A big role for small molecules in mediating *Emiliania huxleyi* – Roseobacter interactions

by

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Abstract

Microalgae are a diverse group of photosynthetic microorganisms that have complex relationships with their surrounding bacteria, which are often controlled by the exchange of bioactive molecules. *Emiliania huxleyi* is a ubiquitous marine microalga, forming massive blooms, driving the marine carbon pump and biogeochemical cycles like the sulphur cycle. Bacterial symbionts of this alga include an abundant group of α-proteobacteria known as roseobacters. These bacteria are known to containing a variety of secondary metabolites that may benefit an algal symbiont; for example, certain members produce a growth-promoting plant hormone, indole-3-acetic acid (IAA) or tropodithietic acid (TDA) that acts to chemically defend their host from further bacterial colonisation. However, at least one species of the roseobacters can switch to produce a pathogenic effect as the alga enters late-stage growth, secreting algaecides called roseobacticides. These are formed in response to a lignin intermediate, *p*-coumaric acid (*p*CA). This duality of mutualism and pathogenicity seems to be controlled by the various bioactive molecules released by the algae and bacteria. This thesis seeks to investigate the interactions between algae and their selected members of their microbiome using controlled laboratory conditions and by doing so, further our understanding of their natural interactions and find novel methods to enhance algae processing for industrial purposes, such as biofuels.

Although *p*CA has been shown to be linked to aging microalgae, the role it plays in the physiology and/or ecology of these organisms is unclear, as is the molecular pathway used to create this compound. Lignin, one of the innovations of land plants, has been found in various algae, raising questions about the evolution of the lignin biosynthetic pathway. To determine the taxonomic distribution of the lignin biosynthesis genes, all publicly available genomes of algae were screened. Genes associated with *p*-coumaryl alcohol (H-monolignol) biosynthesis were found widely present in algae, and therefore postulated to have evolved long before the transition of photosynthetic eukaryotes

to land. The original function of this lignin precursor is therefore unlikely to have been related to water transport. Lignin intermediates are shown to have an antimicrobial action against common marine bacteria, suggesting an early role in the biological defence of some unicellular and multicellular algae.

Roseobacticides production by the roseobacter *Phaeobacter gallaeciensis* was stimulated after addition of a lignin intermediate, *p*CA. However, *p*CA depressed the levels of the antimicrobial tropodithietic acid (TDA) that is normally produced by the bacterium, thereby providing less protection to the alga and *P. gallaeciensis* itself against other bacteria. *P. gallaeciensis* accelerates senescence and selectively kills one cell type from *E. huxleyi*, the coccolith producer, while leaving the bald strain alive.

Indole-3-acetic acid (IAA) is an important auxin influencing plant development, but the production in algae has been contentious. Screening the tryptophan dependent pathway revealed that the biosynthetic potential for IAA is present in various algal groups, especially in *E. huxleyi*. Addition of L-tryptophan to *E. huxleyi* stimulated IAA production, but only in the coccolith-bearing strain. Conversely, addition of exogenous IAA only elicited a physiological response in the bald cell type. A roseobacter *Ruegeria sp*. R11, previously shown to produce IAA, co-cultured with L-tryptophan and both cell types of *E. huxleyi* produced less IAA than the axenic coccolith cell type culture similarly induced. This suggests that IAA plays a novel role signalling between different *E. huxleyi* cell types, rather than between a bacteria and its algal host.

In order to determine a possible commercial application of these findings, the growth and lipid yield of these bacteria and bioactives were measured against *E. huxleyi,* another haptophyte *Isochrysis sp.*, as well as the chlorophyte *Dunaliella tertiolecta*. Only R11 showed early promise in stimulating the lipid content of the green alga, leading to potential industrial applications.

Despite their small size, unicellular organisms such as the microalga *E. huxleyi* are capable of a complex set chemical interactions, both interspecies (e.g. *E. huxleyi* – roseobacter) and even intraspecies

(e.g. *E. huxleyi* coccolith bearing – bald cell types). These interactions and the bioactives that mediate them could be important drivers in shaping the ecology, life history, and bloom-bust interaction of this microalga in the marine environment.

Preface

Some of the research conducted for this thesis forms part of collaborative work, listed below.

The functional analysis performed in Chapter 2 was performed by Dr. Yan Boucher (Figure 2-2). A version of this chapter was published as:

- Labeeuw L**,** Martone PT**,** Boucher Y**,** Case RJ (2015). Ancient origin of the biosynthesis of lignin precursors. *Biology Direct* **10**: 1–21.

The methodology outlined for Chapters 3-4 for use of the Pulse-Amplitude-Modulated (PAM) Fluorometry was determined and optimized by myself and Anna Bramucci, and published as:

- Bramucci AR**,** Labeeuw L**,** Mayers TJ**,** Saby JA**,** Case RJ (2015). A small volume bioassay to assess bacterial/phytoplankton co-culture using WATER-Pulse-Amplitude-Modulated (WATER-PAM) fluorometry. *Journal of Visualized Experiments* **97**: e52455.

In Chapter 3, screening of the bacterium against different *Emiliania huxleyi* strains, as well as the microscopy images taken, was conducted by Dr. Rebecca Case and Alexis Fischer (Table 3-1 and Figure 3-1). The final data collection in Chapter 3 for algal and bacterial co-cultures (Figure 3-2) was collaboration with Anna Bramucci, with initial screening and optimization performed by me.

Chapter 4 was a collaborative work, whereby Dr. Paulina de la Mata optimized and determined the GCxGC-TOFMS settings, while Joleen Khey collaborated on the algal bioinformatics (Table 4-1), and Anna Bramucci collaborated on flow cytometry analysis (Figure 4-6). It has been published as:

- Labeeuw L, Khey J, Bramucci AR, Atwal H, de la Mata P, Harynuk J, Case RJ (2016). Indole-3-acetic acid is produced by *Emiliania huxleyi* coccolith-bearing cells and triggers a physiological response in bald cells. *Frontiers in Microbiology, in press*.

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Lastly, I would like to thank Wenjun Dai and my other roommates for the last few great years, as well as my family, for always supporting me in my pursuits.

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Glossary of Terms

4CL	4-coumarate:CoA ligase
ARF	auxin response factors
AFB	auxin signalling f-box
BBH	bi-directional best hit
C3H	*p*-coumarate 3-hydroxylase
C4H	cinnamate 4-hydroxylase
CAD	cinnamyl alcohol dehydrogenase
CCoAMT	caffeoyl-CoA *O*-methyltransferase
CCR	cinnamoyl-CoA reductase
COMT	caffeic acid *O*-methyltransferase
CSE	caffeoyl shikimate esterase
SUR1	C-S lyase
SUR2	CYP83B1
CYP79B2	cytochrome P450
CYP79B3	cytochrome P450
DMS	dimethyl sulphide
DMSP	dimethylsulfoniopropionate
EGT	endosymbiotic gene transfer
EhV	*Emiliania huxleyi* virus
F5H	ferulate 5-hydroxylase
HCT	hydroxycinnamoyl-CoA
IAAd	indole-3-acetaldehyde
AAO1	indole-3-acetaldehyde oxidase
IAOx	indole-3-acetaldoximine
CYP71A13	indole-acetaldoxime dehydratase
IAM	indole-3-acetamide
AMI1	indole-3-acetamide hydrolase
IAA	indole-3-acetic acid (IAA)
IAN	indole-3-acetonitrile
IPyA	indole-3-pyruvic acid
JGI	Joint Genome Institute
LAC	laccase
LGT	lateral gene transfer
MIC	minimum inhibitory concentration
MKC	minimum killing concentration
MYR1	myrosinase
NCBI	National Centre for Biotechnology Information
NCMA	National Centre for Marine Algae and Microbiota
AHL	N-acyl-homoserine lactone
NIT1	nitralase
PAA	phenylacetic acid
PAL	phenylalanine ammonia-lyase
*p*CA	*p*-coumaric acid
PER	peroxidase
F_v/F_m	potential quantum yield
PAM	pulse-amplitude-modulation
QS	quorum sensing
SAR	stramenopile-alveolate-rhizaria
smF5H	*Selaginella moelledorfii* F5H
SNK	Student-Newman-Keuls
TIR1	transport inhibitor response
TAG	triacylglycerol
TDA	tropodithetic acid
GC×GC-TOFMS	two-dimensional gas chromatography with time-of-flight mass spectrometry
TDC	tyrosine decarboxylase
TAM	tryptamine
YUCCA	tryptamine monooxygenase
TAA1	tryptophan amino transferase
vGSL	viral-glycosphingolipid
ZOI	zone of inhibition

Chapter 1

Introduction

1.1 Background

Phytoplankton are photosynthetic microorganisms, or primary producers, that live planktonically in the water column and are a diverse group that include both eukaryotic algae and cyanobacteria (Litchman *et al.*, 2015). Phytoplankton are important players in global biogeochemical cycles, accounting for approximately 40-50% of the world's carbon fixation and driving the marine carbon pump (Falkowski, 1994). In other words, phytoplankton contribute almost half of the global net primary productivity despite accounting for less than 1% of the world's photosynthetic biomass (Field, 1998; Falkowski, 2012). In addition, phytoplankton are the drivers in their local ecosystems as the base of the food web, thereby affecting ecosystem dynamics and services, and acting as a habitat forming species in the open ocean, where they can form massive blooms (Falkowski, 2012; Stevenson, 2014).

Eukaryotic algae are a polyphyletic group of photosynthetic organisms, ranging from a few micrometres to several metres in size (Round, 1973). The term 'algae' is applied to a large diverse group of organisms only distantly related across the phylogenetic tree (Archibald, 2009; Keeling, 2010). A billion years ago the first endosymbiotic insertion of a cyanobacteria into a non-photosynthetic eukaryote created the chloroplast (Yoon *et al.*, 2004); however the subsequent evolution of plastids becomes murky due to successive transfers to other lineages through secondary and tertiary endosymbiotic events, as well as multiple loss events. This has led to algae being present in nearly every one of the major Eukaryotic supergroups, namely: Archaeplastida, Excavata, and the SAR (stramenopile-alveolate-rhizaria) group (Figure 1-1). However, it should be noted that there is still ongoing debate about restructuring and naming of these supergroups (Keeling, 2013; Burki, 2014; Derelle *et al.*, 2015).

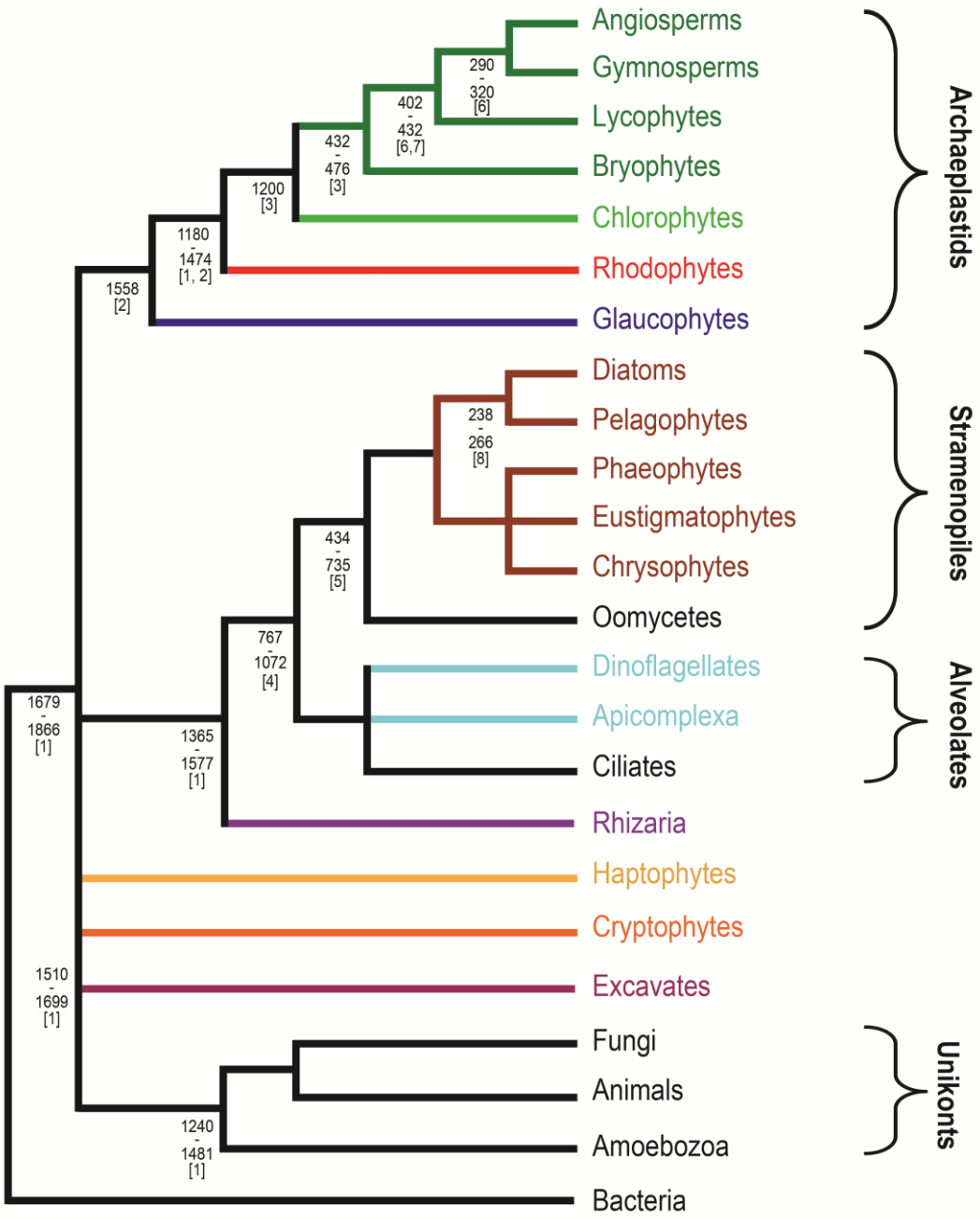

Figure 1-1: Eukaryotic tree with estimated time ranges of divergence.

The tree is a consensus of current phylogenetic analyses of the eukaryotic domain (Kenrick & Crane, 1997; Archibald, 2009; Keeling, 2013). Coloured lines for Eukaryotes indicate that the lineage has at least one photosynthetic organism. The approximate time range for the divergence are given in millions of years ago (mya) before the relevant node. References are given by numbers in square brackets, where [1]**:** (Parfrey *et al.*, 2011), [2]: (Yoon *et al.*, 2004), [3]: (Kenrick & Crane, 1997), [4]: (Douzery *et al.*, 2004), [5]: (Brown & Sorhannus, 2010), [6]: (Bowe *et al.*, 2000), [7]: (Schneider *et al.*, 2004), and [8]: (Kooistra & Medlin, 1996).

Algae are usually relatively simple in cellular composition and differentiation, and can be either uni- or multi-cellular (Mata *et al.*, 2010). Unlike terrestrial plants, algae lack roots, stems, leaves, conducting vessels such as a xylem, or complex sex organs (Round, 1973). They are highly adaptable and are able to thrive under a range of conditions including fresh water, marine and waste-water systems (Bhattacharya & Medlin, 1998).

Microalgae have complex relationships with bacteria inhabiting the space immediately surrounding the algal cell, known as the "phycosphere" (Bell & Mitchell, 1972), which is a habitat that has not been well researched, yet provides a distinctive and diverse habitat for bacteria (Ramanan *et al.*, 2016). The size of the phycosphere is dependent on the size of the algal host, but is determined by the diffusive boundary layer where there is less mixing than the open ocean, which creates an area immediately around the algae (in the µm size) that has higher concentrations of bioactive molecules (Amin et al., 2012). These molecules can affect the interactions between the alga and its surrounding bacterial consortia. The bacterial-algal interactions can range from a mutually beneficial interaction, to a potentially pathogenic one, in which the bacteria induce cell death or lysis in the algal host (Joint *et al.*, 2002; Grossart *et al.*, 2005; Azam & Malfatti, 2007; Amin *et al.*, 2012). One group of bacteria called roseobacters, a clade of α-proteobacteria, have been shown to be highly abundant in the marine environment and are common symbionts of macro and microalgae (Grossart *et al.*, 2005; Azam & Malfatti, 2007). Many of the small molecules mediating the bacterial-algal interactions are not completely elucidated yet. Unlike the interactions between plant roots and their bacteria – the rhizosphere – which has been extensively studied (Jones, 1998; van Loon *et al.*, 1998; Doornbos *et al.*, 2012; Philippot *et al.*, 2013), the interactions and bioactives which control them in the phycosphere have been harder to determine. Algae are a rich source of natural products, with macroalgae alone accounting for 15-20% of the discovered marine natural products (Maschek & Baker, 2008), and phytoplankton accounting for approximately 15% (Hu *et al.*, 2011). All too often, natural products in

microbiology are sought after for their therapeutic and antibiotic uses – communication between microbes is considered secondary (Yim *et al.*, 2007).

My research aims to eavesdrop on the chemical cross-talk occurring between algae and bacteria, which may have a vast potential for new knowledge, relating to understanding bacterial-algal relationships, evolution, and possibly hijacking this communication to better control microbes in commercial systems. My PhD research is to identify how novel bacterial bioactives might control a eukaryotic algal host or vice versa. In this way the bacteria is manipulating the host alga into a more conducive habitat (e.g. algal phycosphere, aquatic biofilms, etc.) for bacterial survival, which is a novel way to look at bacterial-algal symbiosis in natural systems. Far from the 'random interaction' of two microbial organisms from different branches in the tree of life, I propose that bacteria and algae may interact and even shape interactions due to the complex exchange of small bioactive molecules. These bacterial and algal bioactive molecules may prove to be invaluable chemicals in further understanding communication between bacteria and eukaryotes, and might even have biotechnology possibilities.

1.2 Bacterial-algal interactions in marine systems

While not as well studied as plant-microbe systems, the existence of associations between algae and their bacterial assemblage has been known for a long time (Provasoli, 1958). Many of the studies have focused on macroalgae, as the direct interactions with microalgae are harder to determine and they were sometimes assumed to be too small (2-10 μm) and at too low of a concentration (10^4 cells/mL) to play host to a bacterial consortium. However, in recent years, the importance of understanding the role bacteria play in an alga's lifestyle has become increasingly recognized, as bacteria have been shown to affect growth dynamics such as aggregation and sinking, which has important properties for understanding natural blooms (Grossart *et al.*, 2006). Interactions can be classified as: mutualistic,

where both parties benefit; commensal, where one party benefits; or parasitic, whereby the bacteria negatively affect the health and fecundity of algae, or pathogenic, which causes disease and/or death in the host. While competition for resources is another form of parasitism, it shall not be considered here (Ramanan *et al.*, 2016). Small molecules are important mediators in these interactions, yet in many cases they have not yet been identified.

1.2.1 Mutualism

There are many advantages for the bacteria to attach to the algae, as they provide a ready supply of nutrients and close proximity to other bacteria for gene exchange (Geng & Belas, 2010). There are many cases whereby both the algae and the bacteria benefit from an exchange of nutrients to which they might otherwise have limited access to (Goecke et al., 2010). For example, some bacteria that form close associations with algae can modify the type of siderophores (organic molecules that bind to iron and increase its solubility) that they produce, such that the algae can easily use them to scavenge iron (Amin *et al.*, 2009). The bacteria and the algae can also benefit by gaining trace nutrients that they do not otherwise produce themselves. Over half of surveyed algae are auxotrophic for at least one essential vitamin (e.g. B_1, B_{12}, etc.) (Croft *et al.*, 2006). However, this dependence can work both ways; for example, the dinoflagellate *Prorocentrum minimum* provides carbon and vitamins (i.e. B_3) in return for vitamins it cannot produce itself (B_1 and B_{12}) to the roseobacter *Dinoroseobacter shibae* (Wagner-Döbler *et al.*, 2010). The symbiosis can progress to the point where it is necessary for the survival of one or both members. An algal species closely related with the prymnesiophyte *Braarudosphaera bigelowii* has been shown to receive fixed nitrogen from a symbiont, the cyanobacterium UCYN-A, in exchange for organic carbon. What is remarkable about this system is that the cyanobacterium has lost photosystem II and the tricarboxylic acid (TCA) cycle in its genome, while the alga was shown to virtually always carry the bacterium, indicating that both are obligate symbionts (Thompson *et al.*, 2012; Cabello *et al.*, 2015).

