymna scolopes*', *Journal of Bacteriology*, 193(13), pp. 3397–3398. doi: 10.1128/JB.05139-11.

von Dassow, P., John, U., Ogata, H., Probert, I., Bendif, E. M., Kegel, J. U., Audic, S., Wincker, P., Da Silva, C., Claverie, J.-M., *et al.* (2014) 'Life-cycle modification in open oceans accounts for genome variability in a cosmopolitan phytoplankton', *The ISME Journal*, 9(6), pp. 1–13. doi: 10.1038/ismej.2014.221.

Demuez, M., Gonzalez-Fernandez, C. and Ballesteros, M. (2015) 'Algicidal microorganisms and secreted algicides: new tools to induce microalgal cell disruption', *Biotechnology Advances*. Elsevier Inc., 33(8), pp. 1615–1625. doi: 10.1016/j.biotechadv.2015.08.003.

Dep. Energy JGI (2014) '*Emiliania huxleyi* CCMP1516 main genome assembly V1.0. genome', *Portal. http://genome.jgi-psf.org/Emihu1/Emihu1.home.html Dep.*

Durighello, E., Christie-Oleza, J. A. and Armengaud, J. (2014) 'Assessing the exoproteome of marine bacteria, lesson from a RTX-toxin abundantly secreted by *Phaeobacter* strain DSM17395', *PLoS ONE*, 9(2). doi: 10.1371/journal.pone.0089691.

Frada, M. J., Bidle, K. D., Probert, I. and de Vargas, C. (2012) '*In situ* survey of life cycle phases of the coccolithophore *Emiliania huxleyi* (Haptophyta)', *Environmental Microbiology*, 14(6), pp. 1558–1569. doi: 10.1111/j.1462-2920.2012.02745.x.

Frada, M., Probert, I., Allen, M. J., Wilson, W. H. and de Vargas, C. (2008) 'The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(41), pp. 15944–15949. doi: 10.1073/pnas.0807707105.

Gardiner, M., Bournazos, A. M., Maturana-Martinez, C., Zhong, L. and Egan, S. (2017) 'Exoproteome analysis of the seaweed pathogen *Nautella italica* R11 reveals temperature-dependent regulation of RTX-like proteins', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.01203.

Houdan, A., Probert, I., Zatylny, C., Véron, B. and Billard, C. (2006) 'Ecology of oceanic coccolithophores. I. nutritional preferences of the two stages in the life cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*', *Aquatic Microbial Ecology*, 44(3), pp. 291–301. doi: 10.3354/ame044291.

IPCC (2007) 'Climate Change 2007: the physical science basis', *Intergovernmental Panel on Climate Change*, 446(7137), pp. 727–8. doi: 10.1038/446727a.

Jäättelä, M. (2017) 'Programmed Cell Death: Many ways for cells to die decently', 3890(July). doi: 10.1080/078538902321012423.

Kendrick, B. J., DiTullio, G. R., Cyronak, T. J., Fulton, J. M., Van Mooy, B. a. S. and Bidle, K. D. (2014) 'Temperature-induced viral resistance in *Emiliania huxleyi* (Prymnesiophyceae)', *PLoS ONE*, 9(11), p. e112134. doi: 10.1371/journal.pone.0112134.

Mayali, X. and Azam, F. (2004) 'Algicidal bacteria in the sea and their impact on algal blooms', *Journal of Eukaryotic Microbiology*, 51(2), pp. 139–144. doi: 10.1111/j.1550-7408.2004.tb00538.x.

Mayers, T., Bramucci, A., Yakimovich, K. and Case, R. (2016) 'A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*', *Frontiers in Microbiology*, 7(JUN). doi: 10.3389/fmicb.2016.00892.

Miller, T. R., Hnilicka, K., Dziedzic, A., Desplats, P. and Belas, R. (2004) 'Chemotaxis of *Silicibacter* sp. strain TM1040 toward dinoflagellate products', *Society*, 70(8), pp. 4692–4701. doi: 10.1128/AEM.70.8.4692.

Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P., Rickaby, R. E. M., Poulton, A. J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., Gibbs, S., *et al.* (2016) 'Why marine phytoplankton calcify', *Science Advances*, 2(7), pp. e1501822–e1501822. doi: 10.1126/sciadv.1501822.

Perez-Mendoza, D., Coulthurst, S. J., Humphris, S., Campbell, E., Welch, M., Toth, I. K. and Salmond, G. P. C. (2011) 'A multi-repeat adhesin of the phytopathogen, *Pectobacterium atrosepticum*, is secreted by a type I pathway and is subject to complex regulation involving a non-canonical diguanylate cyclase', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2011.07849.x.

Read, B., Kegel, J., Klute, M., Kuo, A., Lefebvre, S., Maumus, F., Mayer, C., Miller, J., Monier, A., Salamov, A., *et al.* (2013) 'Pan genome of the phytoplankton *Emiliania* underpins its global distribution', *Nature*, 499(7457), pp. 209–13. doi: 10.1038/nature12221.

Rokitta, S. D., de Nooijer, L. J., Trimborn, S., de Vargas, C., Rost, B. and John, U. (2011) 'Transcriptome analyses reveal differential gene expression patterns between the life-cycle stages of *Emiliania huxleyi* (Haptophyta) And Reflect Specialization To Different Ecological Niches', *Journal of Phycology*, 47(4), pp. 829–838. doi: 10.1111/j.1529-8817.2011.01014.x.

Rolando, M. and Buchrieser, C. (2014) '*Legionella pneumophila* Type IV effectors hijack the transcription and translation machinery of the host cell', *Trends in Cell Biology*. doi: 10.1016/j.tcb.2014.06.002.

Schatz, D., Shemi, A., Rosenwasser, S., Sabanay, H., Wolf, S. G., Ben-Dor, S. and Vardi, A. (2014) 'Hijacking of an autophagy-like process is critical for the life cycle of a DNA virus infecting oceanic algal blooms', *New Phytologist*, 204(4), pp. 854–863. doi: 10.1111/nph.13008.

Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N., Paulson, J. N., Chai, L., Clardy, J., *et al.* (2016) 'Dynamic metabolic exchange governs a marine algal-bacterial interaction', *eLife*, 5, p. e17473. doi: 10.7554/eLife.17473.

Seyedsayamdost, M. R., Case, R. J., Kolter, R. and Clardy, J. (2011) 'The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*', *Nature chemistry*, 3(4), pp. 331–5. doi: 10.1038/nchem.1002.

Shemi, A., Ben-dor, S. and Vardi, A. (2015) 'Elucidating the composition and conservation of the autophagy pathway in photosynthetic eukaryotes', (April), pp. 701–715.

Slightom, R. N. and Buchan, A. (2009) 'Surface colonization by marine roseobacters: integrating genotype and phenotype', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01508-09.

Thole, S., Kalhoefer, D., Voget, S., Berger, M., Engelhardt, T., Liesegang, H., Wollherr, A., Kjelleberg, S., Daniel, R., Simon, M., *et al.* (2012) '*Phaeobacter gallaeciensis* genomes from globally opposite locations reveal high similarity of adaptation to surface life', *The ISME Journal*. Nature Publishing Group, 6(12), pp. 2229–2244. doi: 10.1038/ismej.2012.62.

Tyrrell, T. and Merico, A. (2004) '*Emiliania huxleyi*: bloom observations and the conditions that induce them'. doi: 10.1007/978-3-662-06278-4_4.

Vardi, A., Haramaty, L., Van Mooy, B., Fredricks, H. F., Kimmance, S., Larsen, A. and Bidle, K. D. (2012) 'Host-virus dynamics and subcellular controls of cell fate in a natural coccolithophore population', *Proceedings of the National Academy of Sciences of the United States of America*, 109(47), pp. 19327–32. doi: 10.1073/pnas.1208895109.

Vergunst, A. C. (2000) 'VirB/D4- dependent protein translocation from *Agrobacterium* into plant cells', *Science*, 290(5493), pp. 979–982. doi: 10.1126/science.290.5493.979.

Vergunst, A. C., van Lier, M. C. M., den Dulk-Ras, A., Grosse Stuve, T. A., Ouwehand, A. and Hooykaas, P. J. J. (2005) 'Positive charge is an important feature of the c-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0406241102.

Wang, R. and Seyedsayamdost, M. R. (2017) 'Roseochelin B, an algaecidal natural product synthesized by the roseobacter *Phaeobacter inhibens* in response to algal sinapic acid', *Organic Letters*, p. acs.orglett.7b02424. doi: 10.1021/acs.orglett.7b02424.

Watanabe, N. and Lam, E. (2005) 'Two *Arabidopsis* metacaspases AtMCP1b and AtMCP2b are arginine/lysine- specific cysteine proteases and activate apoptosis-like cell death in yeast', *Journal of Biological Chemistry*, 280(15), pp. 14691–14699. doi: 10.1074/jbc.M413527200.

Wienhausen, G., Noriega-Ortega, B. E., Niggemann, J., Dittmar, T. and Simon, M. (2017) 'The exometabolome of two model strains of the roseobacter group: A marketplace of microbial metabolites', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.01985.

Zazímalová, E., Murphy, A. S., Yang, H., Hoyerová, K. and Hosek, P. (2010) 'Auxin transporters--why so many?', *Cold Spring Harbor perspectives in biology*. doi: 10.1101/cshperspect.a001552.

Ziveri, P., de Bernardi, B., Baumann, K. H., Stoll, H. M. and Mortyn, P. G. (2007) 'Sinking of coccolith carbonate and potential contribution to organic carbon ballasting in the deep ocean', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 54(5–7), pp. 659–675. doi: 10.1016/j.dsr2.2007.01.006.

Ziveri, P., Broerse, A. T. C., vanHinte, J. E., Westbroek, P. and Honjo, S. (2000) 'The fate of coccoliths at 48 degrees N 21 degrees W, Northeastern Atlantic', *Deep - Sea Research Part II - Topical Studies in Oceanography*.

References

Abramovitch, R. B., Janjusevic, R., Stebbins, C. E. and Martin, G. B. (2006) 'Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0507892103.

Abramovitch, R. B. and Martin, G. B. (2004) 'Strategies used by bacterial pathogens to suppress plant defenses', *Current Opinion in Plant Biology*, 7(4), pp. 356–364. doi: 10.1016/j.pbi.2004.05.002.

Alvarez-Martinez, C. E. and Christie, P. J. (2009) 'Biological diversity of prokaryotic Type IV Secretion Systems', *Microbiol Mol Biol Rev*, 73(4), pp. 775–808. doi: 10.1128/MMBR.00023-09.

Ambastha, V., Tripathy, B. C. and Tiwari, B. S. (2015) 'Programmed Cell Death in plants: a chloroplastic connection', *Plant Signaling & Behavior*, 10(2), p. e989752. doi: 10.4161/15592324.2014.989752.

Amieva, M. R. and El-Omar, E. M. (2008) 'Host-bacterial interactions in *Helicobacter pylori* infection', *Gastroenterology*. doi: 10.1053/j.gastro.2007.11.009.

Angot, A., Vergunst, A., Genin, S. and Peeters, N. (2007) 'Exploitation of Eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial Type III and Type IV Secretion Systems', *PLoS Pathogens*, 3(1), pp. 0001–0013. doi: 10.1371/journal.ppat.0030003.

Atmakuri, K., Cascales, E. and Christie, P. J. (2004) 'Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial Type IV Secretion', *Molecular Microbiology*, 54(5), pp. 1199–1211. doi: 10.1111/j.1365-2958.2004.04345.x.

Avila-Ospina, L., Moison, M., Yoshimoto, K. and Masclaux-Daubresse, C. (2014) 'Autophagy, plant senescence, and nutrient recycling', *Journal of Experimental Botany*, 65(14), pp. 3799–3811. doi: 10.1093/jxb/eru039.

Azam, F. (1998) 'Microbial control of oceanic carbon flux: the plot thickens', *Science*, (280), pp. 694–696.

Azam, F. and Malfatti, F. (2007) 'Microbial structuring of marine ecosystems', *Nature reviews. Microbiology*, 5(10), pp. 782–791. doi: 10.1038/nrmicro1798.

Bach, L. T., Riebesell, U., Sett, S., Febiri, S., Rzepka, P. and Schulz, K. G. (2012) 'An approach for particle sinking velocity measurements in the 3-400 µm size range and considerations on the effect of temperature on sinking rates', *Marine Biology*, 159(8), pp. 1853–1864. doi: 10.1007/s00227-012-1945-2.

Banga, S., Gao, P., Shen, X., Fiscus, V., Zong, W., Chen, L. and Luo, Z. (2007) '*Legionella pneumophila* inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family', *PNAS (Proceedings of the National Academy of Sciences of the United States of America)*, 104(12), pp. 5121–5126.

Baumann, K., Boeckel, B. and Čepek, M. (2008) 'Spatial distribution of living coccolithophores along an East-West transect in the subtropical South Atlantic', *Journal of Nannoplankton Research*, 30(1), pp. 9–21.

Beer, S., Larsson, C., Poryan, O. and Axelsson, L. (2000) 'Photosynthetic rates of *Ulva* (Chlorophyta) measured by Pulse Amplitude Modulated fluorometry', *European journal of phycology*, 35(1), pp. 69–74. doi: 10.1080/09670260010001735641.

Bell, W. H. (1983) ‘Bacterial utilization of algal extracellular products. 3. The specificity of algal-bacterial interaction’, *Limnology and Oceanography*, 28(6), pp. 1131–1143. doi: 10.4319/lo.1983.28.6.1131.

Bell, W. and Mitchell, R. (1972) ‘Chemotactic and growth responses of marine bacteria to algal extracellular products’, *Marine Biological Laboratory*, 143(2), pp. 265–277.

Belogurov, A. A., Delver, E. P., Agafonova, O. V, Belogurova, N. G., Lee, L. Y. and Kado, C. I. (2000) ‘Antirestriction protein Ard (Type C) encoded by IncW plasmid PSa has a high similarity to the “protein transport” domain of TraC1 primase of promiscuous plasmid RP4’, *J Mol Biol*. doi: 10.1006/jmbi.1999.3493.

Bentzon-Tilia, M. and Gram, L. (2017) ‘Biotechnological applications of the roseobacter clade’, in Paterson, R. and Lima, N. (eds) *Bioprospecting: Success, Potential and Constraints*. Cham: Springer International Publishing, pp. 137–166. doi: 10.1007/978-3-319-47935-4_7.

Berges, J. A. and Falkowski, P. G. (1998) ‘Physiological stress and cell death in marine phytoplankton: induction of proteases in response to nitrogen or light limitation’, *Limnology and Oceanography*, 43(1), pp. 129–135. doi: 10.4319/lo.1998.43.1.0129.

Berman-Frank, I., Bidle, K. D., Haramaty, L. and Falkowski, P. G. (2004) ‘The demise of the marine cyanobacterium, *Trichodesmium* spp., Via An Autocatalyzed Cell Death Pathway’, *Limnology and Oceanography*, 49(4), pp. 997–1005. doi: 10.4319/lo.2004.49.4.0997.

Bethke, P. C. (1999) ‘Hormonally regulated programmed cell death in barley aleurone cells’, *The Plant Cell Online*, 11(6), pp. 1033–1046. doi: 10.1105/tpc.11.6.1033.

Bidle, K. D. (2015) ‘The molecular ecophysiology of Programmed Cell Death in marine phytoplankton’, *Annual Review of Marine Science*, 7, p. 24.1–24.35. doi: 10.1146/annurev-marine-010213-135014.

Bidle, K. D. (2016) ‘Programmed Cell Death in unicellular phytoplankton’, *Current Biology*. Elsevier Ltd, 26(13), pp. R594–R607. doi: 10.1016/j.cub.2016.05.056.

Bidle, K. D. and Bender, S. J. (2008) ‘Iron starvation and culture age activate metacaspases and Programmed Cell Death in the marine diatom *Thalassiosira pseudonana*’, *Eukaryotic Cell*, 7(2), pp. 223–236. doi: 10.1128/EC.00296-07.

Bidle, K. D., Haramaty, L., Barcelos E Ramos, J. and Falkowski, P. (2007) ‘Viral Activation And Recruitment Of Metacaspases In The Unicellular Coccolithophore, *Emiliania huxleyi*’, *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), pp. 6049–6054. doi: 10.1073/pnas.0701240104.

Bidle, K. D. and Kwityn, C. J. (2012) ‘Assessing the role of caspase activity and metacaspase expression on viral susceptibility of the coccolithophore, *Emiliania huxleyi* (Haptophyta).’, *Journal of Phycology*, 48(5), pp. 1079–1089. doi: 10.1111/j.1529-8817.2012.01209.x.

Bidle, K. D. and Vardi, A. (2011) ‘A chemical arms race at sea mediates algal host-virus interactions’, *Current opinion in microbiology*. Elsevier Ltd, 14(4), pp. 449–57. doi: 10.1016/j.mib.2011.07.013.

Blaho, J. (2004) ‘Virus infection and apoptosis (issue II) an introduction: cheating death or death as a fact of life?’, *International reviews of immunology*, 23(1–2), pp. 1–6. doi: 10.1080/0883018049026551.

Blaise, C. and Vasseur, P. (2005) ‘Algal microplate toxicity test’, in Blaise, C. and Férard, J.-F. (eds) *Small-scale Freshwater Toxicity Investigations, Vol. 1: Toxicity Test Methods*. Dordrecht, Netherlands: Springer, p. 137−179.

Bleves, S. and Cornelis, G. R. (2000) ‘How to survive in the host: the *Yersinia* lesson’, *Microbes and Infection*, (2), pp. 1451–1560.

Bolch, C. J. S., Subramanian, T. A. and Green, D. H. (2011) 'The toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae) requires marine bacteria for growth', *Journal of Phycology*.

Borchard, C. and Engel, A. (2012) 'Organic matter exudation by *Emiliania huxleyi* under simulated future ocean conditions', *Biogeosciences*, 9(8), pp. 3405–3423. doi: 10.5194/bg-9-3405-2012.

