IN HOT WATER: A BACTERIAL PATHOGEN DISPLAYING TEMPERATURE-ENHANCED VIRULENCE OF THE MICROALGA *EMILIANIA HUXLEYI*

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Ecology

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Abstract

Emiliania huxleyi is a globally abundant microalga that plays a significant role in biogeochemical cycles. Over the next century, sea surface temperatures are predicted to increase drastically, which will likely have significant effects on the survival and ecology of *E. huxleyi*. In a warming ocean, this microalga may become increasingly vulnerable to pathogens, particularly those with temperature-dependent virulence. *Ruegeria* is a genus of Rhodobacteraceae whose population size tracks that of *E. huxleyi* throughout the alga's bloom-bust lifecycle. A representative of this genus, *Ruegeria* sp. R11, is known to cause bleaching disease in a red macroalga at elevated temperatures. To investigate if the pathogenicity of R11 extends to microalgae, it was co-cultured with several cell types of *E. huxleyi* near the alga's optimum (18 °C), and at an elevated temperature (25 °C), known to induce virulence in R11. The algal populations were monitored using flow cytometry and pulse-amplitude modulated fluorometry. Cultures of algae without bacteria remained healthy at 18 °C, but lower cell counts in control cultures at 25 °C indicated some stress at the elevated temperature. Both the C (coccolith-bearing) and S (scale-bearing) cell types of *E. huxleyi* experienced a rapid decline resulting in apparent death when co-cultured with R11 at 25 °C, but had no effect on the N (naked) cell type at either temperature. R11 had no initial negative impact on C and S type *E. huxleyi* population size or health at 18 °C, but caused death in older co-cultures. This differential effect of R11 on its host at 18 °C and 25°C suggests that it is a temperature-enhanced opportunistic pathogen of *E. huxleyi.* This is in contrast to the major viral pathogen of *E. huxleyi – Emiliania Huxleyi* Viruses (EhVs). Given that *E. huxleyi* has recently been shown to have acquired resistance against EhVs at elevated temperature, bacterial pathogens with temperature-enhanced virulence, such as R11, may become much more important in the ecology of *E. huxleyi* in a warming climate.

Preface

Chapter 2 of this thesis has been accepted for publication as Mayers, T. J. Bramucci, A. R. Yakimovich, K. M. Case, R. J. 2016. "A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi,*" *Frontiers in Microbiology*, In Press. R. J. Case was the supervisory author and was involved with concept formation and manuscript composition. Myself and R. J. Case were responsible for the formulation of the study and experimental design, I carried out all data collection and analysis and composed the manuscript. K. M. Yakimovich assisted with data collection in pilot pulse-amplitude modulated fluorometry experiments. A. R. Bramucci assisted with flow cytometry data collection and analysis. K. M. Yakimovich and A.R. Bramucci contributed to the editing of the manuscript.

Acknowledgements

Firstly, I would like to thank Dr. Rebecca Case. She has been not only a wonderful supervisor, but a mentor as well. She has encouraged me to follow my true passions and developed my critical thinking skills. I have learned so much by joining her lab – skills I could have developed nowhere else, and ones that will continue to serve me in the years to come.

I would also like to thank my fellow Case lab members, especially Anna Bramucci and Leen Labeeuw, who have provided immeasurable support, encouragement, and love. They are not only outstanding colleagues, but true life-long friends. Thank you also to the many other graduate students I have had the pleasure of working with over the last 2.67 years – Phil (a.k.a. Paul), Fabini, David, Jessica, Rob, Julia, Stephen, Mo – I have truly enjoyed our discussions, socializing, and comradery!

Thank you, also, to my mom, Lynda Mayers, and dad, Ted Mayers, I love you both very much and I would not be who I am today without your constant encouragement of my dreams, interests, and passions. Your unwavering belief in me provides me with strength and conviction.

Also I would like to thank my partner Evan Scheidt, who has been my constant companion throughout this degree. Thank you for being my friend, confidant, distraction, comfort, motivator, and brewing partner.

Finally, thank you to my committee for their advice and support throughout this process.

Cheers,

Teaghan

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Chapter 1: Introduction

1.1 Tiny Photosynthetic Organisms Altered the Course of History

Phytoplankton are composed of microscopic photosynthetic organisms inhabiting the sunlit surface waters of marine and freshwater habitats, which have dramatically shaped the evolution of life on earth. Early ancestors of modern day cyanobacteria (prokaryotic phytoplankton) evolved the ability to store energy in sugars by splitting water molecules using solar energy – a process known as oxygenic photosynthesis, which produces oxygen and water as byproducts (Dismukes et al., 2001). Thus, approximately 2.8 billion years ago, the accumulation of oxygen in the atmosphere began (Dismukes et al., 2001). During the great oxygenation of the earth, cyanobacteria played a vital role as the most abundant photosynthetic organisms, eventually, more complex eukaryotes began to commandeer the photosynthetic abilities of the cyanobacteria via the engulfment, retention, and enslavement (endosymbiosis) of a prokaryotic phototroph by a eukaryotic heterotrophic host (Bhattacharya et al., 2004). The modern diversity of phytoplankton is due to these events, and subsequent endosymbiosis of these newly autotrophic eukaryotes (Keeling, 2013).

1.2 Tiny organisms, big blooms

In the 2.8 billion years since the evolution of photosynthesis forever changed the future of earthly life, phytoplankton (cyanobacteria and eukaryotic microalgae) have continued to play essential roles in maintaining aerobic atmospheric conditions. Phytoplankton growth accounts for approximately half of global primary productivity – turning inorganic carbon into organic sugars – each year (Behrenfeld et al., 2001). Many phytoplankton display a blooming lifestyle – one in which low levels of a species exist year round – but when environmental conditions are right (when a limiting nutrient becomes available), an explosion of growth is triggered (Behrenfeld and Boss, 2014). Concentrations of phytoplankton during these bloom events can be very high (10^5 cells

per milliliter for some eukaryotic microalgae, higher for blooms of cyanobacteria) (Behrenfeld and Boss, 2014), and in some cases, they cover such extensive areas that they can be seen from space (Brown and Yoder, 1994). Blooms provide seasonal bursts of food to an ecosystem, stimulating the proximate trophic web, due to the production of new organic matter in the form of algal biomass (Legendre and Rassoulzadegan, 1995). This biomass production allows phytoplankton to form a base level of the marine trophic web, constituting the diet of a range of organisms from tiny invertebrates – which in turn feed sequentially larger predators – to giant marine mammals. However, only a fraction of the primary productivity of marine phytoplankton is passed up the food chain in this manner (Azam and Malfatti, 2007). The vast majority - in the form of dissolved organic material (DOM) – is consumed by heterotrophic bacteria (Azam and Malfatti, 2007). This DOM is returned to water column upon the death of the bacteria where it can be re-used by other heterotrophic bacteria, or be taken up by phytoplankton for use in further primary production. This recycling of DOM by heterotrophic bacteria is known as the microbial loop and blooms of phytoplankton are known to stimulate its activity due to the release of algal sugars, secondary metabolites, and carbon rich exudates into the surrounding environment (Azam et al., 1983;Azam, 1998).

While algal blooms are critical for maintaining a functional marine ecosystem, blooms of this magnitude can also have negative consequences. When toxin-producing phytoplankton bloom, for instance, such an influx of toxins can cause mass mortalities of invertebrates, fish, seabirds, and marine mammals to result, due to the special role of phytoplankton in the marine trophic structure (Hallegraeff, 1993). The conditions that trigger a bloom are usually tied to seasonal changes and include increased nutrients, light availability, and temperature (Behrenfeld and Boss, 2014). However, different phytoplankton have specific requirements for bloom formation, and the type of bloom that occurs will depend on those requirements being met.

This variation in the requirements of phytoplankton is logical when considered in a phylogenetic context. The phytoplankton is a polyphyletic group, and its membership is merely defined by the ability to perform photosynthesis, microscopic size, aquatic habitat, and ecological role. The organisms constituting the phytoplankton are extremely

taxonomically diverse, including representatives from both the Eukaryotic and Bacterial domains (Litchman et al., 2015).

Cyanobacteria (domain bacteria), are the monophyletic grouping of prokaryotic phytoplankton (Zhaxybayeva et al., 2006). Extant cyanobacteria are present in nearly all habitats (both terrestrial and aquatic) on earth. In the marine environment, they are ubiquitous in the photic zone, and are the most numerous group of phytoplankton (Johnson et al., 2006). In most regions, cyanobacterial biomass production is less than that of the eukaryotic microalgae. In the oligotrophic tropical and subtropical open-ocean, however, cyanobacterial productivity dominates (Johnson et al., 2006). The most common representative of the cyanobacteria is *Prochlorococcus* – the smallest, yet most abundant, photosynthetic organism in the ocean (Partensky et al., 1999b). Like many cyanobacteria, *Prochlorococcus* thrives in nutrient limited waters and these blooms are also known to be highly dependent on temperature (Partensky et al., 1999b). The second most abundant cyanobacteria is *Synechococcus* – more globally widespread than *Prochlorococcus*, due to its ability to tolerate a wide range of UV, temperature, and salinity and to use multiple sources of nitrogen (Partensky et al., 1999a). Unlike *Prochlorococcus*, *Synechococcus* has been shown to bloom in response to nitrogen input due to spring upwelling (Partensky et al., 1999a) – a trigger commonly associated with eukaryotic phytoplankton blooms.

Representatives of the microalgae (eukaryotic phytoplankton) are distributed across the eukaryotic tree of life (Litchman et al., 2015). The main functional bloom-forming groups are the diatoms (stramenopiles), the dinoflagellates (alveolates), and the haptophytes (unresolved phylogenetic affiliation) (Alvain et al., 2008;Litchman et al., 2015). Diatoms, in particular, have a distinctive seasonal periodicity, in which spring upwelling triggers a long-lasting bloom, producing several generations (Smayda, 2002). These blooms tend to occur in coastal areas where upwelling is strongest (Smayda, 2002). The collapse of these blooms is usually caused by the depletion of silicate ions to such a level that new cells cannot re-form their frustules (without which they cannot survive). In contrast, dinoflagellate and haptophyte blooms tend to appear unpredictably and collapse rapidly (Smayda, 2002) – a sequence referred to as ‘bloom-and-bust’. Blooms of these two groups often succeed diatom blooms as they are both tolerant of the

low nutrient conditions left in the wake of a large diatom bloom (Brown and Yoder, 1994;Smayda, 2002).

The most widespread haptophytes include a distinct group of algae called the coccolithophores. Coccolithophores are unique, as the main cell type (coccolith-bearing, C cells) typically produces elaborate calcium carbonate discs called coccoliths. Coccolithophore blooms can be expansive, turning areas in excess of 10^5 km^2 milky-blue due to the reflection of the calcite coccoliths; these swaths of turquoise can even be distinguished from other algal blooms using satellite photos (Brown and Yoder, 1994). Interestingly, this group is quite numerous in subtropical oligotrophic regions, but regularly blooms in temperate regions with seasonal variation in nutrient availability – again, often following nutrient depletion by the spring diatom blooms in coastal areas (Brown and Yoder, 1994).

The coccolithophores are thought to have evolved in the late Triassic period (~225 million years ago) (Bown et al., 2004). Due to their ability to produce calcium carbonate, an unusually continuous fossil record exists for these algae, and this has been used as a proxy for paleo-planktonic productivity (Bown et al., 2004). The evolution of the coccolithophores transferred the majority of marine calcification from the continental shelf (where corals perform this role) into the open ocean (Bown et al., 2004). The coccolithophores eventually became the most productive calcifying organisms on earth – biological carbon pumps transporting carbon from surface waters to the depths (Rost and Riebesell, 2004). This calcification accumulation on the sea floor is known as 'carbonate ooze', and this sediment is where the carbon sequestered by coccolithophores is deposited. These sediments can only form at depths above the calcium carbonate concentration depth (CCD) – the depth below which calcium carbonate dissolution exceeds its formation. Thus, the geological record of the distribution of the extinct coccolithophores and their contributions to global carbon sequestration are largely determined by the topography of the ocean floor (Baumann et al., 2005).