Certain types of macro green and red algae depend on bacteria to control (or at least help determine) the growth and morphology of the alga throughout its life cycle (Goecke *et al.*, 2010). However, the bioactive compounds that are released to dictate these changes are usually not well known. One exception is the bacterially produced thallusin that has been found to alter the differentiation of the macro green alga *Monostroma oxyspermum* from loose aggregates of single cells to the final differentiated, leafy morphology (Matsuo *et al.*, 2005).

The exchange of nutrients is not the only benefit from living in a symbiosis. Bacteria can also control, to a certain extent, the composition of the phycosphere, as the production of antibiotics by marine bacteria is widespread. This allows the bacteria exclusive access to the alga's secreted nutrients, while providing the alga with a defence mechanism against fouling agents that might otherwise be harmful (Rao *et al.*, 2007). Algae have since evolved mechanisms to turn this antimicrobial biosynthesis to their advantage. Some algae are capable of manipulating their bacterial symbionts. For example, they can secrete compounds that mimic quorum sensing (QS) signalling molecules naturally produced by bacteria, thereby initiating responses in bacteria that work in their favour. QS molecules are autoinducers released by bacteria as a function of their population, and when they reach minimum concentration, they induce gene expression within the bacteria (Waters & Bassler, 2005). One example of algal manipulation of this process is the secretion of riboflavin (vitamin B_2) and its derived version, lumichrome (which are essential for cellular metabolism in normal conditions) by a green microalgae, *Chlamydomonas*. These compounds can then mimic the QS N-acyl-homoserine lactones (AHLs) molecules, which could lead to the bacteria prematurely initiating their virulence factors to protect the algal host against possible pathogens (Teplitski *et al.*, 2004; Rajamani *et al.*, 2008)

1.2.2 Commensalism

The ability of bacteria to switch from a mutualistic to pathogenic interaction is attributed to environmental factors (Valiente-Banuet & Verdú, 2008; Ramanan *et al.*, 2016). One theory is that there is a continuum of interactions, and bacteria pass through a stage of commensalism on the way to pathogenicity (Zapalski, 2011). Another theory put forth is that the end stage of many mutualists and parasites is naturally as commensals (Sachs & Simms, 2006). The study of commensals is complicated, as it is difficult to distinguish between mutualists and commensals. There are often 'satellite' or epiphytic bacterial communities described on algal hosts, but these studies have not investigated how the algae might be benefitting from the relationship (Schäfer & Abbas, 2002; Tujula *et al.*, 2010). Experiments have shown that adding in natural bacterial communities to axenic strains can have a distinct effect on the alga's growth (Grossart *et al.*, 2005). There are many examples of stable communities of bacteria living with algae, with the bacteria benefitting from the leaked nutrients from the algae; however, it is difficult to prove the absence of benefits to the algae, therefore it is difficult to determine whether they are true commensals rather than mutualists (Cole, 1982; Bratbak & Thingstad, 1985).

1.2.3 Parasitism and pathogenicity

There are many forms of parasitism and pathogenicity between algae and bacteria, or even between algal species, as algae can often be the parasite themselves. For example, approximately 10% of red algae are parasites of other, free living, red algae (Hancock *et al.*, 2010). The advantage of bacteria to kill their algal host is that it leads to easy access to the ready-made metabolites and nutrients within their host, and as such, proximity to the dying host should allow for more direct benefit. While for some this is true and close association is a necessity for pathogenicity, with some bacteria residing directly in the algal cell wall (Wang *et al.*, 2010), it has been suggested that only 30% require direct contact (Mayali & Azam, 2004; Demuez *et al.*, 2015). Effects of algaecidal bacteria can include decreased chlorophyll and

photosynthesis, increased caspase production and loss of integrity of the cell wall (Fu *et al.*, 2012). Pathogenicity seems to be a targeted affair, as algaecidal bacteria are often found kill one strain or species but not another (Mayali & Azam, 2004; Demuez *et al.*, 2015). However, unlike viral killing of algae (Bidle & Vardi, 2011), the main mechanisms and compounds used by the bacteria remain mostly uncharacterized (Mayali & Azam, 2004).

Bacteria have the ability to switch from a mutualistic phase into a pathogenic phase, induced by either the aging of the algal host, the presence of a high nutrient media, accumulation of bacterial QS molecules, or other unknown signals (Seyedsayamdost *et al.*, 2011b; Wang *et al.*, 2014c). Chemotaxis to navigate towards algal products such as DMSP is important for mutualists, as it can lead to localized higher concentration around the algae. However, this same advantage is important in achieving the QS signals to potentially switch to pathogenicity (Lovejoy *et al.*, 1998; Wang & Yuan, 2014). One example is the bacteria *Kordia algicida* which has targeted algaecidal effects against selected diatom species, as it only releases the algaecidal proteases once a quorum has been reached (Paul & Pohnert, 2011).

The specific action by which bacteria cause reduced health or death is often not well characterized. The algaecidal bacteria *Alteromonas* sp. and *Thalassobius aestuarii* sp. release enzymes that specifically target the cell wall of the *Alexandrium tamarense*, including chitinase or β-glucosidase (Wang *et al.*, 2010). Specific algaecidal molecules, have been harder to identify, although recently the QS messenger 2-heptyl-4-quinolone (HHQ) (Diggle *et al.*, 2007) produced by *Pseudoalteromonas piscicida* was found to be a potent algaecidal molecule causing the death of the haptophyte *Emiliania huxleyi* (Harvey *et al.*, 2016).

Algae have developed a suite of small molecules that can act as chemical defences, which they can deploy to inhibit colonization, organisms competing for available resources, or predators (Hay, 1996; Wolfe *et al.*, 1997; Potin *et al.*, 2002; Steinberg & de Nys, 2002). Brown algae produce phlorotannins

which are potent antimicrobial metabolites against a range bacteria, fungi and other algae (Eom *et al.*, 2012), although it still has to be conclusively established that these are used *in situ* against local marine bacteria (Steinberg & de Nys, 2002). One of the most studied chemical defence systems has been in the macro red algae *Delisea pulchra* which releases furanones, similar in structure to the QS AHL molecules, thereby regulating the bacterial QS response. The story becomes more complicated since it was also shown that this defence is linked to environmental conditions (Nys *et al.*, 1995; Manefield *et al.*, 1999; Case *et al.*, 2011). Signals for these defences can be components of the alga themselves. When the degradation products of one of the main components of the algal cell wall, agar, is detected, the macro red algae *Gracilaria conferta*, responds with oxidative bursts and halogenating activity against any potentially pathogenic bacteria (Weinberger *et al.*, 1999).

1.3 Haptophyte-Roseobacter as a model system

There are many interactions available between algae and bacteria, and it can vary depending on the alga's physiology. As such, one model system to investigate in further detail is between haptophytes and roseobacters, as this is a highly abundant system and these bacteria are the most common bacteria found to be associated with these microalgae (Goecke *et al.*, 2013). Using these two groups as a model effectively demonstrates the various symbiotic interactions that can take place between algae and their bacterial symbionts, as well as the switch towards pathogenicity that can occur if the delicate balancing act of nutrient exchange is upset.

1.3.1 Description of haptophytes and *Emiliania huxleyi*

Emiliania huxleyi is a globally abundant haptophyte forming seasonal blooms that can cover vast areas of the ocean, yet the alga itself is only about 5µm in size (Holligan *et al.*, 1993). *E. huxleyi* are important players in Earth's biogeochemical cycles including the oxygen, carbon, and sulphur cycles

(Charlson *et al.*, 1987). They are significant carbon fixers, and key for the calcite fossil record (Young & Henriksen, 2003). However, the evolution and phylogeny of haptophytes (prymnesiophytes) is not yet entirely clear. They are thought to form part of the Chromalveolates, where the endosymbiosis of a red alga in an ancestor led to evolution of the heterokonts, stramenopiles, alveolates, cryptophytes and haptophytes (Andersen, 2004; Keeling, 2010; Janouskovec *et al.*, 2010). An alternate hypothesis is that they belong to a unique group, the Hacrobians, which includes cryptophytes and is distinct from the SAR group (Okamoto *et al.*, 2009; Keeling, 2013). However, there is still some debate about whether this is in fact a distinct mono-phyletic group or not (Burki *et al.*, 2012). More recently, haptophytes have been suggested to be closer relatives to stramenopiles than cryptophytes (Miller & Delwiche, 2015). The continuing debate indicates that the exact position of haptophytes in the Eukaryotic tree remains unresolved (Figure 1-1).

E. huxleyi has a haplo-diplontic, hetero-morphogenic life cycle typical of haptophytes, characterized by diploid coccolith non-motile cells (C), diploid bald non-motile cells (N), and haploid motile non-calcified cells (S) which have organic scales. Each of the different cell types has the ability to reproduce through mitosis, and so far little information is available about their ability to switch between the different cell types (Klaveness, 1972; Green *et al.*, 1996; Frada *et al.*, 2012). Meiosis has been reported in other haptophytes (Houdan *et al.*, 2004), and there was an increase of haploid cells observed in a small fraction (<1%) at the end of a natural *E. huxleyi* bloom (Frada *et al.*, 2012). An increase in the proportion of *E. huxleyi* S cells, presumably created through meiosis, has been reported in response to viral infection in C populations (Frada *et al.*, 2008). The cell type has been shown to be important in affecting the life history of the algae, as the different cell types have differences in their transcriptomics (von Dassow *et al.*, 2009), as well as in their responses to viruses (Frada *et al.*, 2008). There is also genetic variability across the different strains of *E. huxleyi* found in different regions around the world, accounting for the differences in physiology (Read *et al.*, 2013; von Dassow *et al.*, 2015).

E. huxleyi is a key driver in biogeochemical cycles and their local ecosystems and has been found to have numerous interactions with the nearby organisms. One of the main methods they do this is by producing the important metabolite dimethylsulfoniopropionate (DMSP), which they use an antioxidant, osmoregulator and cryoprotector (Stefels, 2000; Burkill *et al.*, 2002; Sunda *et al.*, 2002). DMSP, its derivatives, and other small molecules are important for mediating the interactions with grazers and viruses (Wolfe *et al.*, 1997; Vardi *et al.*, 2012; Bidle, 2015). Viruses are one of the key factors responsible for bloom collapse of *E. huxleyi*, and the virally encoded-glycosphingolipids (vGSLs) – glycosphingolipids are common components of a lipid membrane – are one of the key mediators in this interaction (Brussaard *et al.*, 1996; Pagarete *et al.*, 2009; Vardi *et al.*, 2009; Bidle & Vardi, 2011). The other significant cause of bloom collapse are microzooplankton (Wilson *et al.*, 2002b; Fileman *et al.*, 2002). Bacterial numbers increase at bloom termination (Castberg *et al.*, 2001; Wilson *et al.*, 2002a), but are generally not thought to contribute to causing the bloom collapse.

1.3.2 Description of Roseobacter clade

Roseobacters are an abundant group of marine α-proteobacteria, accounting for 15-20% of the bacterioplankton communities. First described in 1991 with the discovery of *Roseobacter litoralis* and *Roseobacter dentrificans,* their distinctive properties was their pink pigment, as well as containing bacteriochlorophyll (Bchl) *a*, which led to their naming (Shiba, 1991). There are now almost 20 genera in the Roseobacter clade, accounting for 41 described species that are physiologically diverse (Buchan *et al.*, 2005; Wagner-Döbler & Biebl, 2006). They are found in varied habitats, ranging from sea ice, open oceans to tropical coral reefs, and can be free-living or particle associated. Commonly described as ecological generalists, they are most frequently found in algal-associated communities (Buchan *et al.*, 2005; Newton *et al.*, 2010). Roseobacters seem to have a biphasic lifestyle whereby they can swim towards or away from certain signalling molecules (Miller *et al.*, 2004; Wadhams & Armitage, 2004), or

switch to form attachments to surfaces as they are rapid colonizers and biofilm formers (Bruhn *et al.*, 2006, 2007; Geng *et al.*, 2008). Some roseobacters have the ability to carry out aerobic anoxygenic photosynthesis (which does not generate oxygen) (Allgaier *et al.*, 2003). One of their most important ecological roles is in converting DMSP into dimethyl sulphide (DMS) (Moran *et al.*, 2003; Malmstrom *et al.*, 2004). DMS forms the basis of cloud condensing nuclei, thereby affecting weather formations and having global relevance in affecting Earth's climate (Howard *et al.*, 2006; Dickschat *et al.*, 2010).

There are several bioactive molecules of interest produced by roseobacters, many produced as a way to communicate with other organisms: this includes production of QS signalling molecules such as AHLs (Moran *et al.*, 2007; Cude & Buchan, 2013). Another way in which they 'talk' to their neighbouring bacteria is through the antibiotic tropodithietic acid (TDA) which is lethal to other bacteria, and which roseobacters produce mainly during their sessile phase (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005). TDA production is controlled by AHLs, and the TDA can itself act as an autoinducer for TDA production (Berger *et al.*, 2011). However, QS is only one of the regulatory systems involved in TDA synthesis, as QS mutants show delayed TDA production, not complete lack of TDA synthesis (Prol García *et al.*, 2013).

1.3.3 Mutualistic roseobacter – *E. huxleyi* interactions

Roseobacters are numerically dominant in *E. huxleyi* blooms (Green *et al.*, 2015), and important for their symbioses. One of the most studied and well known bioactives mediating their interactions is DMSP. The alga is a major producer of the DMSP, which the roseobacters can use as a source of carbon and sulphur or convert it to DMS (González *et al.*, 2000; Kiene *et al.*, 2000; Geng & Belas, 2010). However, there are other symbioses taking place. The presence of a roseobacter has been shown to affect the levels of certain biomarkers such as alkenones in *E. huxleyi* (Segev *et al.*, 2016), thereby affecting models using the alkenones as a proxy for past temperatures (Brassell *et al.*, 1986). In addition,

the roseobacter, *Phaeobacter inhibens*, was shown to reduce the amount of lipid bodies present in the algae (Segev *et al.*, 2016).

The ability to produce several important vitamins, such as vitamin B_{12} (necessary for methionine synthase), is absent from the *E. huxleyi* genome (Read *et al.*, 2013), with bacteria ready to supply this vitamin, thereby benefiting the algae (Croft *et al.*, 2005; Wagner-Döbler *et al.*, 2010). Roseobacters are thought to provide other growth factors to their algal hosts, including a plant hormone indole-3-acetic acid (IAA) (Fernandes *et al.*, 2011), the growth promoting phenylacetic acid (PAA) (Seyedsayamdost *et al.*, 2011b), as well as providing chemical defence from competing pathogenic bacteria through the production of TDA (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005).

1.3.4 Pathogenic switch caused by algaecidal small molecules

Previous research has proposed that the roseobacter *Phaeobacter gallaeciensis* DSM 26640 displays a fascinating switch from mutualism to pathogenicity in response to algal senescence molecules. As the algae age, the bacteria are triggered to produce compounds called roseobacticides that can act as potent algaecides (Seyedsayamdost *et al.*, 2011b). The production of these compounds are stimulated by the proposed senescence molecule *p*-coumaric Acid (*p*CA), a product of algal lignin degradation (Schaefer *et al.*, 2008; Weng & Chapple, 2010). Roseobacticides are assembled from the bacterially produced PAA, algal *p*CA, and Cysteine derived from algal DMSP (Seyedsayamdost *et al*., 2014). These roseobacticides are of interest as they demonstrate a highly specific activity. When tested against selected haptophytes, a green alga, a diatom, and a cryptomonad, only *E. huxleyi* was shown to be susceptible to the algaecidal effects of the bioactive. The diatom *Chaetoceros muelleri* demonstrated morphological changes in response to the roseobacticide. This targeted activity, such as cell lysis resulting in the release of internal compounds, can be useful for commercial processing

(Seyedsayamdost *et al.*, 2011b). These key interactions of both symbiosis and pathogenicity between the alga and the roseobacters are summarized in Figure 1-2.

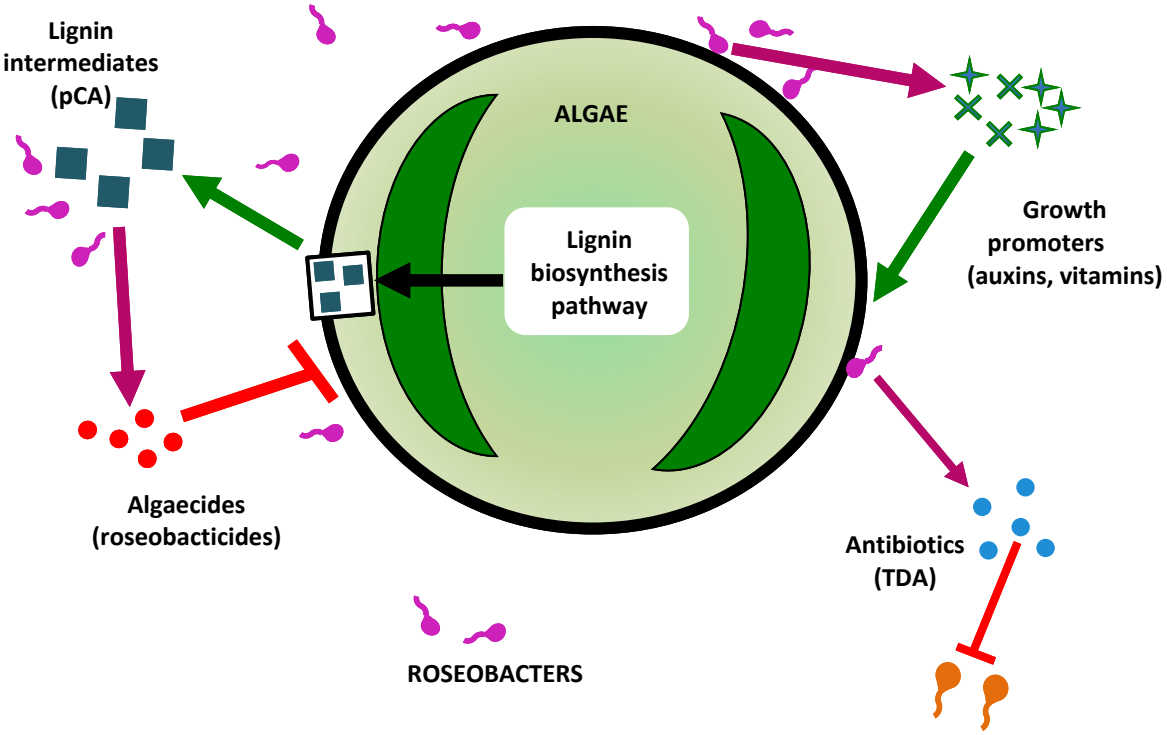

Figure 1-2: Model of proposed interactions between roseobacters and *Emiliania huxleyi*.

Figure adapted from previous literature (Seyedsayamdost *et al.*, 2011b). Central is the algae (green), surrounded by roseobacters (pink) and other potential bacteria (orange). Various small molecules are shown mediating the interactions, where black arrows indicated that internal algal production, green arrows indicate it is produced by the algae, pink arrows produced by the bacteria, and red means it has a negative effect towards the other species.

1.4 Implications of small molecules in commercial applications of algae

Algae are grown commercially for a number of reasons, such as food supplements or for fish feedstocks. The macroalgal market accounted for circa US $6 billion in 2004, with more than 7.5 million tonnes harvested per year, while in the same year the microalgal biomass market generated about US $1.25 billion, with 5,000 tonnes of dry matter harvested (Pulz & Gross, 2004). Algae are also a promising

source of next-generation energy as they can be harvested to produce methane, ethanol, hydrogen, and biofuels (Chisti, 2007; Wang *et al.*, 2008). Algae have many distinct advantages over first and second generation biofuel crops, such as corn and sugarcane. These advantages include higher photosynthetic productivity and yield per unit of land, high lipid content, as well as the ability to produce commercially lucrative by-products (Chisti, 2008; Schenk *et al.*, 2008; Amin, 2009; Brennan & Owende, 2010). Small scale production of algal biofuels has been largely successful (Sheehan, 1998). This has led to an increase in research interest and industry investment in microalgae as a biofuel crop. However, the goal of commercially significant volumes of algal derived biofuels at a competitive market price remains elusive. There are still several barriers to overcome in order to achieve these goals, including scaling issues, optimization of downstream processing, and improving the economics (Mata *et al.*, 2010; Brennan & Owende, 2010; Georgianna & Mayfield, 2012).