Bosch, M. and Franklin-Tong, V. E. (2007) 'Temporal and spatial activation of caspase-like enzymes induced by self-incompatibility in papaver pollen', *Proceedings of the National Academy of Sciences of the United States of America*, 104(46), pp. 18327–18332. doi: 10.1073/pnas.0705826104.

Brady Olson, M., Wuori, T. A., Love, B. A. and Strom, S. L. (2017) 'Ocean acidification effects on haploid and diploid *Emiliania huxleyi* strains: Why changes in cell size matter', *Journal of Experimental Marine Biology and Ecology*. Elsevier B.V., 488, pp. 72–82.

Bratbak, G., Egge, J. K. and Heldal, M. (1993) 'Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms', *Marine Ecology Progress Series*, 93(1–2), pp. 39–48. doi: 10.3354/meps093039.

Bravo-Angel, A. M., Hohn, B. and Tinland, B. (1998) 'The omega sequence of VirD2 is important but not essential for efficient transfer of T-DNA by *Agrobacterium tumefaciens*', *Molecular plant-microbe interactions : MPMI*. doi: 10.1094/MPMI.1998.11.1.57.

Brinkhoff, T., Bach, G., Heidorn, T., Liang, L., Schlingloff, A. and Simon, M. (2004) 'Antibiotic production by a roseobacter clade-affiliated species from the German Wadden Sea and its antagonistic effects on indigenous isolates', *Applied and Environmental Microbiology*, 70(4), pp. 2560–2565. doi: 10.1128/AEM.70.4.2560-2565.2003.

Brinkhoff, T., Giebel, H.-A. and Simon, M. (2008) 'Diversity, ecology, and genomics of the roseobacter clade: A short overview', *Archives of microbiology*, 189(6), pp. 531–9. doi: 10.1007/s00203-008-0353-y.

Broerse, A. T. ., Ziveri, P., van Hinte, J. E. and Honjo, S. (2000) 'Coccolithophore export production, species composition, and coccolith-$CaCO_3$ fluxes in the NE Atlantic (34°N21°W And 48°N21°W)', *Deep Sea Research Part II: Topical Studies in Oceanography*. doi: 10.1016/S0967-0645(00)00010-2.

Brown, C. W. and Yoder, J. a. (1994) 'Coccolithophorid blooms in the global ocean', 99(93), pp. 7467–7482. doi: 10.1029/93JC02156.

Brussaard, C. P. D., Kempers, R. S., Kop, A. J., Riegman, R. and Heldal, M. (1996) 'Virus-like particles in a summer bloom of *Emiliania huxleyi* in the North Sea', *Aquatic Microbial Ecology*, 10, pp. 105–113.

Buchan, A., González, J. M., Moran, M. A. and Gonza, M. (2005) 'Overview of the marine roseobacter lineage', *Society*, 71(10). doi: 10.1128/AEM.71.10.5665.

Buchan, A., LeCleir, G. R., Gulvik, C. a and González, J. M. (2014) 'Master Recyclers: features and functions of bacteria associated with phytoplankton blooms', *Nat Rev Microbiol*. Nature Publishing Group, 12(August), pp. 686–698. doi: 10.1038/nrmicro3326.

Buddruhs, N., Chertkov, O., Petersen, J., Fiebig, A., Chen, A., Pati, A., Ivanova, N., Lapidus, A., Goodwin, L. A., Chain, P., *et al.* (2013) 'Complete genome sequence of the marine methyl-halide oxidizing *Leisingera methylohalidivorans* type strain (DSM 14336T), a representative of the roseobacter clade', *Standards in Genomic Sciences*, 9(1), pp. 128–141. doi: 10.4056/sigs.4297965.

Burki, F. (2016) 'The Eukaryotic tree of life from a global phylogenomic perspective', *Cold Spring Harbor perspectives in biology*, pp. 1–18. doi: 10.1101/cshperspect.a016147.
Bursch, W., Ellinger, a, Gerner, C., Fröhwein, U. and Schulte-Hermann, R. (2000) 'Programmed Cell Death (PCD). Apoptosis, Autophagic PCD, or others?', *Annals of the New York Academy of Sciences*, 926, pp. 1–12. doi: 10.1111/j.1749-6632.2000.tb05594.x.
Buttner, D. (2016) 'Behind the lines-actions of bacterial Type III effector proteins in plant cells', *FEMS Microbiology Reviews*, 40(6), pp. 894–937. doi: 10.1093/femsre/fuw026.
Cascales, E. and Christie, P. J. (2003) 'The versatile bacterial Type IV Secretion Systems', *Nature Reviews Microbiology*. doi: 10.1038/nrmicro753.
Case, R. J., Longford, S. R., Campbell, A. H., Low, A., Tujula, N., Steinberg, P. D. and Kjelleberg, S. (2011) 'Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga', *Environmental Microbiology*, 13(2), pp. 529–537. doi: 10.1111/j.1462-2920.2010.02356.x.
Cemma, M. and Brumell, J. H. (2012) 'Interactions of pathogenic bacteria with autophagy systems', *Current biology : CB*. Elsevier, 22(13), pp. R540-5. doi: 10.1016/j.cub.2012.06.001.
Chang, H. Y. and Yang, X. (2000) 'Proteases for cell suicide: Functions and regulation of caspases', *Microbiology and molecular biology reviews : MMBR*, 64(4), pp. 821–46. doi: 10.1128/MMBR.64.4.821-846.2000.
Chang, J. H., Desveaux, D. and Creason, A. L. (2014) 'The ABCs and 123s of bacterial secretion systems in plant pathogenesis', *Annual Review of Phytopathology*. doi: 10.1146/annurev-phyto-011014-015624.
Chen, M., Tang, H., Ma, H., Holland, T. C., Ng, K. Y. S. and Salley, S. O. (2011) 'Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*', *Bioresource technology*. Elsevier Ltd, 102(2), pp. 1649–55. doi: 10.1016/j.biortech.2010.09.062.
Chen, S. and Dickman, M. B. (2004) 'Bcl-2 Family Members Localize To Tobacco Chloroplasts And Inhibit Programmed Cell Death Induced By Chloroplast-targeted Herbicides', *Journal of Experimental Botany*, 55(408), pp. 2617–2623. doi: 10.1093/jxb/erh275.
Choi, C. J. and Berges, J. a (2013) 'New Types Of Metacaspases In Phytoplankton Reveal Diverse Origins Of Cell Death Proteases.', *Cell death & disease*. Nature Publishing Group, 4(2), p. e490. doi: 10.1038/cddis.2013.21.
Chow, J. S., Lee, C. and Engel, A. (2015) 'The Influence Of Extracellular Polysaccharides, Growth Rate, And Free Coccoliths On The Coagulation Efficiency Of *Emiliania huxleyi*', *Marine Chemistry*. Elsevier B.V., 175, pp. 5–17. doi: 10.1016/j.marchem.2015.04.010.
Chowdhury, I., Tharakan, B. and Bhat, G. K. (2008) 'Caspases - An Update', *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 151(1), pp. 10–27. doi: 10.1016/j.cbpb.2008.05.010.
Christie-Oleza, J. A. and Armengaud, J. (2010) 'In-depth Analysis Of Exoproteomes From Marine Bacteria By Shotgun Liquid Chromatography-tandem Mass Spectrometry: The *Ruegeria pomeroyi* DSS-3 Case-study', *Marine Drugs*. doi: 10.3390/md8082223.
Christie-Oleza, J. A., Pina-Villalonga, J. M., Bosch, R., Nogales, B. and Armengaud, J. (2012) 'Comparative Proteogenomics Of Twelve Roseobacter Exoproteomes Reveals Different Adaptive Strategies Among These Marine Bacteria', *Molecular & Cellular Proteomics*. doi: 10.1074/mcp.M111.013110.

Christie, P. J. (2001) 'Type IV Secretion: Intercellular Transfer Of Macromolecules By Systems Ancestrally Related To Conjugation Machines', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2001.02302.x.
Christie, P. J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S. J. and Cascales, E. (2005) 'Biogenesis, Architecture, And Function Of Bacterial Type IV Secretion Systems', *Annual review of microbiology*.
Christie, P. J., Whitaker, N. and González-Rivera, C. (2014) 'Mechanism And Structure Of The Bacterial Type IV Secretion Systems', *Biochimica et Biophysica Acta - Molecular Cell Research*. doi: 10.1016/j.bbamcr.2013.12.019.
Coburn, J. and Leong, J. M. (2000) 'Microbiology. Arresting Features Of Bacterial Toxins.', *Science (New York, N.Y.)*. doi: 10.1126/science.290.5490.287.
Coffeen, W. C. and Wolpert, T. J. (2004) 'Purification And Characterization of Serine Proteases That Exhibit Caspase-Like Activity and are Associated With Programmed Cell Death in *Avena sativa*', *The Plant Cell Online*, 16(4), pp. 857–873. doi: 10.1105/tpc.017947.
Cole, J. J. (1982) 'Interactions Between Bacteria And Algae In Aquatic Ecosystems', *Annual review of ecological systems*, (13), pp. 291–314.
Collins, A. J., Fullmer, M. S., Gogarten, J. P. and Nyholm, S.V. (2015) 'Comparative Genomics Of Roseobacter Clade Bacteria Isolated From The Accessory Nidamental Gland Of *Euprymna scolopes*', *Frontiers in Microbiology*, 6(FEB), pp. 1–14. doi: 10.3389/fmicb.2015.00123.
Collins, A. J. and Nyholm, S. V. (2011) 'Draft Genome Of *Phaeobacter gallaeciensis* ANG1, A Dominant Member Of The Accessory Nidamental Gland Of *Euprymna scolopes*', *Journal of Bacteriology*, 193(13), pp. 3397–3398. doi: 10.1128/JB.05139-11.
Costello, M. J., Bouchet, P., Boxshall, G., Fauchald, K., Gordon, D., Hoeksema, B. W., Poore, G. C. B., van Soest, R. W. M., Stöhr, S., Walter, T. C., *et al.* (2013) 'Global Coordination And Standardisation In Marine Biodiversity Through The World Register Of Marine Species (WoRMS) And Related Databases', *PLoS ONE*, 8(1). doi: 10.1371/journal.pone.0051629.
Croft, M. T., Warren, M. J. and Smith, A. G. (2006) 'Algae Need Their Vitamins', *Eukaryotic Cell*, 5(8), pp. 1175–1183. doi: 10.1128/EC.00097-06.
D'Alvise, P. W., Lillebø, S., Prol-Garcia, M. J., Wergeland, H. I., Nielsen, K. F., Bergh, Ø. and Gram, L. (2012) '*Phaeobacter gallaeciensis* Reduces *Vibrio anguillarum* In Cultures Of Microalgae And Rotifers, And Prevents Vibriosis In Cod Larvae', *PLoS ONE*, 7(8). doi: 10.1371/journal.pone.0043996.
Dang, H. and Lovell, C. R. (2000) 'Bacterial Primary Colonization And Early Succession On Surfaces In Marine Waters As Determined By Amplified RRNA Gene Restriction Analysis And Sequence Analysis Of 16S RRNA Genes', *Applied and Environmental Microbiology*, 66(2), pp. 467–475. doi: 10.1128/AEM.66.2.467-475.2000.
Danon, A., Delorme, V., Mailhac, N. and Gallois, P. (2000) 'Plant Programmed Cell Death: A Common Way To Die', *Plant Physiology and Biochemistry*, 38(9), pp. 647–655. doi: 10.1016/S0981-9428(00)01178-5.
Darzynkiewicz, Z., Halicka, H. D. and Zhao, H. (2010) 'Analysis Of Cellular DNA Content By Flow And Laser Scanning Cytometry', *Advances in Experimental Medicine and Biology*, 675, pp. 137–147. doi: 10.1007/978-1-4419-6199-0_9.
von Dassow, P., John, U., Ogata, H., Probert, I., Bendif, E. M., Kegel, J. U., Audic, S., Wincker, P., Da Silva, C., Claverie, J.-M., *et al.* (2014) 'Life-cycle Modification In Open Oceans

Accounts For Genome Variability In A Cosmopolitan Phytoplankton', *The ISME Journal*, 9(6), pp. 1–13. doi: 10.1038/ismej.2014.221.
von Dassow, P., Ogata, H., Probert, I., Wincker, P., Da Silva, C., Audic, S., Claverie, J.-M. and de Vargas, C. (2009) 'Transcriptome Analysis Of Functional Differentiation Between Haploid And Diploid Cells Of *Emiliania huxleyi*, A Globally Significant Photosynthetic Calcifying Cell.', *Genome biology*, 10(10), p. R114. doi: 10.1186/gb-2009-10-10-r114.
Demeret, C., Garcia-Carranca, A. and Thierry, F. (2003) 'Transcription-independent Triggering Of The Extrinsic Pathway Of Apoptosis By Human Papillomavirus 18 E2 Protein', *Oncogene*, 22(2), pp. 168–175. doi: 10.1038/sj.onc.1206108.
Demuez, M., Gonzalez-Fernandez, C. and Ballesteros, M. (2015) 'Algicidal Microorganisms And Secreted Algicides: New Tools To Induce Microalgal Cell Disruption', *Biotechnology Advances*. Elsevier Inc., 33(8), pp. 1615–1625. doi: 10.1016/j.biotechadv.2015.08.003.
Dep. Energy JGI (2014) '*Emiliania huxleyi* CCMP1516 Main Genome Assembly V1.0. Genome', *Portal. http://genome.jgi-psf.org/Emihu1/Emihu1.home.html Dep.*
Derelle, R., Torruella, G., Klimeš, V., Brinkmann, H., Kim, E., Vlček, Č., Lang, B. F. and Eliáš, M. (2015) 'Bacterial Proteins Pinpoint A Single Eukaryotic Root', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.1420657112.
Donnenberg, V. S. and Donnenberg, A. D. (2015) 'Coping With Artifact In The Analysis Of Flow Cytometric Data', *Methods*. Elsevier Inc., 82, pp. 3–11. doi: 10.1016/j.ymeth.2015.03.012.
van Doorn, W. G. and Woltering, E. J. (2005) 'Many Ways To Exit? Cell Death Categories In Plants', *Trends in Plant Science*, 10(3), pp. 117–122. doi: 10.1016/j.tplants.2005.01.006.
Durham, B. P., Sharma, S., Luo, H., Smith, C. B., Amin, S. a, Bender, S. J., Dearth, S. P., van Mooy, B. a S., Campagna, S. R., Kujawinski, E. B., *et al.* (2014) 'Cryptic Carbon And Sulfur Cycling Between Surface Ocean Plankton', *Proc Natl Acad Sci U S A*, in review(2), pp. 453–457. doi: 10.1073/pnas.1413137112.
Durighello, E., Christie-Oleza, J. A. and Armengaud, J. (2014) 'Assessing The Exoproteome Of Marine Bacteria, Lesson From A RTX-toxin Abundantly Secreted By Phaeobacter Strain DSM 17395', *PLoS ONE*, 9(2). doi: 10.1371/journal.pone.0089691.
Engel, A., Delille, B., Jacquet, S., Riebesell, U., Rochelle-Newall, E., Terbrüggen, A. and Zondervan, I. (2004) 'Transparent Exopolymer Particles And Dissolved Organic Carbon Production By *Emiliania huxleyi* Exposed To Different CO_2 Concentrations: A Mesocosm Experiment', *Aquatic Microbial Ecology*, 34(1), pp. 93–104. doi: 10.3354/ame034093.
Eppley, R. W., Holmes, R. W. and Strickland, J. D. H. (1967) 'Sinking Rates Of Marine Phytoplankton Measured With A Fluorometer', *Journal of Experimental Marine Biology and Ecology*, 1(2), pp. 191–208. doi: 10.1016/0022-0981(67)90014-7.
Escobar, M. A. and Dandekar, A. M. (2003) '*Agrobacterium tumefaciens* As An Agent Of Disease', *Trends in Plant Science*. doi: 10.1016/S1360-1385(03)00162-6.
Evans, C., Kadner, S. V., Darroch, L. J., Wilson, W. H., Liss, P. S. and Malin, G. (2007) 'The Relative Significance Of Viral Lysis And Microzooplankton Grazing As Pathways Of Dimethylsulfoniopropionate (DMSP) Cleavage: An *Emiliania huxleyi* Culture Study', *Limnology and Oceanography*, 52(3), pp. 1036–1045. doi: 10.4319/lo.2007.52.3.1036.
Evans, C., Malin, G., Mills, G. P. and Wilson, W. H. (2006) 'Viral Infection of *Emiliania huxleyi* (Prymnesiophyceae) Leads To Elevated Production Of Reactive Oxygen Species', *Journal of Phycology*, 42(5), pp. 1040–1047. doi: 10.1111/j.1529-8817.2006.00256.x.

Evans, C. and Wilson, W. H. (2008) 'Preferential Grazing Of *Oxyrrhis Marina* On Virus Infected *Emiliania huxleyi*', *Limnology and Oceanography*, 53(5), pp. 2035–2040. doi: 10.4319/lo.2008.53.5.2035.

Fath, A., Bethke, P., Lonsdale, J., Meza-Romero, R. and Jones, R. (2000) 'Programmed Cell Death In *Cereal aleurone*', *Plant Molecular Biology*, 44(3), pp. 255–266. doi: 10.1023/A:1026584207243.

Fernandes, N., Case, R. J., Longford, S. R., Seyedsayamdost, M. R., Steinberg, P. D., Kjelleberg, S. and Thomas, T. (2011) 'Genomes And Virulence Factors Of Novel Bacterial Pathogens Causing Bleaching Disease In The Marine Red Alga *Delisea pulchra*', *PloS one*, 6(12), p. e27387. doi: 10.1371/journal.pone.0027387.

Fischer, W. (2011) 'Assembly And Molecular Mode Of Action Of The *Helicobacter pylori* cag Type IV Secretion Apparatus', *FEBS Journal*. doi: 10.1111/j.1742-4658.2011.08036.x.

Frada, M. J., Bidle, K. D., Probert, I. and de Vargas, C. (2012) 'In Situ Survey Of Life Cycle Phases Of The Coccolithophore *Emiliania huxleyi* (Haptophyta)', *Environmental Microbiology*, 14(6), pp. 1558–1569. doi: 10.1111/j.1462-2920.2012.02745.x.