Extant coccolithophores continue to play this unique role in carbon cycling. Due to their coccolith production, they actually remove carbon from surface waters. During the natural senescence (Voss et al., 1998;Chow et al., 2015) and pathogen-induced death of a coccolithophore cell (Wilson et al., 2002), its coccoliths are shed. These coccoliths

are denser than the surrounding seawater, so they sink and are eventually deposited in the deep ocean where the carbon they contain is essentially removed from the carbon cycle (Schmidt et al., 2013).

Coccolithophores also significantly impact the sulfur cycle. As major producers of dimethylsulfoniopropionate (DMSP), the most abundant source of organic sulfur in the ocean, coccolithophores are hypothesized to play a role in regulating earth's climate (Charlson et al., 1987;Ayers and Cainey, 2007). Under conditions that promote increased growth (increased temperature, increased availability of carbon for photosynthesis, etc.), DMSP production is increased (Ayers and Cainey, 2007). DMSP is degraded to dimethyl sulfide (DMS) by bacteria and other phytoplankton, and this serves as the main contributor of cloud condensation nuclei (CCN) in marine environments (Charlson et al., 1987;Ayers and Cainey, 2007;Reisch et al., 2011). An increase in CCN production may lead to increased cloud cover and a subsequent increase in albedo, which has a cooling effect on atmospheric temperature (Charlson et al., 1987;Ayers and Cainey, 2007). However, this feedback hypothesis is highly debated (Quinn and Bates, 2011).

DMSP also plays an important role in driving marine food web dynamics. The open ocean represents a vast expanse of territory devoid of the diversity of life seen in nearshore ecosystems. Planktonic community distribution is heterogeneous (Azam, 1998), and areas of condensed plankton are often where peak trophic activity occurs (Seymour et al., 2010). The DMSP produced by phytoplankton (especially coccolithophores in the oligotrophic open ocean) acts as a chemoattractant to zooplankton, fish, marine birds and mammals, and even marine bacteria which are capable of utilizing DMSP as a source of carbon and sulfur (Seymour et al., 2010).

Despite their major roles in the marine environment, the extant coccolithophores are not a diverse group, and today only a few species make up the vast majority of their biomass globally (Young et al., 2005). By far, the most abundant of these is *Emiliania huxleyi* (Hay and Mohler 1967).

1.3 *Emiliania huxleyi* as a model coccolithophore

Emiliania huxleyi (Prymnesiophyta) is the most common representative of the extant coccolithophores. It is present across their entire range (except for the Arctic and

Antarctic regions), and is able to grow under a wide variety of nutrient regimes, temperatures, and salinities (Paasche, 2002). This species has been shown to overproduce coccoliths under certain environmental conditions – an unusual trait among the coccolithophores (Paasche, 2002). Its blooms – occurring most often in the north Atlantic and Pacific oceans, and in many near-shore seas such as the Black Sea and the Tasman Sea – are thought to be related to low phosphorus, high irradiance conditions, a regimen which discourages the growth of other phytoplankton species, but to which *E. huxleyi* is remarkably tolerant (Paasche, 2002). The life cycle of *E. huxleyi* alternates between diploid C type cells (non-motile coccolith bearing) and haploid S type cells (flagellated with organic scales), both of which are capable of propagating indefinitely via mitosis (Paasche, 2002). There is indirect evidence that S cells are generated through meiosis of C cells, and conversely, C cells are regenerated through syngamy of S cells. However, neither of these processes has been directly observed (Green et al., 1996;Paasche, 2002;von Dassow et al., 2009). A third cell type also exists – diploid non-motile N (naked) cells. These are thought to be naturally occurring variants of C cells lacking the ability to produce coccoliths (Paasche, 2002;Frada et al., 2012).

Although there are many strains of *E. huxleyi* maintained in culture collections, several lines are of particular interest. Strain CCMP1516 – originally a diploid coccolith bearing C type cell – was collected in the South Pacific off the coast of Brazil in 1991, and currently exists in non-axenic culture only. Attempts to make the culture axenic resulted in a permanent switch to the diploid naked N cell type. Interestingly, the non-axenic culture has recently also experienced that same switch to the N cell type. The axenic N cell culture is called CCMP2090 and exists as a pure culture perpetuating itself via mitosis, and no reversion to the C type cell has been reported (Paasche, 2002). Strain CCMP3266 – a C type cell – was collected in the Tasman Sea off the coast of New Zealand in 1998. CCMP3266 was successfully isolated into axenic culture and the coccolith-bearing cell type was retained. Soon after this, the original culture of CCMP3266 was observed to undergo a partial shift to an S type haploid flagellated cell (Frada et al., 2008;von Dassow et al., 2009). The haploid cell was isolated in 1999 and mitotically perpetuates itself in a pure culture. It has been assigned the strain code CCMP3268 (Frada et al., 2008;von Dassow et al., 2009).

In nature, *E. huxleyi* lives in close association with a diverse assemblage of planktonic microbes (Green et al., 2015). Its main predators are members of the microzooplankton (ciliates and heterotrophic Dinoflagellates) (Olson and Strom, 2002). However, the dominant coccolith-bearing cell type of *E. huxleyi* is thought to be a sub-optimal prey species, due to their low nutritional value:size ratio (Olson and Strom, 2002;Harvey et al., 2015). In fact, one study has shown that the consumption of a diet composed of C-type cells limits predator growth rate and allows the continued increase of an *E. huxleyi* population (Harvey et al., 2015).

E. huxleyi also lives in close association with many bacteria and viruses. In particular, bloom collapse has been attributed to outbreaks of EhVs (*Emiliania huxleyi* viruses) – members of the *Phycodnaviridae*, a group of viruses known to infect microalgae (Castberg et al., 2002;Wilson et al., 2009). The EhVs elicit death in *E. huxleyi* by orchestrating the production of a glycosphingolipid within the algal cell, which, once reaching a threshold concentration, triggers algal - programmed cell death (PCD) of the alga (Bidle et al., 2007). The role of PCD in the immune response of land plants to viral and bacterial pathogens has been repeatedly characterized (Lam et al., 2001). The same type of response has been suggested for *E. huxleyi* to prevent outbreaks of disease in large clonal populations (Bidle et al., 2007). Interestingly, the S cell type of *E. huxleyi* is resistant to viral infection by EhVs due to lack of recognition by the viral particles, and may represent an escape strategy (Frada et al., 2008). Additionally, susceptible *E. huxleyi* cells have been shown to become resistant to EhVs with only a 3 °C increase in temperature, due to acquired alterations in the membrane lipids – those involved in viral recognition (Kendrick et al., 2014). In the context of a warming ocean, this acquired resistance potentially leaves an open niche for new invading pathogens (Kendrick et al., 2014).

Interactions between *E. huxleyi* and its bacterial consortia are poorly understood, apart from a handful of examples. For instance, one known mutualistic interaction is that *E. huxleyi* lacks the ability to synthesize vitamin B12 – a nutrient essential to its growth – however, it has been shown that *E. huxleyi* is able to survive in vitamin deficient conditions due to the exogenous production of vitamin B12 by a closely associated bacterium (Helliwell et al., 2011). Most of the bacteria living with *E. huxleyi* are assumed

to be commensals – having no obvious benefit nor posing any detriment to the alga, but gaining a benefit from the algal exudates (Gonzalez et al., 2000).

The main bacterial groups associated with blooms of *E. huxleyi* are the SAR86 clade, the SAR11 clade, and the roseobacter lineage (all Proteobacteria) (Gonzalez et al., 2000). The role of these SAR clades in the ecology of *E. huxleyi* is unclear, however both are thought to use DMSP as a sulfur source (Tripp et al., 2008;Dupont et al., 2012). Representatives of the roseobacter lineage have been shown to possess both known pathways of DMSP degradation, and are hypothesized to play a major role in the sulfur cycling performed by *E. huxleyi* (Varaljay et al., 2012).

1.4 The roseobacter clade as important marine symbionts and pathogens

As previously mentioned, the roseobacter clade (α-Proteobacteria) is one of the most abundant bacterial groups present during *E. huxleyi* blooms (second only to SAR lineages) (Gonzalez et al., 2000). The roseobacter clade represents one of the most abundant heterotrophic bacterial groups occupying the upper photic zone of the ocean (Rappe et al., 2000) and its members are often the primary colonizers of substrates in the marine environment (Dang and Lovell, 2000). There are also many representatives with symbiotic affiliations (Luo and Moran, 2014). Roseobacters have been implicated as both probionts and pathogens in aquaculture (Boettcher et al., 2000;D'Alvise et al., 2012) and coral systems (Raina et al., 2009;Sunagawa et al., 2009;Rypien et al., 2010;Luo and Moran, 2014). There are also a few reports in the literature of roseobacters acting as pathogens on algal species. Roseobacters are known to be the causative agent in the formation of gall-like tumors on the red macroalga, *Prionitis lanceolata* (Ashen and Goff, 1998). Additionally, the roseobacter clade also contains the only known bacterial pathogen of *E. huxleyi – Phaeobacter gallaeciensis (Seyedsayamdost et al., 2011) –* which produces potent algaecides in response to products of algal senescence (Seyedsayamdost et al., 2011).

Another example of an algaecidal roseobacter is *Ruegeria* sp. R11 (hereafter referred to as R11), a strain known to cause bleaching disease in the habitat forming red macroalga, *Delisea pulchra* (Case et al., 2011). *D. pulchra* is an alga native to the waters surrounding southern Australia and the Tasman Sea, and is known to produce several

anti-microbial furanone compounds (De Nys et al., 1995;Manefield et al., 1999). The ability of R11 to cause bleaching events in *D. pulchra* has been demonstrated in both field and laboratory experiments to be temperature-dependent, with the disease only presenting at elevated temperature (Case et al., 2011). R11 was originally isolated from diseased *D. pulchra* in the Tasman Sea (Case et al., 2011) – an area overlapping with one of the global hot spots for *E. huxleyi* blooms (Brown and Yoder, 1994). Thus, it is possible that R11 could also act as a pathogen to *E. huxleyi*, especially given that the only known bacterial pathogen of *E. huxleyi*, *P. gallaeciensis*, is closely related to R11.

1.5 Thesis Objectives

There are many studies that detail bacterial-macroalgal interactions (Weinberger et al., 1994;Ashen and Goff, 1998;Matsuo et al., 2005;Case et al., 2011;Egan et al., 2013). Although the study of bacterial-microalgal interactions is also gaining traction, this is an area of research that warrants increased attention due to the essential role played by this group in global processes (Legendre and Rassoulzadegan, 1995;Ayers and Cainey, 2007). In light of the rapidly changing climate, it is more important than ever to understand the interactions of ecologically important organisms, such as *E. huxleyi*, in the context of a warming ocean. Since pathogen attacks are predicted to increase as SST rises (Harvell et al., 2002), understanding pathogen-microalgal host dynamics is especially important. The research comprising this thesis has two main aims:

1. To test the pathogenicity of the known macroalgal pathogen, *Ruegeria* sp. R11, on the ecologically important coccolithophore *Emiliania huxleyi*, and
2. To assess the role a moderate temperature increase (from 18 °C to 25 °C) will have in these interactions.

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Chapter 2: A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*

2.1 Introduction

Ocean warming is one of the largest contemporary threats to the stability of the marine ecosystem. Since the 1990's, the average global sea surface temperature (SST) has been reported to be rising – an increase as dramatic as 4 °C in some regions – and the rate of increase continues to climb (Smith et al., 2008). In the last century, there has been a global decline in phytoplankton which is strongly correlated to increasing SST (Boyce et al., 2010). This trend is disturbing, given the fact that phytoplankton form the base of the marine food web, and account for over half of the earth's primary productivity annually (Behrenfeld et al., 2001).