Large scale growth of microalgae is conducted in open or closed systems or photobioreactors (Chisti, 2007; Brennan & Owende, 2010). Downstream processing includes a number of steps to concentrate the algal biomass for harvest. Several strategies are available for the conversion of the algal biomass to biofuel, often adapted from other biofuel crop systems, which include thermochemical or biochemical conversion steps (Brennan & Owende, 2010). Various studies and life cycle assessments have indicated that one of the major steps that can be improved upon in terms of energy consumption and economics is the harvesting stage of the biomass (Uppán, 2002; Molina Grima *et al.*, 2003; Levine *et al.*, 2009; Clarens *et al.*, 2010). Harvesting is also an important step when the aim is to collect valuable algal metabolites that can be used and/or sold for additional profit (Greenwell *et al.*, 2010). The current methods most commonly employed are: centrifugation; gravity sedimentation; filtration; and flocculation (Molina Grima *et al.*, 2003; Chisti, 2007; Brennan & Owende, 2010; Zhang et al., 2014). All of these methods can be problematic to scale-up. One area that can be further investigated for improvement is flocculation, which often precedes other harvesting steps. Currently, flocculation tends

to use multivalent metal salts (such as Ferric Chloride or Aluminium Sulphate) to coagulate the cells (Molina Grima *et al.*, 2003). However, there is still an issue with toxicity, cost, and scale-up. Auto-flocculation of algae and the use of bioflocculants – flocculent producing bacteria – to coagulate the algae is an attractive alternative (Oh *et al.*, 2001; Gutzeit *et al.*, 2005; Wang *et al.*, 2012; González-Fernández & Ballesteros, 2013).

Removing bacteria from algal species has been shown to drastically reduce their flocculating ability, and further research in key species would help improve this field (Lee *et al.*, 2013; Ramanan *et al.*, 2016). This also demonstrates the importance of understanding the natural communities present in algal systems, and the possibilities in artificially creating a community ('synthetic ecology') to increase the robustness and productivity of a commercial algal system (Kazamia *et al.*, 2012; Cho *et al.*, 2015). This can prevent algal crashes from occurring, which causes delays in production and increases in cost. Understanding the underlying interactions occurring in this community, as well as the bioactive molecules involved, will allow for control over the systems, including increased lipid production (Keshtacher-Liebso *et al.*, 1995; Lenneman *et al.*, 2014; Cho *et al.*, 2015). Alternatively, addition of bacteria, or bioactive molecules that cause a change in the symbiosis of the bacteria towards the algae, could allow for timed death within the system. The impact and possibilities of bacteria in commercial systems has been greatly underestimated, and only recently is the potential of bacteria starting to be recognized (Wang *et al.*, 2014a), although the role of the metabolites involved is still greatly overlooked.

1.5 Thesis objectives and outline

The fact that *P. gallaeciensis* responds to *p*CA, a lignin intermediary, is an evolutionary puzzle; lignin is a complex and highly recalcitrant – difficult to break down – form of carbon often thought to be one of the key evolutionary advancements allowing the movement of plants from marine habitats to terrestrial ecosystems, essential for structural support and water retention (Boerjan *et al.*, 2003; Weng

& Chapple, 2010). The discovery of lignin and its intermediates in algae present the intriguing question as to their role (Martone *et al.*, 2009; Seyedsayamdost *et al.*, 2011b; Goiris *et al.*, 2014). *p*CA has also been shown to be the precursor to a novel class of homoserine lactone QS molecule (Schaefer *et al.*, 2008). That, and other intermediates, has led to the theory that they may play a role in microbial defence and be important in the algal arsenal against bacteria in plants (Boudet, 2000; Tronchet *et al.*, 2010), and this potential role in algae warrants further investigation.

Plant hormones (phytohormones) are well characterized, and are known to play a role in plant-microbe interactions. Algae have been suggested to produce a range of plant hormones (Tarakhovskaya *et al.*, 2007; Lu & Xu, 2015), although their presence in algae is still debated (Lau *et al.*, 2009; Ross & Reid, 2010). Plant hormones may play a role in an alga's stress response, as suggested for the chlorophyte *Klebsormidium crenluatum* (Holzinger & Becker, 2015). One important class of plant hormones, IAA, was reported to be produced by an axenic brown macroalgae, *Ectocarpus siliculosus* and affected the growth of the alga (Le Bail *et al.*, 2010). However, subsequent research indicated that an un-culturable microbe associated with the alga could be responsible for the IAA production (Dittami *et al.*, 2014). The bacteria is thought to complement the biosynthesis of IAA in the algae, as the algae has more of the initial genes and the bacteria has the later genes in the biosynthetic pathway necessary to produce IAA (Dittami *et al.*, 2014). IAA has been shown to play important roles in the virulence of plant associated bacteria (Zhu *et al.*, 2000; Liu & Nester, 2006). Bacteria are also capable of producing some of these phytohormones (Kudoyarova *et al.*, 2015), including roseobacters and other marine bacteria (Ashen *et al.*, 1999; Fernandes *et al.*, 2011). This would suggest that IAA is an important bioactive in the cross-talk between algae and marine bacteria, similar to their function between plants and their bacteria (Patten & Glick, 2002; Spaepen *et al.*, 2007). This was further demonstrated by the diatom *Pseudo-nitzschia multiseries* which provided its associated roseobacter, *Sulfitobacter sp.* SA11 with tryptophan, which the bacteria converted into IAA, which in turn impacted the growth of the diatom (Amin *et al.*,

2015). There are still plenty research needed on these, and other, possible roles for algal-bacterial interaction.

1.5.1 Reducing a complex system to its component parts to reveal the individual interactions

In order to tease apart some of the interactions between algae and bacteria, a model system using a single alga-bacterium is useful as it breaks down a complex system to its component parts. This is the methodology that shall be followed in this thesis. However, this system is not without its own caveats. Field experiments can be unwieldy, costly, and multifaceted, while a simple model system can provide a lot of advantages, including: better experimental control, limitation of the variables, and easier replication (Jessup *et al.*, 2004). However, these very advantages have led to criticisms of model systems; namely that they lose relevance and do not accurately represent the ecological system across different temporal or spatial ranges (Carpenter, 1996). For algae, larger scales can affect the algal characteristics, as the turbulence, light penetration, nutrient availability, and the bacterial assemblage present becomes less homogenous, and this heterogeneity can make extrapolations difficult from smaller scale (Grossart, 1999; Fon Sing *et al.*, 2013; Lohrer *et al.*, 2015).

Reducing an ecosystem down to its individual components may minimize some of the complexity present, but in doing so there is the added benefit of identifying how subsets of the consortia directly interact. A microbial community will commonly consist of various subsets, some symbiotically working together, while others benefit directly from certain subsets, or compete with other subsets of the population to gain dominance of the population. In this way more complex communities will have symbioses, mutualism, and pathogenesis all occurring between various organisms within the community, which may not be directly observed by a single model system. Further complicating the system, some bacteria may break down products produced by other members of the community, which

will have novel impacts on the system being studied. As a final level of complexity, the environmental conditions may affect the interactions (Grossart, 1999). Understanding the limits of the relevance of conclusions drawn, and the relative ability to be scaled up is crucial in designing a good model system (Lawton, 1995; Jessup *et al.*, 2004). While recent advances in 'omics reveal a new level of detail in our understanding in microbial ecology (Jansson *et al.*, 2012; Cooper & Smith, 2015), they can be used in conjunction with lab systems or to better inform new hypothesises for new experiments (Amin *et al.*, 2015; Hom *et al.*, 2015). Model systems can act as a link between theory and nature, and there is a constant interplay between model driven insights and the relative complexity of the model and field observations (Drake *et al.*, 1996).

1.5.2 Thesis objectives

My general aim is to assess the role of small molecules in algal-bacterial interactions and determine the molecules that may mediate these interactions and elicit physiological changes in algae. Ultimately, these bioactive compounds are screened for properties that have the potential to improve algal lipid and growth yields. My research focuses on the identification of bacterially derived small bioactive molecules which may affect algal physiology. I also assess the effect of the algae on the growth and metabolism of associated bacteria to determine the nature of reciprocity in the relationship. Using the large amount of publically available genomic data available, novel biosynthetic pathways can be determined in both alga and bacteria. The hypothesized relationship dyamics of microalgae and associated bacteria are summarized in Figure 1-2, with some of the various bioactive small molecules that play a part in this complex interaction shown.

The specific hypotheses of my project are:

1. Given the presence of lignin in red algae and the role of the intermediates in algal-bacterial interactions, it is hypothesized that the presence of homologues of the lignin biosynthesis pathway will be present in the genomes of various algae;
2. The roseobacter, *Phaeobacter gallaciencsis*, should kill the algae *Emiliania huxleyi* in a manner similar to the roseobacticides it produces, while the role of the lignin intermediate *p*CA in regulating the production of the various bioactives the bacterium produces shall be investigated;
3. Since the phytohormone indole-3-acetic acid has been shown to mediate algal-bacterial interactions, a similar role may be present between an IAA producing roseobacter and *Emiliania huxleyi*; and,
4. The bioactive molecules or the roseobacters themselves may increase lipid production for commercial applications, such as for biofuel production.

1.5.3 Thesis outline

My hypotheses shall be addressed in the following chapters:

Chapter 2 will review the current knowledge of the presence of lignin in algae, as several species have been found to have either lignin or its intermediates. Looking at the available public genomes, I found that the presence of the H-lignin biosynthesis pathway was more widely distributed in algae than previously thought. Some of these intermediates are shown to have inhibitory effects on common algal bacterial symbionts.

Chapter 3 investigates the interaction between the roseobacter, *Phaeobacter gallaeciensis* and its algal host *E. huxleyi.* The bacterium was found to be highly specific in killing only the coccolith bearing strain of *E. huxleyi*, while allowing the bald strain to survive. The production of known algaecides

(roseobacticides) by the bacterium was stimulated by the addition of a lignin intermediate, *p*-coumaric acid.

Chapter 4 looks at another set of small molecules traditionally only associated with terrestrial systems, specifically the phytohormone indole-3-acetic acid (IAA). A review of the publicly available algal genomes revealed that algae may have the presence of the IAA biosynthetic pathway; as such, *E. huxleyi* was stimulated with the IAA precursor, L-tryptophan, then screened for the presence of IAA. The coccolith bearing strain of *E. huxleyi* was shown to produce IAA, while the bald strain was not. However, the bald strain was shown to be more sensitive to the addition of exogenous IAA. Addition of L-tryptophan or IAA did not significantly impact the alga's relationship with a bacterium, *Ruegeria sp.* R11, which has been shown to produce IAA previously.

Chapter 5 looks at the effect on lipid and accumulation of auxin and bacteria, with limited potential being shown by these screened products for commercialization.

Chapter 6 is the general discussion of my thesis. The presence, role and evolutionary considerations of the bioactives researched in this thesis are discussed, and the proposed model of interactions is updated in light of the results. Possible future avenues of research are discussed.

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Chapter 2

Ancient origin of the biosynthesis of lignin precursors

2.1 Introduction

Lignin is a complex and highly recalcitrant form of carbon often thought to be one of the key innovations of land plants, allowing the movement of plants from aquatic habitats to terrestrial ecosystems (Kenrick & Crane, 1997; Weng & Chapple, 2010). It is the second most abundant biopolymer on earth, after cellulose (Boerjan *et al.*, 2003). In land plants, lignin is deposited in the secondary cell wall and provides structural support, giving rigidity and strength to stems (Campbell & Sederoff, 1996; Espiñeira *et al.*, 2011), as demonstrated by lignin-deficient mutants with low structural support (Jones *et al.*, 2001; Bonawitz & Chapple, 2010). The hydrophobic nature of lignin makes it impermeable to water and is thus crucial for a vascular system, enabling transport of water throughout the plant (Campbell & Sederoff, 1996). In addition, lignin has been suggested to protect plants from biological attacks. Being recalcitrant to biological degradation, increased lignification can offer protection when a plant is damaged (Moura *et al.*, 2010). The precursors of lignin (*p*-coumaric acid, coumaroyl-CoA, coumaraldehyde) and its monomers (monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) have also been suggested to have antimicrobial properties (Keen & Littlefield, 1979; Tronchet *et al.*, 2010).

Lignin biosynthesis starts with the general phenylpropanoid pathway, which can generate precursors to a diverse group of compounds including flavonoids, coumarins, quinones, and monolignols (Figure 2-1). The starting point of this pathway is the amino acid phenylalanine, which is deaminated to form cinnamic acid, followed by *p*-coumaric acid and coumaroyl-CoA (Zhong & Ye, 2009). The lignin specific

pathway then uses coumaroyl-CoA to produce the simplest monolignol, H monolignol (*p*-coumaryl alcohol), through a series of reduction reactions that modify the side chain (Bonawitz & Chapple, 2010) (Figure 2-1). Production of the more structurally complex G (coniferyl alcohol) and S (sinapyl alcohol) monolignols requires additional enzymes using intermediates of H monolignol synthesis as substrates, which perform O-methylation and hydroxylation at various sites on the phenolic ring (Weng & Chapple, 2010). After their biosynthesis inside the cell, the monolignols are transported to the cell wall through an unknown mechanism, before undergoing cross-linking of the monomers to form the lignin polymer. This process, called lignification, is not well understood but involves simple radical coupling reactions between monolignols and a growing lignin polymer (Lewis & Yamamoto, 1990). The simple coupling reactions are believed to be carried out by peroxidases and laccases, enzymes that are diverse and abundant in eukaryotes (Boerjan *et al.*, 2003; Vanholme *et al.*, 2010).

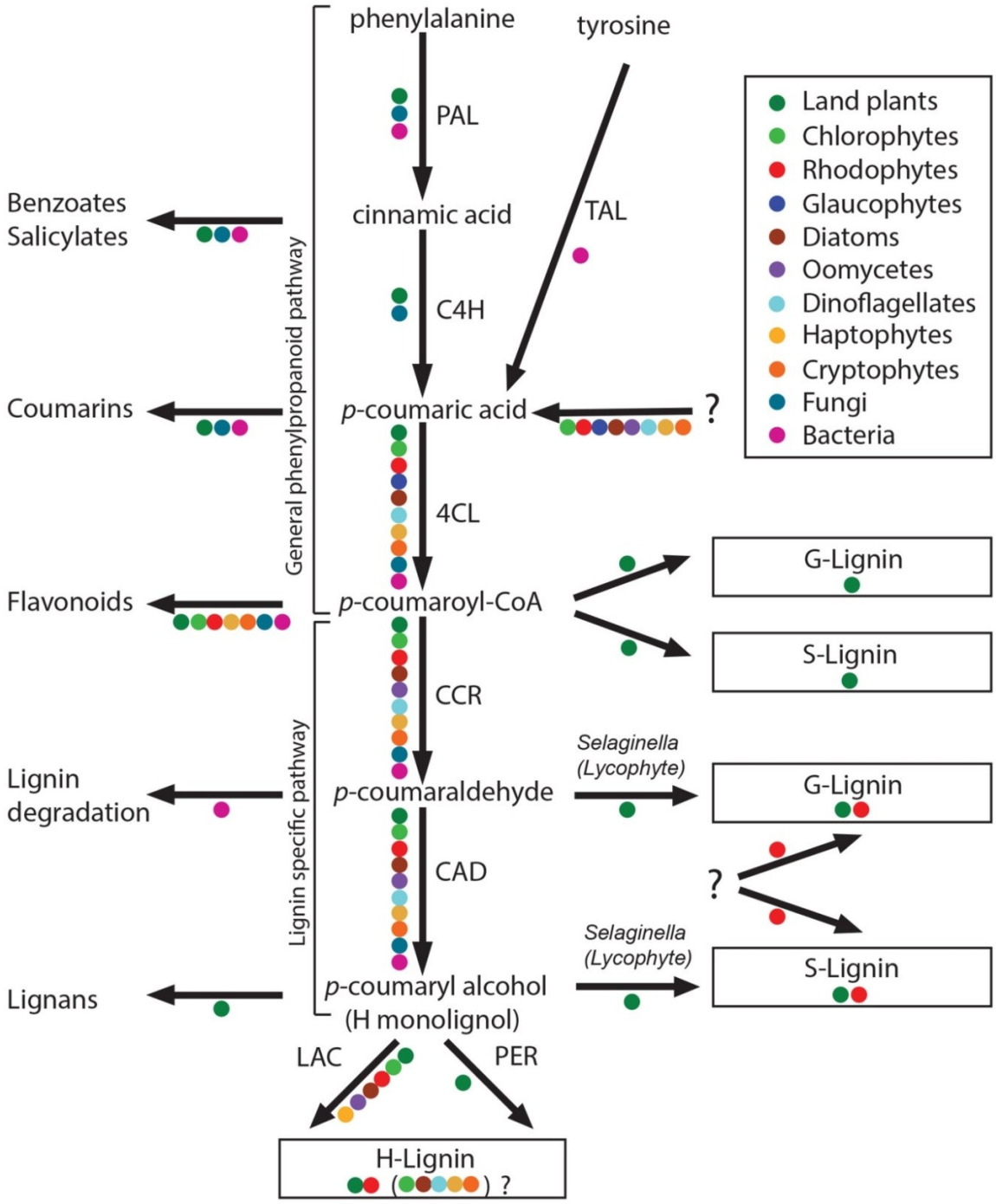

Figure 2-1: The lignin biosynthesis pathway as currently conceptualized in land plants.

Coloured dots represent the presence of a given enzyme in a specific taxonomic group. The abbreviations used for the enzyme are: phenylalanine ammonia-lyase (PAL); cinnamate 4-hydroxylase (C4H); 4-coumarate:CoA ligase (4CL); cinnamoyl-CoA reductase (CCR); cinnamyl alcohol dehydrogenase (CAD); peroxidase (PER); and laccase (LAC). Question marks (?) indicate that the enzyme responsible for a specific function or its substrate is unknown, or that the presence of a particular compound in a given taxonomic group is hypothesized but has not been demonstrated.

The lignin biosynthesis pathway has been extensively studied in vascular plants, and was thought to be a hallmark of this group (Weng *et al.*, 2008a). Gymnosperms are usually primarily composed of G lignin (Uzal *et al.*, 2009), angiosperm eudicots are composed of predominantly G and S lignins, while monocots (such as grasses) usually have more H lignin content (Boerjan *et al.*, 2003; Vanholme *et al.*, 2010). Investigation of so called "lower" (non-vascular) plants revealed the evolution of lignin biosynthesis to be more ancient than the origin of vascular plants. *Selaginella moellendorfii* (spikemoss), which is part of the most ancient lineage of vascular plants, the lycophytes, can synthesize all three types of lignin, including the supposedly-derived S lignin, which is synthesized using an enzyme that is absent from all other land plants (Weng *et al.*, 2008b, 2010). Therefore, *S. moellendorfii* likely developed this ability independently of angiosperms, which were previously the only organisms believed capable of synthesizing S lignin. Even the moss *Physcomitrella patens*, a representative of the most ancient group of land plants, the non-vascular bryophytes, encodes all lignin biosynthesis enzyme necessary to synthesize H and G lignins in its genome (Espiñeira *et al.*, 2011). Although true polymerized lignin has not been found in this organism, it contains monolignols and lignin-like molecules (Ros *et al.*, 2007; Xu *et al.*, 2009; Espiñeira *et al.*, 2011). More surprising still is that H, G and S lignins have recently been identified in the cell walls of the calcified red alga *Calliarthron cheilosporioides* (Rhodophyta) (Martone *et al.*, 2009). Given that land plants are separated by a large evolutionary distance from rhodophytes, convergent evolution of lignin biosynthetic genes has been suggested as an explanation of how *C. cheilosporioides* acquired lignified cells (Vanholme *et al.*, 2010). There is some evidence that lignin might also be found in other algae besides rhodophytes, but it is still heavily debated. Homologs of some of the genes for lignin biosynthesis have also been found in green algae and diatom species (Xu *et al.*, 2009). Charophytes, a class of freshwater streptophyte algae, have been suggested to contain lignin-like compounds (Delwiche *et al.*, 1989; Kroken *et al.*, 1996), as have some brown algae (Reznikov *et al.*, 1978; Dovgan & Medvedeva, 1983; Dovgan *et al.*, 1983). Later studies argued against this, claiming that

brown algae contain only phenol compounds and not specifically lignin (Ragan, 1984; Lewis & Yamamoto, 1990).