Frada, M., Probert, I., Allen, M. J., Wilson, W. H. and de Vargas, C. (2008) 'The "Cheshire Cat" escape Strategy Of The Coccolithophore *Emiliania huxleyi* In Response To Viral Infection.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(41), pp. 15944–15949. doi: 10.1073/pnas.0807707105.

Frank, O., Michael, V., Pauker, O., Boedeker, C., Jogler, C., Rohde, M. and Petersen, J. (2015) 'Plasmid Curing And The Loss Of Grip - The 65-kb Replicon Of *Phaeobacter inhibens* DSM 17395 Is Required For Biofilm Formation, Motility And The Colonization Of Marine Algae', *Systematic and Applied Microbiology*. Elsevier GmbH., 38(2), pp. 120–127. doi: 10.1016/j.syapm.2014.12.001.

Frank, O., Pradella, S., Rohde, M., Scheuner, C., Klenk, H.-P., Göker, M. and Petersen, J. (2014) 'Complete Genome Sequence Of The *Phaeobacter gallaeciensis* Type Strain CIP 105210T (= DSM 26640T = BS107T)', *Standards in Genomic Sciences*. doi: 10.4056/sigs.5179110.

Franklin, D. J., Airs, R. L., Fernandes, D. J., Bell, T. G., Bongaerts, R. J., Berges, J. A. and Malin, G. (2012) 'Identification Of Senescence And Death In *Emiliania huxleyi* And *Thalassiosira pseudonana*: Cell Staining, Chlorophyll Alterations, And Dimethylsulfoniopropionate (DMSP) Metabolism', *Limnology and oceanography*, 57(1), pp. 305–317. doi: 10.4319/lo.2012.57.1.0305.

Franklin, D. J. and Berges, J. a (2004) 'Mortality In Cultures Of The Dinoflagellate *Amphidinium carterae* During Culture Senescence And Darkness.', *Proceedings. Biological sciences / The Royal Society*, 271(1553), pp. 2099–107. doi: 10.1098/rspb.2004.2810.

Franklin, D. J., Brussaard, C. P. D. and Berges, J. a. (2006) 'What Is The Role And Nature Of Programmed Cell Death In Phytoplankton Ecology?', *European Journal of Phycology*, 41(2006), pp. 1–14. doi: 10.1080/09670260500505433.

Fritz, J. J. (1999) 'Carbon Fixation And Coccolith Detachment In The Coccolithophore *Emiliania huxleyi* In Nitrate-limited Cyclostats', *Marine Biology*, 133(3), pp. 509–518. doi: 10.1007/s002270050491.

Fritz, J. J. and Balch, W. M. (1996) 'A Light-limited Continuous Culture Study Of *Emiliania huxleyi*: Determination Of Coccolith Detachment And Its Relevance To Cell Sinking', *Journal of Experimental Marine Biology and Ecology*. doi: 10.1016/S0022-0981(96)02633-0.

Fronzes, R., Christie, P. J. and Waksman, G. (2009) 'The Structural Biology Of Type IV Secretion Systems.', *Nature reviews. Microbiology*, 7(10), pp. 703–14. doi: 10.1038/nrmicro2218.

Fullner, K. J. (1998) 'Role Of *Agrobacterium* VirB Genes In Transfer Of T Complexes And RSF1010', *Journal of Bacteriology*.

Gao, L. Y. and Abu Kwaik, Y. (2000) 'Hijacking Of Apoptotic Pathways By Bacterial Pathogens', *Microbes and Infection*, 2(14), pp. 1705–1719. doi: 10.1016/S1286-4579(00)01326-5.

Gardiner, M., Bournazos, A. M., Maturana-Martinez, C., Zhong, L. and Egan, S. (2017) 'Exoproteome Analysis Of The Seaweed Pathogen Nautella Italica R11 Reveals Temperature-dependent Regulation Of RTX-like Proteins', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.01203.

Gelvin, S. B. (1998) '*Agrobacterium* VirE2 Proteins Can Form A Complex With T Strands In The Plant Cytoplasm', *Journal of Bacteriology*.

Geng, H. and Belas, R. (2010) 'Molecular Mechanisms Underlying Roseobacter-phytoplankton Symbioses.', *Current opinion in biotechnology*. Elsevier Ltd, 21(3), pp. 332–8. doi: 10.1016/j.copbio.2010.03.013.

Gibbs, S. J., Poulton, A. J., Bown, P. R., Daniels, C. J., Hopkins, J., Young, J. R., Jones, H. L., Thiemann, G. J., O'Dea, S. A. and Newsam, C. (2013) 'Species-specific Growth Response Of Coccolithophores To Palaeocene–Eocene Environmental Change', *Nature Geoscience*. doi: 10.1038/ngeo1719.

González, J. M., Simó, R., Massana, R., Covert, J. S., Casamayor, E. O., Pedrós-Alió, C. and Moran, M. a (2000) 'Bacterial Community Structure Associated With A Dimethylsulfoniopropionate-producing North Atlantic Algal Bloom.', *Applied and environmental microbiology*, 66(10), pp. 4237–46. doi: 10.1128/AEM.66.10.4237-4246.2000.

Gray, M. W. and Doolittle, W. F. (1982) 'Has The Endosymbiont Hypothesis Been Proven?', *Microbiological reviews*, 46(1), pp. 1–42.

Green, D. H., Echavarri-Bravo, V., Brennan, D. and Hart, M. C. (2015) 'Bacterial Diversity Associated With The Coccolithophorid Algae *Emiliania huxleyi* And *Coccolithus pelagicus F. braarudii*', *BioMed Research International*, 2015. doi: 10.1155/2015/194540.

Green, E. R. and Mecsas, J. (2016) 'Bacterial Secretion Systems: An Overview', in *Virulence Mechanisms of Bacterial Pathogens, Fifth Edition*. doi: 10.1128/microbiolspec.VMBF-0012-2015.

Green, J. C., Course, P. A. and Tarran, G. A. (1996) 'The Life-cycle Of *Emiliania huxleyi*: A Brief Review And A Study Of Relative Ploidy Levels Analysed By Flow Cytometry', *Journal of Marine Systems*, 9(1–2), pp. 33–44. doi: 10.1016/0924-7963(96)00014-0.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P., Dourala, N., Nielsen, K. F. and Gram, L. (2015) 'Isolation Of TDA-producing *Phaeobacter* Strains From Sea Bass Larval Rearing Units And Their Probiotic Effect Against Pathogenic *Vibrio* Spp. In *Artemia* Cultures', *Systematic and Applied Microbiology*, 39, pp. 180–188. doi: 10.1016/j.syapm.2016.01.005.

Guillard, R. R. L. and Hargraves, P. E. (1993) '*Stichochrysis immobilis* Is a diatom, not a chrysophyte', *Phycologia*, 32(3), pp. 234–236. doi: 10.2216/i0031-8884-32-3-234.1.

Gunasinghe, S. D., Webb, C. T., Elgass, K. D., Hay, I. D. and Lithgow, T. (2017) 'Super-resolution imaging of protein secretion systems and the cell surface of Gram-negative bacteria', *Frontiers in cellular and infection microbiology*. doi: 10.3389/fcimb.2017.00220.

Hancock, L., Goff, L. and Lane, C. (2010) 'Red algae lose key mitochondrial genes in response to becoming parasitic', *Genome biology and evolution*, 2(September), pp. 897–910. doi: 10.1093/gbe/evq075.
Harvey, E. L., Bidle, K. D. and Johnson, M. D. (2015) 'Consequences of strain variability and calcification in *Emiliania huxleyi* on microzooplankton grazing', *Journal of Plankton Research*, 0(0), pp. 1–12. doi: 10.1093/plankt/fbv081.
Hatsugai, N., Iwasaki, S., Tamura, K., Kondo, M., Fuji, K., Ogasawara, K., Nishimura, M. and Hara-Nishimura, I. (2009) 'A novel membrane fusion-mediated plant immunity against bacterial pathogens', *Genes and Development*. doi: 10.1101/gad.1825209.
Hatsugai, N., Yamada, K., Goto-Yamada, S. and Hara-Nishimura, I. (2015) 'Vacuolar processing enzyme in plant progammed cell death', *Frontiers in Plant Science*, 6(April), pp. 1–11. doi: 10.3389/fpls.2015.00234.
Hay, W. W., Mohler, H. P., Roth, P. H., Schmidt, R. R. and Boudreau, J. E. (1967) 'Calcareous nannoplankton zonation of the Cenozoic of the Gulf Coast and Caribbean-Antillean area, and Transoceanic', *Planktonic zonation of tertiary*, XVII.
Henriksen, K., Stipp, S. L. S., Young, J. R. and Bown, P. R. (2003) 'Tailoring calcite: Nanoscale AFM of coccolith biocrystals', *American Mineralogist*, 88(11–12 PART 2), pp. 2040–2044.
Herigstad, B., Hamilton, M. and Heersink, J. (2001) 'How to optimize the drop plate method for enumerating bacteria', *Journal of microbiological methods*, 44(2), pp. 121–9.
Hersh, D., Monack, D., Smith, M., Ghori, N., Falkw, S. and Sychlisnski, A. (1999) 'The *Salmonella invasin* SipB induces macrophage apoptosis by binding to Caspase-1', *Proc Natl Acad Sci U S A*, 96(March), pp. 2396–2401.
Hilbi, H., Moss, J. E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., Flavell, R. A., Yuan, J., Sansonetti, P. J. and Zychlinsky, A. (1998) '*Shigella*-induced apoptosis is dependent on Caspase-1 which binds to IpaB*', 273(49), pp. 32895–32900.
Holligan, P. M., Fernandez, E., Aiken, J., Balch, W. M., Boyd, P., Burkill, P., Finch, M., Groom, S., Malin, G., Muller, K., *et al.* (1993) 'A biogeochemical study of the coccolithopore, *Emiliania huxleyi*, in the North Atlantic', 7(4), pp. 879–900.
Houdan, A., Probert, I., Zatylny, C., Véron, B. and Billard, C. (2006) 'Ecology of oceanic coccolithophores. I. nutritional preferences of the two stages in the life cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*', *Aquatic Microbial Ecology*, 44(3), pp. 291–301. doi: 10.3354/ame044291.
Hubber, A. and Roy, C. R. (2010) 'Modulation of host cell function by *Legionella pneumophila* Type IV Effectors'. doi: 10.1146/annurev-cellbio-100109-104034.
Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J. and Polz, M. F. (2008) 'Resource partitioning and sympatric differentiation among closely related bacterioplankton', *Science*, 320(5879), pp. 1081–1085. doi: 10.1126/science.1157890.
Iakimova, E. T., Sobiczewski, P., Michalczuk, L., Wegrzynowicz-Lesiak, E., Mikiciński, A. and Woltering, E. J. (2013) 'Morphological and biochemical characterization of *Erwinia amylovora*-induced hypersensitive cell death in apple leaves', *Plant Physiology and Biochemistry*, 63, pp. 292–305. doi: 10.1016/j.plaphy.2012.12.006.
Ingvardsen, C. and Veierskov, B. (2001) 'Ubiquitin- and proteasome-dependent proteolysis in plants', *Physiologia Plantarum*. doi: 10.1034/j.1399-3054.2001.1120401.x.
IPCC (2007) 'Climate Change 2007: the physical science basis', *Intergovernmental Panel on Climate Change*, 446(7137), pp. 727–8. doi: 10.1038/446727a.

Isberg, R. R., O'Connor, T. J. and Heidtman, M. (2009) 'The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells', *Nature Reviews Microbiology*, 7(1), pp. 13–24. doi: 10.1038/nrmicro1967.

Ishida, H., Izumi, M., Wada, S. and Makino, A. (2014) 'Roles of autophagy in chloroplast recycling', *Biochimica et Biophysica Acta - Bioenergetics*. doi: 10.1016/j.bbabio.2013.11.009.

Jäättelä, M. (2017) 'Programmed Cell Death: Many ways for cells to die decently', 3890(July). doi: 10.1080/078538902321012423.

Jan, N., Hussain, M. and Andrabi, K. (2008) 'Programmed Cell Death or apoptosis: do animals and plants share anything in common', *Biotechnol Mol Biol Rev*, 3(5), pp. 111–126.

Jesenberger, V., Jentsch, S., V, J. and S, J. (2002) 'Deadly Encounter: Ubiquitin Meets Apoptosis.', *Nature reviews. Molecular cell biology*. doi: 10.1038/nrm731.

Jiménez, C., Capasso, J. M., Edelstein, C. L., Rivard, C. J., Lucia, S., Breusegem, S., Berl, T. and Segovia, M. (2009) 'Different ways to die: Cell death modes of the unicellular Chlorophyte *Dunaliella viridis* exposed to various environmental stresses are mediated by the caspase-like activity DEVDase', *Journal of Experimental Botany*, 60(3), pp. 815–828. doi: 10.1093/jxb/ern330.

Jones, A. R. (2000) 'Does the plant mitochondrion integrate cellular stress and regulate Programmed Cell Death?', *Trends in Plant Science*, 5(5), pp. 225–230. doi: 10.1016/S1360-1385(00)01605-8.

Jones, R. J., Ward, S., Amri, A. Y. and Hoegh-Guldber, O. (2000) 'Changes in quantum efficiency of Photosystem II of symbiotic dinoflagellates of corals after heat stress, and of bleached corals sampled after the 1998 Great Barrier Reef mass bleaching event', *Marine and freshwater research* , 51(1), pp. 63–71. doi: 10.1071/MF99100.

De Jong, A. J., Yakimova, E. T., Kapchina, V. M. and Woltering, E. J. (2002) 'A critical role for ethylene in hydrogen peroxide release during programmed cell death in tomato suspension cells', *Planta*, 214(4), pp. 537–545. doi: 10.1007/s004250100654.

Juhas, M., Crook, D. W. and Hood, D. W. (2008) 'Type IV Secretion Systems: Tools of bacterial horizontal gene transfer and virulence', *Cellular Microbiology*. doi: 10.1111/j.1462-5822.2008.01187.x.

Ke, Y., Wang, Y., Li, W. and Chen, Z. (2015) 'Type IV Secretion System of *Brucella* spp. and its effectors', *Front Cell Infect Microbiol*.

Keeling, P. J. (2013) 'The number, speed, and impact of plastid endosymbioses in Eukaryotic evolution', *Annual Review of Plant Biology*, 64(1), pp. 583–607. doi: 10.1146/annurev-arplant-050312-120144.

Kendrick, B. J., DiTullio, G. R., Cyronak, T. J., Fulton, J. M., Van Mooy, B. a. S. and Bidle, K. D. (2014) 'Temperature-induced viral resistance in *Emiliania huxleyi* (Prymnesiophyceae)', *PLoS ONE*, 9(11), p. e112134. doi: 10.1371/journal.pone.0112134.

Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) 'Apoptosis: A basic biological phenomenon with wide- ranging implications in tissue kinetics', *Journal of Internal Medicine*, 258(6), pp. 479–517. doi: 10.1111/j.1365-2796.2005.01570.x.

Kim, M., Ahn, J. W., Jin, U. H., Choi, D., Paek, K. H. and Pai, H. S. (2003) 'Activation of the programmed cell death pathway by inhibition of proteasome function in plants', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M210539200.

Klais, R., Norros, V., Lehtinen, S., Tamminen, T. and Olli, K. (2016) 'Community assembly and drivers of phytoplankton functional structure', *Functional Ecology*, pp. 760–767. doi: 10.1111/1365-2435.12784.

Klaveness, D. (1972a) '*Coccolithus huxleyi* (Lohm.) Kamptn II. The flagellate cell, aberrant cell types, vegetative propagation and life cycles', *British Phycological Journal*, 7(3), pp. 309–318. doi: 10.1080/00071617200650321.

Klaveness, D. (1972b) '*Coccolitus huxleyi* (Lohmann) Kamptner I. Morphological investigations on the vegetative cell and the process of coccolith formation', *Protistologica*, 8(3), pp. 335–346.

Kloft, N., Busch, T., Neukirch, C., Weis, S., Boukhallouk, F., Bobkiewicz, W., Cibis, I., Bhakdi, S. and Husmann, M. (2009) 'Biochemical and biophysical research communications pore-forming toxins activate MAPK P38 by causing loss of cellular potassium', *Biochemical and Biophysical Research Communications*. Elsevier Inc., 385(4), pp. 503–506. doi: 10.1016/j.bbrc.2009.05.121.

Kolb, A. and Strom, S. (2013) 'An inducible antipredatory defense in haploid cells of the marine microalga *Emiliania huxleyi* (Prymnesiophyceae)', *Limnology and Oceanography*, 58(3), pp. 932–944. doi: 10.4319/lo.2013.58.3.0932.

Krishnamurthy, K. V., Krishnaraj, R., Chozhavendan, R. and Samuel Christopher, F. (2000) 'The programme of cell death in plants and animals: A comparison', *Current Science*, 79(9), pp. 1169–1181.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E., Baehrecke, E., Blagosklonny, M., El-Deiry, W., Golstein, P., Green, D., *et al.* (2009) 'Classification of cell death: recommendations of the nomenclature committee on cell death 2009', *Cell death and differentiation*, 16(1), pp. 3–11. doi: 10.1038/cdd.2008.150.

Kubori, T., Hyakutake, A. and Nagai, H. (2008) '*Legionella* translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2008.06124.x.

Kurokawa, M. and Kornbluth, S. (2010) 'Stalling in mitosis and releasing the apoptotic brake', *The EMBO Journal*, 29, pp. 2255–2257. doi: 10.1038/emboj.2010.150.

Kwak, Y., Vikström, E., Magnusson, K., Vécsey-semjén, B., Colque-navarro, P. and Möllby, R. (2012) 'The *Staphylococcus aureus* alpha-toxin perturbs the barrier function in Caco-2 epithelial cell monolayers by altering junctional integrity', pp. 1670–1680. doi: 10.1128/IAI.00001-12.