Emiliania huxleyi (Prymnesiophyta) is a ubiquitous marine phytoplankton and is the most common representative of the extant coccolithophores (Paasche, 2002). The life cycle of *E. huxleyi* alternates between diploid C type cells (non-motile coccolith bearing) and haploid S type cells (flagellated with organic scales), both of which are capable of propagating indefinitely via mitosis (Paasche, 2002). There is indirect evidence that S cells are generated through meiosis of C cells, and conversely, C cells are regenerated through syngamy of S cells; however, neither of these processes has been directly observed (Green et al., 1996;Paasche, 2002;von Dassow et al., 2009). A third, less common cell type, also exists – diploid non-motile N (naked) cells. These are thought to be naturally occurring mutants of C cells lacking the ability to produce coccoliths (Paasche, 2002;Frada et al., 2012).

E. huxleyi is a model organism and has been studied extensively due to its significant role in global biogeochemical cycles (Simó, 2004). As a major producer of dimethylsulfoniopropionate (DMSP), the most abundant source of organic sulfur in the

ocean, *E. huxleyi* is hypothesized to play a role in regulating earth's climate (Charlson et al., 1987;Ayers and Cainey, 2007). Under conditions that promote increased growth in *E. huxleyi* (increased temperature, increased availability of carbon for photosynthesis, etc.), DMSP production is increased (Ayers and Cainey, 2007). DMSP is degraded to dimethyl sulfide (DMS) by bacteria and phytoplankton, and this serves as the main contributor of cloud condensation nuclei (CCN) in marine environments (Charlson et al., 1987;Ayers and Cainey, 2007;Reisch et al., 2011). An increase in CCN production may lead to increased cloud cover and a subsequent increase in albedo, which has a cooling effect on atmospheric temperature (Charlson et al., 1987;Ayers and Cainey, 2007). However, this feedback hypothesis is highly debated (Quinn and Bates, 2011). Recent work has shown that the production of DMS by *E. huxleyi* cultures decreases at high CO_2 concentrations in mesocosm experiments (Webb et al., 2015). This indicates that one of the marine environments temperature regulation mechanisms may be hindered by anthropogenic CO_2 emissions.

E. huxleyi also plays a unique role in carbon cycling. In addition to organic photosynthate production, *E. huxleyi* produces elaborate calcium carbonate disks (coccoliths) that cover its cells. Although the function of these coccoliths remains unclear, they may aid in UV protection (Gao et al., 2009). During the natural senescence (Voss et al., 1998;Chow et al., 2015) and virally induced (Wilson et al., 2002) death of an *E. huxleyi* cell, its coccoliths are shed. These calcite coccoliths are denser than the surrounding seawater, so they sink and are eventually deposited in the deep ocean where they are essentially removed from the carbon cycle (Schmidt et al., 2013). Since *E. huxleyi* displays a lifestyle in which expansive blooms – sometimes hundreds of thousands of square kilometers in size – appear suddenly and unpredictably, then collapse rapidly (Brown and Yoder, 1994), the influence of this phytoplankton on the sulfur and carbon cycles, as well as the proximate biological ecosystem, is maximized during these bloom events. Because of these major roles in global processes, it is essential to understand the ecology of such an influential organism.

E. huxleyi lives in close association with a diverse assemblage of microbes (Green et al., 2015). This microbial community is defined by complex and intimate metabolic exchange and communication (Sapp et al., 2007). Some members of this microbial

consortium are mutualistic. For example, *E. huxleyi* lacks the ability to synthesize vitamin B12 – a nutrient essential to its growth – however, it has been shown that it is able to survive in culture due to the exogenous production of vitamin B12 by a closely associated bacterium (Helliwell et al., 2011).

Conversely, several microbes associated with *E. huxleyi* are pathogenic. Bloom collapse has been attributed to outbreaks of EhVs – members of the *Phycodnaviridae*, a group of viruses known to infect microalgae (Wilson et al., 2009). The EhVs elicit death in *E. huxleyi* by up-regulating metacaspase activity and causing the associated caspase-like programmed cell death (PCD) of the alga (Bidle et al., 2007). The role of metacaspases and PCD in the immune response of land plants to microbial pathogens has been repeatedly characterized (Lam et al., 2001). The same type of response has been suggested for *E. huxleyi* to prevent outbreaks of disease in large clonal populations (Bidle et al., 2007).

The roseobacter clade (α-Proteobacteria) is one of the most abundant bacterial groups present during *E. huxleyi* blooms (second only to SAR lineages) (Gonzalez et al., 2000), and contains several pathogenic representatives. One such representative has been demonstrated to be the causative agent in the formation of gall-like tumors on *Prionitis lanceolata* (Rhodophyta) (Ashen and Goff, 1998). Additionally, the roseobacter clade also contains the only known bacterial pathogen of *E. huxleyi – Phaeobacter gallaeciensis (Seyedsayamdost et al., 2011)* – which produces potent algaecides in response to products of algal senescence (Seyedsayamdost et al., 2011).

Another example of an algaecidal roseobacter is *Ruegeria* sp. R11, which is a strain known to cause bleaching disease in the habitat forming *Delisea pulchra* – a red macroalgae native to the waters surrounding southern Australia (Case et al., 2011). The ability of R11 to cause bleaching events in *D. pulchra* has been demonstrated in both field and laboratory experiments to be temperature-dependent, with the disease only presenting at elevated temperature (Case et al., 2011). R11 was originally isolated from *D. pulchra* in the Tasman Sea (Case et al., 2011), one of the global hot spots for *E. huxleyi* blooms (Brown and Yoder, 1994). The present study aims to test the pathogenicity of R11 on *E. huxleyi*, a ubiquitous microalgae, which blooms in regions overlaping with *D. pulchra*'s geographical distribution (western and southern Australia,

and New Zealand) (Huisman, 2000), and to assess the role a warming ocean might play in this interaction. Bacterial-macroalgal symbioses have been studied in detail, however fewer bacterial-microalgal interactions have been described (Egan et al., 2013).

It has been predicted that ocean warming will increase the frequency and severity of pathogenic attacks (Harvell et al., 2002). Consequently, it is essential to study the effects of shifting temperature on the biotic interactions of ecologically important organisms, like *E. huxleyi*. In the present study, we demonstrate that R11 is a temperature-enhanced pathogen of both the C and S cell types of *E. huxleyi*, but not N cell type.

2.2 Materials and Methods

2.2.1 Growth and maintenance of algal and bacterial strains

Three axenic strains of *Emiliania huxleyi* were obtained from the Provasoli-Guillard National Centre for Marine Algae and Microbiota (NCMA): a C type diploid coccolith-bearing strain – CCMP3266; an S type haploid sexual strain – CCMP3268; and an N type diploid bald strain – CCMP2090. All strains were maintained in L1-Si media (Guillard and Hargraves, 1993) at 18 °C in a diurnal incubator (8:16 hour dark-light cycle). Algal cultures and media were checked for bacterial contamination prior to use in experiments by microscopic observations and by inoculation onto ½ marine agar (18.7 g Difco Marine Broth 2216 supplemented 9 g NaCl and 15 g Difco agar in 1L). All strains were grown statically for 5 d in the 18 °C incubator under the same light-dark regimen under which they were maintained. These incubation periods allowed the cultures to reach early-log phase prior to the start of an experiment.

The bacterium, *Ruegeria* sp. R11, was maintained on ½ marine agar at 30 °C. It was grown to stationary phase in 5 mL ½ marine broth (18.7 g Difco Marine Broth 2216 supplemented 9 g NaCl) in a shaking incubator (160 rpm) at 21.5 °C for 24 hr prior to experiments.

2.2.2 Control cultures and co-cultures

For each algal strain tested, control cultures of the algae alone and *Ruegeria* sp. R11 alone, as well as a co-culture of R11 and algae, were prepared as previously described by Bramucci *et al.* (Bramucci et al., 2015). Briefly, a stationary phase culture of R11 was grown, washed twice by centrifugation and re-suspended in L1-Si media before undergoing a serial dilution in L1-Si to the target cell concentration within the range of $1x10^2 – 5x10^2$ cfu/mL. To prepare the co-culture, an early log-phase culture (5 d old, $10^4 – 10^5$ cells/mL) was mixed volumetrically 1:1 with the 10^2 cfu/mL R11. Control cultures of both R11 and the algae were prepared by mixing the respective culture volumetrically 1:1 with sterile L1-Si medium, to account for the ½ dilution of the co-culture. The controls and co-culture were then aliquoted in 1 mL volumes into 48-well

microtiter plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Aliquots were dispensed in such a way that three independent replicates of each culture type could be sampled and sacrificed for each time point. This method allowed a time course experiment to be conducted using sacrificial sampling, eliminating the need for re-sampling and reducing the error involved in an experiment with diminishing culture volume (Bramucci et al., 2015).

Half of the microtiter plates were incubated at 18 °C, while the other half were incubated at 25 °C. The microtiter plates were then incubated statically (8:16 hour dark-light cycle) at the respective temperatures for 24 d. This protocol was carried out for each of the three algal strains tested.

2.2.3 Fluorescence measurements

A pulse-amplitude-modulation (PAM) fluorometer (WATER-PAM, Waltz, Effeltrich, Germany) was used to measure photosynthetic yield (Fv/Fm) of cultures containing algae (Schreiber et al., 1986). On sampling days, all samples were taken at the mid-point of the dark cycle (at 4 h) and diluted in L1-Si media to within the detection range of the PAM fluorometer. Samples were kept in the dark and at the appropriate temperature (18 °C or 25 °C) throughout sampling. For each sample, an initial dark adaption period of 3 min was administered, after which a saturating pulse was applied and the fluorescence readings were taken twice at intervals of 1 min 30 sec to calculate the Photosystem II (PSII) potential quantum yield (Fv/Fm) – which indicates the efficiency of PSII (van Kooten and Snel, 1990). Duplicate readings of each sample were averaged and this average was used to determine the Fv/Fm of each sample (in triplicate). After culture death occurred, artificial yield values were detected for some samples. Severe damage to the chloroplasts and calvin cycle has been shown to result in an artificially high yield (1998), and for this reason yield data were reported as not detectable for samples where both chlorophyll content and cell number indicated that the culture was dead. Data were analyzed using SigmaPlot 12.

2.2.4 Enumerating algal and bacterial population density

Algal samples were prepared for flow cytometry from control cultures and co-cultures. Cells were fixed for flow cytometry by incubating in the dark for 10 min with 0.6% glutaraldehyde. Cells were then flash-frozen in liquid nitrogen and stored at -80 °C until flow cytometry was performed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). A single replicate was analyzed for each experimental day. A 488 nm laser was used for excitation and a 670 nm laser was used for detection of chlorophyll. Chlorophyll autofluorescence was used for cellular enumeration. Cells were subsequently stained with SYBR-I (Life Technologies, Carlsbad, CA, USA) for DNA detection (520 nm). Data were processed using FlowJo 9.2. The R11 population density from co-culture experiments was enumerated by counting colony forming units (cfu) to enumerate planktonic R11 cells and those attached to *E. huxleyi* cells. Samples were first vortexed vigorously to remove R11 cells from *E. huxleyi* and reduce bacterial cell clumping. Then a dilution series was prepared in L1-Si media, plated on ½ marine agar and incubated for 2 d at 30 °C.

2.3 Results

2.3.1 Population dynamics of *Emiliania huxleyi* and *Ruegeria* sp. R11 in co-culture

Three cell types of *E. huxleyi* were tested for their interaction with R11 at 18 °C and at 25 °C. For each algal strain tested, control cultures of the algae alone and *Ruegeria* sp. R11 alone, as well as a co-culture of R11 and algae, were prepared as previously described by Bramucci et al (2015).