Recent research has found that the lignin precursor, *p*-coumaric acid, as well as key genes of the lignin biosynthesis pathway are present in the haptophyte *Emiliania huxleyi* (Seyedsayamdost *et al.*, 2011b). Another study also found *p*-coumaric acid as well as flavonoid compounds derived from it, in a diatom (*Phaedactylum tricornutum*), a haptophyte (*Diacronema lutheri*) and several green algae (*Tetraselmis suecica, Chlorella vulgaris* and *Haematococcus pluviaris*) (Goiris *et al.*, 2014). It is surprising that haptophytes and diatoms contain a lignin precursor, as well as the genes potentially encoding enzymes to convert it into monolignol. Haptophytes and diatoms are evolutionarily very distant to plants, red and green algae and to each other. The potential for these two phylogenetically disparate phytoplankton to synthesize monolignols suggests that the pathway for lignin synthesis might have evolved in ancient oceans and may vastly predate the origin of land plants. To investigate this possibility, we screened all available algal genomes for the presence of the lignin biosynthesis pathway, in addition to a selection of representative species of land plants and other eukaryotes belonging to various supergroups (unikonts, archaeplastids, stramenopiles, alveolates, cryptophytes, haptophytes). Although known genes required for the synthesis of G and S lignins were mostly absent outside of land plants, homologs of those needed for making the monomer of the simplest form, *p*-coumaryl alcohol (H monolignol), were found in green algae, red algae and glaucophytes, as well as multiple organisms in supergroups other than Archaeplastida. Phylogenetic analysis of the *p*-coumaryl alcohol biosynthesis genes suggests an early origin for this metabolic process, followed by at least three examples of independently evolved enzymes allowing organisms to make more complex derivatives, G and S monolignols, in red algae, club mosses and the ancestor of vascular plants.

2.2 Methods

2.2.1 Database search

Sequences for the enzymes in the phenylpropanoid and lignin specific pathways of *Arabidopsis thaliana* were found in the Kyoto Encyclopedia of Genes and Genomes *(*KEGG) database (http://www.genome.jp/kegg/), as well as previous literature (Xu *et al.*, 2009; Barakat *et al.*, 2011; Vanholme *et al.*, 2013). Additional enzymes from the lignin pathway found only in *Selaginella moellendorffii* were obtained from the KEGG database and previous literature (Weng *et al.*, 2008b). Whenever possible, proteins that have been biochemically characterized were used as queries. These protein sequences [NCBI: NP_179765, NCBI: NP_188576, NCBI: NP_173872, NCBI: NP_001077697, NCBI: NP_173047, NCBI: NP_181241, NCBI: NP_180607, NCBI: NP_199704, NCBI: NP_200227, NCBI: NP_850337, NCBI: NP_195345, NCBI: XP_002963471, NCBI: AAB09228, NCBI: XP_002992167] were used to query the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) for homologs, using BLASTp and tBLASTn searches of the protein, genome and expressed sequence tags (EST) databases. An e-value of 10^{-30} or less was used as a stringent cut-off for homology. A bi-directional best hit (BBH) BLASTp search of the hits on *Arabidopsis thaliana* proteins was then performed to confirm orthology. Searches of the algal genomes were carried out using the Department of Energy (DoE) Joint Genome Institute (JGI) database (http://genome.jgi.doe.gov) using the latest releases as of April 2014 of the dataset created with 'all models' gene prediction algorithms. The algal genomes included were: *Chamydomonas reinhardtii, Chlorella sp.* NC64A*, Micromonas sp.* RCC299*, Micromonas pusilla CCMP 1545, Ostreococcus lucimanarinus, Ostreococcus sp.* RCC809*, Volvox carteri f. nagariensis, Fragilariopsis cylindrus, Thalassiosira pseudonana, Phaeodactylum tricornutum, Aureococcus anophagefferens,* and *Emiliania huxleyi* CCMP 1516. Additional algal genomes were retrieved from NCBI, including: *Bathycoccus prasinos, Botryococcus braunii, Chlorella variablilis,*

Coccomyxa subellipsoidea, Dunaliella salina, Haematococcus pluvialis, Ostreococcus lucimarinus, Ostreococcus tauri, Polytomella sp., Polytomella parva, Prototheca wickerhamii, Chondrus crispus, Galdiera sulphararia, Ectocarpus siliculosus, Nannochloropsis gaditana, Ochromonas danica, Guillardia theta, and *Hemiselmis andersenii.* The genome of the dinoflagellate *Symbiodinium minutum* was searched using http://marinegenomics.oist.jp. Additionally, the genomes of the following reference land plants were searched in NCBI: *Oryza sativa, Physcomitrella patens, Selaginella moellendorffii.* The genomes from non-photosynthetic species closely related to chloroplast-bearing taxa were also specifically searched: *Albugo laibachii, Phytophthora infestans, Phytophthora sojae, Cryptosporidium muris, Cryptosporidium parvum, Theileria parva, Toxoplasma gondii,* and *Tetrahymena thermophile. Paramecium tetraurelia*, using http://paramecium.cgm.cnrs-gif.fr/db/tool and *Picea abies* using http://congenie.org/blastsearch, were also searched. Local searches against red algal genomes were carried out for *Calliarthron tuberculosum, Cyanidioschyzon merolae,* and *Porphyridium cruentum* (Chan *et al.*, 2011). *C. tuberculosum* is very closely related to the other species of *Calliarthron*, *C. cheilosporioides*, in which lignin was physically identified (Gabrielson *et al.*, 2011). In addition, Dr Adrian Reyes performed a local search on *Cyanophora paradoxa* genome (Price *et al.*, 2012).

2.2.2 Functional Prediction

Functional prediction was performed on all homologs of enzymes in the phenylpropanoid and lignin specific pathways found in our public database searches. Two protein function prediction packages were used, Argot2 (Falda *et al.*, 2012) as well as ESG (Chitale *et al.*, 2009). These are two of the top programs for protein function prediction according to the ongoing Critical Assessment of protein Function Annotation (CAFA) study (Radivojac *et al.*, 2013). Argot2 performs BLAST and HMMer searches of sequence databases and then annotates the results with GO (Gene Ontology) terms retrieved from the UniProtKB-GOA database and terms which are then weighted using the e-values from BLAST and

HMMer. The weighted GO terms, which can also be provided directly, are processed according to both their semantic similarity relations described by the Gene Ontology and their associated score. ESG recursively performs PSI-BLAST searches from sequence hits obtained in the initial search from the target sequence, thereby performing multi-level exploration of the sequence similarity space around the target protein. Each sequence hit in a search is assigned a weight that is computed as the proportion of the log(e-value) of the sequence relative to the sum of log(e-value) from all the sequence hits considered in the search of the same level, and this weight is assigned for GO terms annotating the sequence hit. The weights for GO terms found in the second level search are computed in the same fashion. Ultimately, the score for a GO term is computed as the total weight from the two levels of the searches. The score for each GO term ranges from 0 to 1.0.

2.2.3 Phylogenetic analysis

As only land plants contained most genes found in the phenylpropanoid and lignin biosynthesis pathways, only the three core enzymes of the *p*-coumaryl alcohol biosynthesis pathway were further analyzed: cinnamyl alcohol dehydrogenase (CAD), 4-coumarate:CoA ligase (4CL), and cinnamoyl-CoA reductase (CCR). In order to ensure the sequence dataset was complete, additional sequences of enzymes from reference plant genomes characterized in previous literature were added to the datasets (Ehlting *et al.*, 1999; Lauvergeat *et al.*, 2001; Larroy *et al.*, 2002; Raes *et al.*, 2003; Costa *et al.*, 2003; Sibout *et al.*, 2005; Mee *et al.*, 2005; Baltas *et al.*, 2005; Silber *et al.*, 2008; Barakat *et al.*, 2009; Xu *et al.*, 2009; Barakat *et al.*, 2011; Guo *et al.*, 2010; Fraser & Chapple, 2011). Protein sequences were imported into Geneious Pro v5.5 (Biomatters, New Zealand) (Drummond *et al.*, 2011) and multiple alignments were constructed using MUSCLE (Edgar, 2004). The alignments were then edited in Geneious. Core functional domains and motifs were determined using the NCBI conserved domain search on the *Arabidopsis thaliana* proteins as well as previous literature (Ehlting *et al.*, 2001; Guo *et al.*, 2010).

Proteins in which these core motifs were not conserved were eliminated. Poorly aligned regions were manually edited. The alignments were then imported into Randomized Axelerated Maximum Likelihood v.7.0.4 (RAxML) (Stamatakis, 2006) (http://sco.h-its.org/exelixis/software.html) to create a maximum likelihood phylogenetic trees (WAG substitution model, 100 bootstrap replicates, gamma distribution parameter estimated). The tree was formatted for presentation in FigTree v.1.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.2.4 Bacterial strains

The bacteria *Ruegeria sp.* R11, *Phaeobacter gallaeciensis* 2.10 *Vibrio cholerae* 11EO7, and *Vibrio fischeri* MY-1 were maintained at 30 °C on ½ YTSS agar (2g Bacto yeast, 1.25g tryptone, 20g sea salts, and 15g Bacto agar in 1L, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) then grown on a rotating drum to stationary phase in 5 mL ½ YTSS broth (2g Bacto yeast, 1.25g tryptone, 20g sea salts, in 1L, Becton, Dickinson and Company) for 24 hr before experiments.

2.2.5 Determination of minimum inhibition and killing concentration

p-coumaric acid (*p*CA), ferulic acid, and cinnamic acid (Sigma-Aldrich, St. Louis, MO, USA) were freshly prepared by creating a 128 mM stock solutions for the first two, and 32mM for the latter in bacterial media, pH adjusted to 7, and subsequent filter sterilization.

A 1/100 dilution was created of the bacteria in fresh ½ YTSS broth. This was then aliquoted into 96-well plates (Corning, Sigma-Aldrich, St. Louis, MO, USA). Blank media was also aliquoted as a control. The plates were grown at 30°C with low level shaking and with the OD at 600nm measured every 20 minutes in a Molecular Devices ThermoMax plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) and KC4 microplate software (Bio-Tek, Winooski, VT, USA) with settings adapted from previous literature (Toussaint & Conconi, 2006). At the mid-point of the exponential growth phase, half the

volume of each well was removed and replaced with various concentrations for the compounds in the ½ YTSS medium (0, 1, 2, 4, 8, 16, 32 and 64mM in triplicates for *p*CA and ferulic acid, and 0, 0.25, 0.5, 1, 2, 4, 8, and 16mM for cinnamic acid), then placed back for incubation for 24 hr and OD measurements every 20 min. The minimum inhibitory concentration (MIC) was then determined to be the concentration at which growth was reduced to half the 0mM growth. After 24 hrs, 5 replicate drops of 20µL were placed on ½ YTSS agar to determine the minimum killing concentration (MKC).

In addition, *Phaeobacter gallaeciensis* 2.10 was grown in ½ YTSS with 0, 0.1, 1 and 10 mg of lignin (Sigma-Aldrich) for 24 hrs, after which the bacteria was enumerated by counting colony forming units (cfu) on ½ YTSS agar after 2 d of incubation at 30 °C.

2.3 Results and Discussion

2.3.1 The lignin biosynthetic pathway has a conserved and taxonomically widespread core

An extensive screen for homologs of the known lignin biosynthesis genes was performed across all domains of life, with a specific focus on eukaryotes (Table 2-1 and Table 2-2). Previous research has focused on lignin biosynthesis in *Arabidopsis* (Raes *et al.*, 2003; Goujon *et al.*, 2003; Costa *et al.*, 2003; Fraser & Chapple, 2011) and other model land plants (Yokoyama & Nishitani, 2004; Hamberger *et al.*, 2007; Xu *et al.*, 2009; Weng & Chapple, 2010; Carocha *et al.*, 2015), so only representative species of this group have been included in our search. As expected, all the lignin biosynthesis genes were found in all land plants for which genomes are available, with the exception of ferulate 5-hydroxylase (F5H), which is absent from the only bryophyte (moss) in our dataset, *Physcomitrella patens* (Bonawitz & Chapple, 2010). Surprisingly, four gene families had a wide distribution across the various eukaryotic supergroups and were not restricted to land plants. These include: 4-coumarate:CoA ligase (4CL); cinnamoyl-CoA

reductase (CCR); cinnamyl alcohol dehydrogenase (CAD); and caffeoyl-CoA O-methyltransferase (CCoAMT) (Table 2-1 and Table 2-2).

The first three of these enzymes (4CL, CCR and CAD) catalyze consecutive steps in lignin biosynthesis and are sufficient to produce *p*-coumaryl alcohol from *p*-coumaric acid (Figure 2-1). Homologs of all three enzymes have similar taxonomic distributions, being found mostly in marine photosynthetic algae in addition to land plants. Representatives of green algae, red algae, glaucophytes, diatoms, dinoflagellates, haptophytes and cryptophytes, as well as the non-photosynthetic oomycetes, harbor homologs of these three enzymes. Although oomycetes are non-photosynthetic, they are believed to share a photosynthetic ancestor with other stramenopiles such as brown algae and diatoms (Keeling, 2013). If the 4CL, CCR and CAD homologs in these diverse eukaryotes indeed catalyze the same biochemical reactions as their plant homologs, the capacity to make at least the precursor of the simplest form of lignin (H lignin) would be much more widespread than currently thought (land plants and red algae).

We have performed functional prediction analysis on all homologs of 4CL, CCR and CAD homologs using the Argot2 (Falda *et al.*, 2012) and ESG (Chitale *et al.*, 2009) packages, which are among the best performing functional annotation programs available (Radivojac *et al.*, 2013) (Figure 2-2). Homologs of plant CCR and CAD were predicted to have a conserved function with moderate to high confidence in at least one representative of the green algae, red algae, diatoms, dinoflagellates, haptophytes and cryptophytes. For CAD, alcohol dehydrogenase function was often predicted along with cinnamyl alcohol dehydrogenase function with comparable confidence. The prediction for the specific function of 4-coumarate:CoA ligase was difficult for 4CL homologs, being weak even for enzymes that have been biochemically characterised as having that function (such as several of *Arabidopsis thaliana* paralogous 4CL enzymes) (Bonawitz & Chapple, 2010). However, ligase function was predicted with very high or high confidence for all organisms with predicted CCR and CAD functions. Although homologs of all three

enzymes are also found in glaucophytes and oomycetes, functional predictions were weak or absent for one or more of these enzymes in these two taxonomic groups.

Most enzymes involved in lignin biosynthesis are multifunctional or have multiple, slightly divergent, paralogous copies with different functions, either with the direction of the reaction catalyzed reversed or a change in substrate affinity. This is especially true for CAD, which catalyzes the last step in monolignol biosynthesis. SAD (sinapyl alcohol dehydrogenases) cannot be differentiated from CAD phylogenetically and both enzymes display some of the other's specific activity (Barakat *et al.*, 2009; Guo *et al.*, 2010). Also, there are many enzymes with CAD activity (oxidation of an aldehyde to an alcohol) as well as alcohol dehydrogenase activity (reduction of an alcohol to an aldehyde), such as yeast ADH6 and ADH7 enzymes (Larroy *et al.*, 2002). Given the functional flexibility in these protein families, functional switches are likely to have occurred frequently throughout eukaryotic evolution, making exact functional predictions difficult without biochemical data.

Table 2-1: Distribution of lignin biosynthesis genes in archaeplastid genomes[1]

Organism	Phylum	PAL	C4H	4CL	CCR	CAD	HCT	C3H	COMT	CCoAMT	F5H	CSE	*sm*F5H	PER	LAC
ARCHAEPLASTIDS															
Land plants															
Arabidopsis thaliana	Streptophyta	+	+	+	+	+	+	+	+	+	+	+		+	+
Oryza sativa	Streptophyta	+	+	+	+	+	+	+	+	+	+	+		+	+
Physcomitrella patens	Streptophyta	+	+	+	+	+	+	+	+	+		+		+	+
Picea abies	Streptophyta	+	+	+	+	+	+	+	+	+	+	+		+	+
Selaginella moellendorffii	Streptophyta	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Green algae															
Bathycoccus prasinos	Chlorophyta					+[G]				+[G]					
Botryococcus braunii	Chlorophyta					+[E]				+[E]					
C. reinhardtii	Chlorophyta				+	+				+[G]					+[G]
Chlorella sp. NC64A	Chlorophyta				+[G]	+[G]				+[G]					+[G]
Chlorella variablilis	Chlorophyta			+[G]	+[G]	+[G]				+[G]					+[G]
Coccomyxa subellipsoidea	Chlorophyta			+[G]	+	+				+			+[G]		+[G]
Dunaliella salina	Chlorophyta					+[E]									
Haematococcus pluvialis	Chlorophyta					+[E]									
M. pusilla CCMP1545	Chlorophyta					+[G]				+[G]					
Micromonas sp. RCC299	Chlorophyta					+[G]				+[G]					
O. lucimarinus	Chlorophyta					+									
Ostreococcus sp.RCC809	Chlorophyta					+[G]									
Ostreococcus tauri	Chlorophyta					+[G]				+[G]					
Polytomella sp.	Chlorophyta					+[G]									
Polytomella parva	Chlorophyta					+[E]									
Prototheca wickerhamii	Chlorophyta					+[E]									
Volvox carteri	Chlorophyta				+[G]	+				+[G]					
Red algae															
C. tuberculosum	Rhodophyta			+[G]	+[G]	+[G]				+[G]					+[G]
Chondrus crispus	Rhodophyta			+[G]	+[G]	+[G]									+[G]
Cyanidioschyzon merolae	Rhodophyta				+[G]	+[G]				+[G]					
Galdiera sulphararia	Rhodophyta			+[G]		+[G]									
Porphyridium cruentum	Rhodophyta			+[E]	+[E]	+[E]				+[E]					
Glaucophytes															
Cyanophora paradoxa	Glaucophyta			+[G]	+[G]	+[G]									

Table 2-2: Distribution of lignin biosynthesis genes in non-archaeplastid genomes[2]

Organism	Phylum	PAL	C4H	4CL	CCR	CAD	HCT	C3H	COMT	CCoAMT	F5H	CSE	*sm*F5H	PER	LAC
STRAMENOPILES															
Diatoms															
Fragilariopsis cylindrus	Bacillariophyta			+	+[E]	+									+
Phaeodactylum tricornutum	Bacillariophyta			+	+	+									+[E]
T. pseudonana	Bacillariophyta			+[G]											
Pelagophyte															
A. anophagefferens	Heterokontophyta			+[G]		+[G]									
Brown algae															
Ectocarpus siliculosus	Phaeophyceae			+[G]											
Eustigmatophytes															
N. gaditana	Eustigmatophyceae			+[G]	+[G]										
Chrysophytes															
Ochromonas danica	Chrysoophyceae									+[E]					
Oomycetes															
Albugo laibachii	Heterokontophyta			+[G]	+[G]	+[G]									+
Phytophthora infestans	Heterokontophyta			+	+	+									+
Phytophthora sojae	Heterokontophyta			+		+									+
ALVEOLATES															
Apicomplexa															
Cryptosporidium muris	Apicomplexa				+[G]	+									
Cryptosporidium parvum	Apicomplexa				+[G]	+									
Theileria parva	Apicomplexa														
Toxoplasma gondii	Apicomplexa														
Dinoflagellates															
Symbiodinium minutum	Dinoflagellata			+[G]	+[G]	+[G]									
Ciliates															
Paramecium tetraurelia	Ciliophora			+[G]		+									
T. thermophila	Ciliophora			+[G]	+[G]										
HAPTOPHYTES															
E. huxleyi CCMP1516	Haptophyta			+	+	+				+					+[G]
CRYPTOPHYTES															
Guillardia theta	Cryptophyta			+[G]	+[G]	+[G]				+[G]					
Hemiselmis andersenii	Cryptophyta														

[1 and 2] Footnotes = + Present in both genome sequence and EST library, $+^{E}$ Present in EST library only, $+^{G}$ Present in genome sequence only. Presence (+) is determined by a bi-directional best hit (BBH) BLASTP hit with an E-value < $1x10^{-30}$ or less using the characterized land plant *A. thaliana* or *S. moellendorffii* gene as a query, searching the NCBI (http://www.ncbi.nlm.nih.gov/), JGI (http://genome.jgi.doe.gov/), Congenie (http://congenie.org/blastsearch), OIST Marine Genomics Unit (http://marinegenomics.oist.jp/genomes/gallery/) and *Paramecium* (http://paramecium.cgm.cnrs-gif.fr/) databases. The abbreviations used for the enzyme can be described as follows: phenylalanine ammonia-lyase (PAL); cinnamate 4-hydroxylase (C4H); 4-coumarate:CoA ligase (4CL); cinnamoyl-CoA reductase (CCR); cinnamyl alcohol dehydrogenase (CAD); *p*-hydroxycinnamoyl-CoA (HCT); *p*-coumarate 3-hydroxylase (C3H); caffeic acid O-methyltransferase (COMT); caffeoyl-CoA O-methyltransferase (CCoAMT); ferulate 5-hydroxylase (F5H); caffeoyl shikimate esterase (CSE) and *Selaginella moelledorfii* F5H (smF5H); peroxidase (PER); and laccase (LAC).