Labeeuw, L., Khey, J., Bramucci, A. R., Atwal, H., De La Mata, A. P., Harynuk, J. and Case, R. J. (2016) 'Indole-3-acetic acid is produced by *Emiliania huxleyi* coccolith-bearing cells and triggers a physiological response in bald cells', *Frontiers in Microbiology*, 7(JUN). doi: 10.3389/fmicb.2016.00828.

Lacroix, B. and Citovsky, V. (2011) 'Extracellular VirB5 enhances T-DNA transfer from *Agrobacterium* to the host plant', *PLoS ONE*. doi: 10.1371/journal.pone.0025578.

Laguna, R., Romo, J., Read, B. a and Wahlund, M. (2001) 'Induction of phase variation events in the life cycle of the marine coccolithophorid *Emiliania huxleyi* induction of phase variation events in the life cycle of the marine coccolithophorid *Emiliania huxleyi*', 67(9), pp. 3824–3831. doi: 10.1128/AEM.67.9.3824.

Lampert, W. (1978) 'Release Of Dissolved Organic Carbon By Grazing Zooplankton', *Limnology and Oceanography*, 23(4), pp. 831–834. doi: 10.4319/lo.1978.23.4.0831.

Langer, G., Nehrke, G., Probert, I., Ly, J. and Ziveri, P. (2009) 'Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry', *Biogeosciences Discussions*, 6(2), pp. 4361–4383. doi: 10.5194/bgd-6-4361-2009.

Lawen, A. (2003) 'Apoptosis: An introduction', *BioEssays*, 25(9), pp. 888–896. doi: 10.1002/bies.10329.

Lee, S., Lee, M., Cherla, R. P. and Tesh, V. L. (2008) '*Shiga* toxin 1 induces apoptosis through the Endoplasmic Reticulum stress response in human monocytic cells', (2007), pp. 770–780. doi: 10.1111/j.1462-5822.2007.01083.x.

Levine, A., Pennell, R. I., Alvarez, M. E., Palmer, R. and Lamb, C. (1996) 'Calcium-mediated apoptosis in a plant hypersensitive disease resistance response', *Current biology : CB*, 6(4), pp. 427–437. doi: 10.1016/S0960-9822(02)00510-9.

Li, G., Froehlich, J. E., Elowsky, C., Msanne, J., Ostosh, A. C., Zhang, C., Awada, T. and Alfano, J. R. (2014) 'Distinct *Pseudomonas* Type-III Effectors use a cleavable transit peptide to target chloroplasts', *Plant Journal*. doi: 10.1111/tpj.12396.

Locht, C., Coutte, L. and Mielcarek, N. (2011) 'The ins and outs of *Pertussis* toxin', *Febs Journal*. doi: 10.1111/j.1742-4658.2011.08237.x.

Luo, H. and Moran, M. A. (2014) 'Evolutionary ecology of the marine roseobacter clade', *Microbiology and Molecular Biology Reviews*, 78(4), pp. 573–587. doi: 10.1128/MMBR.00020-14.

Luo, H., Swan, B. K., Stepanauskas, R., Hughes, A. L. and Moran, M. A. (2014) 'Comparing effective population sizes of dominant marine alphaproteobacteria lineages', *Environmental Microbiology Reports*, 6(2), pp. 167–172. doi: 10.1111/1758-2229.12129.

MacIntyre, D. L., Miyata, S. T., Kitaoka, M. and Pukatzki, S. (2010) 'The *Vibrio cholerae* Type VI Secretion System displays antimicrobial properties', *Proc. Natl. Acad. Sci. USA*. doi: 10.1073/pnas.1012931107.

MacIntyre, H. L. and Cullen, J. J. (2005) 'Using cultures to investigate the physiological ecology of microalgae', in Anderson, R. (ed.) *Algal Culturing Techniques*. 1st Editio. Burlington, MA: Elsevier Academic Press, pp. 287–326.

Mackinder, L. C. M., Worthy, C. A., Biggi, G., Hall, M., Ryan, K. P., Varsani, A., Harper, G. M., Wilson, W. H., Brownlee, C. and Schroeder, D. C. (2009) 'A unicellular algal virus, *Emiliania huxleyi* virus 86, exploits an animal-like infection strategy', *Journal of General Virology*. doi: 10.1099/vir.0.011635-0.

Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S., *et al.* (2002) 'A caspase-related protease regulates apoptosis in yeast', *Molecular Cell*, 9(4), pp. 911–917. doi: 10.1016/S1097-2765(02)00501-4.

Martens, T., Heidorn, T., Pukal, R., Simon, M., Tindall, B. J. and Brinkhoff, T. (2006) 'Reclassification of *Roseobacter gallaeciensis* Ruiz-Ponte *et al*. 1998 As *Phaeobacter gallaeciensis* gen. nov., comb. nov., description of *Phaeobacter inhibens* sp. nov., reclassification of *Ruegeria algicola* Lafay *et al.* 1995) Uchino *et al.* 19', *International Journal of Systematic and Evolutionary Microbiology*, 56(6), pp. 1293–1304. doi: 10.1099/ijs.0.63724-0.

Martínez-Fábregas, J., Díaz-Moreno, I., González-Arzola, K., Díaz-Quintana, A. and De la Rosa, M. A. (2014) 'A common signalosome for programmed cell death in humans and plants', *Cell Death and Disease*. doi: 10.1038/cddis.2014.280.

Maxwell, K. and Johnson, G. N. (2000) 'Chlorophyll fluorescence-a practical guide', *Journal of experimental botany*, 51(345), pp. 659–668. doi: 10.1093/jexbot/51.345.659.

Mayali, X. and Azam, F. (2004) 'Algicidal bacteria in the sea and their impact on algal blooms', *Journal of Eukaryotic Microbiology*, 51(2), pp. 139–144. doi: 10.1111/j.1550-7408.2004.tb00538.x.
Mayali, X., Franks, P. J. S. and Azam, F. (2008) 'Cultivation and ecosystem role of a marine roseobacter clade-affiliated cluster bacterium', *Applied and Environmental Microbiology*, 74(9), pp. 2595–2603. doi: 10.1128/AEM.02191-07.
Mayers, T., Bramucci, A., Yakimovich, K. and Case, R. (2016) 'A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*', *Frontiers in Microbiology*, 7(JUN). doi: 10.3389/fmicb.2016.00892.
Medlin, L. K., Barker, G. L. A., Campbell, L., Green, J. C., Hayes, P. K., Marie, D., Wrieden, S. and Vaulot, D. (1996) 'Genetic Characterisation Of *Emiliania huxleyi* (Haptophyta)', *Journal of Marine Systems*. doi: 10.1016/0924-7963(96)00013-9.
Michael, V., Frank, O., Bartling, P., Scheuner, C., Göker, M., Brinkmann, H. and Petersen, J. (2016) 'Biofilm Plasmids With A Rhamnose Operon Are Widely Distributed Determinants Of The "swim-or-stick" Lifestyle In Roseobacters', *The ISME Journal*, pp. 1–16. doi: 10.1038/ismej.2016.30.
Miller, T. R. and Belas, R. (2004) 'Dimethylsulfoniopropionate Metabolism By Pfiesteria - Associated Roseobacter Spp .', *Applied and environmental microbiology*, 70(6), pp. 3383–3391. doi: 10.1128/AEM.70.6.3383.
Miller, T. R. and Belas, R. (2006) 'Motility Is Involved In *Silicibacter* sp. TM1040 Interaction With Dinoflagellates', *Environmental Microbiology*, 8(9), pp. 1648–1659. doi: 10.1111/j.1462-2920.2006.01071.x.
Miller, T. R., Hnilicka, K., Dziedzic, A., Desplats, P. and Belas, R. (2004) 'Chemotaxis of *Silicibacter* sp. strain TM1040 toward dinoflagellate products', *Society*, 70(8), pp. 4692–4701. doi: 10.1128/AEM.70.8.4692.
Mitchell, J. G., Yamazaki, H., Seuront, L., Wolk, F. and Li, H. (2008) 'Phytoplankton Patch Patterns: Seascape Anatomy In A Turbulent Ocean', *Journal of Marine Systems*, 69, pp. 247–253. doi: 10.1016/j.jmarsys.2006.01.019.
Moharikar, S., D'Souza, J. S., Kulkarni, A. B. and Rao, B. J. (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(2), pp. 423–433. doi: 10.1111/j.1529-8817.2006.00207.x.
Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P., Rickaby, R. E. M., Poulton, A. J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., Gibbs, S., *et al.* (2016) 'Why marine phytoplankton calcify', *Science Advances*, 2(7), pp. e1501822–e1501822. doi: 10.1126/sciadv.1501822.
Moore, C. M., Mills, M. M., Arrigo, K. R., Berman-Frank, I., Bopp, L., Boyd, P. W., Galbraith, E. D., Geider, R. J., Guieu, C., Jaccard, S. L., *et al.* (2013) 'Processes And Patterns Of Oceanic Nutrient Limitation', *Nature Geoscience*. Nature Publishing Group, 6(9), pp. 701–710. doi: 10.1038/ngeo1765.
Moore, L. R., Goericke, R. and Chisholm, S. W. (1995) 'Comparative Physiology Of *Synechococcus* And *Prochlorococcus*: Influence Of Light And Temperature On Growth, Pigments, Fluorescence And Absorptive', *Marine Ecology Progress Series*, 116, pp. 259–275. doi: 10.3354/meps116259.
Moran, M. A., Belas, R., Schell, M. A., González, J. M., Sun, F., Sun, S., Binder, B. J., Edmonds, J., Ye, W., Orcutt, B., *et al.* (2007) 'Ecological Genomics Of Marine

Roseobacters', *Applied and Environmental Microbiology*, 73(14), pp. 4559–4569. doi: 10.1128/AEM.02580-06.
Moran, M. A., González, J. M. and Kiene, R. P. (2003) 'Linking A Bacterial Taxon To Sulfur Cycling In The Sea: Studies Of The Marine Roseobacter Group', *Geomicrobiology Journal*, 20(4), pp. 375–388. doi: 10.1080/01490450303901.
Moran, M. A., Reisch, C. R., Kiene, R. P. and Whitman, W. B. (2012) 'Genomic Insights Into Bacterial DMSP Transformations', *Annual Review of Marine Science*, 4(1), pp. 523–542. doi: 10.1146/annurev-marine-120710-100827.
Mossey, P., Hudacek, A. and Das, A. (2010) '*Agrobacterium tumefaciens* Type IV Secretion Protein VirB3 Is An Inner Membrane Protein And Requires VirB4, VirB7, And VirB8 For Stabilization', *Journal of Bacteriology*, 192(11), pp. 2830–2838. doi: 10.1128/JB.01331-09.
Mu, A., Gu, D., Du, F., Naumann, M., Meyer, T. F. and Rudel, T. (1999) '*Neisserial* Porin (PorB) Causes Rapid Calcium Influx In Target Cells And Induces Apoptosis By The Activation Of Cysteine Proteases', 18(2), pp. 339–352.
Mukhtar, M. S., McCormack, M. E., Argueso, C. T. and Pajerowska-Mukhtar, K. M. (2016) 'Pathogen Tactics To Manipulate Plant Cell Death', *Current Biology*. Elsevier Ltd, 26(13), pp. R608–R619. doi: 10.1016/j.cub.2016.02.051.
Müller, A. and Rudel, T. (2001) 'Modification Of Host Cell Apoptosis By Viral And Bacterial Pathogens.', *International journal of medical microbiology : IJMM*, 291(3), pp. 197–207. doi: 10.1078/1438-4221-00125.
Müller, M. N., Antia, A. N. and LaRoche, J. (2008) 'Influence Of Cell Cycle Phase On Calcification In The Coccolithophore *Emiliania huxleyi*', *Limnology and Oceanography*. doi: 10.4319/lo.2008.53.2.0506.
Nagai, H. and Kubori, T. (2011) 'Type IVB Secretion Systems Of Legionella And Other Gram-negative Bacteria', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2011.00136.
Nagai, T., Taya, K., Annoh, H. and Ishihara, S. (2013) 'Application Of A Fluorometric Microplate Algal Toxicity Assay For Riverine Periphytic Algal Species', *Ecotoxicology and Environmental Safety*, 94, pp. 37–44. doi: 10.1016/j.ecoenv.2013.04.020.
Nevitt, G. A., Veit, R. R. and Kareiva, P. (1995) 'Dimethyl Sulphide As A Foraging Cue For Antarctic *Procellariiform* Seabirds', *Nature*, pp. 680–682. doi: http://dx.doi.org/10.1038/376680a0.
Newton, H. J., Kohler, L. J., McDonough, J. A., Temoche-Diaz, M., Crabill, E., Hartland, E. L. and Roy, C. R. (2014) 'A Screen Of Coxiella Burnetii Mutants Reveals Important Roles For Dot/Icm Effectors And Host Autophagy In Vacuole Biogenesis', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1004286.
Newton, R. J., Griffin, L. E., Bowles, K. M., Meile, C., Gifford, S., Givens, C. E., Howard, E. C., King, E., Oakley, C. A., Reisch, C. R., *et al.* (2010) 'Genome Characteristics Of A Generalist Marine Bacterial Lineage.', *The ISME journal*. Nature Publishing Group, 4(6), pp. 784–98. doi: 10.1038/ismej.2009.150.
Niu, H., Kozjak-pavlovic, V., Rudel, T. and Rikihisa, Y. (2010) 'Anaplasma Phagocytophilum Ats-1 Is Imported Into Host Cell Mitochondria And Interferes With Apoptosis Induction', 6(2). doi: 10.1371/journal.ppat.1000774.
Oberhammer, F., Wilson, J., Dive, C., Morris, I., Hickman, J., Wakeling, A., Walker, P. and Sikorska, M. (1993) 'Apoptotic Death In Epithelial Cells: Cleavage Of DNA To 300 And/or 50 Kb Fragments Prior To Or In The Absence Of Internucleosomal Fragmentation', *The EMBO journal*, 12(9), pp. 3679–3684.

Olson, M. B. and Strom, S. L. (2002) 'Phytoplankton Growth, Microzooplankton Herbivory And Community Structure In The Southeast Bering Sea: Insight Into The Formation And Temporal Persistence Of An *Emiliania huxleyi* Bloom', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 49(26), pp. 5969–5990. doi: 10.1016/S0967-0645(02)00329-6.

Paasche, E. (1998) 'Roles Of Nitrogen And Phosphorus In Coccolith Formation In *Emiliania huxleyi* (Prymnesiophyceae)', *European Journal of Phycology*, 33(1), pp. 33–42. doi: 10.1017/S0967026297001480.

Paasche, E. (2002) 'A Review Of The Coccolithophorid *Emiliania huxleyi*.pdf', *Phycologia*, 40(6), pp. 503–529.

Paasche, E. and Klaveness, D. (1970) 'A Physiological Comparison Of Coccolith-forming And Naked Cells Of Coccolithus huxleyi', *Arch. Mikrobiol.*, 73(2), pp. 143–152. doi: 10.1007/BF00410317.

Păcurar, D. I., Thordal-Christensen, H., Păcurar, M. L., Pamfil, D., Botez, M. L. and Bellini, C. (2011) '*Agrobacterium tumefaciens*: From Crown Gall Tumors To Genetic Transformation', *Physiological and Molecular Plant Pathology*, 76(2), pp. 76–81. doi: 10.1016/j.pmpp.2011.06.004.

Park, S., Park, J. M., Jung, Y. T., Won, S. M. and Yoon, J. H. (2015) '*Primorskyibacter Insulae* Sp. Nov., Isolated From The Junction Between The Ocean And A Freshwater Spring', *International Journal of Systematic and Evolutionary Microbiology*, 65(11), pp. 3971–3976. doi: 10.1099/ijsem.0.000522.

Parkhill, J. P., Maillet, G. and Cullen, J. J. (2001) 'Fluorescence-based Maximal Quantum Yield For PSII As A Diagnostic Of Nutrient Stress', *Journal of Phycology*. doi: 10.1046/j.1529-8817.2001.037004517.x.

Patzelt, D., Michael, V., Päuker, O., Ebert, M., Tielen, P., Jahn, D., Tomasch, J., Petersen, J. and Wagner-Döbler, I. (2016) 'Gene Flow Across Genus Barriers - Conjugation Of *Dinoroseobacter Shibae*'s 191-kb Killer Plasmid Into *Phaeobacter Inhibens* And AHL-mediated Expression Of Type IV Secretion Systems', *Frontiers in Microbiology*, 7(MAY), pp. 1–12. doi: 10.3389/fmicb.2016.00742.

Perez-Mendoza, D., Coulthurst, S. J., Humphris, S., Campbell, E., Welch, M., Toth, I. K. and Salmond, G. P. C. (2011) 'A multi-repeat adhesin of the phytopathogen, *Pectobacterium atrosepticum*, is secreted by a type I pathway and is subject to complex regulation involving a non-canonical diguanylate cyclase', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2011.07849.x.

Perez-Perez, M. E., Lemaire, S. D. and Crespo, J. L. (2012) 'Reactive oxygen species and autophagy in plants and algae', *Plant Physiology*, 160(1), pp. 156–164. doi: 10.1104/pp.112.199992.

Persson, O. P., Pinhassi, J., Riemann, L., Marklund, B. I., Rhen, M., Normark, S., González, J. M. and Hagström, Å. (2009) 'High abundance of virulence gene homologues in marine bacteria', *Environmental Microbiology*, pp. 1348–1357. doi: 10.1111/j.1462-2920.2008.01861.x.

Pitzschke, A. and Hirt, H. (2010) 'New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation', *EMBO*.

Podack, E. R. and Munson, G. P. (2016) 'Killing of microbes and cancer by the immune system with three mammalian pore-forming killer proteins', *Frontiers in Immunology*. doi: 10.3389/fimmu.2016.00464.

Poulton, A. J., Charalampopoulou, A., Young, J. R., Tarran, G. a., Lucas, M. I. and Quartly, G. D. (2010) 'Coccolithophore dynamics in non-bloom conditions during late summer in the central iceland basin (July-August 2007)', 55(4), pp. 1601–1613. doi: 10.4319/lo.2010.55.4.1601.