Coccolith-bearing C type E. huxleyi. At 18 °C, C type *E. huxleyi* (CCMP3266) in co-culture with R11 remained healthy until 14 d, when death of CCMP3266 was observed (Fig. 1A & C). The Photosystem II (PSII) potential quantum yield (Fv/Fm) – a measure of photosynthetic efficiency hereafter referred to as yield (Schreiber, 1998) – of CCMP3266 in co-culture began to drop from 12-14 d, and continued dropping until the damage to PSII resulted in an undetectable yield at 20 d, and did not recover at any subsequent time in the experiment (Fig. 1A). Algal cell numbers followed a similar pattern, with a small decrease occurring from 12-14 d, and a greater decrease to near zero values between 16-20 d (Fig. 1C). In contrast, control cultures of CCMP3266 at 18 °C retained a consistently high yield and cell density throughout the experiment (Fig. 1A & C).

Death was observed much earlier in the co-culture of CCMP3266 with R11 grown at 25 °C compared to 18 °C (Fig. 1B & D). At 25 °C, the yield (Fig. 1B) and algal cell density (Fig. 1D) began to decline by 2 d and reached an undetectable level by 4 d (compared to 14 d and 20 d at 18 °C). A small resurgence in cell density with high yield values was observed on 8 d, which was again undetectable by 12 d and remained so through the experiment (Fig. 1B & D). Like those grown at 18 °C, control cultures of CCMP3266 at 25 °C retained a consistently high yield throughout the experiment (Fig. 1B). CCMP3266 cell density in control culture at 25 °C was initially similar to the control culture at 18 °C ($1.4x10^6$ cells/mL and $1.5x10^6$ cells/mL respectively, on 4 d), but later decreased on 6 d and 8 d to approximately half of it's peak cell density and then experienced large oscillations around this number from 10 d to 24 d (Fig. 1D).

The R11 population density in co-culture with CCMP3266 at 18 °C and 25 °C both increased from 10^2 cfu/mL to 10^7 cfu/mL (Fig. 1E & F).

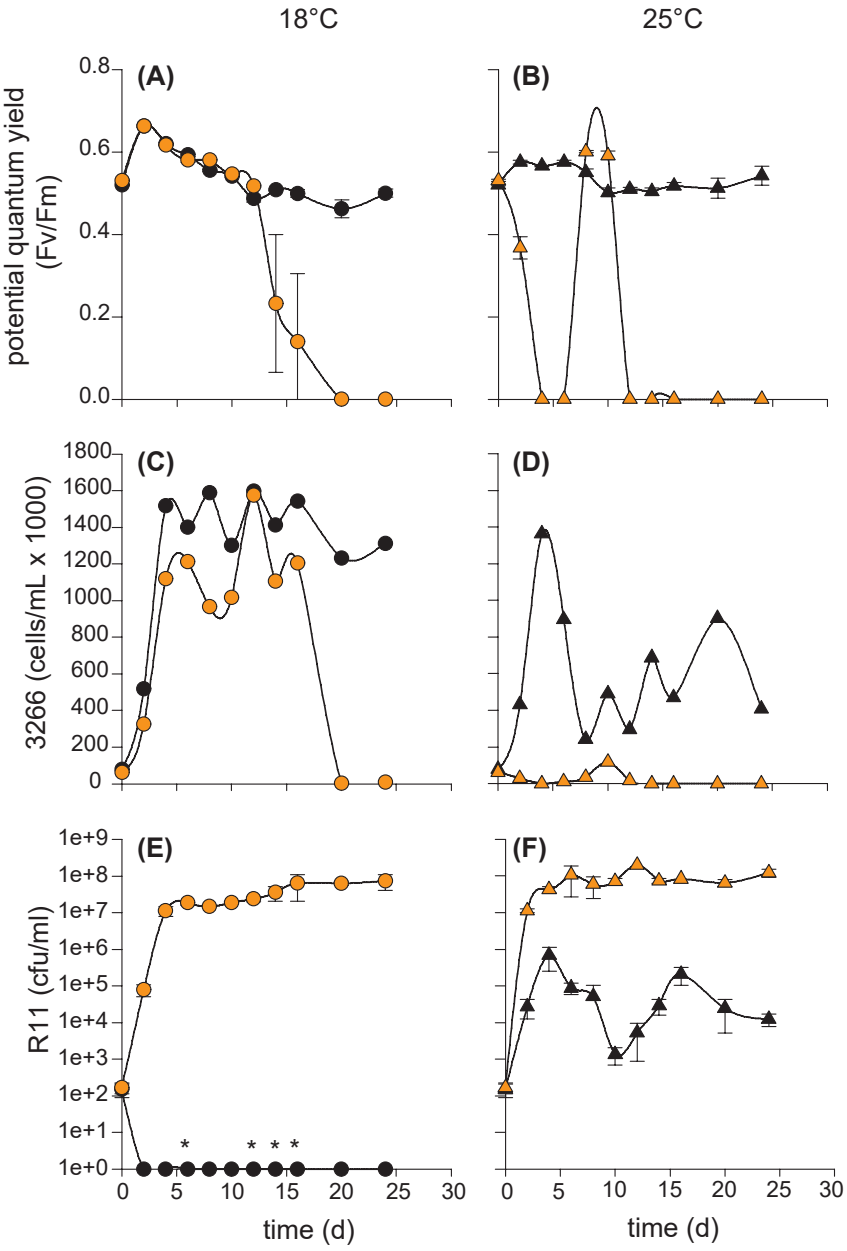

Figure 1: Influence of temperature on co-cultures of *Ruegeria* sp. R11 with C type *Emiliania huxleyi* (CCMP3266). R11 (10^2 cells/ml) was co-cultured with CCMP3266 (10^5 cells/ml) at 18 °C and 25 °C and monitored over 24 days to determine the influence of temperature on co-cultures. For co-cultures and CCMP3266 grown alone, the potential quantum yield (Fv/Fm) was measured at 18 °C **(A)** and 25 °C **(B)**. Algal cell counts (cells/ml) were measured using flow cytometry for co-cultures and CCMP3266 alone at 18 °C **(C)** and 25 °C **(D)**. Bacterial enumeration (cfu/ml) was performed for R11 alone and in co-culture with CCMP3266 in L1 medium at 18 °C **(E)** and 25 °C **(F)**. Data for R11-CCMP3266 co-cultures are indicated with orange data points and control cultures (R11 or CCMP3266 grown alone) with black data points. All control cultures and co-cultures were performed in triplicate. Error bars = ± SE. Asterisks signify days on which R11 colonies were detected, however the cfu numbers were insufficient to count.

However, the 25 °C co-cultures reached this cell density faster (on 2 d) than the co-culture at 18 °C (on 4 d) (Fig. 1E & F). At both temperatures, the R11 populations benefited from the presence of CCMP3266. At 18 °C, control R11 (bacteria alone) population density crashed by 2 d (Fig. 1E), while at 25 °C, the R11 population remained present, but experienced large oscillations from 10^3-10^6 cfu/mL for the remainder of the experiment (Fig. 1F). After the population density crashed at 18 °C on 2 d, occasional R11 colonies was detected in some bacterial control samples, however the cfu numbers were insufficient to count. The fact that the R11 population in pure bacterial cultures remained present at a significant level throughout the experiment at 25 °C but not 18 °C, when it grows well in ½ marine broth at 18 °C, suggests that R11 does not thrive in L1-Si media at 18 °C due to the additive effects of a low nutrient medium and low temperature. *Scale-bearing swarming S type E. huxleyi.* Similar to the C cell type (CCMP3266), the co-culture of the S cell type (CCMP3268) with R11 at 18 °C remained healthy until 10 d, after which death was observed (Fig. 2A & C). The yield began to decline on 10 d, becoming negligible by 14 d and no recovery being observed by 24 d (Fig. 2A). Both control (CCMP3268 alone) and co-culture S cell density experienced a rapid increase from 0-4 d, but declined after 6 d (Fig. 2C). The CCMP3268 control culture cell density remained steady at this lower level for the remainder of the experiment (Fig. 2C). However, the algal cell density of the co-culture kept declining, approaching zero by 12 d, where it remained until the end of the experiment (Fig. 2C).

Death was observed much earlier in the co-culture of CCMP3268 with R11 at 25 °C in comparison to 18 °C (Fig. 2B & D). At this higher temperature, the yield (Fig. 2B) and cell count (Fig. 2D) of the co-culture were similar to control values (no R11) on 2 d, but had crashed by 4 d. Algal cell density remained near zero for the remainder of the experiment (Fig. 2D). Co-culture yield values also remained undetectable, except for an anomaly on 16 d where a single replicate gave a detectable reading (Fig. 2B). This type of outlier is due to the nature of the sacrificial sampling method and was observed in replicate experiments (results not shown). On a given sampling day, three replicates of the 1 mL wells are sacrificed and sampled for each culture type (algal control, bacterial control, and co-culture). At 25 °C, control cultures of CCMP3268 retained a high yield for 10 d after the death of the co-culture, falling significantly only on 16 d (Fig. 2B).

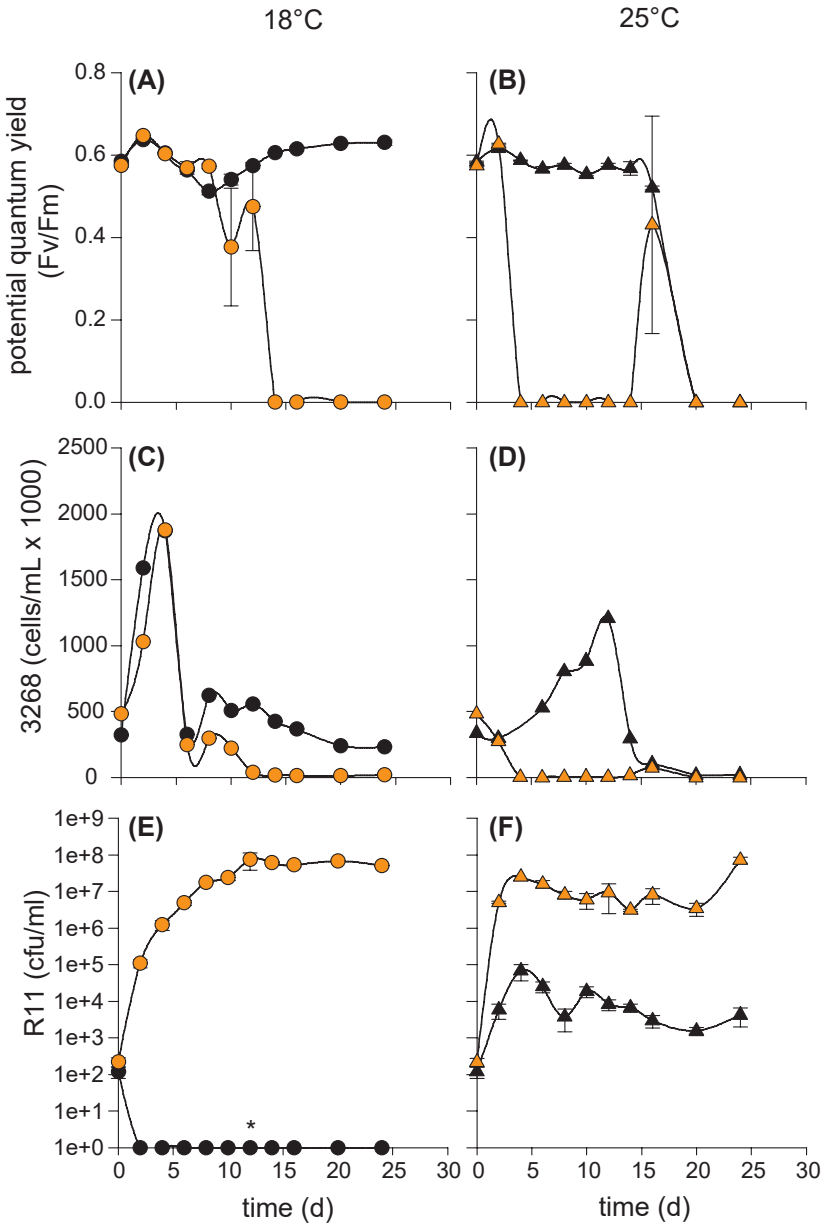

Figure 2: Influence of temperature on co-cultures of *Ruegeria* sp. R11 with S type *Emiliania huxleyi* (CCMP3268). R11 (10^2 cells/ml) was co-cultured with CCMP3268 (10^5 cells/ml) at 18 °C and 25 °C and monitored over 24 days to determine the influence of temperature on co-cultures. For co-cultures and CCMP3268 grown alone, the potential quantum yield (Fv/Fm) was measured at 18 °C **(A)** and 25 °C **(B)**. Algal cell counts (cells/ml) were measured using flow cytometry for co-cultures and CCMP3268 alone at 18 °C **(C)** and 25 °C **(D)**. Bacterial enumeration (cfu/ml) was performed for R11 alone and in co-culture with CCMP3268 in L1 medium at 18 °C **(E)** and 25 °C **(F)**. Data for R11-CCMP3268 co-cultures are indicated with orange data points and control cultures (R11 or CCMP3268 grown alone) with black data points. All control cultures and co-cultures were performed in triplicate. Error bars = ± SE. Asterisks signify days on which R11 colonies were detected, however the cfu numbers were insufficient to count.