2.3.2 *p*-coumaryl alcohol synthesis is likely common in photosynthetic eukaryotes

Homologs for all genes of the 4CL-CCR-CAD pathway, responsible for the synthesis of *p*-coumaryl alcohol from *p*-coumaric acid, occur in several widely divergent eukaryotic taxonomic groups (stramenopiles, haptophytes, cryptophytes, dinoflagellates and archaeplastids). This does not agree with a previously proposed origin in early land plants (Boerjan *et al.*, 2003). Several alternative hypotheses could explain this distribution. The genes of this pathway (which will be called the *p*-coumaryl alcohol biosynthesis pathway henceforth) could have been present in eukaryotic ancestors predating the origin of some or all of these groups, and lost in lineages in which the pathway is absent. The superfamilies to which 4CL (adenylate-forming enzymes), CCR (adenylate reductase) and CAD (medium-chain dehydrogenase/reductases) belong are most certainly ancestral to eukaryotes, being widespread in all eukaryotic supergroups (Fulda *et al.*, 1994; Cukovic *et al.*, 2001; Nordling *et al.*, 2002; Persson *et al.*, 2009; Barakat *et al.*, 2011; Carocha *et al.*, 2015). However, close homologs to characterized plant 4CL, CCR and CAD enzymes with a correctly predicted function are not so ubiquitous. The complete *p*-coumaryl alcohol biosynthesis pathway is present almost solely in photosynthetic eukaryotes, making a scenario in which they evolve in an ancient eukaryotic ancestor and subsequently lost in most groups except photosynthetic eukaryotes unlikely. A more parsimonious explanation could be that this pathway

would have originated in an ancestor of green and red algae and subsequently been transferred to other taxonomic groups in the same way they acquired the capacity to photosynthesize: through taking up a red or green alga in a secondary endosymbiotic event (endosymbiotic gene transfer or EGT) (Keeling, 2013). Another possibility is that several genes in this pathway were acquired independently by LGT from bacteria or heterotrophic eukaryotes in various photosynthetic lineages. Phylogenetic analysis of the 4CL, CCR and CAD enzymes were performed to differentiate between these hypotheses on the origin(s) of the *p*-coumaryl alcohol biosynthesis pathway.

2.3.3 *p*-coumaryl alcohol biosynthesis could have originated in an ancient archaeplastid

The *p*-coumaryl alcohol biosynthesis pathway seems to be ancestral to green algae, but with frequent loss of this metabolic function throughout this taxonomic group (Figure 2-2). All green algal species are found within a single well-supported (97%) clade in the 4CL tree (mixed with red algae and secondarily photosynthetic organisms) (Figure 2-3) and in two clades in the CCR tree and three clades in the CAD tree (Figure 2-4 and Figure 2-5). The multiple green algal clades in the CAD tree likely represent conservation of a different paralog in two major green algal lineages; prasinophytes and the core chlorophytes (Leliaert *et al.*, 2011). The core chlorophytes are monophyletic and group with land plants, while the prasinophytes are divided in two clades, the result of multiple divergent paralogs being present in *Bathycoccus prasinos* and *Ostreococcus lucimarinus*. In the CCR tree, the two green algal clades are both composed of core chlorophytes, as well as overlapping in their species content and are proximal to each other. They likely represent paralogy originating after the divergence of core chlorophytes from ancestral green algae. Despite paralogy being observed in green algae for all three genes, the presence of *p*-coumaryl alcohol biosynthesis gene homologs in various species from two major green algal groups suggests the presence of this pathway is ancestral. Green algae are never part of clades containing organisms from other taxonomic groups besides secondarily photosynthetic species

and red algae, suggesting the absence of LGT for *p*-coumaryl alcohol biosynthesis gene homologs in this lineage. The fact that only two of fifteen green algal genomes or EST libraries screened contain homologs to all three *p*-coumaryl alcohol biosynthesis enzymes suggests this function has been frequently lost and is likely non-essential, as compared to its crucial role in most land plants.

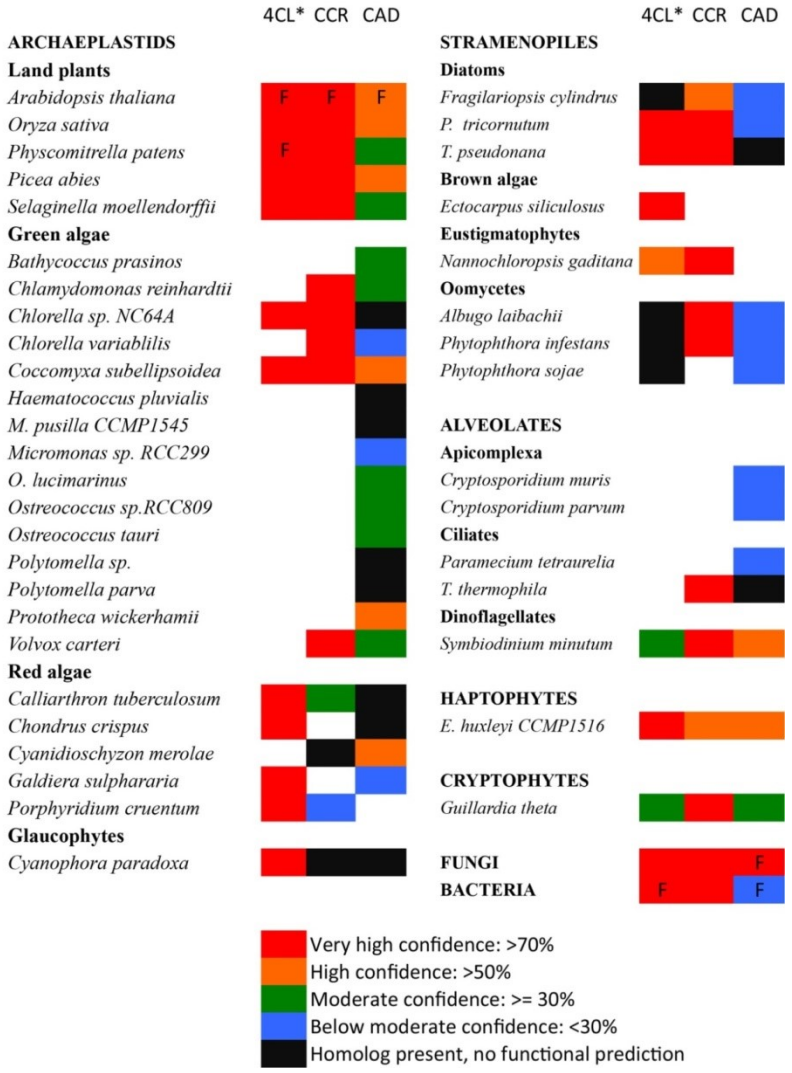

Figure 2-2: Functional prediction of the *p*-coumaryl alcohol biosynthesis pathways genes.

Both programs used for functional prediction (Argot 2 and ESG) need to predict the correct function for it to be annotated as such. An "F" indicates that an enzyme from that taxonomic group has been biochemically characterized. No single fungi or bacteria harbour homologs for all three enzymes, but all the enzymes are found individually in some representatives of these taxonomic groups. An empty space indicates that no homolog is found in a particular species. Confidence values are derived from the ESG software. * for 4CL, as even enzymes with biochemically demonstrated function are not annotated as such with significant confidence by the softwares used, the figure indicates prediction of ligase activity.

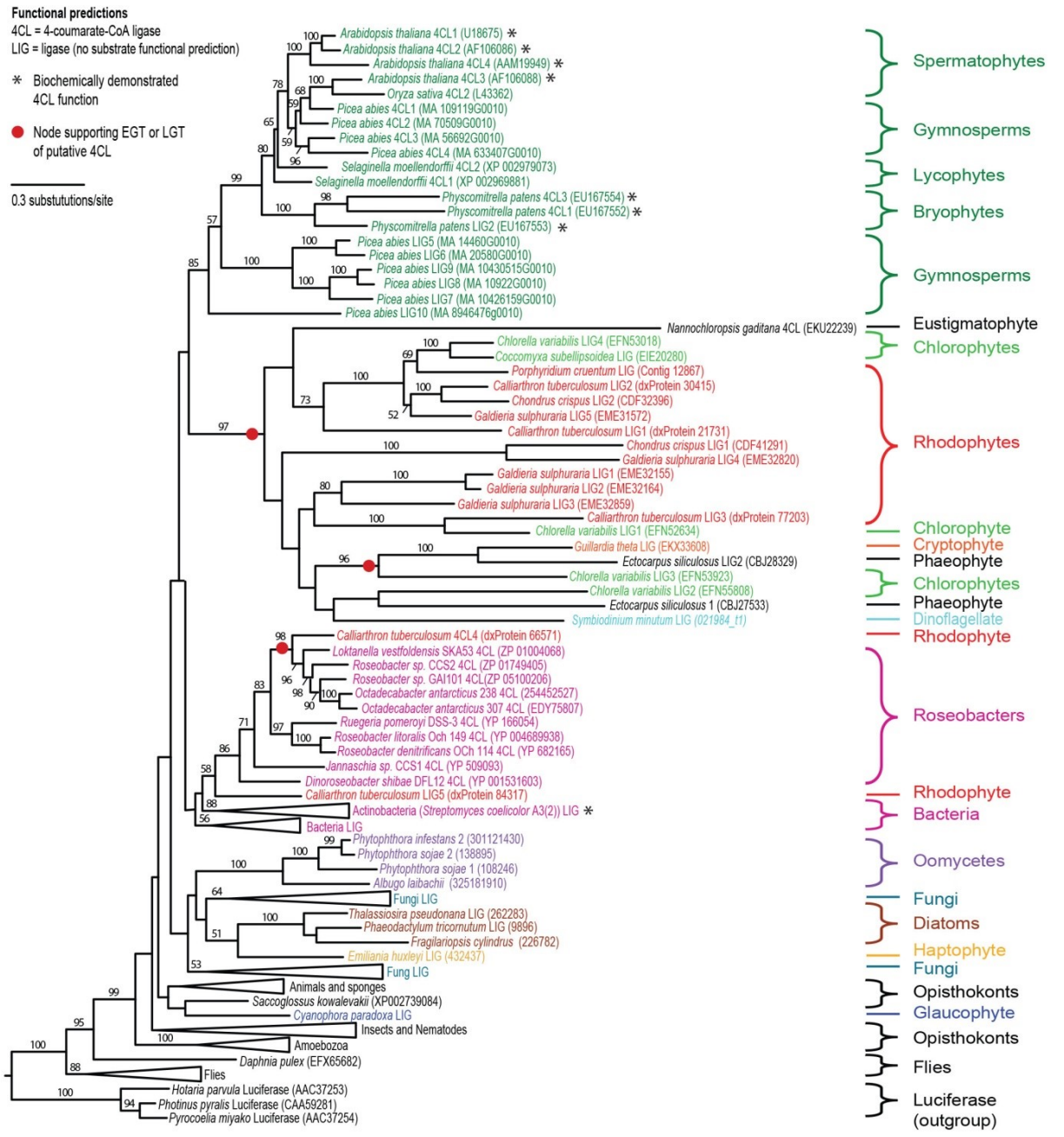

Figure 2-3: Maximum likelihood phylogeny of the 4-coumarate:CoA ligase (4CL) from the *p*-coumaryl alcohol biosynthesis pathway.

Amino acid sequences were aligned with MUSCLE and the tree compiled using RaxML. Numbers above branches refer to bootstrap values above 50%. Luciferase, a 4CL homolog (Conti et al., 1996), was used as the outgroup. Gene names are included next to taxa when function could be predicted. * indicates that the enzyme has been biochemically characterized in this organism (Ehlting et al., 1999; Raes et al., 2003; Silber et al., 2008).

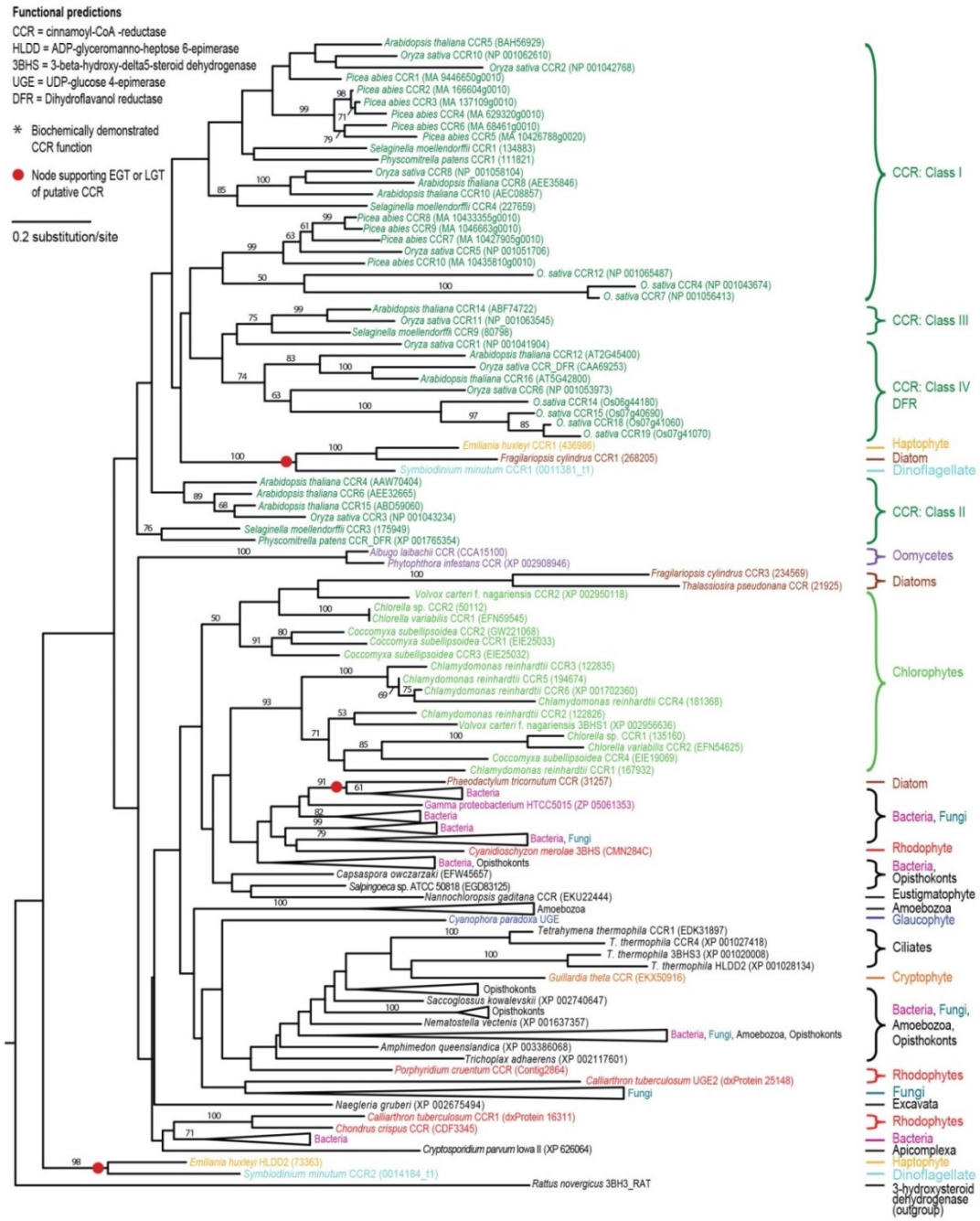

Figure 2-4: Maximum likelihood tree of cinnamoyl-CoA reductase (CCR) from the *p*-coumaryl alcohol biosynthesis pathway.

Amino acid sequences were aligned with MUSCLE and the tree compiled using RaxML. Numbers above branches refer to bootstrap values above 50%. 3-hydroxysteroid dehydrogenase, a CCR homolog (Lacombe et al., 1997), was used as the outgroup. The various classes of CCR are shown based on previous research including bona fide CCR compared to CCR or CCR-like genes. In addition, genes showing high similarity to dihydroflavonol reductase (DFR) genes were included (Xu et al., 2009; Barakat et al., 2011). Gene names are included next to taxa when function could be predicted. * indicates that the enzyme has been biochemically characterized in this organism (Lauvergeat et al., 2001; Costa et al., 2003; Baltas et al., 2005).

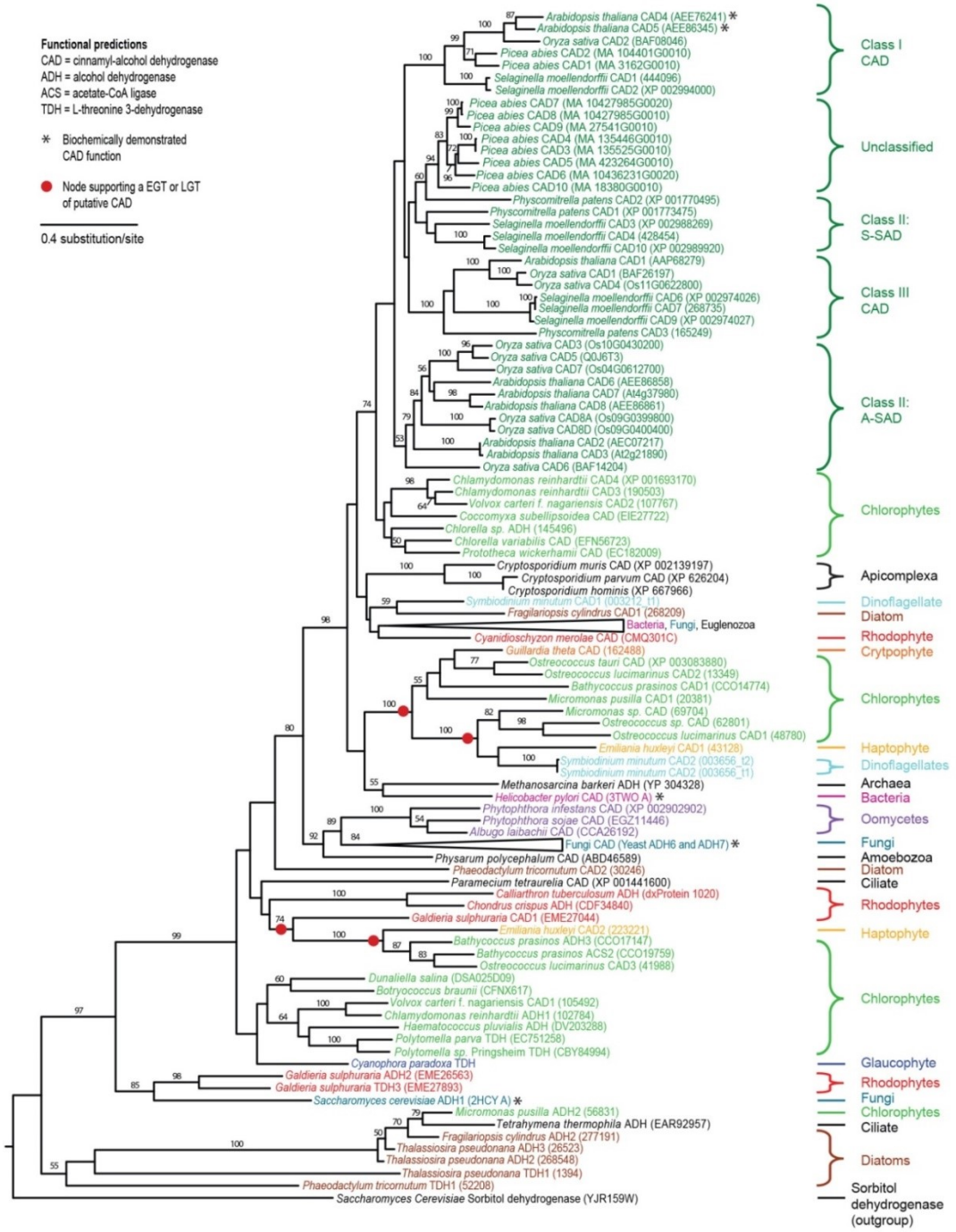

Figure 2-5: Maximum likelihood tree of cinnamyl alcohol dehydrogenase (CAD) from the *p*-coumaryl alcohol biosynthesis pathway.