Poulton, A. J., Young, J. R., Bates, N. R. and Balch, W. M. (2011) 'Biometry of detached *Emiliania huxleyi* along the Patagonian Shelf', *Marine Ecology Progress Series*, 443, pp. 1–17. doi: 10.3354/meps09445.

Pozarowski, P., Huang, X., Halicka, D. H., Lee, B., Johnson, G. and Darzynkiewicz, Z. (2003) 'Interactions Of Fluorochrome-labeled Caspase Inhibitors With Apoptotic Cells: A Caution In Data Interpretation.', *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 55(1), pp. 50–60. doi: 10.1002/cyto.a.10074.

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D. and Mekalanos, J. J. (2007) 'Type VI Secretion System Translocates A Phage Tail Spike-like Protein Into Target Cells Where It Cross-links Actin', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0706532104.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F. and Mekalanos, J. J. (2006) 'Identification Of A Conserved Bacterial Protein Secretion System In Vibrio Cholerae Using The Dictyostelium Host Model System.', *Proceedings of the National Academy of Sciences of the United States of America*, 103(5), pp. 1528–1533. doi: 10.1073/pnas.0510322103.

Ramanan, R., Kang, Z., Kim, B.-H., Cho, D.-H., Jin, L., Oh, H.-M. and Kim, H.-S. (2015) 'Phycosphere Bacterial Diversity In Green Algae Reveals An Apparent Similarity Across Habitats', *Algal Research*. Elsevier B.V., 8, pp. 140–144. doi: 10.1016/j.algal.2015.02.003.

Ramanan, R., Kim, B. H., Cho, D. H., Oh, H. M. and Kim, H. S. (2016) 'Algae-bacteria Interactions: Evolution, Ecology And Emerging Applications', *Biotechnology Advances*. doi: 10.1016/j.biotechadv.2015.12.003.

Rao, D., Webb, J. S., Holmström, C., Case, R., Low, A., Steinberg, P. and Kjelleberg, S. (2007) 'Low Densities Of Epiphytic Bacteria From The Marine Alga Ulva Australis Inhibit Settlement Of Fouling Organisms', *Applied and Environmental Microbiology*, 73(24), pp. 7844–7852. doi: 10.1128/AEM.01543-07.

Rao, D., Webb, J. S. and Kjelleberg, S. (2006) 'Microbial colonization and competition on the marine alga *Ulva australis*', *Applied and Environmental Microbiology*, 72(8), pp. 5547–5555. doi: 10.1128/AEM.00449-06.

Rastorguev, S. M. and Zavilgelsky, G. B. (2003) 'Role of "antirestriction" motif in functional activity of antirestriction protein ArdA PKM101 (IncN)', *Russian Journal of Genetics*. doi: 10.1023/A:1022444212370

Ray, J. L., Haramaty, L., Thyrhaug, R., Fredricks, H. F., Van Mooy, B. A. S., Larsen, A., Bidle, K. D. and Sandaa, R. A. (2014) 'Virus infection of *Haptolina ericina* and *Phaeocystis pouchetii* implicates evolutionary conservation of programmed cell death induction in marine Haptophyte-virus interactions', *Journal of Plankton Research*, 36(4), pp. 943–955. doi: 10.1093/plankt/fbu029.

Read, B., Kegel, J., Klute, M., Kuo, A., Lefebvre, S., Maumus, F., Mayer, C., Miller, J., Monier, A., Salamov, A., *et al.* (2013) 'Pan genome of the phytoplankton *Emiliania* underpins its global distribution', *Nature*, 499(7457), pp. 209–13. doi: 10.1038/nature12221.

Reape, T. J. and McCabe, P. F. (2008) 'Apoptotic-like programmed cell death in plants', *New Phytologist*, 180(1), pp. 13–26. doi: 10.1111/j.1469-8137.2008.02549.x.

Reape, T. J., Molony, E. M. and McCabe, P. F. (2008) 'Programmed cell death in plants: distinguishing between different modes', *Journal of Experimental Botany*, 59(3), pp. 435–444. doi: 10.1093/jxb/erm258.

Ribet, D. and Cossart, P. (2010) 'Pathogen-mediated posttranslational modifications: a re-emerging field', *Cell*. doi: 10.1016/j.cell.2010.11.019.

Rokitta, S. D., de Nooijer, L. J., Trimborn, S., de Vargas, C., Rost, B. and John, U. (2011) 'Transcriptome analyses reveal differential gene expression patterns between the life-cycle stages of *Emiliania huxleyi* (Haptophyta) And Reflect Specialization To Different Ecological Niches', *Journal of Phycology*, 47(4), pp. 829–838. doi: 10.1111/j.1529-8817.2011.01014.x.

Rolando, M. and Buchrieser, C. (2014) '*Legionella pneumophila* Type IV effectors hijack the transcription and translation machinery of the host cell', *Trends in Cell Biology*. doi: 10.1016/j.tcb.2014.06.002.

Rosenwasser, S., Mausz, M. a, Schatz, D., Sheyn, U., Malitsky, S., Aharoni, A., Weinstock, E., Tzfadia, O., Ben-Dor, S., Feldmesser, E., *et al.* (2014) 'Rewiring host lipid metabolism by large viruses determines the fate of *Emiliania huxleyi*, a bloom-forming alga in the ocean', *The Plant cell*, 26(June), pp. 2689–2707. doi: 10.1105/tpc.114.125641.

Ruiz-Ponte, C., Samain, J. J. F., Sánchez, J. L. J. and Nicolas, J. J. L. (1999) 'The benefit of a roseobacter species on the survival of scallop larvae', *Marine biotechnology*, 1(1), pp. 52–59. doi: 10.1007/PL00011751.

Russell, A. B., Peterson, S. B. and Mougous, J. D. (2014) 'Type VI Secretion System effectors: poisons with a purpose', *Nature Reviews Microbiology*. doi: 10.1038/nrmicro3185.

Rutkowski, D. T., Kaufman, R. J., Stirling, P. C., Harding, H. P., Kaufman, R. J., Harding, H. P., Bertolotti, A., *et al.* (2004) 'A trip to the ER: Coping with stress', *Trends in cell biology*. doi: 10.1016/J.TCB.2003.11.001.

Sapp, M., Schwaderer, A. S., Wiltshire, K. H., Hoppe, H. G., Gerdts, G. and Wichels, A. (2007) 'Species-specific bacterial communities in the phycosphere of microalgae?', *Microbial Ecology*, 53(4), pp. 683–699. doi: 10.1007/s00248-006-9162-5.

Scarratt, M. G., Marchetti, A., Hale, M. S., Rivkin, R. B., Michaud, S., Matthews, P., Levasseur, M., Sherry, N., Merzouk, A., Li, W. K. W. W., *et al.* (2006) 'Assessing microbial responses to iron enrichment in the subarctic northeast pacific: do microcosms reproduce the *in situ* condition?', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 53(20–22), pp. 2182–2200. doi: 10.1016/j.dsr2.2006.05.035.

Schaechter, M., Ingraham, J. L. and Neidhardt, F. C. (2007) 'Eukaryotic Microbes', in *Microbe - Das Original mit Übersetzungshilfen*.

Schatz, D., Shemi, A., Rosenwasser, S., Sabanay, H., Wolf, S. G., Ben-Dor, S. and Vardi, A. (2014) 'Hijacking of an autophagy-like process is critical for the life cycle of a DNA virus infecting oceanic algal blooms', *New Phytologist*, 204(4), pp. 854–863. doi: 10.1111/nph.13008.

Schreiber, U. (1998) 'Chlorophyll fluorescence: new instruments for special applications', *Photosynthesis: mechanisms and effects*, V, pp. 4253–4258.

Schreiber, U. (2004) 'Pulse-Amplitude-Modulation (PAM) fluorometry and saturation pulse method: an overview', in Papageorgiou, G. C. and Govindjee (eds) *Chlorophyll a Fluorescence: A Signature of Photosynthesis*. Netherlands: Springer, pp. 279–319. doi: 10.1007/978-1-4020-3218-9.

Schulein, R., Guye, P., Rhomberg, T. A., Schmid, M. C., Schröder, G., Vergunst, A. C., Carena, I. and Dehio, C. (2005) 'A bipartite signal mediates the transfer of Type IV Secretion

Substrates of *Bartonella henselae* into human cells', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0406796102.
Schulein, R., Guye, P., Schmid, M. C., Scheidegger, F., Dehio, M., Chennakesava, C. S., Biedermann, B. and Dehio, C. (2006) 'A translocated bacterial protein protects vascular endothelial cells from apoptosis', 2(11). doi: 10.1371/journal.ppat.0020115.
Segev, E., Tellez, A., Vlamakis, H. and Kolter, R. (2015) 'Morphological heterogeneity and attachment of *Phaeobacter inhibens*', *PLoS ONE*, 10(11), pp. 1–13. doi: 10.1371/journal.pone.0141300.
Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N., Paulson, J. N., Chai, L., Clardy, J., *et al.* (2016) 'Dynamic metabolic exchange governs a marine algal-bacterial interaction', *eLife*, 5, p. e17473. doi: 10.7554/eLife.17473.
Segovia, M., Haramaty, L., Berges, J. a and Falkowski, P. G. (2003) 'Cell death in the unicellular Chlorophyte *Dunaliella tertiolecta*. a hypothesis on the evolution of apoptosis in higher plants and metazoans', *Plant physiology*, 132(1), pp. 99–105. doi: 10.1104/pp.102.017129.
Seubert, A., Hiestand, R., De La Cruz, F. and Dehio, C. (2003) 'A bacterial conjugation machinery recruited for pathogenesis', *Molecular Microbiology*. doi: 10.1046/j.1365-2958.2003.03650.x.
Seyedsayamdost, M. R., Case, R. J., Kolter, R. and Clardy, J. (2011) 'The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*', *Nature chemistry*, 3(4), pp. 331–5. doi: 10.1038/nchem.1002.
Seymour, J. R., Amin, S. A., Raina, J.-B. and Stocker, R. (2017) 'Zooming in on the phycosphere: The ecological interface for phytoplankton–bacteria relationships', *Nature Microbiology*, 2(May), p. 17065. doi: 10.1038/nmicrobiol.2017.65.
Seymour, J. R., Simo, R., Ahmed, T. and Stocker, R. (2010) 'Chemoattraction to dimethylsulfoniopropionate throughout the marine microbial food web', *Science*, 329(July), pp. 342–346.
Shabek, N. and Ciechanover, A. (2010) 'Degradation of ubiquitin: The fate of the cellular reaper', *Cell Cycle*, 9(3), pp. 523–530. doi: 10.4161/cc.9.3.11152.
Shao, F. (2003) 'Cleavage of *Arabidopsis* PBS1 by a bacterial Type III Effector', *Science*. doi: 10.1126/science.1085671.
Shemi, A., Ben-dor, S. and Vardi, A. (2015) 'Elucidating the composition and conservation of the autophagy pathway in photosynthetic eukaryotes', (April), pp. 701–715.
Sheyn, U., Rosenwasser, S., Ben-Dor, S., Porat, Z. and Vardi, A. (2016) 'Modulation of host ROS metabolism is essential for viral infection of a bloom-forming coccolithophore in the ocean', *The ISME journal*. Nature Publishing Group, 10(7), pp. 1–13. doi: 10.1038/ismej.2015.228.
Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B. and Tsujimoto, Y. (2004) 'Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes.', *Nature cell biology*, 6(12), pp. 1221–1228. doi: 10.1038/ncb1192.
Si, M., Zhao, C., Burkinshaw, B., Zhang, B., Wei, D., Wang, Y., Dong, T. G. and Shen, X. (2017) 'Manganese scavenging and oxidative stress response mediated by Type VI Secretion system in *Burkholderia thailandensis*', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1614902114.

Signorini, S. R. and McClain, C. R. (2009) 'Environmental factors controlling the Barents Sea spring-summer phytoplankton blooms', *Geophysical Research Letters*, 36(10), pp. 1–5. doi: 10.1029/2009GL037695.
Sikora, A. E. (2013) 'Proteins secreted via the Type II Secretion System: smart strategies of *Vibrio cholerae* to maintain fitness in different ecological niches', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1003126.
Silhavy, T. J., Kahne, D. and Walker, S. (2010) 'The bacterial cell envelope', *Cold Spring Harbor perspectives in biology*, 2(5), p. a000414. doi: 10.1101/cshperspect.a000414.
da Silva, J. M., da Silva, A. B. and Pádua, M. (2007) 'Modulated chlorophyll *a* fluorescence: A tool for teaching photosynthesis', *Journal of Biological Education*, 41(4), pp. 178–183. doi: 10.1080/00219266.2007.9656094.
Singer, A. U., Schulze, S., Skarina, T., Xu, X., Cui, H., Eschen-Lippold, L., Egler, M., Srikumar, T., Raught, B., Lee, J., *et al.* (2013) 'A pathogen Type III Effector with a novel E3 ubiquitin ligase architecture', *PLoS Pathogens*. doi: 10.1371/journal.ppat.1003121.
Skjelbred, B., Edvardsen, B. and Andersen, T. (2012) 'A high-throughput method for measuring growth and loss rates in microalgal cultures', *Journal of applied phycology*, 24, pp. 1589–1599. doi: 10.1007/s10811-012-9819-z.
Slightom, R. N. and Buchan, A. (2009) 'Surface colonization by marine roseobacters: integrating genotype and phenotype', *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01508-09.
Smriga, S., Fernandez, V. I., Mitchell, J. G. and Stocker, R. (2016) 'Chemotaxis toward phytoplankton drives organic matter partitioning among marine bacteria', *Proceedings of the National Academy of Sciences of the United States of America*, 113(6), pp. 1576–81. doi: 10.1073/pnas.1512307113.
Stennicke, H. and Salvesen, G. (1999) 'Catalytic properties of the caspases', *Cell Death and Differentiation*, (6), pp. 1054–1059.
Stocker, R. (2012) 'Marine microbes see a sea of gradients', *Science*, 338(6107), pp. 628–633. doi: 10.1126/science.1208929.
Stocker, R., Seymour, J. R., Samadani, A., Hunt, D. E. and Polz, M. F. (2008) 'Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches', *Proceedings of the National Academy of Sciences of the United States of America*, 105(11), pp. 4209–4214. doi: 10.1073/pnas.0709765105.
Strom, S., Wolfe, G., Slajer, A., Lambert, S. and Clough, J. (2003) 'Chemical defense in the microplankton II: Inhibition of protist feeding by β-dimethylsulfoniopropionate (DMSP)', *Limnology and oceanography*. American Society of Limnology and Oceanography, 48(1), pp. 230–237.
Suchéras-Marx, B. and Henderiks, J. (2014) 'Testing the impact of calcareous nannofossil size evolution on pelagic carbonate burial over the past 17 million years', *Global and Planetary Change*. Elsevier B.V., 123, pp. 94–109. doi: 10.1016/j.gloplacha.2014.10.015.
Sunda, W., Kieber, D. J., Kiene, R. P. and Huntsman, S. (2002) 'An antioxidant function for DMSP and DMS in marine algae', *Nature*, 418(6895), pp. 317–20. doi: 10.1038/nature00851.
Sundström, J. F., Vaculova, A., Smertenko, A. P., Savenkov, E. I., Golovko, A., Minina, E., Tiwari, B. S., Rodriguez-Nieto, S., Zamyatnin, A. a, Välineva, T., *et al.* (2009) 'Tudor staphylococcal nuclease is an evolutionarily conserved component of the Programmed Cell

Death degradome', *Nature cell biology*. Nature Publishing Group, 11(11), pp. 1347–1354. doi: 10.1038/ncb1979.
Suttle, C. A., López-Bueno, A., Pearce, D. A., Alcamí, A., Suttle, C. A., Ignacio-Espinoza, J. C., Solonenko, S. A., Sullivan, M. B., Fuhrman, J. A., Mann, N. H., *et al.* (2005) 'Viruses in the sea', *Nature*. doi: 10.1038/nature04160.
Thole, S., Kalhoefer, D., Voget, S., Berger, M., Engelhardt, T., Liesegang, H., Wollherr, A., Kjelleberg, S., Daniel, R., Simon, M., *et al.* (2012) '*Phaeobacter gallaeciensis* genomes from globally opposite locations reveal high similarity of adaptation to surface life', *The ISME Journal*. Nature Publishing Group, 6(12), pp. 2229–2244. doi: 10.1038/ismej.2012.62.
Thornton, D. C. O. (2014) 'Dissolved Organic Matter (DOM) release by phytoplankton in the contemporary and future ocean', *European Journal of Phycology*. © 2014 British Phycological Society, 49(1), pp. 20–46. doi: 10.1080/09670262.2013.875596.
Thrower, J. S. (2000) 'Recognition of the polyubiquitin proteolytic signal', *The EMBO Journal*. doi: 10.1093/emboj/19.1.94.
Tyrrell, T., Holligan, P. M. and Mobley, C. D. (1999) 'Optical impacts of oceanic coccolithophore blooms', *Journal of Geophysical Research*, 104(C2), p. 3223. doi: 10.1029/1998JC900052.
Tyrrell, T. and Merico, A. (2004) '*Emiliania huxleyi*: bloom observations and the conditions that induce them'. doi: 10.1007/978-3-662-06278-4_4.
Tyrrell, T. and Young, J. R. (2009) 'Coccolithophores', *Encyclopedia of Ocean Sciences*, (c), pp. 606–614. doi: 10.1016/B978-012374473-9.00662-7.
Tzfira, T., Vaidya, M. and Citovsky, V. (2004) 'Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*', *Nature*. doi: 10.1038/nature02857.
Vardi, A., Haramaty, L., Van Mooy, B., Fredricks, H. F., Kimmance, S., Larsen, A. and Bidle, K. D. (2012) 'Host-virus dynamics and subcellular controls of cell fate in a natural coccolithophore population', *Proceedings of the National Academy of Sciences of the United States of America*, 109(47), pp. 19327–32. doi: 10.1073/pnas.1208895109.
Vardi, A., Mooy, B. A. S. Van, Fredricks, H. F., Popendorf, K. J., Ossolinski, J. E., Haramaty, L. and Bidle, K. D. (2009) 'Viral glycosphingolipids induce lytic infection and cell death in marine phytoplankton', *AAAS*, 326(5954), pp. 861–865.
Vartapetian, A., Tuzhikov, A., Chichkova, N., Taliansky, M. and Wolpert, T. (2011) 'A plant alternative to animal caspases: Subtilisin-like proteases', *Cell death and differentiation*. Nature Publishing Group, 18(8), pp. 1289–1297. doi: 10.1038/cdd.2011.49.
Vergunst, A. C. (2000) 'VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells', *Science*, 290(5493), pp. 979–982. doi: 10.1126/science.290.5493.979.
Vergunst, A. C., van Lier, M. C. M., den Dulk-Ras, A., Grosse Stuve, T. A., Ouwehand, A. and Hooykaas, P. J. J. (2005) 'Positive Charge Is An Important Feature Of The C-terminal Transport Signal Of The VirB/D4-translocated Proteins Of Agrobacterium', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0406241102.
Vieira, S., Ribeiro, L., Jesus, B., Cartaxana, P. and da Silva, J. M. (2013) 'Photosynthesis Assessment In Microphytobenthos Using Conventional And Imaging Pulse Amplitude Modulation Fluorometry.', *Photochemistry and photobiology*, 89(1), pp. 97–102. doi: 10.1111/j.1751-1097.2012.01224.x.
Voth, D. E., Broederdorf, L. J. and Graham, J. G. (2012) 'Bacterial Type IV Secretion Systems: versatile virulence machines', 7(2), pp. 241–257. doi: 10.2217/fmb.11.150.Bacterial.