Compared to the control culture at 18 °C, which grew to ~1.5x10^6 cells/mL by 4 d and maintained this cell density throughout the experiment, the cell density of the CCMP3268 control culture at 25 °C increased slowly, peaking at ~1.3x10^6 cells/mL on 12 d, after which it followed the same pattern as the yield (Fig. 2D). Neither the yield, nor the cell count recovered by the end of the experiment.

R11 populations attained equally high density (10^7 cfu/mL) in co-culture with CCMP3268 at both 18 °C and 25 °C (Fig. 2E & F). Similar to the co-culture with CCMP3266, this level was achieved twice as quickly at 25 °C as at 18 °C (Fig. 2E & F). Control populations of R11 reached the same density as control bacterial populations from the CCMP3266 co-culture experiment, crashing rapidly at 18 °C (on 2 d) and maintaining their population at 25 °C at a lower level than in the co-culture (Fig. 2E & F).

Bald N type E. huxleyi. At 18 °C and 25 °C, both the co-culture and control culture of the N type *E. huxleyi* cells (CCMP2090) retained a high yield through 24 d (Fig. 3A & B). Death was never observed in these co-cultures. Co-cultures were established with the same density of R11 (10^2 cfu/mL) and reached the same peak density (10^7 cfu/mL) as the CCMP3266 and CCMP3268 co-cultures. Absolute numbers of R11 were not quantified throughout the experiment, but its presence was confirmed with drop plating of the co-culture on ½ marine agar at every sampling point. Flow cytometry was not run for this cell type, as no effect of co-culturing on yield or minimum fluorescence (a proxy for chlorophyll fluorescence, data not shown) was observed.

2.3.2 Observation of algal bleaching in *E. huxleyi* and *Ruegeria* sp. R11 co-cultures

Since R11 is known to cause bleaching in the macroalga *D. pulchra* (Case et al., 2011), the bleaching effect of R11 on *E. huxleyi* was assessed using flow cytometry. R11 pathogenesis of *E. huxleyi* caused the loss of chlorophyll autofluorescence, or bleaching, of CCMP3266 (Fig. 4) and CCMP3268 (Fig. 5). At 18 °C, co-culture populations of CCMP3266 and CCMP3268 with R11 are indistinguishable from control populations (algae alone) at 12 d and 8 d respectively, when the chlorophyll autofluorescence (670 nm) of cells was plotted against the forward scatter for both populations (Fig. 4A & 5A).

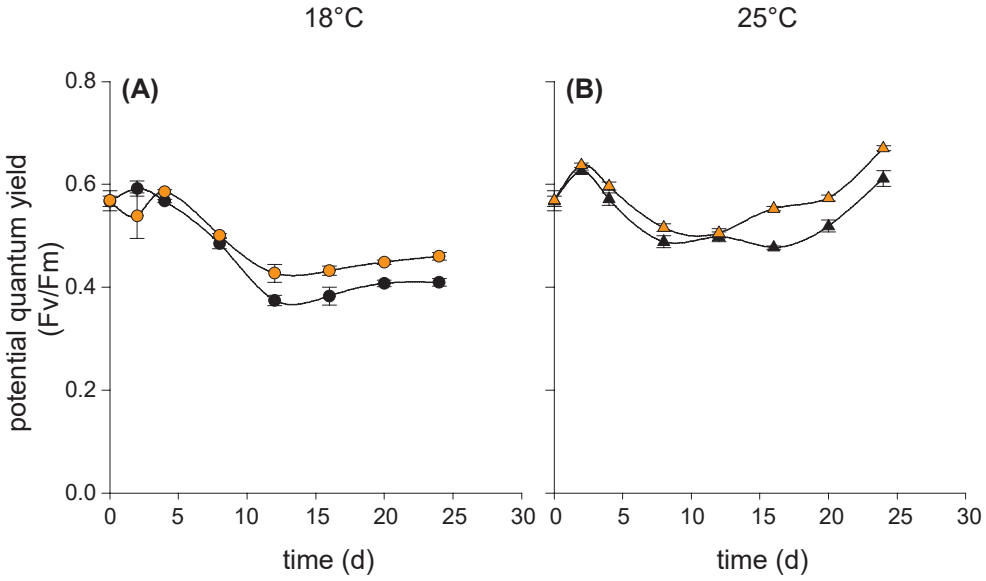

Figure 3: Influence of temperature on co-cultures of *Ruegeria* sp. R11 with N type *Emiliania huxleyi* (CCMP2090). R11 (10^2 cells/ml) was co-cultured with CCMP2090 at 18 °C and 25 °C and monitored over 24 days to determine the influence of temperature on co-cultures. For co-cultures and CCM2090 grown alone, the potential quantum yield (Fv/Fm) was measured at 18 °C **(A)** and 25 °C **(B)**. Data for R11-CCMP2090 co-cultures are indicated with orange data points and control cultures (CCMP2090 grown alone) with black data points. All control cultures and co-cultures were performed in triplicate. Error bars = ± SE.

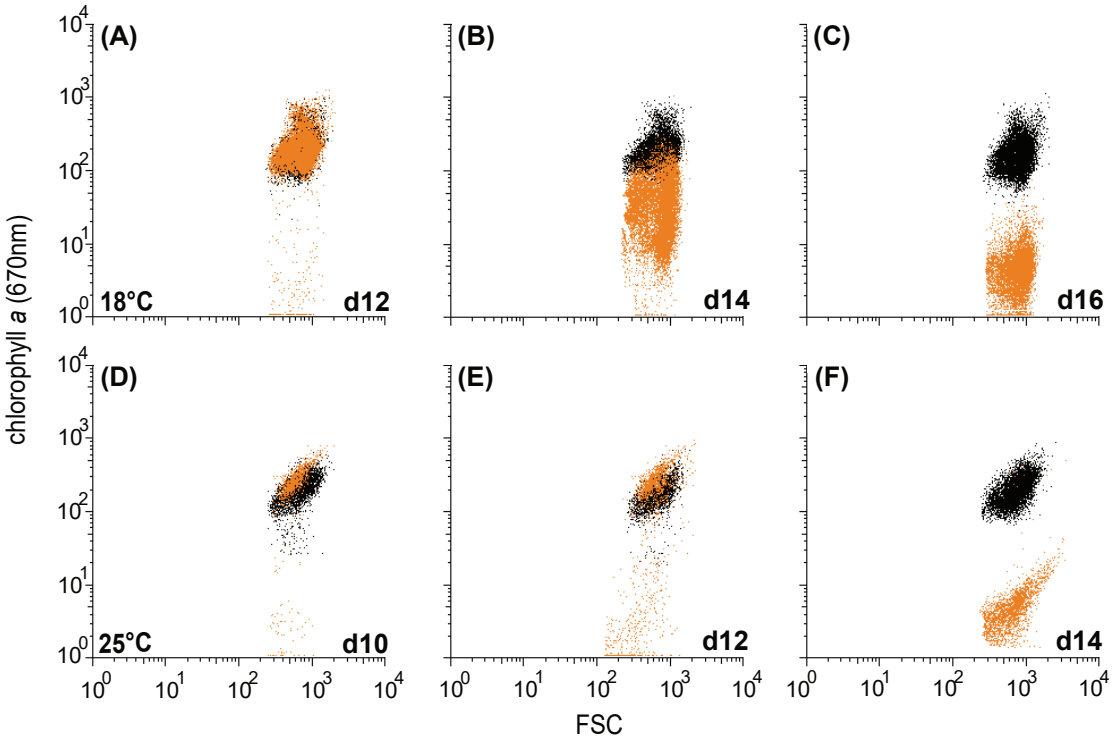

Figure 4: Bleaching in C type *Emiliania huxleyi* (CCMP3266) in co-culture with *Ruegeria* sp. R11. Control cultures of CCMP3266 and co-cultures of CCMP3266 with R11 were assessed for chlorophyll content using flow cytometry at 18 °C on 12 d **(A)**, 14 d **(B)**, and 16 d **(C)**; and at 25 °C on 10 d **(D)**, d 12 **(E)**, and d 14 **(F).** Data for R11-CCMP3266 co-cultures are indicated with orange data points and control cultures (CCMP3266 grown alone) with black data points.

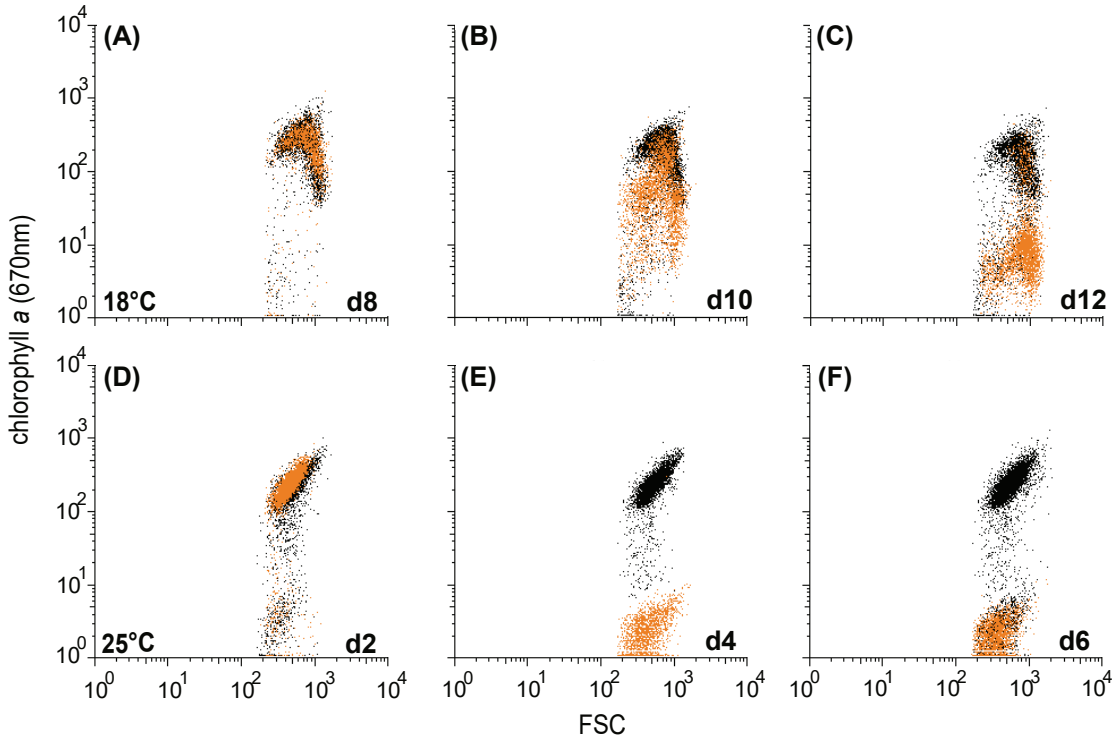

Figure 5: Bleaching in S type *Emiliania huxleyi* (CCMP3268) in co-culture with *Ruegeria* sp. R11. Control cultures of CCMP3268 and co-cultures of CCMP3268 with R11 were assessed for chlorophyll content using flow cytometry at 18 °C on 8 d **(A)**, 10 d **(B)**, and 12 d **(C)**; and at 25 °C on 2 d **(D)**, 4 d **(E)**, and 6 d **(F).** Data for R11-CCMP3268 co-cultures are indicated with orange data points and control cultures (CCMP3268 grown alone) with black data points.