Amino acid sequences were aligned with MUSCLE and the tree compiled using RaxML. Numbers above branches refer to bootstrap values above 50%. Sorbitol dehydrogenase, a CAD homolog (McKie et al., 1993), was used as the outgroup. The various classes of CAD are shown based on previous research, including the sinapyl alchohol dehydrogenases (SAD), which share some specific activity with CAD (Barakat et al., 2009; Guo et al., 2010). Gene names are included next to taxa when function could be predicted. * indicates that the gene has been biochemically characterized in this organism (Larroy et al., 2002; Sibout et al., 2005; Mee et al., 2005; Fraser & Chapple, 2011).

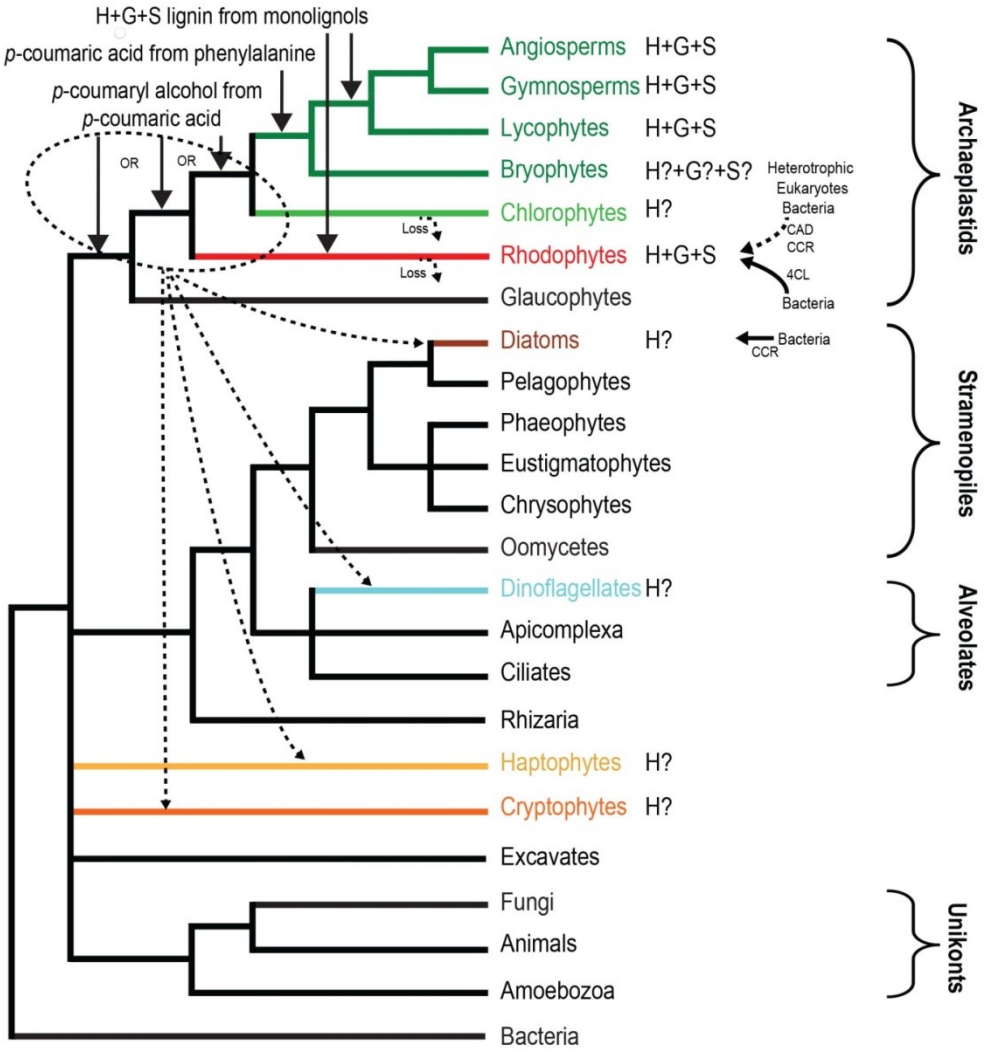

Figure 2-6: Hypothesized major evolutionary events in the lignin biosynthetic pathway across the eukaryotic tree.

The tree is a consensus of current phylogenetic analyses of the eukaryotic domain (Kenrick & Crane, 1997; Archibald, 2009; Keeling, 2013). Major events indicated by labelled arrows on the tree are hypothesized from our genome survey and phylogenetic analyses of the putative *p*-coumaryl alcohol biosynthesis enzymes. Taxonomic groups in which three or more enzymes catalysing consecutive steps in the lignin biosynthesis pathway were found are coloured. The chemical detection of polymerized lignin is indicated in the margin for each taxonomic group, with the type of lignin (H, G or S) specified. A question mark (?) indicates that some putative lignin biosynthetic enzymes are found but that there is currently no biochemical evidence of polymerized lignin. Arrows indicate the origin and direction of putative EGT and LGT events (solid arrows are used for events that are conclusive, dashed arrows when events are hypothesized).

The story is different for red algae. In this group, only 4CL seems to be ancestral, being found in a single clade, which also contains green algae and secondarily photosynthetic organisms (Figure 2-3). The only exceptions to this are two of the five 4CL paralogs in *Calliarthron tuberculosum* clustering in bacterial clades, one of them very strongly with a marine α-proteobacteria group known as the roseobacter clade, indicating a likely LGT from bacteria. For both CCR and CAD, red algae are polyphyletic, with some paralogs weakly clustering with homologs from bacteria or heterotrophic eukaryotes. It is therefore difficult to say whether the pathway was ancestrally found in red algae or some of its enzymes were acquired by LGT. Like in green algae, only a small proportion of red algal genomes investigated have homologs for all three enzymes (one in five). Although the fact that the red alga *Calliarthron cheilosporioides*, a close relative to *C. tuberculosum*, can synthesize *p*-coumaryl alcohol is well established (Martone *et al.*, 2009), this function does not seem to be an essential feature of red algae, making the loss of it likely. This is perhaps visible in the specialized role of lignin in the uncommon lignified joints, genicula, of *C. cheilosporioides* (Martone *et al.*, 2009).

These results suggest that all three genes of the *p*-coumaryl alcohol biosynthesis pathway are likely to have been present in at least the shared ancestor of plants and green algae, but possibly earlier, before the speciation of the red algal ancestor (Figure 2-6). A few additional features of the phylogenies support an earlier origin than the ancestor of land plants. The CAD homologs found in core chlorophytes cluster with land plants (albeit with modest 74% bootstrap support). Also, although both the CCR and CAD phylogenies likely contain hidden ancestral paralogy and differential loss, this is not the case for the 4CL tree. Paralogy is evident only for red and green algae and confined to a single clade (with the exception of copies likely acquired from bacteria by *C. tuberculosum*). As red and green algal species are polyphyletic inside this clade, duplication(s) of the 4CL homolog likely occurred in their ancestor and therefore would predate these lineages. It is difficult to say if the pathway could have been present in the archaeplastid ancestor itself, before divergence of the glaucophytes. Glaucophyte is the earliest

branching lineage in archaeplastids (Archibald, 2009) and could potentially be quite informative on the origin of the *p*-coumaryl alcohol biosynthesis pathway in this group. Unfortunately, although it harbours homologs for all three enzymes of the pathway, none of them has strong functional prediction and their position in phylogenies is unresolved. It is therefore not possible to determine if the pathway is ancestral to archaeplastids with information currently available.

2.3.4 The *p*-coumaryl alcohol biosynthesis pathway likely spread through endosymbiotic gene transfer

Four very disparate groups of secondarily photosynthetic organisms have at least one representative with all three enzymes of the *p*-coumaryl alcohol biosynthesis pathway with the correct functional predictions: dinoflagellates, diatoms, haptophytes and cryptophytes. The large majority of 4CL, CCR and CAD homologs found in these organisms cluster with each other (despite being from widely divergent taxonomic groups) or with red or green algae.

There is no observed paralogy of the 4CL gene in secondarily photosynthetic organisms. Diatoms and the haptophyte weakly cluster together (51%), the cryptophyte clusters strongly (100%) with another secondarily photosynthetic organism (*Ectocarpus silicosis*, a brown algae) and with green algae, while the dinoflagellate is found inside the same well-supported (97%) mixed green and red algal clade. For CCR, the haptophyte has two paralogs, one clustering strongly with the dinoflagellate (98%) and the other with both the dinoflagellate and the diatom *Fragilariopsis cylindrus* (100% support). Other diatom CCR paralogs weakly cluster with green algae (50% support), while the single CCR homolog found in the diatom *Phaeodactylum tricornutum* strongly groups with bacteria (91%) and was likely acquired from them by LGT. The cryptophyte single CCR homolog position in the tree is unresolved. The CAD homologs of secondarily photosynthetic organisms show a similar pattern to 4CL and CCR. A dinoflagellate and a haptophyte paralog strongly cluster together within a green algal clade that also includes the

cryptophyte (100% support). The second dinoflagellate CAD paralog weakly clusters with the diatom *F. cylindrus* single CAD homolog (59% support) and the second haptophyte paralog groups strongly with green algae (100%).

The underlying pattern is clear: a recurring clustering of secondarily photosynthetic organisms from disparate taxonomic groups with each other or with green or red algae. Although the exact pattern of species clustering varies between phylogenies (a likely result of ancient paralogy and differential loss), it suggests that most 4CL, CCR and CAD homologs present in secondarily photosynthetic species have been acquired by EGT from a red or green algae or their ancestors. This also implies that the *p*-coumaryl alcohol biosynthesis pathway, or at the very least its component genes, are ancient, predating the diversification of various major eukaryotic taxonomic groups such as the dinoflagellates, haptophytes, cryptophytes and diatoms. Although ancient paralogy coupled with differential loss can often make phylogenies misleading, it is very unlikely that it would result in similar patterns of taxonomically unrelated secondarily photosynthetic organisms clustering with each other or with green/red algae for three different genes. The recurrent co-clustering of dinoflagellates, haptophytes and diatoms in 4CL, CCR and CAD phylogenies is more parsimoniously explained by a common origin. The cryptophyte, *Gulliardia theta*, on the other hand, does not cluster directly with these other secondarily photosynthetic organisms in any phylogeny, suggesting an independent origin. More evidence is needed to confirm the exact origin of these genes and the number of events in which they might have been acquired by secondarily photosynthetic organisms.

2.3.5 LGT could have impacted the evolution of lignin precursors biosynthesis

Previous studies have suggested that at least one gene in the pathway, phenylalanine ammonia lyase (PAL) (Figure 2-1), was likely acquired through LGT from soil bacteria to an ancestor of land plants (Emiliani *et al.*, 2009). LGT is likely to also have influenced the evolution of *p*-coumaryl alcohol

biosynthesis. Some of the 4CL homologs present in the red alga *C. tuberculosum* might have been affected by this phenomenon. Indeed, the 4CL phylogeny contains a strongly supported clade composed of the red alga *C. tuberculosum* grouping with bacteria, including many sequences derived from roseobacters (Figure 2-3). This suggests a horizontal 4CL gene transfer from a roseobacter to a red alga (Figure 2-3), giving *C. tuberculosum* extra copies of 4CL in addition to those it inherited from archaeplastid ancestors. Close physical associations have been shown to promote LGT, and two roseobacter species are known to live intracellularly and intercellularly within red macroalgae (Ashen & Goff, 1996; Case *et al.*, 2011). Some red algal species even depend on bacteria for growth or morphogenesis, highlighting how close this relationship is (Fries, 1970; Hanzawa *et al.*, 2010). This bacteria-red algae clade in the 4CL tree also includes a functionally characterized gene from *Streptomyces coelicolor*, which has been shown to have 4CL activity (Kaneko *et al.*, 2003). Argot2 and ESG also predict all roseobacter and *C. tuberculosum* 4CL homologs in this clade to have 4CL function, a prediction only made for some land plants enzymes in our datasets besides these bacteria and red alga. This makes it likely that these laterally transferred homologs have true 4-coumarate:CoA ligase function. For a LGT event from a bacterium to a eukaryote to be confirmed, bacterial genes need to be found inserted next to genuine eukaryotic genes. Unfortunately, the *C. tuberculosum* 4CL homologs are found on very small contigs in the alga's genome and further upstream and downstream sequence data would be needed to determine if they have a bacterial or algal context. It is therefore not possible to exclude that the *C. tuberculosum* 4CL genes clustering with roseobacters represent bacterial contamination present in its genome sequence, despite systematic screening of sequence data to remove it (Chan *et al.*, 2011). Regardless of whether LGT between roseobacters and *C. tuberculosum* has taken place, the presence of 4CL in these bacteria raises the possibility that they could provide intermediates for the production of *p*-coumaryl alcohol to their algal host. If roseobacters can make *p*-coumaric acid (none of

the known genes for doing so have yet been found in this group), the presence of 4CL would theoretically enable them to produce coumaroyl-CoA and potentially provide it to their algal host.

Another likely case of LGT is the acquisition of a putative CCR (with very high confidence functional prediction) by the diatom *P. tricornutum* from bacteria. The former is found nested inside a bacterial clade with strong support (91%). As other diatoms' putative CCRs cluster with green algae, it is likely that a bacterial CCR displaced the homolog from algal origin previously present in *P. tricornutum*. Although LGT did not bring a novel gene to either *C. tuberculosum* or *P. tricornutum* (both had an existing homolog prior to LGT which was either displaced or complemented), it likely had an effect on their secondary metabolism.

2.3.6 The phenylpropanoid pathway is unique to land plants and fungi

The phenylpropanoid pathway enzymes responsible for the production of *p*-coumaric acid from the amino acid phenylalanine, PAL and cinnamate 4-hydroxylase (C4H), were only found in land plants and fungi and are clearly missing from all other eukaryotic genomes screened (Table 2-1 and Table 2-2, Figure 2-1). How is it possible for organisms to synthesize monolignols without these enzymes? The red alga *Calliarthron* can produce all types of lignin (Martone *et al.*, 2009) and we could not find these enzymes encoded in the *C. tuberculosum* genome sequence (Table 2-1). The product made by PAL and C4H from phenylalanine, *p*-coumaric acid, has been found in an axenic culture of the haptophyte E. huxleyi (Seyedsayamdost *et al.*, 2011b), which also lacks PAL and C4H (Table 2-2). Since both *Calliarthron* and *E. huxleyi* have 4CL, CCR and CAD homologs, there must be other, yet to be described, enzyme(s) capable of synthesizing *p*-coumaric acid and provide it as a substrate to the 4CL-CCR-CAD pathway to produce *p*-coumaryl alcohol. Furthermore, we could not find PAL or C4H genes in the genomes of any green alga or diatom (Table 2-1 and Table 2-2), although *p*-coumaric acid has been found in species from both of these groups (Goiris *et al.*, 2014).

PAL was likely acquired from bacteria by the ancestor of land plants or fungi and later horizontally transferred between these two groups (Emiliani *et al.*, 2009). C4H is also uniquely found in land plants and fungi, with distant bacterial homologs (data not shown). The combination of these two genes is therefore likely a late invention of land plants and/or fungi. Land plants added two genes (C4H and PAL) to the 4CL, CCR and CAD already present in their ancestor (Figure 2-6). Whether PAL and C4H displaced an ancestral enzyme(s) synthesizing *p*-coumaric acid or those enzyme(s) are still present in land plant genomes is currently unknown. The only other enzyme known to synthesize *p*-coumaric acid is tyrosine ammonia lyase (TAL), which has only been found in a few bacteria (Emiliani *et al.*, 2009). It can by itself convert the amino acid tyrosine to *p*-coumaric acid, suggesting that a single unknown enzyme could carry the same function in eukaryotes lacking PAL and C4H but which have 4CL, CCR and CAD, such as the haptophyte *E. huxleyi* and the red alga *Calliarthron*. It is also possible that enzymes analogous to PAL and/or C4H exist, as PAL activity has been found in the green alga *Chlorella pyrenoidosa* (Chen *et al.*, 2003), but we could not find PAL homologous to plant enzymes in any of the *Chlorella* genomes screened (Table 2-1).

2.3.7 Expansion of the lignin biosynthesis pathway has occurred multiple times independently on land and in the sea

Screening of eukaryotic genomes revealed that except for 4CL, CCR and CAD, all other lignin biosynthesis genes found in land plants are missing from *Calliarthron* (Table 2-1), despite the clear presence of all three lignin types in this red alga (Martone *et al.*, 2009). Assuming the capacity to produce 4CL's substrate *p*-coumaric acid, the presence of 4CL, CCR and CAD genes theoretically enables the synthesis of *p*-coumaryl alcohol as well as its intermediates, which can be used as substrates for the synthesis of G and S lignins (Figure 2-1). This makes convergent evolution of the ability to synthesize G and S lignins in *Calliarthron* simple, as it would only require the addition of two more enzymes to this core pathway. For example, the lycophyte *S. moellendorfii* only needed to add caffeic acid O-

methyltransferase (COMT) and ferulate 5-hydroxylase (smF5H) to the enzymes needed for *p*-coumaryl alcohol synthesis to be able to make both G and S lignins (Weng *et al.*, 2010). Also, not all land plants can make S lignin, and the presence of both producers and non-producers in various groups of plants suggests that this ability has been gained and lost multiple times in land plants. For example, most gymnosperms do not produce S lignin, but some can, such as *Ginkgo biloba* (maidenhair tree) (Uzal *et al.*, 2009). The fact that modifications of lignin production can easily evolve from a genetic background found in various photosynthetic eukaryotic lineages lends insight into how the red alga *Calliarthron* likely evolved the ability to produce G and S lignins. Whether this type of convergent evolution has also happened in other lineages is an open question. Provisional biochemical evidence for the presence of *p*-coumaryl alcohol in brown and green algae, but absence of G and S variants (Espiñeira *et al.*, 2011) suggests that numerous eukaryotes could have the capacity to only synthesize *p*-coumaryl alcohol or H lignin.