Wagner-Döbler, I., Ballhausen, B., Berger, M., Brinkhoff, T., Buchholz, I., Bunk, B., Cypionka, H., Daniel, R., Drepper, T., Gerdts, G., *et al.* (2010) 'The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: A hitchhiker's guide to life in the sea', *The ISME journal*, 4(1), pp. 61–77. doi: 10.1038/ismej.2009.94.

Wagner-Döbler, I. and Biebl, H. (2006) 'Environmental biology of the marine roseobacter lineage.', *Annual review of microbiology*, 60, pp. 255–280. doi: 10.1146/annurev.micro.60.080805.142115.

van der Wal, P., de Jong, E. W., Westbroek, P., de Bruijn, W. C. and Mulder-Stapel, A. A. (1983) 'Ultrastructural polysaccharide localization in calcifying and naked cells of the coccolithophorid *Emiliania huxleyi*', *Protoplasma*, 118(2), pp. 157–168. doi: 10.1007/BF01293073.

Wallden, K., Rivera-Calzada, A. and Waksman, G. (2010) 'Type IV Secretion Systems: Versatility and diversity in function', *Cellular Microbiology*, 12(9), pp. 1203–1212. doi: 10.1111/j.1462-5822.2010.01499.x.

Wang, H., Tomasch, J., Michael, V., Bhuju, S., Jarek, M., Petersen, J. and Wagner-Döbler, I. (2015) 'Identification of genetic modules mediating the Jekyll and Hyde interaction of *Dinoroseobacter shibae* with the dinoflagellate *Prorocentrum minimum*', *Frontiers in Microbiology*, 6(NOV), pp. 1–8. doi: 10.3389/fmicb.2015.01262.

Wang, R., Gallant, É. and Seyedsayamdost, M. R. (2016) 'Investigation of the genetics and biochemistry of roseobacticide production in the roseobacter clade bacterium *Phaeobacter inhibens*', *mBio*, 7(2), pp. 1–10. doi: 10.1128/mBio.02118-15.

Wang, R. and Seyedsayamdost, M. R. (2017) 'Roseochelin B, an algaecidal natural product synthesized by the roseobacter *Phaeobacter inhibens* in response to algal sinapic acid', *Organic Letters*, p. acs.orglett.7b02424. doi: 10.1021/acs.orglett.7b02424.

Watanabe, N. and Lam, E. (2005) 'Two *Arabidopsis* metacaspases AtMCP1b and AtMCP2b are arginine/lysine- specific cysteine proteases and activate apoptosis-like cell death in yeast', *Journal of Biological Chemistry*, 280(15), pp. 14691–14699. doi: 10.1074/jbc.M413527200.

Welchman, R. L., Gordon, C. and Mayer, R. J. (2005) 'Ubiquitin And ubiquitin-like proteins as multifunctional signals', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm1700.

Wetmore, K. M., Price, M. N., Waters, R. J., Lamson, J. S., He, J., Hoover, C. A., Blow, M. J., Bristow, J., Butland, G., Arkin, A. P., *et al.* (2015) 'Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons', *mBio*, 6(3), pp. 1–15. doi: 10.1128/mBio.00306-15.

Whitman, W., Coleman, D. C. and Wiebe, W. J. (1998) 'Prokaryotes: The unseen majority', *Proceedings of the …*, 95(June), pp. 6578–6583.

Wienhausen, G., Noriega-Ortega, B. E., Niggemann, J., Dittmar, T. and Simon, M. (2017) 'The exometabolome of two model strains of the roseobacter group: A marketplace of microbial metabolites', *Frontiers in Microbiology*. doi: 10.3389/fmicb.2017.01985.

Wilhelm, S. W. and Suttle, C. A. (1999) 'Viruses and nutrient cycles in the sea', *BioScience*, 49(10), pp. 781–788. doi: 10.2307/1313569.

Wilson, W. H., Schroeder, D. C., Allen, M. J., Holden, M. T. G., Parkhill, J., Barrell, B. G., Churcher, C., Hamlin, N., Mungall, K., Quail, M. A., *et al.* (2015) 'Complete genome sequence and lytic phase transcription profile of a coccolithovirus', *Science*, 309, pp. 1090–1092.

Wilson, W. H., Tarran, G., Schroeder, D., Cox, M., Oke, J. and Malin, G. (2002) 'Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel', *Journal of the Marine Biological Association of the United Kingdom*, 82, pp. 369–377.
Wilson, W. H., Tarran, G. and Zubkov, M. V (2002) 'Virus dynamics in a coccolithophore-dominated bloom in the North Sea', *Deep Sea Research Part II: Topical Studies in Oceanography*, 49(15), pp. 2951–2963. doi: 10.1016/S0967-0645(02)00065-6.
Winter, A., Henderiks, J., Beaufort, L., Rickaby, R. E. M. and Brown, C. W. (2014) 'Poleward expansion of the coccolithophore *Emiliania huxleyi*', *Journal of Plankton Research*, 36(2), pp. 316–325. doi: 10.1093/plankt/fbt110.
Wlodkowic, D., Skommer, J. and Darzynkiewicz, Z. (2009) 'Flow cytometry-based apoptosis detection', 559(0), pp. 1–14. doi: 10.1007/978-1-60327-017-5.
Wolfe, G. V, Sherr, E. B. and Sherr, B. F. (1994) 'Release and consumption of DMSP from *Emiliania huxleyi* during grazing by *Oxyrrhi marina*', *Marine Ecology-Progress Series*. doi: 10.3354/meps111111.
Woltering, E. J. (2004) 'Death proteases come alive', *Trends in Plant Science*, 9(10), pp. 469–472. doi: 10.1016/j.tplants.2004.08.001.
Worden, A. Z., Cuvelier, M. L. and Bartlett, D. H. (2006) 'In-depth analysis of marine microbial community genomics', *Trends in Microbiology*. doi: 10.1016/j.tim.2006.06.008.
Wyllie, A. H. and Golstein, P. (2001) 'More than one way to go', *PNAS*, 98(26), pp. 11–13. doi: 10.1073/pnas.98.1.11.
Xu, Q. and Zhang, L. (2009) 'Plant caspase-like proteases in plant programmed cell death', *Plant signaling & behavior*, 4(9), pp. 902–904. doi: 10.4161/psb.4.9.9531.
Yao, N., Tada, Y., Sakamoto, M., Nakayashiki, H., Park, P., Tosa, Y. and Mayama, S. (2002) 'Mitochondrial oxidative burst involved in apoptotic response in oats', *Plant Journal*, 30(5), pp. 567–579. doi: 10.1046/j.1365-313X.2002.01314.x.
Yi, H., Lim, Y. W. and Chun, J. (2007) 'Taxonomic evaluation of the genera *Ruegeria* and *Silicibacter*: A proposal to transfer the genus *Silicibacter* Petursdottir and Kristjansson 1999 to the genus *Ruegeria* Uchino *et al.* 1999', *International Journal of Systematic and Evolutionary Microbiology*. doi: 10.1099/ijs.0.64568-0.
Young, J., Geisen, M., Cros, L., Kleijne, A., Sprengel, C., Probert, I. and Østergaard, J. (2003) 'A guide to extant coccolithophore taxonomy', *Journal of Nannoplankton Research*, p. 125.
Young, J. and Henriksen, K. (2003) 'Biomineralization within vesicles: The calcite of coccoliths', *Biomineralisaton. Reviews in Mineralogy and Geochemistry*, pp. 189–215. doi: 10.2113/0540189.
Yu, L., Lenardo, M. J. and Baehrecke, E. H. (2004) 'Authophagy and caspases: A new cell death program', *Cell Cycle*, 3(9–September), pp. 1124–1126. doi: 10.4161/cc.3.11.1203.
Zazímalová, E., Murphy, A. S., Yang, H., Hoyerová, K. and Hosek, P. (2010) 'Auxin transporters--why so many?', *Cold Spring Harbor perspectives in biology*. doi: 10.1101/cshperspect.a001552.
Zechner, E. L., Lang, S. and Schildbach, J. F. (2012) 'Assembly and mechanisms of bacterial Type IV Secretion machines', *Philosophical Transactions of the Royal Society B: Biological Sciences*.
Zhang, D. C., Li, H. R., Xin, Y. H., Liu, H. C., Chi, Z. M., Zhou, P. J. and Yu, Y. (2008) '*Phaeobacter arcticus* sp. nov., a psychrophilic bacterium isolated from the arctic', *International Journal of Systematic and Evolutionary Microbiology*, 58(6), pp. 1384–1387. doi: 10.1099/ijs.0.65708-0.

Zhang, X., Gamarra, J., Castro, S., Carrasco, E., Hernandez, A., Mock, T., Hadaegh, A. R. and Read, B. A. (2016) 'Characterization of the small RNA transcriptome of the marine coccolithophorid, *Emiliania huxleyi*', *PLoS ONE*. doi: 10.1371/journal.pone.0154279.
Zhang, Y., Higashide, W., Dai, S., Sherman, D. M. and Zhou, D. (2005) 'Recognition and ubiquitination of *Salmonella* Type III Effector SopA by a ubiquitin E3 ligase, HsRMA1', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M506309200.
Zhang, Y., Higashide, W. M., McCormick, B. A., Chen, J. and Zhou, D. (2006) 'The inflammation-associated *Salmonella* SopA Is A HECT-like E3 ubiquitin ligase', *Molecular Microbiology*.
Zhao, W., Dao, C., Karim, M., Gomez-Chiarri, M., Rowley, D. and Nelson, D. R. (2016) 'Contributions of tropodithietic acid and biofilm formation to the probiotic activity of *Phaeobacter inhibens*', *BMC Microbiology*, 16(1), p. 1. doi: 10.1186/s12866-015-0617-z.
Zhou, Y. and Zhu, Y. (2015) 'Diversity of bacterial manipulation of the host ubiquitin pathways', *Cellular Microbiology*. doi: 10.1111/cmi.12384.
Ziveri, P., de Bernardi, B., Baumann, K. H., Stoll, H. M. and Mortyn, P. G. (2007) 'Sinking of coccolith carbonate and potential contribution to organic carbon ballasting in the deep ocean', *Deep-Sea Research Part II: Topical Studies in Oceanography*, 54(5–7), pp. 659–675.
Ziveri, P., Broerse, A. T. C., vanHinte, J. E., Westbroek, P. and Honjo, S. (2000) 'The fate of coccoliths at 48 degrees N 21 degrees W, Northeastern Atlantic', *Deep - Sea Research Part II*.
Zubkov, M. V., Fuchs, B. M., Archer, S. D., Kiene, R. P., Amann, R. and Burkill, P. H. (2001) 'Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea', *Environmental Microbiology*, 3(5), pp. 304–311. doi: 10.1046/j.1462-2920.2001.00196.x.
Zupan, J., Hackworth, C. A., Aguilar, J., Ward, D. and Zambryski, P. (2007) 'VirB1* promotes T-pilus formation in the Vir-type IV Secretion System Of *Agrobacterium tumefaciens*', *Journal of Bacteriology*. doi: 10.1128/JB.00480-07.

Appendix A: supplemental for Chapter 1

This supplemental information accompanies Chapter 1: Introduction, pg.1.

Throughout the duration of my Ph.D. I have contributed to collaborative works that were not included in this thesis. The following is a list of those publications and my contribution.

Mayers T., **Bramucci A.R.**, Yakimovich K., Case R. (2016). A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*. Front. Microbiol. 7:892 10.3389/fmicb.2016.00892

This was a collabrotive work in TM and AB did the flow cytometry and growth curves of the algal strains at various temperatures. The manuscript was drafted by TM, AB, and RC.

Labeeuw L., Khey J., **Bramucci A.R.**, Atwal H., de la Mata P., Haryunk J., and Case R.J. (2016). Indole-3-acetic acid is produced by *Emiliania huxleyi* cocolith-bearing cells and triggers a physiological response in bald cells. *Front. Microbiol.* 7:82810.3389/fmicb.2016.00828

My contribution to this work was conducting the flow cytometry and contributing the flow analysis and figure.

Other publications that are either chapters or are not peer reviewed are listed below:

Labeeuw L., **Bramucci A.R.**, Case R. (2017). Bioactive small molecules mediate microalgal-bacterial interactions. In: Systems Biology of Marine Ecosystems. 1st edition Springer International Publishing (pg. 279-300).

Orata FD, Rosana ARR, Xu Y, Simkus DN, **Bramucci A.R.**, Boucher Y, Case RJ. (2016). Draft genome sequences of four bacterial strains isolated from a polymicrobial culture of naked (N-Type) *Emiliania huxleyi* CCMP1516. Genome Announce. 4(4).

Rosana ARR, Orata FD, Xu Y, Simkus DN, **Bramucci A.R.**, Boucher Y, Case RJ. (2016). Draft genome sequences of seven bacterial strains isolated from a polymicrobial culture of coccolith-bearing (C-Type) *Emiliania huxleyi* M217. Genome Announce. 4(4).

Table A-1. **Summary of known bacterial secretion systems.** Abbreviations: IM=intracellular membrane; PS=periplasmic space; OM=outer membrane; MM=Mycomembrane.

Secretion system	membrane(s) substrate crosses	Possible substrates	Final destination	Gram negative or Gram positive
Sec (general secretory translocase)[1]	1 membrane; bacterial IM	Unfolded proteins	can be inserted into OM or folded in PS and exported via T2/5SS	both[2]
Tat (twin-arginine targeting translocase)[1]	1 membrane; bacterial IM	Folded proteins	can be inserted into OM or exported via T2SS	both[2]
T1SS (ATP-binding cassette translocase)	2 membranes; bacterial IM and OM	polypeptide cargo (i.e. antimicrobial peptides or larger proteins with repeats-in-toxins (RTX) motifs)[3]	extracellular milieu	Gram-negative bacteria[2]
T2SS[4]	1-2 membranes; bacterial IM and OM; or from PS out OM	Folded proteins: Sec proteins folded in PS, Tat proteins, toxins, proteases, cellulases, and lipases	extracellular milieu	Gram-negative bacteria[2]
T3SS	2-3 membranes; bacterial IM, OM, host cytoplasm†; bacterial IM, OM, and eukaryotic OM‡	variety of unfolded proteinaceous substrates	variety of proteinaceous substrates	Gram-negative bacteria[2]
T4SS (conjugation related transporters)	2-3 membranes; bacterial IM, OM, host cytoplasm†; bacterial IM, OM, and eukaryotic OM‡	Conjugation T4SS: ssDNA or entire plasmids[5]	inserted into the target bacterial cells	both[6]
		DNA release/uptake T4SS: ssDNA or dsDNA[7]	extracellular milieu	both[7]
		Effector translocator T4SS: single proteins, protein/protein complexes, protein/DNA complexes[4]	inserted into the target eukaryotic cells	Gram negative[8]

Secretion system	membrane(s) substrate crosses	Possible substrates	Final destination	Gram negative or Gram positive
T5SS (sec-dependent autotransporters)	1 membrane; bacterial OM	unfolded Sec proteins, folded in periplasm	can be inserted into OM or exported extracellular milieu	Gram-negative bacteria[2]
T6SS	2-3 membranes; bacterial IM, OM, extracellular milieu; bacterial IM, OM, and target bacterial OM	Proteins	directly into the cytoplasm of target bacteria	Gram-negative bacteria[24]
T7SS	1-2 membranes; bacterial IM, extracellular milieu; bacterial IM, MM, into extracellular milieu	Proteins and export of Type 1 pili[9]	inserted into OM or extracellular milieu	Gram-positive bacteria and Mycobacteria[2]
T8SS (curli biogenesis pathway)[10]	1-2 membranes; bacterial PS to OM bacterial IM and OM	amyloids called curli, that mediate adhesion to surfaces and biofilm formation	curli are exported and inserted into OM	Gram-negative bacteria[11]
T9SS[12]	1 membrane; bacterial OM to extracellular milieu	Sec transported proteins from the PS	extracellular milieu; or exported and covalently attached to bacterial OM	Gram-negative bacteria

[†]intracellular pathogen

[‡]extracellular pathogen

References used in Table A-1.