On 14 d and 10 d, when yield and cell density indicated the start of algal decline (Fig. 1A & C; Fig. 2A & C), CCMP3266 and CCMP3268 cells lost chlorophyll autofluorescence, but retained forward scatter values (Fig. 4B & 5B). This shows that the algae lose chlorophyll autofluorescence before cell size (i.e. lysis). This decrease in chlorophyll autofluorescence happened relatively gradually (compared to 25 °C), resulting in a 'smear' of cells in the process of losing chlorophyll on the scatter plot. On days where yield and cell density were near zero, a population of cells was present with the same forward scatter as control cultures, but almost all fluorescence (chlorophyll) was gone (Fig. 4C & 5C).

At 25 °C, the decrease in chlorophyll content occurred much more quickly, and the gradual loss observed at 18 °C is not present (Fig. 4 & 5). Instead, the co-cultures appear to experience a rapid loss of fluorescence (chlorophyll) from populations similar to control cultures with no bacteria (same forward scatter and chlorophyll values), to populations of cells with the same forward scatter as control cultures, but a low level of fluorescence (chlorophyll) by the next time point (Fig. 4 & 5).

As a secondary measure of culture death, the integrity of the DNA was measured using flow cytometry throughout the experiment. DNA integrity was lost in both CCMP3266 (Fig. S1) and CCMP 3268 (Fig. S2) following the same general pattern as the chlorophyll bleaching.

2.4 Discussion

2.4.1 *Ruegeria sp.* R11 pathogenicity varies between cell types of *Emiliania huxleyi*

The present study demonstrates that *Ruegeria* sp. R11 is a pathogen of *E. huxleyi*, but that this pathogenicity, or host resistance, is strain dependent. Both *E. huxleyi* CCMP3266 and CCMP3268 are killed when in co-culture with R11 (Fig. 1 & 2, respectively). CCMP3268, an S type haploid flagellated cell, was originally isolated from cultures of CCMP3266, a C type diploid coccolith bearing cell isolated from the Tasman Sea, after part of the original culture of CCMP3266 was observed to undergo a shift to this haploid cell type (Frada et al., 2008;von Dassow et al., 2009). It is hypothesized that CCMP3268 is the sexual cell of CCMP3266. However, since neither meiosis nor syngamy has ever been directly observed, this cannot be confirmed. Given this relationship between CCMP3266 and CCMP3268, it follows that they should be similar in their sensitivity to pathogens unless the cell type conveyed resistance. Transcriptomic analyses have shown that these strains display ~50% transcript similarity, with the major functional differences relating to motility and biogenic $CaCO_3$ production – the haploid CCMP3268 cells are flagellated while the diploid CCMP3266 cells are coccolith bearing (von Dassow et al., 2009). However, it has been shown that while CCMP3266 is sensitive to the bloom-collapse causing EhVs, CCMP3268 is resistant due to a lack of host recognition by the virus (Frada et al., 2008). As opposed to the C or S type strains, bald N type *E. huxleyi* CCMP2090 is seemingly resistant to infection by R11 (Fig. 3), although interestingly, CCMP2090 is susceptible to two of the major strains of EhV (EhV1 and EhV86) (Fulton et al., 2014). Since the bacteria infects different cell types than the previously described EhVs, the complex interplay between these two algal pathogens will be important to understand as the ecology of *E. huxleyi* blooms.

It is unclear from the present study what is the key difference between the *E. huxleyi* strains that causes the observed variability in susceptibility to R11 infection, but there are several possibilities. CCMP2090 is the diploid axenic non-coccolith bearing (bald) isolate of the diploid coccolith bearing CCMP1516, which was collected off the coast of Ecuador, making it geographically distant from both CCMP3266 and R11, which were both isolated from the Tasman Sea. The ability of phylogenetically closely related

strains of the Roseobacter clade to induce gall formation on species of *Prionitis* has been shown to be geographically specific – strains of gall-forming roseobacter isolated from infected *Prionitis* in one geographical area were unable to induce gall formation in closely related *Prionitis* from a distant geographical location (Ashen and Goff, 2000). Similarly, it has been demonstrated in land plants that geographically distant subpopulations of a single species can differ in their resistance/susceptibility to pathogens, likely due to decreased interbreeding (Thrall et al., 2001). Additionally, the nature of the CCMP2090 cell – naked, lacking both coccoliths and organic scales – may contribute to the differences in sensitivity between strains. It may be that R11 has different levels of attachment and/or colonization of the naked, organic or calcite liths covering *E. huxleyi's* cell surface.

N type cells, like CCMP2090, are thought to be a rare natural variant of C type cells, such as CCMP3266 (Paasche, 2002). However, if the mutation that causes the non-calcifying N cell type to occur provides an escape strategy from pathogens, the abundance and distribution patterns of this cell type may change in the future, and this would have consequences for the carbon sequestration role of *E. huxleyi*.

Although the mode of R11's pathogenesis is not currently known, several virulence factors have been identified, including the production of ammonia (inhibits photosynthesis), cytolytic toxins (lyses cells) (Fernandes et al., 2011), and glutathione peroxidase (resists oxidative bursts from the host) (Gardiner et al., 2015). It has also been hypothesized that the virulence of R11 may be related to its production of idole-3-acetic acid (IAA) – a phytohormone with various roles in the growth and development of land plants, known to be produced by R11 (Fernandes et al., 2011). An extracellular excess of IAA causes hypertrophy and may increase the amount of algal exudates available to R11 (Fernandes et al., 2011). Interestingly, it has recently been shown that the exogenous addition of IAA causes increased cell permeability in CCMP2090, but not in CCMP3266 (Labeeuw et al., 2016). Thus, the role of IAA in the virulence of R11 on *E. huxleyi* may be more complex than previously thought, since CCMP2090 is sensitive to the effects of IAA, but is not susceptible to the virulence of R11.

2.4.2 The virulence of R11 towards *E. huxleyi* is temperature-enhanced

The decrease in CCMP3266 and CCMP3268 health observed when grown in co-culture with R11 at 25 °C compared to 18 °C indicates that the pathogenicity of this bacterium towards them is temperature-enhanced. While R11 ultimately causes the death of CCMP3266 and CCMP3268 at both 18 °C and 25 °C, the course of the infection is accelerated at elevated temperature (Fig. 1A-D; Fig. 2A-D). This increase in the pathogenicity of R11 at elevated temperature cannot be explained by differential bacterial loads, as the R11 populations reached the same order of magnitude (10^7 cfu/mL) in co-culture with both algal strains at both temperatures (Fig. 1E & F; Fig. 2E & F). Although R11 attains this carrying capacity 2-4 days earlier at 25 °C than at 18 °C, this is also insufficient to explain the differences (Fig. 1E & F; Fig. 2E & F). With CCMP3266, the initial drop in algal yield at 25 °C occurs on the same day that R11 cell density reach their carrying capacity, while at 18 °C, algal death in co-culture does not begin until 10 days after the carrying capacity of R11 is reached (Fig. 1). With CCMP3268, the timelines are slightly closer together, with the death of the 25 °C co-culture beginning 2 days after R11 cell density reached carrying capacity and the death of the 18 °C co-culture beginning 4 days after R11 carrying capacity had been reached (Fig. 2).

The differences in timeline leading to death also cannot be explained by differences in the photosynthetic health of the algae at the two temperatures. CCMP3266 control cultures displayed equal yield values at both temperatures for the duration of the experiment (Fig. 1A & B). CCMP3268 control cultures also maintained equivalent yield values at both 18 °C and 25 °C, until the control culture experienced death starting on 16 d (Fig. 2A & B).

Taken together, these results support the hypothesis that the virulence of R11 on *E. huxleyi* is temperature-enhanced. This is in keeping with the original *Delisea pulchra*-R11 model of virulence in which R11 was pathogenic to *D. pulchra* at 24 °C, but not pathogenic at 19 °C (Case et al., 2011). Temperature-enhanced bacterial pathogens have been linked to several other algal diseases including 'white tip disease' in *Gracilaria conferta* – in which a bacterial isolate was found to be the causative agent and that increasing temperature above 20 °C increased the rate of infection (Weinberger et al., 1994). Another example of temperature-enhanced virulence in the marine environment

can be found in the bleaching of the coral *Pocillopora damicornis* by the bacterium *Vibrio coralliilyticus*, triggered by elevated temperature (Kushmaro et al., 1996;Ben-Haim et al., 2003;Rosenberg et al., 2009). In fact, this temperature-induced bleaching results from an attack by *V. coralliitilyricus* on the zooxanthellae algal symbionts living within the coral tissue (Ben-Haim et al., 2003). It appears that the increased pathogenicity in this case was due to both the increased expression of virulence factors and a possible increase in sensitivity of the algae to pathogen attack due to temperature stress (Kushmaro et al., 1996;Ben-Haim et al., 2003;Rosenberg et al., 2009).

In the present study, there is evidence of temperature stress in CCMP3266 and CCMP3268 at 25 °C, as there were marked differences in the algal population size and dynamics at 18 °C and 25 °C. For CCMP3266 at 25 °C, the population size initially followed the same trajectory as the culture at 18 °C, reaching nearly the same peak cell density, but subsequently dropping to around half the density of the 18 °C culture, where it stabilized for the remainder of the experiment (Fig. 1C & D). For CCMP3268, the control culture at 25 °C followed a completely different trajectory to the 18 °C control culture, slowly increasing to a peak only two thirds the density of the maximum at 18 °C, 8 days later (Fig. 2C & D). After this peak, cell density dropped sharply and remained near zero for the remainder of the experiment.

The fact that the cell densities were lower at 25 °C for both CCMP3266 and CCMP3268 likely indicates temperature stress. The reported temperature range of *E. huxleyi* is highly variable (spanning 6-26 °C) (Rhodes et al., 1995;Paasche, 2002;Daniels et al., 2014), with marked differences in temperature optima reported even between strain clones (Paasche, 2002). In the present study, while cultures of both CCMP3266 and 3268 grow normally at 18 °C (with a rapid log phase and a stable stationary phase), they both display altered dynamics at 25 °C (slow initial growth rate and low or un-sustained stationary phase), which is near the upper limit of the species' temperature range. However, 25 °C is an ecologically relevant temperature for these strains, as current SST in the Tasman Sea, where both CCMP3266/3268 and R11 originate, regularly reaches 25 °C in the austral summer. This area – sometimes referred to as the 'Tasman Hot Spot' – is predicted to have a rate of SST warming 3-4 times the global average (Oliver et al., 2014). Additionally, a metagenomic study has shown that EhVs are absent from

populations of *E. huxleyi* in warm equatorial waters (von Dassow et al., 2015). This raises the possibility that regions likely to be even warmer in the future, such as the Tasman Sea, which currently host populations of *E. huxleyi* infected with EhVs, may soon represent a niche open to new pathogens such as R11.