2.3.8 Anti-microbial properties of lignin intermediates

The function of *p*-coumaryl alcohol in unicellular marine photosynthetic eukaryotes such as diatoms, dinoflagellates, haptophytes, cryptophytes and some green and red algae, is unclear. The two main roles of lignins derived from *p*-coumaryl alcohol and other monolignols in land plants are water transport and structural support. Water transport systems are absent in unicellular algae. If the *p*-coumaryl alcohol likely produced by unicellular and photosynthetic eukaryotes is polymerized as lignin or lignans, it could also contribute to their structural strength, although other compounds such as silica and cellulose are already known fulfil this function in such organisms (Popper & Tuohy, 2010). More likely functions that can be fulfilled by *p*-coumaryl alcohol are UV protection and microbial defence. Intermediates of the lignin biosynthesis pathway have been shown to have antimicrobial properties against terrestrial bacteria (Gunnison & Alexander, 1975; Keen & Littlefield, 1979; Cowan, 1999; Barber *et al.*, 2000). The

antimicrobial properties against marine bacteria of some intermediates of the H, G and S lignin biosynthesis pathway is shown in Table 2-3. The concentration was generally slightly higher than that required to inhibit terrestrial bacteria (2-6 mM), although the same or lower than required to inhibit yeast (≥ 8 mM) (Herald & Davidson, 1983; Barber *et al.*, 2000). As with terrestrial bacteria, while there was an inhibitory effect, they were generally not able to kill the bacteria at concentrations less than 16 mM, if at all (Barber *et al.*, 2000). Addition of lignin to *Phaeobacter gallaeciensis* 2.10 at concentrations up to 10mg/L did not cause a change in cfu numbers (between 6.7-9.5 x 10^7 cfu/mL). These concentrations are relatively high to be achieved in a dilute ocean, but near the alga (in the phycosphere), it would be conceivably possibly to reach these concentrations of these hydrophobic compounds.

Table 2-3: Antimicrobial properties of lignin biosynthesis intermediates against marine bacteria

Organism	*p*-Coumaric Acid		Ferulic Acid		Cinnamic Acid	
	MIC[a]	MKC[b]	MIC[a]	MKC[b]	MIC[a]	MKC[b]
***Ruegeria sp.* R11**	8 mM	>64 mM	8 mM	>64 mM	8 mM	>16 mM
***Vibrio cholera* 11E01**	8 mM	64 mM	8 mM	64 mM	8 mM	>16 mM
***Vibrio fischeri* MY-1**	16 mM	>64 mM	16 mM	>64 mM	>8 mM	>16 mM
***Phaeobacter gallaeciencis* 2.10**	8 mM	>64 mM	8mM	64 mM	4 mM	>8 mM

[a] minimum inhibitory concentration (MIC)
[b] minimum killing concentration (MKC)

Phenolic compounds such as *p*-coumaric acid and its derivatives exhibit high UV absorptivity and could potentially protect an organism against the damaging effects of sunlight (Gitz *et al.*, 2004). The lignin biosynthetic pathway has also been implicated in the defence system of plants, as individual enzymes (e.g., CAD, CCR and CCoAMT) have been shown to defend against microbial attacks (Lacombe *et al.*, 1997; Boudet, 2000; Tronchet *et al.*, 2010; Moura *et al.*, 2010). Such a role in host defense or UV protection may have provided selection for the early evolution of lignin biosynthetic pathway in the

ocean, which was then co-opted for water transport and structural strength in land plants faced with new selective pressures of an air-land environment.

2.4 Conclusion

The widespread distribution of *p*-coumaryl biosynthesis gene homologs across various eukaryotic supergroups suggests an ancient origin for this pathway. Although we cannot dismiss the possibility of its presence in an ancient eukaryotic ancestor and subsequent loss in all lineages in which it is absent, an origin in archaeplastids is more parsimonious. The ancient pathway for *p*-coumaryl alcohol synthesis should contain one or more gene(s) that precede 4CL, CCR and CAD, as it requires a source of *p*-coumaric acid, but these have yet to be discovered. Since *p*-coumaric acid is found in the haptophytes *E. huxleyi* and *D. lutheri*, the diatom *P. tricornutum* and the green alga *C. vulgaris* (Seyedsayamdost *et al.*, 2011b; Goiris *et al.*, 2014), and none of these lineages contains any homologs of PAL and C4H, there is little doubt in the existence of enzyme(s) with an analogous function(s). It is not implied that any organisms carrying this ancient pathway can necessarily polymerize *p*-coumaryl alcohol (H monolignol) to form H lignin or even synthesize the more complex G and S monolignols, but they are likely able to synthesize at least *p*-coumaryl alcohol. All the secondary photosynthetic organisms investigated as well as most green and red algae are marine organisms, so it is intriguing to consider an authentic marine source of monolignols or lignin, with a possible original role as a defensive mechanism. As these compounds and their degradation products are used as biomarkers to calibrate for terrestrial carbon input into marine systems (Opsahl & Benner, 1997; Hedges *et al.*, 1997), marine sources of monolignols or lignin therefore have the potential to redefine our understanding of the marine carbon cycle.

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Chapter 3

A bacterial symbiont shapes the life history of its host

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). Motile microbes capable of directly associating with phytoplankton thereby expose themselves to a continual stream of algal metabolites leaked from their algal hosts (Sapp *et al.*, 2007; Geng & Belas, 2010; Sule & Belas, 2013). These algal metabolites are at their highest concentrations in the phycosphere (Bell & Mitchell, 1972), the area immediately surrounding an algal cell, which means algal-associated bacteria experience a nutrient benefit.

Microbial metabolic activity is highest on marine particulate matter and many essential biogeochemical processes likely occur at the surface of phytoplankton or within the phycosphere, as metabolites from primary producers are leaked from the cell to the epiphytic and phycosphere communities (Mitchell *et al.*, 1985; Baines & Pace, 1991; Breckels *et al.*, 2010). These communities represent the fraction of the microbial loop that will first encounter these metabolites and where metabolic rates are highest (Smith *et al.*, 1992). While many bacteria simply use metabolites leaked by algae, some microbes have fine-tuned their ability to sense and respond to molecules essential to marine biogeochemical cycles (Miller *et al.*, 2004; Miller & Belas, 2006; Tripp *et al.*, 2008; Seymour *et al.*, 2010). Bacteria engage in dynamic relationships with their algal host, producing small molecules key to symbiosis, such as vitamins or small molecules beneficial or even required by the host alga (Bolch *et al.*, 2011). Other bacteria use bioactive molecules in pathogenesis to co-ordinate virulence or kill their host

(Ashen *et al.*, 1999; Seyedsayamdost *et al.*, 2011b; Fernandes *et al.*, 2011; Case *et al.*, 2011). To counteract the ocean's diluting influence, bioactive molecules are often implicated in bacterial-algal interactions at the cell-cell interface (Dworjanyn *et al.*, 2006; Wagner-Döbler & Biebl, 2006).

The haptophyte *Emiliana huxleyi* is a numerically abundant phytoplankton forming dense populations (>10^6 cells/mL) (Tyrrell & Merico, 2004) in short time scales (days to weeks) over vast expanses of the upper ocean (>250,000 km^2) (Holligan *et al.*, 1993). Its bloom-bust life cycle dramatically restructures the marine ecosystem as, despite its tiny size, its large-scale populations make it an habitat forming species that is both abundant and important in marine ecosystems where it is present. Bloom formation has been associated with a variety of environmental factors (Tyrrell & Merico, 2004), while bloom collapse is frequently attributed to viral infection or eukaryote grazing (Wolfe *et al.*, 1994, 1997; Wilson *et al.*, 2002a).

The ubiquity and abundance of *E. huxleyi* in the ocean, as well as its production of important intermediates in both the carbon and the sulphur biogeochemical cycles has made it a model phytoplankton species. It produces three variant cell types: non-motile bald cells (Type N: diploid), motile swarming cells (Type S: haploid), and non-motile coccolith-bearing cells (Type C: diploid) (Klaveness, 1972; Laguna *et al.*, 2001). All three cell types are tiny, 5 μM, and photosynthetic, but only the coccolith-bearing cells numerically dominate blooms and mineralise calcite (Frada *et al.*, 2012), which is deposited in the deep ocean, forming the marine paleobotanic record (Young *et al.*, 2003). It is not presently understood how these three cell types form its life history, but each cell type is capable of proliferation (Klaveness, 1972). Chemical cues might induce cell type switches from diploid coccolith formers to haploid swarming cells (Laguna *et al.*, 2001; Frada *et al.*, 2012), which supports the suggestion that the alga's lifecycle involves both asexual reproduction and meiosis of diploid cells generating haploid S cells (Klaveness, 1972). However, how these different cell types impact the ecology, interactions and biogeochemistry of *E. huxleyi* is largely unknown, except in the context of its

interaction with *Emiliania huxleyi* viruses (EhV)s. EhVs kill both diploid cell types (C and N) while S cells are resistant to infection by this virus (Wilson *et al.*, 2002a; Frada *et al.*, 2008). Once an EhV infects the diploid cell, it proliferates and produces the viral glycosphingolipids (vGSLs) that kill the algal cell. Viral infection triggers caspase-like activity in *E. huxleyi* (likely from metacaspases), suggesting that the virus is hijacking algal programmed cell death (PCD) machinery to kill the host (Bidle *et al.*, 2007).

Although its viral predators and grazers (Wolfe *et al.*, 1994; Wilson *et al.*, 2002a) have been studied, the association of bacteria with *E. huxleyi* is largely unexplored, despite their potential connections in biogeochemical cycles (Seymour *et al.*, 2010; Curson *et al.*, 2011). To gain insights in such bacterial-algal relationships, we experimentally investigated how *E. huxleyi* interacts with the marine α-proteobacteria, *Phaeobacter gallaeciensis*, a member of the Roseobacter clade that is commonly associated with the alga (González *et al.*, 2000; Green *et al.*, 2015). It also produces a number of novel bioactives, including the antibiotic tropodithetic acid (TDA) (Geng *et al.*, 2008; Berger *et al.*, 2011; Thole *et al.*, 2012) and potent algaecides called roseobacticides, which have been shown to have specific activity against *E. huxleyi* (Seyedsayamdost *et al.*, 2011b). These bioactives might allow *P. gallaeciensis* to live a duplicitous lifestyle as both pathogen and symbiont, as roseobacticides could facilitate a pathogenic lifestyle and TDA has been implicated in *P. gallaeciensis'* chemical defence of various hosts. *P. gallaeciensis* is a symbiont of *Ulva australis*, chemically defending this ubiquitous seaweed from colonisation (Rao *et al.*, 2007). It can also act as a probiotic for turbot cod larvae, protecting it against *Vibrio anguillarum* infections (Planas *et al.*, 2006). However, *P. gallaeciensis* has not been shown to be a pathogen.

We show that an *E. huxleyi* cell can harbour a small number of bacterial cells on its surface. The attachment of *P. gallaeciensis* to *E. huxleyi* persists throughout the host cells life. We also demonstrate that *P. gallaeciensis* selectively causes the precipitous death of aged *E. huxleyi* coccolith-bearing C populations while leaving bald N populations alive. The ability of *P. gallaeciensis* to cause the collapse of the entire C cell population during senescence has a direct benefit on its own population size, as it

increases during its host's population collapse. The bacterium would appear to attack different fractions of the *E. huxleyi* population than EhVs, which target both diploid cell types (C and N) and do not kill the S cell population (Frada *et al.*, 2008). This suggests that *E. huxleyi* hosts a bacterium and a virus with competing interests; and that its life history and bloom dynamics have several layers of complexity.

3.2 Methods

3.2.1 Algal and bacterial strains

All *Emiliania huxleyi* strains were obtained from the Provasoli-Guillard National Centre for Marine Algae and Microbiota (NCMA): the bald diploid (N cell) strains CCMP370 (isolated form Oslo Fjord, Norway, North Atlantic), CCMP372 (Sargasso Sea, North Atlantic), CCMP374 (Gulf of Maine, North Atlantic), CCMP379 (English Channel, North Atlantic), CCMP2090 (coast of South America, South Pacific), and the coccolith-producing (C cell) strain CCMP2231 (Gulf of Maine, North Atlantic) and CCMP3266 (Tasman Sea, South Pacific) (Table 3-1). All strains, except for CCMP2231, were axenic. The axenic strains are all from unique geographical locations spanning the biogeography of all available axenic diploid *E. huxleyi* cultures. They were maintained in L1-Si medium (Guillard & Hargraves, 1993) at 18 °C in a diurnal incubator (8:16 hr dark-light cycle) and 41.51 +/- 11.15 µmol m^{-2} s^{-1}. Algal cultures and medium were checked for bacterial contamination by microscopic observations and by inoculation onto marine agar (37.4 g Difco Marine Broth 2216 supplemented with 15 g Difco agar in 1 l) followed by incubation at 18 °C for 2 d. *E. huxleyi* was grown statically for 5 d to 10^4 cells/mL (early-log) for experiments.

The bacteria *Phaeobacter gallaeciensis* DSM 26640 (Frank *et al.*, 2014) (previously known as BS107 (Seyedsayamdost *et al.*, 2011b)), was maintained at 30 °C on marine agar. *Vibrio celticus* J1-22 (Lemire *et al.*, 2014) was maintained on ½ marine agar (18.7 g Difco Marine Broth 2216 supplemented 9 g NaCl and

15 g Difco agar in 1 l). The bacteria were grown to stationary phase in their respective broth on a rolling drum at room temperature for 24 hr prior to experimentation.

3.2.2 Algal metabolite stimulation and inhibition of *P. gallaeciensis* production of bioactives

E. huxleyi produces *p*-coumaric acid (*p*CA) that stimulates secondary metabolite production in DSM 26640 (Seyedsayamdost *et al.*, 2011b). *P. gallaeciensis* was grown in 25 mL of marine minimal medium (Holmstrom *et al.*, 1998) in a 250 mL Erlenmeyer flask with and without 1 mM of *p*CA dissolved in ethanol (an equal volume of solvent was added to the control) (Sigma-Aldrich, St. Louis, MO, USA) at 30 °C shaking at 160 rpm in triplicate using a 1% inoculum. Roseobacticides and tropodithetic acid (TDA) are bioactives produced by *P. gallaeciensis* and were detected by spectrometry as previously described (Bruhn *et al.*, 2005; Seyedsayamdost *et al.*, 2011b). Briefly, samples of cultures were taken at 24, 48, 72 and 96 hr, and their absorbance measured by spectrophotometry (Ultrospec 3100 Pro, Fisher Scientific, Hampton, NH, USA) for growth (600nm), roseobacticides (430nm) and TDA (356 nm) (Bruhn *et al.*, 2005; Seyedsayamdost *et al.*, 2011b). Although TDA can be measured at 304 nm (Bruhn *et al.*, 2005), the absorbance of *p*CA overlaps with this wavelength (Challice & Williams, 1965) and 356 nm was therefore used to detect this compound. Data were processed using Sigmaplot 12.

To determine if *p*CA inhibition of TDA would reduce *P. gallaeciensis* inhibition of the TDA-sensitive bacterium *V. celticus* J1-22, DSM 26640 (-/+ 1 mM of *p*CA) and J1-22 were grown with a 1% inoculum in 25 mL of ½ marine broth in a 250 mL Erlenmeyer flask at 30 °C for 24 h and 160 rpm. J1-22 was diluted to a 20% inoculum in ½ marine soft agar plates (0.7%) -/+ 1mM *p*CA. The J1-22 lawn was spotted with 10 µl of DSM 26640 (-/+ 1 mM of *p*CA respectively) and incubated at 30 °C for 24 h (n=12). The zone of inhibition (ZOI) was calculated for each spot and photographed using ImageJ (http://imagej.nih.gov/ij/) (Schneider *et al.*, 2012). Statistical significance was determined using a one-way ANOVA and Tukey test.

3.2.3 Bacterial and algal co-cultivation

Bacterial-algal co-cultivation was performed as previously described (Bramucci *et al.*, 2015). Briefly, stationary phase bacterial cultures were washed twice by centrifugation and re-suspended in L1-Si medium before further centrifugation and re-suspension to the target cell concentration (10^2 cfu/mL). *E. huxleyi* and *P. gallaeciensis* were mixed 1:1 (volume:volume) with 10^2 cfu/mL bacteria and 10^4 cells/mL algae, then 1 mL of this co-culture was aliquoted in 48-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The same cell densities of the bacterium and the alga were inoculated as monocultures in L1-Si medium and aliquoted into the microtitre plate. L1-Si medium alone served as a sterility control. All controls/co-cultures were performed in triplicate. The microtitre plates were incubated in a diurnal incubator (8:16 hr dark-light cycle) at 18 °C for all experiments.

3.2.4 Fluorescence measurements

A pulse-amplitude-modulation (PAM) fluorometer (WATER-PAM, Waltz, Effeltrich, Germany) was used to measure chlorophyll fluorescence and photosynthetic potential quantum yield (Schreiber *et al.*, 1986). All samples were taken at the mid-point of the dark cycle (4 h) and diluted in L1-SI medium to within the detection range of the PAM fluorometer. Samples were maintained at 18 °C throughout handling. A dark adaption period of 3 min was determined, after which a saturating pulse was applied and the fluorescence readings were taken in triplicate at intervals of 1 min 30 sec to calculate the minimal dark fluorescence (F_0), the maximum dark fluorescence (F_m) and the Photosystem II (PSII) potential quantum yield (F_v/F_m), $F_v/F_m = (F_m - F_0)/F_m$ (Schreiber *et al.*, 1986; van Kooten & Snel, 1990). Triplicate readings of each sample were averaged and the three microtitre wells were treated as replicates to determine the potential quantum yield. Data were subsequently processed using SigmaPlot 12.

3.2.5 Flow cytometry and bacterial counts

E. huxleyi from co-cultivation experiments was fixed for flow cytometry by incubating cells in the dark for 10 min with 0.15% glutaraldehyde (Sigma-Aldrich). 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 488nm laser was used for excitation. Samples were first run using chlorophyll fluorescence (670nm) for detection and then stained with SYBR-I (Life Technologies, Carlsbad, CA, USA) (520nm) to enumerate cells. Data were processed using FlowJo 9.2.

The bacterial population density from co-cultivation experiments was enumerated by counting colony forming units (cfu) on marine agar after 2 d of incubation at 30 °C. The latter technique was used to enumerate bacteria, as those attached to *E. huxleyi* would not be counted using flow cytometry. Although *P. gallaeciensis* forms biofilms on *E. huxleyi*, attached bacterial cells were not observed after vigorous vortexing (and the odd bacterium attached to *E. huxleyi* will form a cfu). Prior to inoculation, samples were therefore vortexed vigorously to reduce aggregates of bacterial cells.

3.3 Results and Discussion

3.3.1 *Emiliania huxleyi*, despite its tiny size, can host a bacterial community on its surface

Much of the work on deciphering *E. huxleyi's* cell biology has been done in axenic cultures of the bald diploid cells (N) (Fulton *et al.*, 2014; Rose *et al.*, 2014), which are readily available. This focus on axenic cultures of a single cell type means that knowledge about the interactions of bacteria with *E. huxleyi* is limited, including the influence of host cell type on this relationship. In the ocean, *E. huxleyi* populations are dominated by the coccolith-bearing diploid cell type (C), which can be found attached to one another and to haploid swarmer cells (S), forming clumps or biofilms (Bidle *et al.*, 2007; Frada *et al.*, 2012).

Cultures of C type *E. huxleyi* that have not been treated with antibiotics (to remove their bacterial symbionts) produce clumps that contain bacterial biofilms coating *E. huxleyi* cells (Figure 3-1 A). Despite its 5 μm size, *E. huxleyi* can therefore host a surface associated bacterial community (Figure 3-1 A). To investigate the host-symbiont relationship in a controlled environment, we have compared the interactions of the type N (CCMP2090) and C (CCMP3266) axenic *E. huxleyi* cultures with our model marine bacterium *Phaeobacter gallaeciensis* DSM 26640. *P. gallaeciensis* readily attaches to *E. huxleyi* cells in co-culture, which is best visualized on N type cells, as it is difficult to readily differentiate a coccolith from a bacterial cell, due to their similar size (Figure 3-1 B & C). *P. gallaeciensis* attaches to *E. huxleyi* at its pole (Figure 3-1 C & D) and can form short chains (Figure 3-1 C).