1. Natale, P., Brüser, T. & Driessen, A. J. M. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane-Distinct translocases and mechanisms. *Biochimica et Biophysica Acta - Biomembranes* (2008). doi:10.1016/j.bbamem.2007.07.015
2. Green, E. R. & Mecsas, J. Bacterial Secretion Systems: An Overview. in *Virulence Mechanisms of Bacterial Pathogens, Fifth Edition* (2016). doi:10.1128/microbiolspec.VMBF-0012-2015
3. Chang, J. H., Desveaux, D. & Creason, A. L. The ABCs and 123s of bacterial secretion systems in plant pathogenesis. *Annu. Rev. Phytopathol.* (2014). doi:10.1146/annurev-phyto-011014-015624
4. Fronzes, R., Christie, P. J. & Waksman, G. The structural biology of type IV secretion systems. *Nat. Rev. Microbiol.* **7,** 703–14 (2009).
5. Christie, P. J., Whitaker, N. & González-Rivera, C. Mechanism and structure of the bacterial type IV secretion systems. *Biochimica et Biophysica Acta - Molecular Cell Research* (2014). doi:10.1016/j.bbamcr.2013.12.019
6. Wallden, K., Rivera-Calzada, A. & Waksman, G. Type IV secretion systems: Versatility and diversity in function. *Cell. Microbiol.* **12,** 1203–1212 (2010).
7. Chen, I. & Dubnau, D. DNA uptake during bacterial transformation. *Nat. Rev. Microbiol.* (2004). doi:10.1038/nrmicro844
8. Bhatty, M., Laverde Gomez, J. A. & Christie, P. J. The expanding bacterial type IV secretion lexicon. *Res. Microbiol.* (2013). doi:10.1016/j.resmic.2013.03.012
9. Abdallah, A. M. *et al.* Type VII secretion — mycobacteria show the way. *Nat. Rev. Microbiol.* (2007). doi:10.1038/nrmicro1773
10. Cao, B. *et al.* Structure of the nonameric bacterial amyloid secretion channel. *Proc. Natl. Acad. Sci.* (2014). doi:10.1073/pnas.1411942111
11. Barnhart, M. M. & Chapman, M. R. Curli Biogenesis and Function. *Annu. Rev. Microbiol.* (2006). doi:10.1146/annurev.micro.60.080805.142106
12. Lasica, A. M., Ksiazek, M., Madej, M. & Potempa, J. The Type IX Secretion System (T9SS): Highlights and Recent Insights into Its Structure and Function. *Front. Cell. Infect. Microbiol.* (2017). doi:10.3389/fcimb.2017.00215

Table A-2. **Characterization of metazoan apoptosis relies heavily on morphological and physiological features that are not always paralleled in plant and/or algal systems dying of AL-PCD.** This table demonstrates the distribution of various morphologies and physiologies in metazoans, plants, phytoplankton, and *E. huxleyi*. PS = phosphatidylserine. Areas where published information is lacking are noted by (--) symbol.

Apoptosis Morphology/Physiology		**metazoans**	**plants**	**phytoplankton**	***E. huxleyi***
Plasma Membrane	plasma membrane integrity maintained	R[1-5]	R	R[6,7]	--
	Externalized PS residues	R[1,2,4,5,8]	A[9]	P[6,10]	--
Cytoplasmic	Cell shrinkage	R[1,3,5]	P[9,11,12]	P[6]	--
	Condensation of cytoplasm	R[1-3,5]	P[9]	--	--
Nuclear	Nuclear blebbing & fragmentation of nuclear membrane	R[3,13]	P[14,15]	P[6,7,16]	--
	DNA nicking (Tunel Assay)†	R[13,29]	R[9,17-19]	P[6,7,16,20]/A[21]	--
	Internucleosomal cleavage of DNA (DNA laddering)‡	P[1,5]/A[4]	P[17-19] /A[22,23]	P[20]/A[24]	A[25]
	loss of DNA content per cell	N/A	P[22]	P[6]	P[26]
Late stage	cytoplasm released in apoptotic bodies (late-stage)	R[1,3,4]	A[9,11,13,27]	P[16]/A[6]	A[25]
	Lack of inflammatory response	R[1,3]	R[27]	R†	R†
	abrupt loss of PM integrity (late-stage)	A[1,3,4,13]	P[9,22]	A[16]/P	P[28]

†By definition apoptosis and AL-PCD processes should not propagate to nearby or adjoining cells (Danon *et al.*, 2000).

Works Cited in Table A-2.

1. Elmore, S. Toxicol. Pathol. (2007). doi:10.1080/01926230701320337
2. Melino, G., Knight, R. A. & Nicotera, P. Cell Death Differ. 12, 1457–1462 (2005).
3. Kerr, J. F. R., Wyllie, A. H. & Currie, A. R. J. Intern. Med. 258, 479–517 (1972).
4. Kroemer, G. et al. Cell Death Differ. 16, 3–11 (2009).
5. Lockshin, R. A. & Zakeri, Z. Int. J. Biochem. Cell Biol. 36, 2405–2419 (2004).
6. Moharikar, S., D'Souza, J. S., Kulkarni, A. B. & Rao, B. J. J. Phycol. 42, 423–433 (2006).
7. Segovia, M., Haramaty, L., Berges, J. a & Falkowski, P. G. Plant Physiol. 132, 99–105 (2003).
8. Lauber, K., Blumenthal, S. G., Waibel, M. & Wesselborg, S. Mol. Cell 14, 277–287 (2004).
9. Kasuba, K. C., Vavilala, S. L. & D'Souza, J. S. Algal Res. 12, 126–133 (2015).
10. Bidle, K. D. & Bender, S. J. Eukaryot. Cell 7, 223–236 (2008).
11. Reape, T. J. & McCabe, P. F. Apoptosis 15, 249–256 (2010).
12. McCabe, P. F., Levine, A., Meijer, P.-J., Tapon, N. A. & Pennell, R. I. Plant J. (1997). doi:10.1046/j.1365-313X.1997.12020267.x
13. Van Doorn, W. G. & Woltering, E. J. Trends Plant Sci. 10, 117–122 (2005).
14. Wang, H., Li, J., Bostock, R. & Gilchrist, D. Plant Cell (1996). doi:10.1105/tpc.8.3.375
15. Danon, A. & Gallois, P. FEBS Lett. (1998). doi:10.1016/S0014-5793(98)01208-3
16. Jiménez, C. et al. J. Exp. Bot. 60, 815–828 (2009).
17. Chen, S. & Dickman, M. B. J. Exp. Bot. 55, 2617–2623 (2004).
18. Kim, K. S., Min, J.-Y. & Dickman, M. B. Mol. Plant. Microbe. Interact. 21, 605–612 (2008).
19. Li, W., Kabbage, M. & Dickman, M. B. Physiol. Mol. Plant Pathol. 74, 363–375 (2010).
20. Vavilala, S. L., Sinha, M., Gawde, K. K., Shirolikar, S. M. & D'Souza, J. S. Phycologia (2016). doi:10.2216/15-103.1
21. Berges, J. a. & Falkowski, P. G. Limnol. Oceanogr. 43, 129–135 (1998).
22. Bethke, P. C. Plant Cell Online 11, 1033–1046 (1999).
23. Simeonova, E., Sikora, A., Charzyńska, M. & Mostowska, A. Protoplasma 214, 93–101 (2000).
24. Leu, K. L. & Hsu, B. D. Plant Sci. 168, 145–152 (2005).
25. Bidle, K. D., Haramaty, L., Barcelos E Ramos, J. & Falkowski, P. Proc. Natl. Acad. Sci. U. S. A. 104, 6049–6054 (2007).
26. Mayers, T., Bramucci, A., Yakimovich, K. & Case, R. Front. Microbiol. 7, (2016).
27. Danon, A., Delorme, V., Mailhac, N. & Gallois, P. Plant Physiol. Biochem. 38, 647–655 (2000).
28. Bidle, K. D. Ann. Rev. Mar. Sci. 7, 24.1–24.35 (2015).

Appendix B: supplemental for Chapter 2

This supplemental information accompanies Chapter 2: A small volume bioassay to assess bacterial-phytoplankton co-culture using Pulse-Amplitude-Modulated fluorometry, pg. 42.

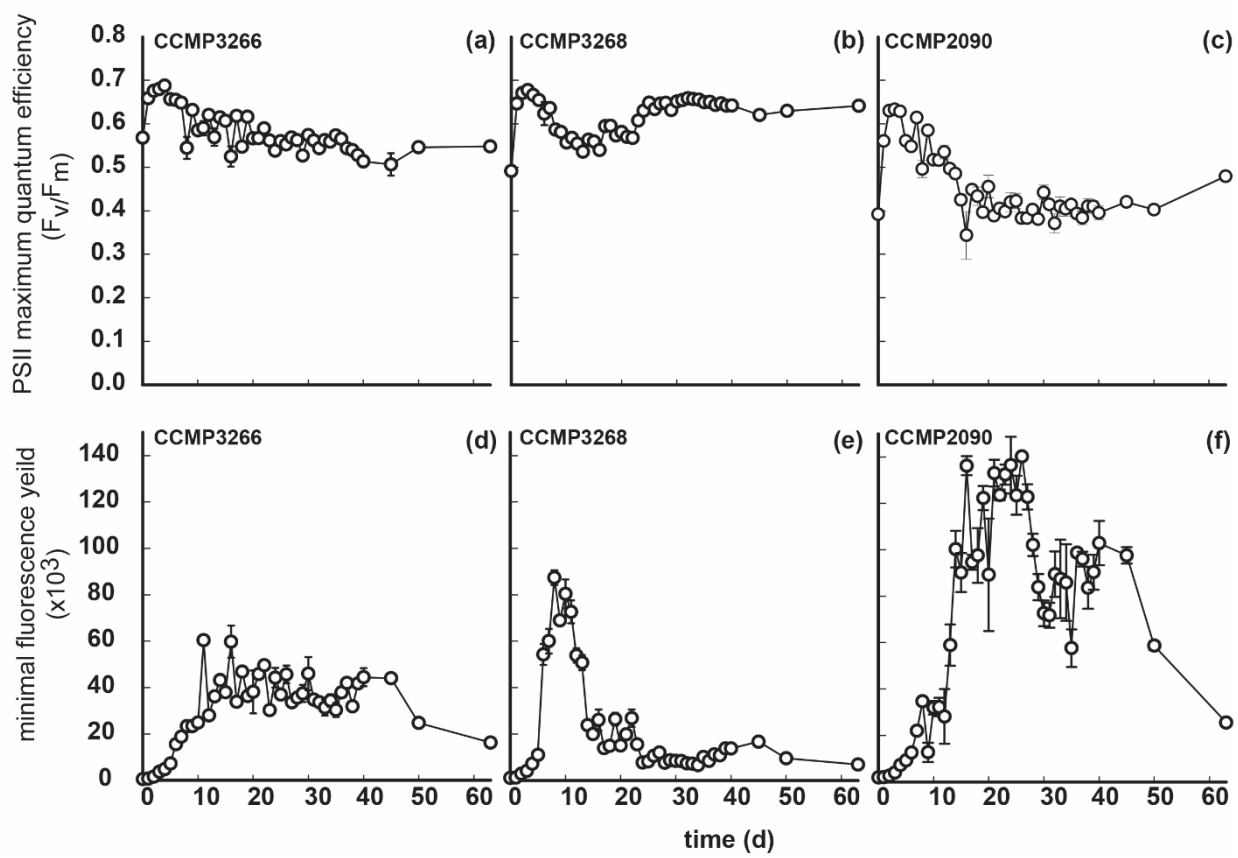

Figure B-1. **Representative PAM fluorometry graphs of a 60 d growth curve of axenic *Emiliania huxleyi* calcifying diploid CCMP3266, non-calcifying haploid CCMP3268, and non-calcifying diploid CCMP2090.** Readings for the potential quantum yield (F_v/F_m) for the three tested *E. huxleyi* cell types (white circles) calcifying CCMP3266 (a), non-calcifying haploid CCMP3268 (b), and non-calcifying diploid CCMP2090 (c). The initial algal fluorescence (F_0) for the three tested *E. huxleyi* cell types calcifying CCMP3266 (d), non-calcifying haploid CCMP3268 (e), and non-calcifying diploid CCMP2090 (f). Error bars represent the standard error between triplicate wells

Appendix C: supplemental for Chapter 3

This supplemental information accompanies Chapter 3: Roseobacter *Phaeobacter inhibens* is a selective pathogen of its algal host *Emiliania huxleyi*, pg.58.

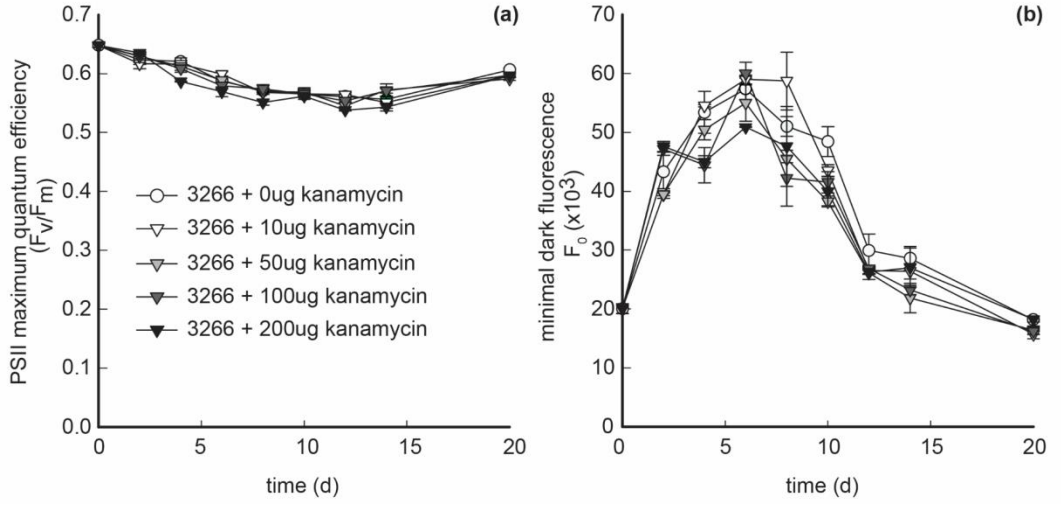

Figure C-1. **There is no effect effect of kanamycin (0-200 ug/ml) on calcifying *Emiliania huxleyi* CCMP3266.** (a) Potential quantum yield (F_v/F_m) of axenic *E. huxleyi* control cultures amended with 0 µg/mL kanamycin (white circles), *E. huxleyi* amended with 50 µg/mL kanamycin (inverted white triangles), *E. huxleyi* amended with 100 µg/mL kanamycin (inverted light grey triangles), *E. huxleyi* amended with 150 µg/mL kanamycin (inverted grey triangles), *E. huxleyi* amended with 200 µg/mL kanamycin (inverted black triangles). And (b) dark adapted state constant fluorescent yield (F_0) of axenic *E. huxleyi* control cultures amended with 0 µg/mL kanamycin (white circles), 10 µg/mL kanamycin (inverted white triangles), 50 µg/mL kanamycin (inverted light grey triangles), 100 µg/mL kanamycin (inverted grey triangles), and 200 µg/mL kanamycin (inverted black triangles).

Appendix D: supplemental for Chapter 5

This supplemental information accompanies Chapter 5: *Phaeobacter inhibens* Type IV Secretion System facilitates pathogenesis of calcifying *Emiliania huxleyi*, pg.112.

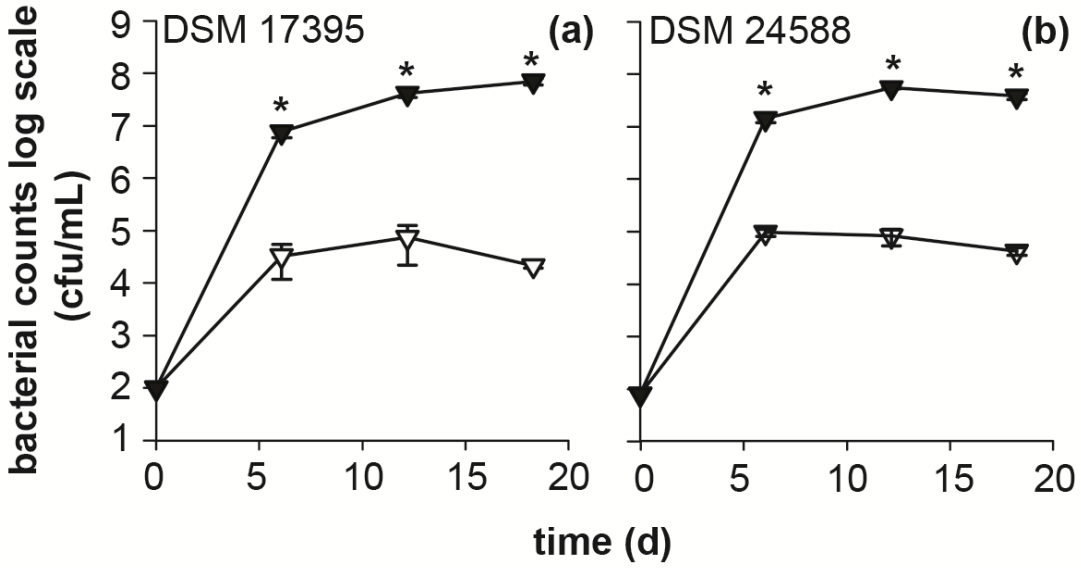

Figure D-1. **Population-wide benefit to *Phaeobacter inhibens* when grown in co-culture with an algal host at 18 °C.** Enumeration of bacteria grown alone in algal media L1-Si (white triangles) and in co-culture with *E. huxleyi* (black triangles), using colony forming units (cfu). (a) *P. inhibens* strain DSM17395 grown alone and in co-culture with *E. huxleyi*, (b) *P. inhibens* DSM24588 grown alone and in co-culture with *E. huxleyi*. Error bars = ± SE of counts for triplicate wells. An asterix (*) over a time point indicates that the cfu/mL of the control and co-culture are statistically different. Statistics done using SigmaPlot 12 with a Student's T-test, p value <0.001.