It is unclear from the data presented whether the cause of the increase in pathogenicity of R11 at 25 °C was the result of increased susceptibility of *E. huxleyi*, or was due to an increase in the production of virulence factors by R11 at elevated temperature, or a combination of the two factors. Plant pathogens are known to be triggered by temperatures outside the optimal range for host growth – in other words, by temperatures at which the defenses of the host may be compromised (Smirnova et al., 2001). For example, the blight pathogen *Pseudomonas syringae* significantly increases production of a phytotoxin at 18 °C (7-10 °C below the growth optimum of its host) (Budde and Ullrich, 2000).

It is possible that R11 is an opportunistic pathogen, as its host range appears to be broad – including a red macroalga (Case et al., 2011) and a haptophyte (present study). For a pathogen with diverse hosts, a versatile strategy of triggering virulence might be to sense the stress of a host directly, instead of sensing the conditions that would cause a host's defenses to be compromised. This is a mechanism known to exist in *Phaeobacter gallaeciensis* BS107, another member of the Roseobacter clade. *P. gallaeciensis* produces algaecides in response to *p*-coumaric acid (*p*CA) – produced by E. huxleyi and thought to be a product of senescence (Seyedsayamdost et al., 2011). However, R11 is unlikely to use this particular molecule as a cue, since the addition of *p*CA did not stimulate a change in its production of small molecules (Seyedsayamdost et al., 2011). The evidence from the present study – the fact that R11 displays a broad host range and increased virulence under conditions at which the host displays evidence of temperature stress – supports the hypothesis that R11 is an opportunistic pathogen.

2.4.3 *Ruegeria* sp. R11 causes bleaching in *E. huxleyi*

Bleaching – the loss of pigmentation – is a common phenomenon in marine corals and macroalgae (Jenkins et al., 1999;Douglas, 2003;Egan et al., 2013). In corals, this color loss refers to the death or loss of the symbiotic algae that live within the coral's

tissue – a temperature-dependent effect often linked to bacterial infection – that ultimately leads to the death of coral host (Kushmaro et al., 1996;Ben-Haim et al., 2003;Rosenberg et al., 2009). In macroalgae, the bleaching effect is due to the degradation of photosynthetic pigment that, depending of the extent of the bleaching, may lead to the death of the whole organism. In the present study, a color change was clearly visible in dead or dying cultures of *E. huxleyi*. R11-*E. huxleyi* co-culture wells changed from green to white. This bleaching was also evident from the flow cytometry results (Fig. 4; Fig. 5). In the case of both CCMP3266 (Fig. 4) and CCMP3268 (Fig. 5), during culture death, cells maintained their size (forward scatter) but lost their chlorophyll α content over 2 days at 25 °C (Fig. 4D-F; Fig. 5D-F) or 4 days at 18 °C (Fig. 4A-C; Fig. 5A-C).

Algal bleaching has been mostly attributed to temperature or UV stress alone (Jenkins et al., 1999), except in the case of *D. pulchra*, in which R11 is the temperature-dependent causative agent of the bleaching disease – *D. pulchra* grown without R11 at high temperature does not exhibit bleaching (Case et al., 2011). With the mentioned exception of *D. pulchra*, these studies do not assess the microbial community component of the system, and as such, bacterially-mediated temperature induced bleaching in marine algae could be far more common than the literature reports. Here we demonstrate that it occurs in a microscopic unicellular haptophyte, distantly related, both phylogenetically and physiologically, to the red macroalgae in which it was previously found.

2.5 Conclusion

Natural blooms of *E. huxleyi* often experience a rapid collapse that has been attributed to lytic EhV infections causing PCD in the blooming algae (Vardi et al., 2009). However, it has recently been demonstrated that EhV strains become avirulent at increased temperature due to a change in the structure of the glycosphingolipid required for viral recognition (Kendrick et al., 2014). This algal resistance is gained with only a 3 °C increase in temperature – from 18 °C to 21 °C (Kendrick et al., 2014).

In the context of a rapidly warming ocean, the emergence of temperature-induced resistance in *E. huxleyi* to its major pathogen may present an ecological gap. Our findings indicate that opportunistic bacterial pathogens like R11 with temperature-enhanced

virulence have the ability to fill this gap and a transition between viral and bacterial disease outbreaks in *E. huxleyi* may be observed as SST continues to rise.

2.6 References

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Chapter 3: Discussion

3.1 Synthesis of results

Photosynthetic microbes have been shaping the earth for the past 2.8 billion years (Dismukes et al., 2001). Today, despite their phylogenetic diversity, phytoplankton share common ecological roles – they are a vast source of primary productivity, form the base of the marine trophic cascade, and are major players in global biogeochemical cycling (Behrenfeld et al., 2001). The coccolithophores, ubiquitous calcifying marine microalgae, are especially important members of temperate planktonic communities, where they can form enormous blooms appearing suddenly and collapsing rapidly (Brown and Yoder, 1994). This thesis has explored the interaction between *Emiliania huxleyi* – the most abundant of the coccolithophores – and *Ruegeria* sp. R11 – a bacterium in the roseobacter clade known to cause bleaching disease in a red macroalga at elevated temperatures (Case et al., 2011). My work has shown that R11 displays temperature-enhanced pathogenesis of *E. huxleyi,* but that not all host cell types are equally susceptible. The findings presented here indicate that the *E. huxleyi* pathogen R11 kills the coccolith bearing diploid cell and the flagellated haploid cell, but not the bald diploid cell type. This type of pathogenic niche differentiation has been demonstrated before in the *E. huxleyi*—EhV interaction, but in that case, the virus was found to kill coccolith bearing diploid cells, while the haploid cells escaped (Frada et al., 2008). This further complicates the concept of niche differentiation, even during interactions between tiny pathogens (e.g. EhV or roseobacters) and their 5 um algal hosts (e.g. *E. huxleyi* cell types: CCMP3266-coccolith, CCMP3268-haploid, and CCMP2090-bald). Such dramatic niche

partitioning of pathogens may have major ecological implications in terms of *E. huxleyi* bloom dynamics in the future, particularly in regard to how increasing sea surface temperatures impact these interactions.

3.2 The effects of climate change on *Emiliania huxleyi*

The results of this research are especially interesting when considered in the context of modern, anthropogenically driven climate change (Harvell et al., 2002;Gattuso et al., 2015). The endeavors of the scientific community to understand the potential effects of this climate change have focused on two of its major anticipated and documented effects: warming and acidification (Gattuso et al., 2015).

3.2.1 Warming

One of the major consequences of modern climate change is rising SST. A decline in overall phytoplankton productivity, significantly correlated with increasing SST, has been recorded over the last century (Boyce et al., 2010). This is especially pronounced in tropical and sub-tropical regions, as species already live close to their thermal optima – after which growth rates drop sharply (Thomas et al., 2012). A gradient of biodiversity loss – highest at the equator and decreasing with increased latitude – is predicted with increased warming, and species are expected to shift their niches further towards polar regions (Thomas et al., 2012). This trend has already been observed for *E. huxleyi*, which has been documented to shift its range from subtropical and temperate regions into polar waters over the last decade (Winter et al., 2013). This has been hypothesized to be in part, due to a lengthened growing season at higher latitudes, prolonging the low nutrient, high light intensity conditions that favor *E. huxleyi* blooms (Winter et al., 2013). However, it has also been suggested that a geographic shift in the thermal temperature niche of *E.*

huxleyi is the simplest explanation for the observed range alteration (Thomas et al., 2012;Winter et al., 2013). The current trend in ocean warming is not wholly unprecedented. During the Paleocene-Eocene Thermal Maximum (56 million years ago) ocean temperatures were thought to have risen by 4-5 °C and there was a drastic reduction in the range of the coccolithophores (Gibbs et al., 2016). The dominant species, *Zyghrablithus bijugatus*, disappeared from low latitudes, and became concentrated at the colder, higher latitudes – a change correlated to temperature increase (not ocean acidification) (Gibbs et al., 2016), and mirrored by the observed shifts in *E. huxleyi* today (Winter et al., 2013).

It has recently been demonstrated that *E. huxleyi* is capable of rapid adaptation to increasing temperature (Schluter et al., 2014), and so it seems that the physical environment is only part of the story when it comes to ocean warming. Pathogen attacks are predicted to become more common with increased temperature due to both increased virulence and decreased host resistance due to thermal stress (Harvell et al., 2002). Bacterial thermal ranges tend to be wider than eukaryotic ranges (Harvell et al., 2002). Thus, as temperatures increase, the bacterial pathogen likely remains healthy and uncompromised, while the host's defenses are increasingly weakened by the pressure of thermal stress. The R11-*E. huxleyi* system explored in this thesis (Chapter 2) is a demonstration of this type of opportunistic infection – one triggered in a compromised host (Egan et al., 2014). Although R11 is capable of causing precipitous population death at a lower temperature, death happens almost immediately at elevated temperature – one at which *E. huxleyi* experienced decreased health (Chapter 2). If this pattern of temperature-enhanced pathogenesis is prevalent in the roseobacters (at least one other

related species is a known pathogen of *E. huxleyi* and there are many more members pathogenic to other organisms (Ashen and Goff, 1998;Boettcher et al., 2000;Sunagawa et al., 2009;Seyedsayamdost et al., 2011;Luo and Moran, 2014)), it is possible that a decline in the large bloom events at high latitudes typical of this species may occur. Instead, the year round low level pattern of distribution currently seen at lower latitudes may become the norm across the range of *E. huxleyi.* Limited blooms in northern waters, where *E. huxleyi* is one of the dominant primary producers, will impact northern ecosystems in addition to affecting the species role as a surface-to-depth carbon pump. Additionally, the importance of EhVs in the demise of *E. huxleyi* blooms may decrease in populations of *E. huxleyi* as temperatures increase, as a rise of only 3 °C causes *E. huxleyi* to become resistant to viral infection.

3.2.2 Acidification

As the major calcifying organisms in the open ocean, considerable attention has been paid to the calcification ability of the coccolithophores under increased atmospheric CO_2 partial pressure (pCO_2) conditions. The results of this line of investigation have been mixed, with some studies demonstrating decreased calcification with the predicted higher pCO_2 conditions (Meyer and Riebesell, 2015), while other studies have shown surprisingly rapid adaptation to these projected levels (Schluter et al., 2014). The general view of these mixed results is that the response of coccolithophores to increased pCO_2 is not only species dependent, but also likely to vary with strain and ecotype as well (Meyer and Riebesell, 2015). A recent meta-analysis has indicated a slight decrease in calcification for *E. huxleyi* under increased pCO_2 conditions, but no net effect on rates of photosynthesis (Meyer and Riebesell, 2015). However, this result is especially complex

for *E. huxleyi*, given the fact that its populations, while dominated by calcifying cells, also contain two non-calcifying cell types, both able to reproduce asexually (Paasche, 2002). It is possible that the non-calcifying cell type may benefit from increased pCO_2, as this causes a subsequent increase in bicarbonate ions – the primary source of carbon used in the coccolithophore's photosynthesis (Paasche, 2002). Since the non-calcifying cell type does not invest energetically in producing coccoliths, it may not experience the same downsides of acidification as its coccolith-bearing relatives – increased energy expenditure to make up for increased calcium carbonate dissolution in acidic water. To my knowledge, the effects of increasing pCO_2 have not been investigated in non-calcifying *E. huxleyi*. This is an area that merits further research, especially considering the mounting evidence that pathogens of *E. huxleyi* display specificity for certain cell types (Chapter 2) (Frada et al., 2008). EhVs have been shown to display pathogenicity towards diploid calcifying and diploid bald cells, while the haploid scaly cells are resistant to infection due to differences in the membrane of the haploid cell which render the virus unable to recognize the host cell (Frada et al., 2008). The work presented in this thesis demonstrates that *Ruegeria* sp. R11 is pathogenic to the diploid calcifying cells, while the diploid non-calcifying naked cells are resistant to it – the mechanism is unclear, though there are several differences between the coccolith bearing and bald diploid cells (Chapter 2). In both these cases (EhV and R11), the calcifying cells are susceptible, while one of the non-calcifying cell type escapes (the haploid scaly cell for EhV, the diploid bald cell for R11). This apparent trend in host specificity, in addition to any competitive advantages conferred by non-calcifying cell types under high pCO_2 conditions may lead

to increased proportions of non-calcifying cells in *E. huxleyi* populations in the future, and may have implications for the role of these algae in the carbon cycle.

3.3 Future Directions

Despite the apparent ability of *E. huxleyi* to adapt to a rapidly changing physical environment (Thomas et al., 2012;Schluter et al., 2014), the species is exhibiting a range shift towards cooler polar waters (Winter et al., 2013). This, in combination with mounting evidence that increasing temperature will mean increased bacterial pathogen attacks for algal species (Chapter 2)(Weinberger et al., 1994;Rosenberg et al., 2009;Case et al., 2011), suggests that bacterial—algal interactions will play a major role in determining the prospective bloom dynamics and habitat range of *E. huxleyi.* Further research is needed in order to understand the interaction between these factors. Firstly, it would be useful to perform the co-culture experiments from Chapter 2 over the known thermal range of *E. huxleyi* to determine if there is a temperature at which *E. huxleyi* becomes resistant to R11, and to compare this data to a growth optimum curve (to my knowledge this curve has not been generated for the strains used for this thesis). This information could be used to predict potential cool water refuges for *E. huxleyi* from temperature-dependent bacterially mediated pathogens (such as R11 or other roseobacters), and hypothetical boundaries at which EhVs and bacterial pathogens might alternately fill the role of the major pathogen of *E. huxleyi*. Elucidating the mechanism behind the virulence of R11 would also be useful, as this trait could then be identified in metagenomic data from global initiatives (TARA oceans, for example (Pesant et al., 2015)), locating populations of potentially virulent roseobacters and comparing this to that of the EhVs.

Additionally, tracking the ratios over time of calcifying to non-calcifying cells in *E. huxleyi* blooms in areas in which attacks by selective pathogens (roseobacters and EhVs) are hypothesized to skew cell type ratios could elucidate the role of these pathogens in the future of carbon sequestration by the coccolithophores.

Finally, phytoplankton like *E. huxleyi* do not live axenically in nature. As previously discussed (Chapter 1), there is a diverse assemblage of microbes associated with their blooms. This community may contain other bacterial pathogens (potentially other pathogenic roseobacters), but also likely contains mutualistic symbionts. Several members of the roseobacters are known to produce a potent antibiotic (tropodithietic acid), and have been hypothesized to defend their host from other bacteria through its production (Thiel et al., 2010;Seyedsayamdost et al., 2011). Performing mesocosm experiments, in which the natural microbial consortium of *E. huxleyi* is present, would test the ability of an opportunistic pathogen (such as R11) to invade a natural community and cause disease in a host.

3.4 Conclusion

Although the abiotic effects of climate change on *E. huxleyi* have been extensively studied, the effects of these environmental changes on biotic interactions have been largely neglected. This is an area of research that should be explored further given the results of this thesis (and others suggesting the importance of temperature to pathogen-*E. huxleyi* interactions), the wide geographic range of the roseobacters, and the global importance of *E. huxleyi*. While *E. huxleyi* has proven to be resilient to a changing physical environment thus far (Schluter et al., 2014), pathogens like R11 may further

compromise populations, and complicate the ability of this important organism to adapt to a rapidly changing ocean.

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APPENDIX A

Fig S1: DNA loss in C type Emiliania huxleyi (CCMP3266) in co-culture with Ruegeria sp. R11. Control cultures of CCMP3266 and co-cultures of CCMP3266 with R11 were assessed for DNA content using flow cytometry at 18 °C on 10 d (A), 14 d (B), and 16 d (C); and at 25 °C on 14 d (D), d 20 (E), and d 24 (F). Data for R11-CCMP3266 co-cultures are indicated with blue contour lines and control cultures (CCMP3266 grown alone) with black contour lines. The density of the lines is proportional to the density of detection events.

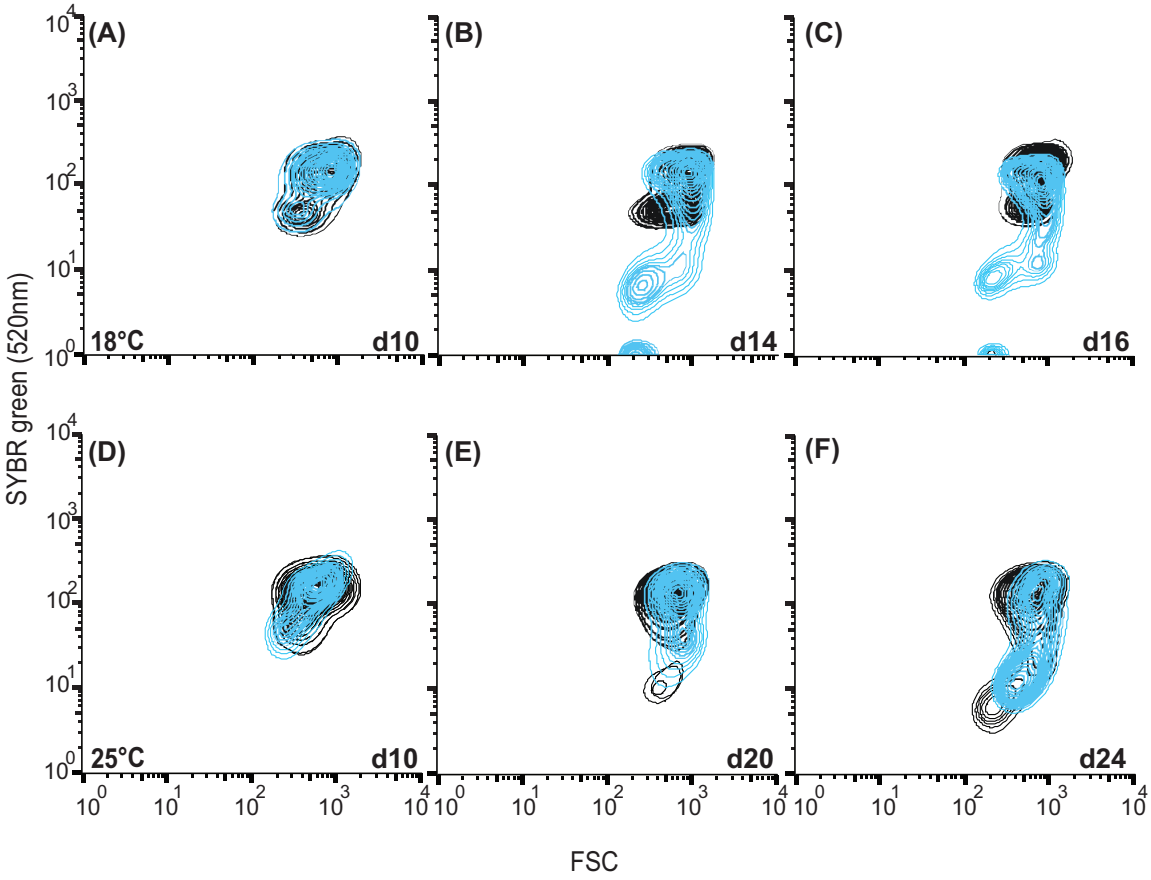
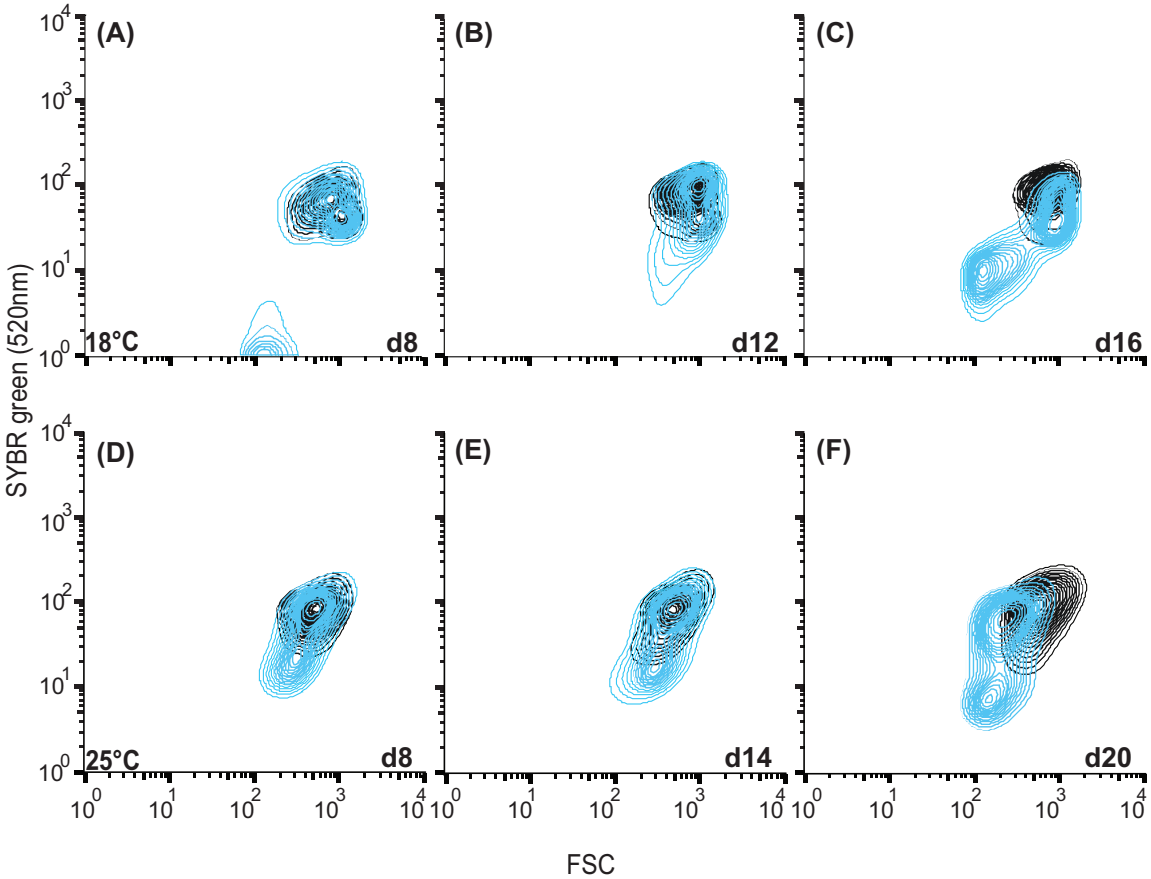


Fig S2: DNA loss in S type Emiliania huxleyi (CCMP3268) in co-culture with Ruegeria sp. R11. Control cultures of CCMP3268 and co-cultures of CCMP3268 with R11 were assessed for DNA content using flow cytometry at 18 °C on 8 d (A), 12 d (B), and 16 d (C); and at 25 °C on 8 d (D), d 14 (E), and d 20 (F). Data for R11-CCMP3266 co-cultures are indicated with blue contour lines and control cultures (CCMP3266 grown alone) with black contour lines. The density of the lines is proportional to the density of detection events.



Suggestions for Future Statistical Analysis

Statistics were not included in the analysis of the data for this thesis, as death was rapid and produced a clear, observable, and quantifiable change in the cultures with little, if any, variation among independent replicates. However, in the future, for experiments in which differences between treatments are more subtle, statistical analysis of this type of data is certainly possible, due to the true biological replication inherent in the method. For experiments in which only two groups are compared (a single control to a single experimental group), a t-test could be used. For experiments in which multiple groups are being compared (a single control and multiple experimental groups), an analysis of variance (ANOVA), followed by a Tukey HSD test would be appropriate.