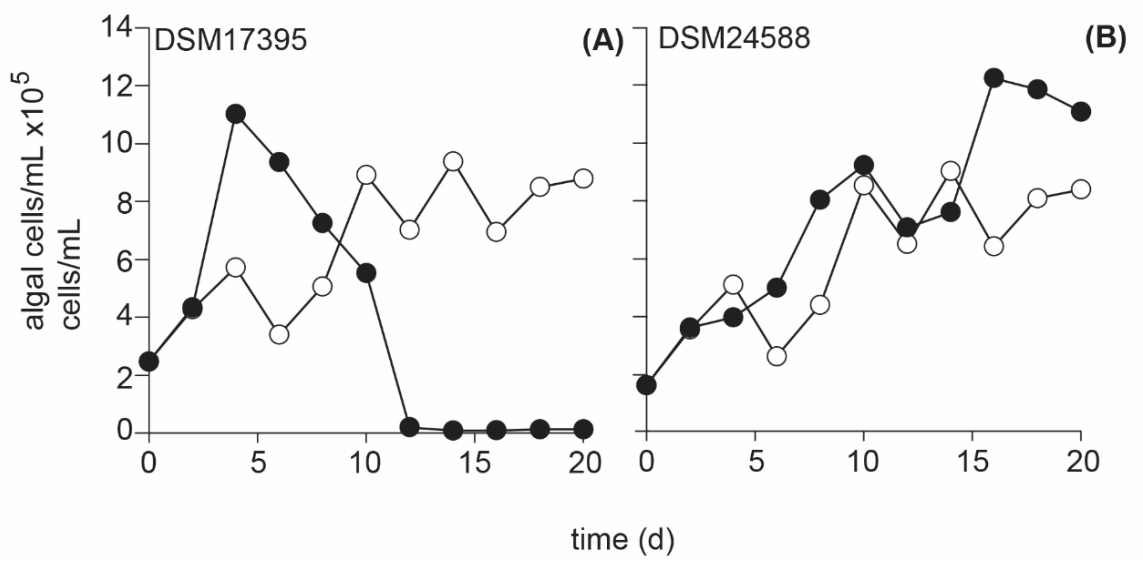

Figure D-2. **Pathogenic *Phaeobacter inhibens* DSM17395 induces decline of *Emiliania huxleyi* CCMP3266 population cells/mL.** Control cultures of E. huxleyi and co-cultures were enumerated using flow cytometry to count SYBR stained algal cells. (a) Co-culture of *E. huxleyi* with *P. inhibens* DSM17395; and (b) Co-culture of *E. huxleyi* with *P. inhibens* DSM24588.

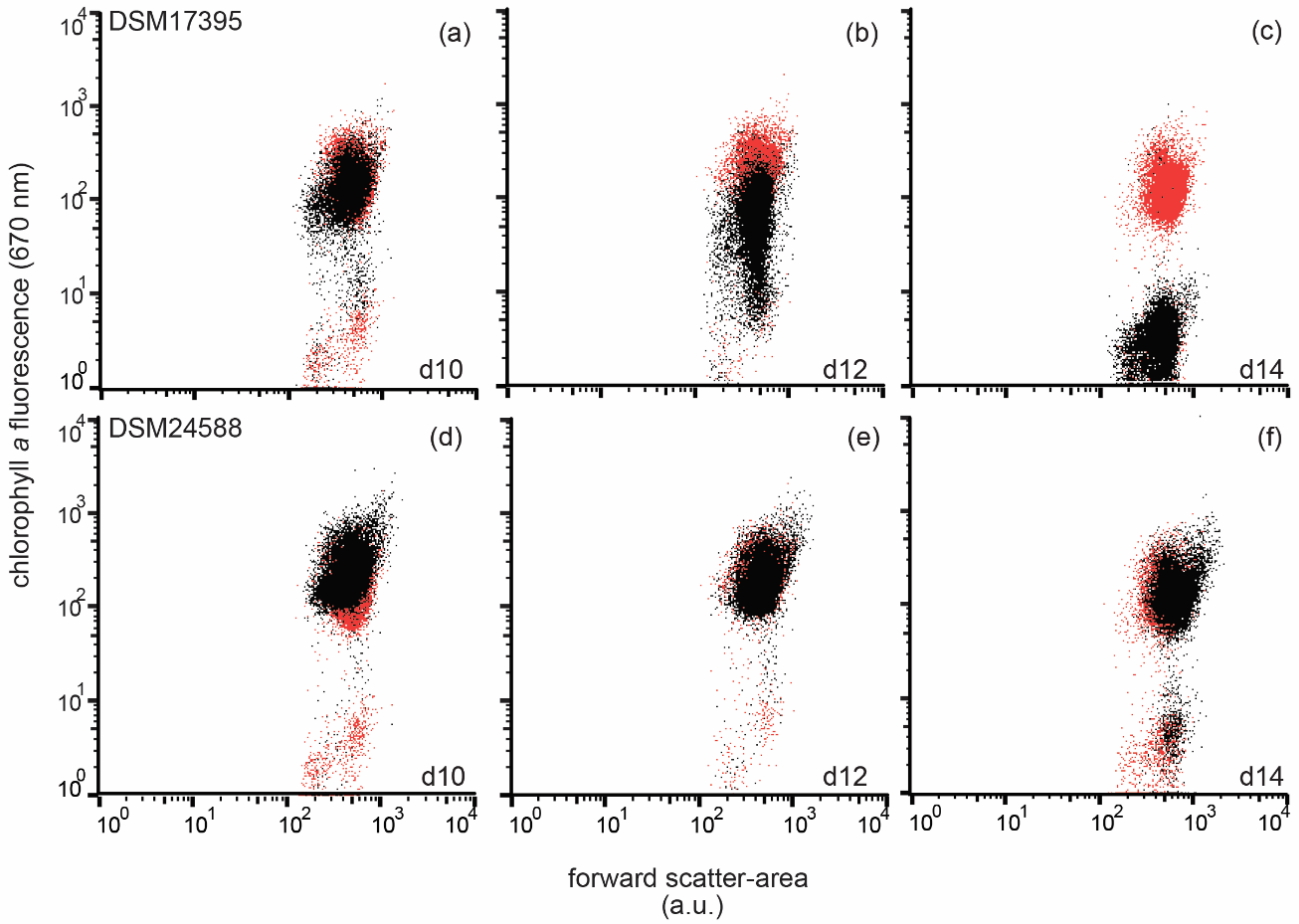

Figure D-3. **Pathogenic *Phaeobacter inhibens* causes a loss of chlorophyll *a* in calcifying *Emiliania huxleyi* CCMP3266.** Control cultures of *E. huxleyi* and co-cultures were assessed for Chlorophyll *a* excitement (488 nm) and emission (670 nm) and cell size (forward scatter area) using flow cytometry. Co-culture of *E. huxleyi* with *P. inhibens* DSM17395 on 10 d (a), 12 d (b), and 14 d (c); and non-pathogenic P. inhibens DSM24588 on 10 d (d), d 12 (e), and d 14 (f). Data for co-cultures are indicated with black dots and control cultures (*E. huxleyi* grown alone) with red dots. The density of the dots is proportional to the density of detection events.

Methods for Figure D-4 (below). Biofilm assay of *P. inhibens* DSM17395 WT and T4SS transposon mutants.

P. inhibens DSM17395 transposon mutants within the *virB* and *virD* operons of the T4SS were maintained on ½ MA amended with 200 µg/mL kanamycin, WT *P. inhibens* DSM17395 was maintained on ½ MA without kanamycin. Cultures were then grown in 5 mL ½ MB (for mutants ½ MB was amended with 200 µg/mL kanamycin). Liquid cultures were then inoculated into fresh 5 mL ½ MB (for mutants ½ MB was amended with 200 µg/mL kanamycin) in borosilicate glass tubes (Fisher Scientific) and grown to early stationary phase at 18 °C in shaking incubator (160 rpm, 30 hr). Biofilm assay, following previously described method was followed (Zhao *et al.*, 2016). Briefly, pellicle and liquid culture was removed (liquid culture was measured for growth at OD600 nm) (Ultrospec 3100 Pro, Fisher Scientific). Then tubes were gently rinsed twice to remove non-attached cells, without disrupting the biofilm. The remaining biofilm was stained with 5 mL of crystal violet for 20 min at room temperature. Unbound dye was discarded and biofilm was rinsed 3 times to ensure all unbound dye was removed and the glass walls without biofilm were clean. Tubes were then allowed to air dry at room temperature. CV stained biofilm was then eluted in 5 mL 2% sodium dodecyl sulphate (SDS) for 30 minutes. CV measurements (absorption: 595 nm) were normalized to the corresponding OD of the culture. Error bars = ± SE for 6 biological replicates.

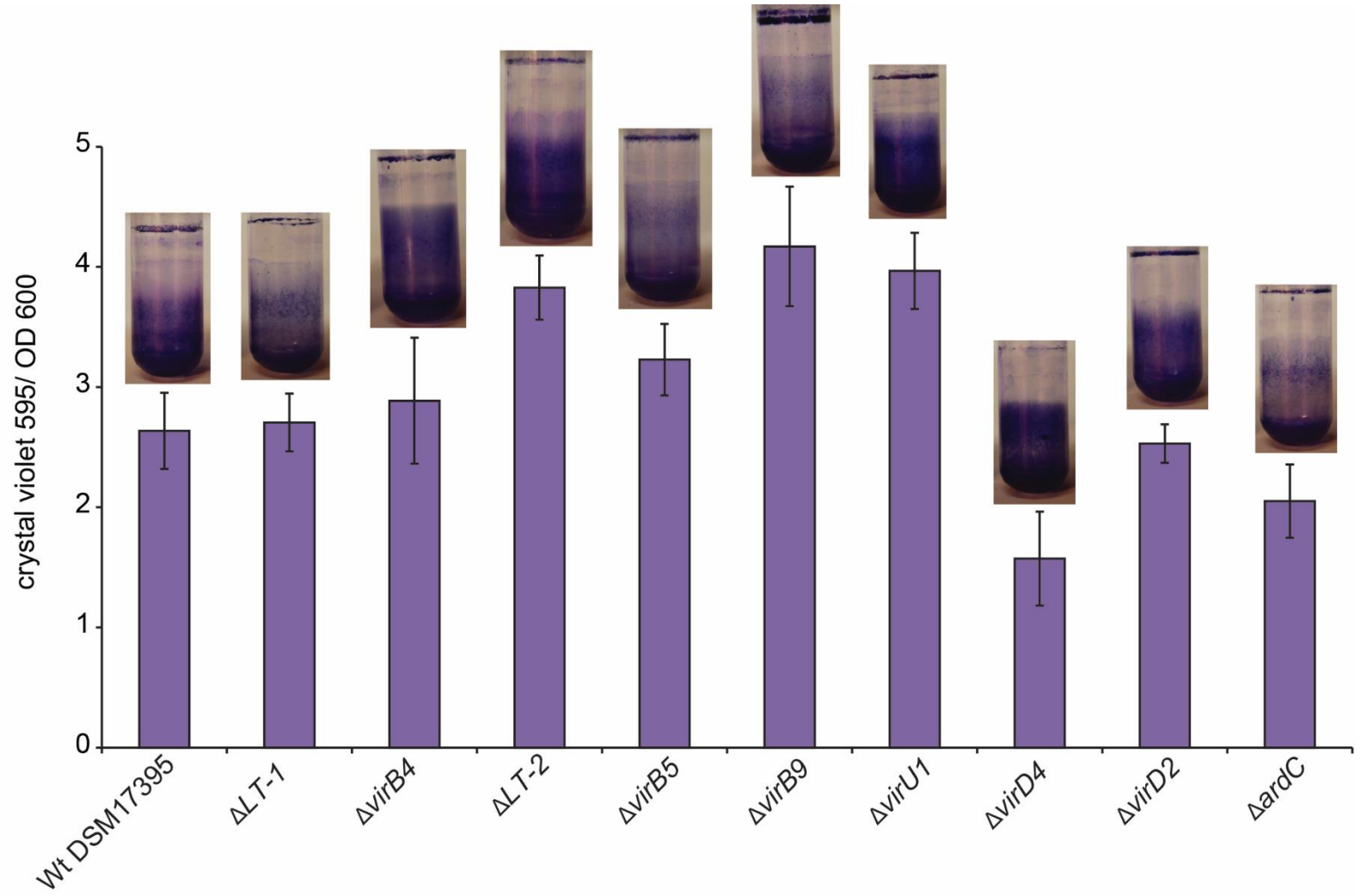

Figure D-4. **Biofilm formation of WT *Phaeobacter inhibens* DSM17395 and *Phaeobacter inhibens* T4SS mutants.** Crystal violet (CV) biofilm assay of *P. inhibens* DSM17395 WT and transposon mutants amended with 200 µg/mL kanamycin grown to early stationary phase at 18 °C in shaking incubator (160 rpm, 30 hr). CV measurements (absorption: 595 nm) were normalized to the corresponding OD of the culture. Error bars = ± SE for 6 biological replicates.

Appendix E: supplemental for Chapter 6

This supplemental information accompanies Chapter 6: General Conclusion, pg.140.

Strains used for Figure E-1 detailed below: The roseobacter strains used in co-culture with calcifying *Emiliania huxleyi* CCMP 3266 include: 1) a more distant endosymbiotic roseobacter 6) *Leisingera* spp. ANG-1 (previously known as *P. gallaeciensis* ANG-1 (Collins *et al.*, 2011)); 2) *Silicibacter* spp. TM1040 (Miller and Belas, 2004), 3) *Ruegeria pomeroyi* DSM15171 (=DSS-3) (formerly *Silicibacter pomeroyi* DSS-3) (Yi *et al.*, 2007). All bacterial strains were grown in co-culture with CCMP3266 as described in Chapter 5.

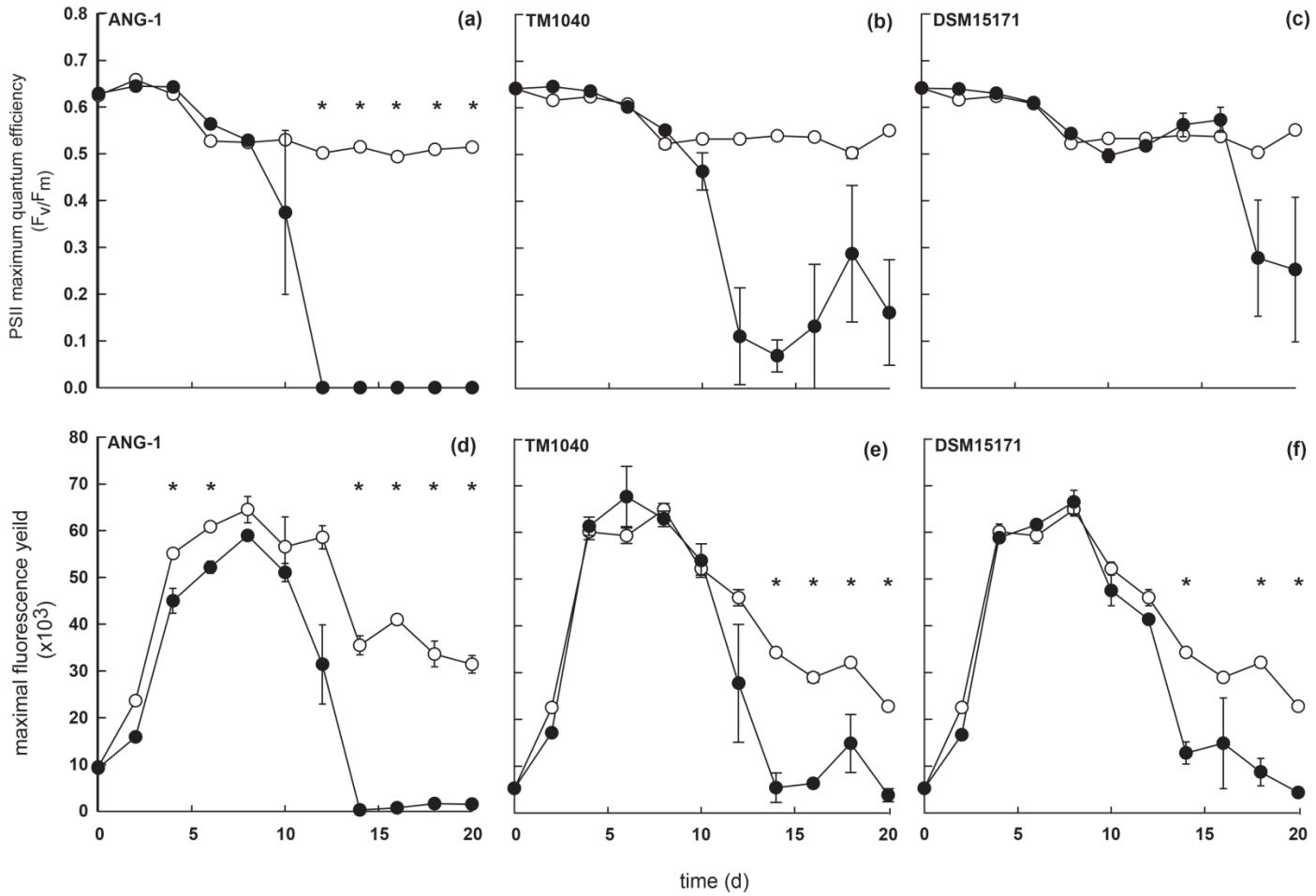

Figure E-1. **Differential pathogenesis of roseobacters on *Emiliania huxleyi* CCMP3266.** Roseobacters were inoculated (concentration: 10-100 cfu/mL) with *E. huxleyi* (concentration: 10^4-10^5 cells/ml) statically at 18 °C for 20 d. Potential quantum yield (F_v/F_m) of axenic *E. huxleyi* (control) (white circles) and co-cultures (black circles): (a) co-culture with *Leisingera* spp. ANG-1 and (b) co-culture with *Silicibacter* spp. TM1040 (black circles), and (c) *Ruegeria pomeroyi* DSM15171 (=DSS-3). A dark adapted state constant fluorescent yield (F_0) of (d) co-culture with ANG-1, (e) TM1040, and (f) DSM15171. Error bars = ± SE. An asterix (*) indicates that the quantum potential yield of the control and co-culture are statistically different. SigmaPlot 12 was used to perform a one-way ANOVA followed by a Tukey test, p value <0.05.