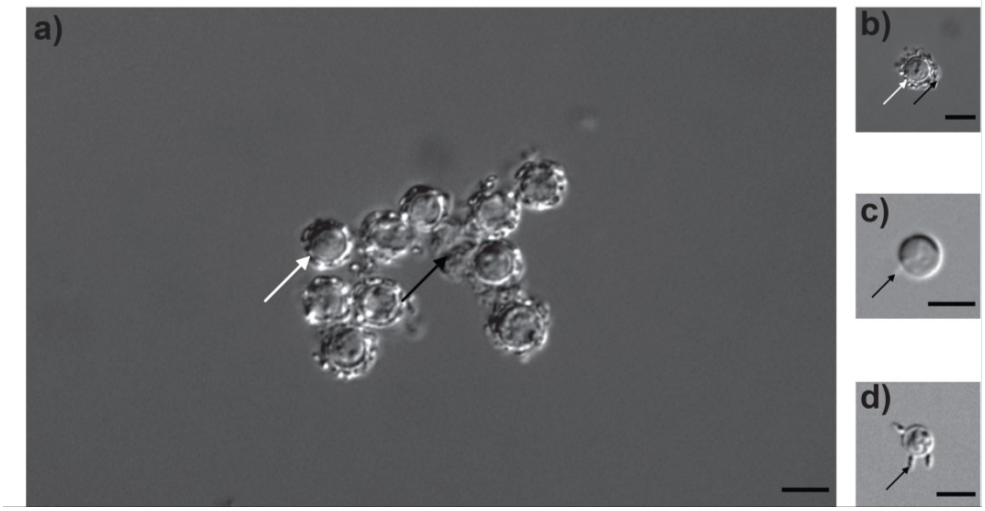

Figure 3-1: DIC Microscopic observations of *Emiliania huxleyi* and its bacterial epiphytes.

C type cultures of *E. huxleyi* (CCMP2231) commonly has bacterial symbionts that live epiphytically on *E. huxleyi,* forming large aggregates or biofilms (a), however, C type *E. huxleyi* cells (CCMP3266) co-cultured with *Phaeobacter gallaeciensis* DSM 26640 can live as individual cells coated in DSM 26640 (b). N type *E. huxleyi* cells (CCMP372) co-cultured with DSM 26640 show various levels of colonisation from an individual cell that has formed a cell chain (denoted by the black arrow) (c) to three DSM 26640 cells (d). The black arrow denotes bacteria attached to *E. huxleyi* and the white arrow denotes coccoliths attached to *E. huxleyi's* surface. The scale bar represents 5 μM. *E. huxleyi* cell type is the main determinant of its interaction with *P. gallaeciensis*

The recently published pangenome of *E. huxleyi* found that gene content seems to be the main factor controlling strain variability in regard to phenotype and physiological characteristics between genome-sequenced *E. huxleyi* strains isolated across its global distribution (Read *et al.*, 2013). The ecology of *E. huxleyi* is known to be complex and dynamic within its bloom-bust lifestyle, with several described interactions including grazing and viral infection, both of which are mediated by bioactive small molecules (Wolfe *et al.*, 1997; Bidle & Falkowski, 2004; Bidle *et al.*, 2007). *E. huxleyi's* interactions with its viruses (EhVs) have been extensively studied and are known to be variable between strains (Bidle *et al.*, 2007; Frada *et al.*, 2008; Vardi *et al.*, 2012). To determine if there was a different interaction with bacteria based on the origin or cell type of the host, we co-cultured all the axenic diploid *E. huxleyi* strains from unique locations across the globe available from the National Centre for Marine Algae and Microbiota (NCMA) with *P. gallaeciensis* (CCMP370, 372, 374, 379, 2090 and 3266). Given the global distribution and genomic plasticity of *E. huxleyi*, we would expect broad variability between *E. huxleyi* strains in their interaction with *P. gallaeciensis*. However, only one of these strains, the C type CCMP3266, was killed by *P. gallaeciensis* (Table 3-1). This result was surprising, as strains other than CCMP3266, such as CCMP372, are killed by roseobacticides, the algaecides produced by *P. gallaeciensis* (Seyedsayamdost *et al.*, 2011b). Also, although microscopic observation of *P. gallaeciensis* co-cultured with N type *E. huxleyi* cells (CCMP372) identifies *P. gallaeciensis* attached to a few senescent and dead *E. huxleyi* (Figure 3-1), the bacterium was unable to cause population wide collapse of the N type cultures as it did the C type (Table 3-1, Figure 3-2).

Table 3-1: *Emiliania huxleyi* strains and their susceptibility to *P. gallaeciensis* DSM 26640 pathogenesis

CCMP strain code	RCC strain code	Isolation location	Axenic	Cell type*	Killed by DSM 26640%
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	RCC1731^	Coast of South America, South Pacific	Yes#	N	No
CCMP2231		Gulf of Maine, North Atlantic	No	C	n/a
CCMP3266	RCC1216	Tasman Sea, South Pacific	Yes	C	Yes

* Coccolith bearing diploid cell (C) and naked diploid cell (N)
# (from genome sequenced strain CCMP1516)
^this strain is not axenic
%Co-culture experiments were observed over 30 d and no loss of chlorophyll or cell death was observed
n/a denotes that this experiment was not conducted as CCMP2231 Is not an axenic strain

To further investigate this relationship, the N type strain CCMP2090 (which is an axenic culture derived from the genome-sequenced CCMP1516) and the axenic C type strain, CCMP3266, were chosen for further in depth analysis. Populations of C type CCMP3266 and N type CCMP2090 respond differently in co-culture with *P. gallaeciensis*. CCMP2090 and *P. gallaeciensis* have a mutually positive (or symbiotic) interaction, where both the bacterium and alga reach higher population densities when grown together rather than alone (Figure 3-2B & D). In contrast, the interaction between *P. gallaeciensis* and CCMP3266 appears antagonistic, as *P. gallaeciensis* population density is greater in co-culture than when grown alone while CCMP3326's cell density is lower in co-culture compared to the control (Figure 3-2 A & C).

Populations of *P. gallaeciensis* and *E. huxleyi* were grown alone and in co-culture to determine the influence they have on each other's population density, health and survival. The bacterial and algal

populations where monitored using cfu counts and flow cytometry, while PAM fluorometry was used to determine the health of *E. huxleyi's* Photosystem II (PSII) (Figure 3-2).

P. gallaeciensis was inoculated at a very low cell density (10^2 cfu/mL), to ensure that the effect of *P. gallaeciensis* on *E. huxleyi* was not due to an artificially high cell density. Under the experimental conditions used (an algal medium, L1-SI, with limited carbon), *P. gallaeciensis* is able to grow to a cell density of 10^5 cfu/mL without a host (Figure 3-2 A & B). However, its growth is greatly enhanced by the presence of its host, *E. huxleyi*. The bacterium not only has a higher density throughout the experiment, but also survives longer in co-culture and reaches one hundred times the density (10^7 cfu/mL after 14d in co-culture), compared to *P. gallaeciensis* cells inoculated in L1-SI medium without a host (control), which have died by 14 d. (Figure 3-2 A & B). This effect is similar when *P. gallaeciensis* is co-cultured with either C type (CCMP3266) or N type (CCMP2090) *E. huxleyi* cultures during the algae's log and stationary phase growth. This persistence and greater population density when *P. gallaeciensis* is co-cultured with *E. huxleyi* is likely the result of nutrients made available by *E. huxleyi* exudate (Borchard & Engel, 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 (Mitchell *et al.*, 1985; Baines & Pace, 1991; Miller *et al.*, 2004; Miller & Belas, 2006). The continued survival of *P. gallaeciensis* when co-cultured with *E. huxleyi* demonstrates their ability to co-exist and most importantly, the resulting increase in population density confirms the benefit derived from it by this bacterium (Figure 3-2).

Although the growth dynamic of *P. gallaeciensis* is initially similar regardless of the *E. huxleyi* cell type it is co-cultured with, displaying an increased population density and longevity (Figure 3-2), the situation is different when the algae becomes senescent. After killing C type (CCMP3266) at 20-25 d, the population density of *P. gallaeciensis* increases a further eight fold, an increase not seen when co-cultured with N type (CCMP2090), which it does not kill (Figure 3-2). This increase in population density

suggests that *P. gallaeciensis* is able to increase division in response to killing its host, presumably due to the nutrients released by algal cell lysis (Kolb *et al.*, 2013). The benefit to *P. gallaeciensis* in killing its host makes it a pathogen rather than a parasite, as parasites can reduce the health of their host but benefit from prolonging the host's life, while *P. gallaeciensis* causes CCMP3266 to die prematurely. This killing is unique to its interaction with CCMP3266, suggesting that *P. gallaeciensis* is able to distinguish between bald and coccolith-bearing diploid *E. huxleyi* strains and only kill the later, or that N strains have a resistance mechanism against *P. gallaeciensis*.

As CCMP3266 ages (identified as a decline in cell density after 14 d), *P. gallaeciensis* uncouples its fate from its dying host by killing it. *P. gallaeciensis* cells attached to *E. huxleyi* loose motility as biofilm cells (Figure 3-1 C & D) and therefore cannot simply swim away from their host after it dies. Killing their host provides them not only with a sudden burst of nutrients, but also an exit strategy. This is achieved by *P. gallaeciensis* through production of roseobacticides, which cause cell lysis in *E. huxleyi* (Seyedsayamdost *et al.*, 2011b). Cell lysis releases *P. gallaeciensis* from a dying host, whose fate is to sink through the water column (Chow *et al.*, 2015).

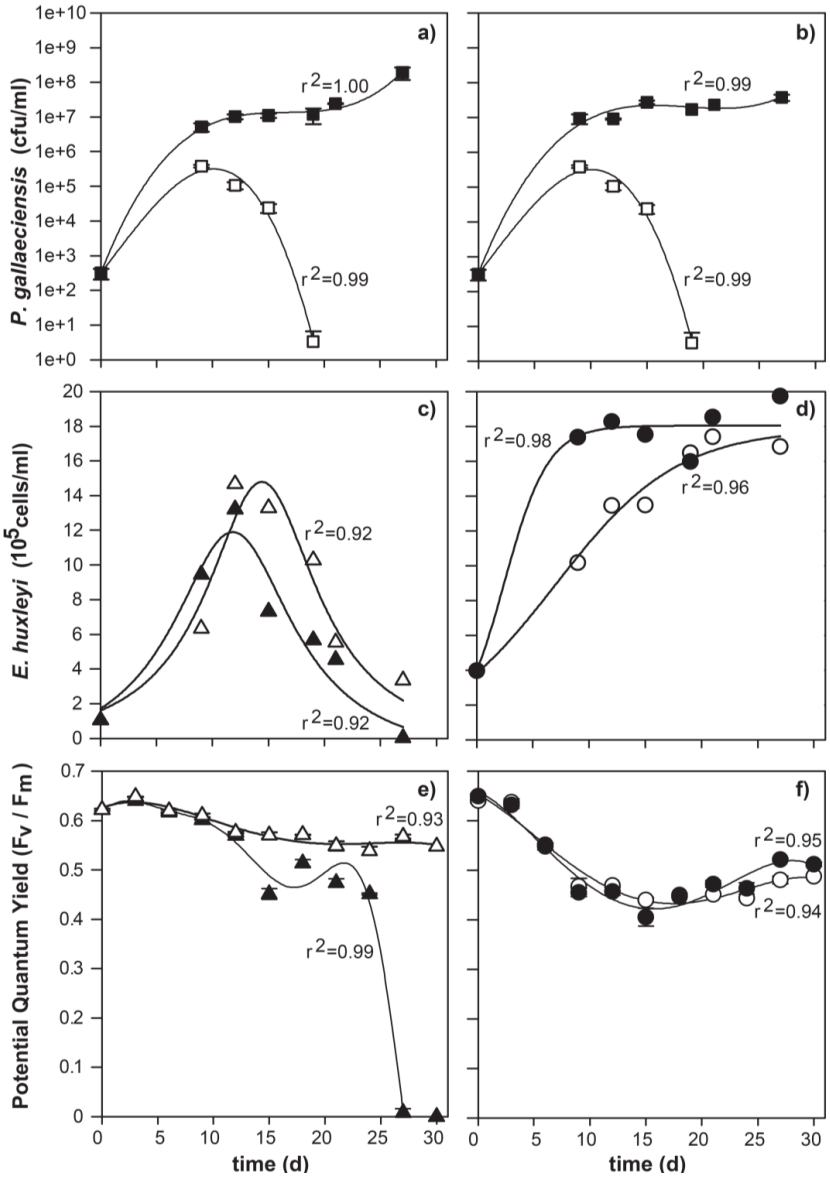

Figure 3-2: Co-culturing experiment of *Phaeobacter gallaeciensis* DSM 26640 with axenic diploid cultures of C (CCMP3266) and N (CCMP2090) *Emiliania huxleyi* strains.

DSM 26640 (10^2 cells/mL) was co-cultured with algal strains (10^5 cells/mL) separately and monitored over 29 d to determine the influence of the bacterium on the different *E. huxleyi* cell types. Bacterial counts (cfu/mL) were performed on DSM 26640 cultured in L1-SI and co-cultured with CCMP3266 (a) and CCMP2090 (b), where the line of best fit was a 3rd order polynomial. Data for DSM 26640 is shown as squares. Algal cell counts (cells/mL) were performed using flow cytometry for CCMP3266 (c), with a line of best fit as a 4 parameter log normal curve, and CCMP2090 (d) with a 3 parameter sigmoidal curve. The potential quantum yield (F_v/F_m) is shown for CCMP3266 (e) and 2090 (f) , where the line of best fit was a 4th order polynomial. For all panels, data for CCMP3266 are shown as triangles, and data for CCMP2090 are shown as circles. Data representing single species experiments are represented by white triangles, circles and squares whereas data from co-cultured experiments are shown as black triangles, circles and squares in all panels.

3.3.2 Antagonistic interaction between *P. gallaeciensis* and coccolith-bearing *E. huxleyi*

CCMP3266 grown alone undergoes exponential growth until entering stationary phase by 12 d. The stationary phase is short lived (between 12-15 d) and cultures enter the death phase by 15 d, indicated by a declining population density (Figure 3-2 C). There are several indicators that *P. gallaeciensis* accelerates senescence in CCMP3266, showing a steeper decline in cell density (Figure 3-2 C) and a sustained drop in photosynthetic potential quantum yield between 15-25 d compared to CCMP3266 grown alone (Figure 3-2 E). These parameters indicate that the aging process in CCMP3266 populations is accelerated by *P. gallaeciensis*, as CCMP3266 undergoes an earlier and faster decline in cell density during the death phase when in co-culture compared to when it is grown alone. However, CCMP3266 death in co-culture is not a progression of senescence, as its death is marked by a sudden drop in photosynthetic potential quantum yield to zero on 27 d (Figure 3-2 E). CCMP3266 grown alone did not die in this experiment and senescent CCMP3266 has been maintained in this microtitre format for >60 d when evaporation of the medium (not death) limits its continued growth (Bramucci *et al.*, 2015). The sudden decline is accompanied by a complete loss of chlorophyll, similar to what is observed in EhV killing of *E. huxleyi* (Bidle *et al.*, 2007). This suggests that senescence is a feature of host physiology that is sensed and exploited by *P. gallaeciensis*, but that its killing mechanism is distinct from its ability to accelerate senescence (Figure 3-2).

The rapid decline in potential quantum yield and loss of chlorophyll is consistent with the physiological response of *E. huxleyi* to roseobacticides, where death is marked by chloroplast blebbing and formation of apoptotic-like bodies (Seyedsayamdost *et al.*, 2011b). EhVs are thought to co-opt the Programmed Cell Death (PCD) pathway in *E. huxleyi* to produce an autodestruct response. This results in a release of EhVs and initiates a chain of infection and PCD in *E. huxleyi*, which ultimately leads to bloom termination (Frada *et al.*, 2012). Bidle (2015) has hypothesised that EhV resistance in *E. huxleyi* may have its basis in components of viral replication being locked up in the subcellular molecular armor

around the PCD machinery. The resistance mechanism to *P. gallaeciensis* is likely to be different, as it does not rely on its host for replication (as do EhVs), but it is thought to use the host metabolite, DMSP, to synthesize roseobacticides (Sule & Belas, 2013). As N type strain CCMP372 is susceptible to roseobacticides but resistant to *P. gallaeciensis*, the resistance mechanism is likely to impede progression of the interaction before roseobacticide production. One possible mechanism could involve the reactive oxygen species (ROS) produced in apoptosis, which can react with DMSP, leading to a PCD response 'locking up' host metabolites driving this interaction by depleting the local DMSP pool needed for roseobacticide synthesis (Bidle & Vardi, 2011; Seyedsayamdost *et al.*, 2011a). Determining if *P. gallaeciensis* elicits a PCD response and generation of ROS in *E. huxleyi* could test this hypothesis.

3.3.3 Symbiotic interaction between *P. gallaeciensis* and bald *E. huxleyi*

In contrast to its fatal influence on CCMP3266, *P. gallaeciensis* has a stimulatory effect on CCMP2090 population density compared to when this host is grown alone (Figure 3-2 D). This effect on density is ongoing throughout the experiment, but there is no effect on PSII potential quantum yield (Figure 3-2 D & F). The increase in population density for CCMP2090 is not as large as for *P. gallaeciensis*, whose long-term survival in co-culture is dependent on *E. huxleyi*, but still very significant, with more than 1.7 fold increase in cell density seen in log phase (9 d) (Figure 3-2 D). This symbiotic interaction between CCMP2090 and *P. gallaeciensis* may explain the increase in the proportion of N cells in the later stages of *E. huxleyi* blooms (Frada *et al.*, 2012), which coincides with the dominance of roseobacter cells during algal blooms (Pinhassi *et al.*, 2004).

The death phase and senescence are not observed in CCMP2090 grown alone or in co-culture with *P. gallaeciensis*. Rather, there is a prolonged stationary phase that persists for CCMP2090 grown alone and in co-culture maintained for 60 d, which is considered the limitation of the microtitre plate format, as evaporation causes well-well variation in the liquid volume of the experiment (T.J. Mayers and A.R.

Bramucci, unpublished). Microscopic examination of N type cells (CCMP372 and CCMP3266) co-cultured with *P. gallaeciensis* showed that *P. gallaeciensis* did attach to N type cells, and that a loss of chlorophyll and cell integrity was associated with the number of *P. gallaeciensis* cells attached to the N cell (Figure 3-1). This means that *P. gallaeciensis* has the ability to kill individual N type cells, but at a frequency low enough that its stimulating effect on N cell population outweighs this negative impact so that the overall effect is a denser N cell population with no loss of potential quantum yield (Figure 3-2).

The symbiosis between *P. gallaeciensis* and N type *E. huxleyi* could be mediated through bacterially produced bioactive small molecules, as bacteria have been shown to stimulate, or be required, for algal growth due to the production of vitamins and phytohormones (Croft *et al.*, 2005; Bertrand & Allen, 2012; Amin *et al.*, 2015). One possibility for such a bioactive is tropodithetic acid (TDA), a molecule with antimicrobial activity produced by *P. gallaeciensis*.

3.3.4 A lifestyle switch mediated by bioactive small molecules

It has been previously proposed that *P. gallaeciensis* can transition between symbiosis and pathogenesis of its host, *E. huxleyi* (Seyedsayamdost *et al.*, 2011b). This lifestyle switch is likely based on the production of bioactives that influence other organisms within aggregate bacterial-algal communities. The proposed lifestyle switch by *P. gallaeciensis* from symbiosis to pathogenesis is cued by the presence of *p*-coumaric acid (*p*CA), the latter triggering production of the pathogenic bioactives, roseobacticides, which are algaecides capable of killing the host (Seyedsayamdost *et al.*, 2011b). The molecule(s) involved in the symbiotic stage of *P. gallaeciensis* with *E. huxleyi*, and whether its production is also influenced by *p*CA, has yet to be determined. TDA is a likely candidate, as it has been implicated in symbiosis of *P. gallaeciensis* with various other hosts, including the seaweed *Ulva australis* and juvenile scallop and turbot cod larvae (Ruiz-Ponte *et al.*, 1998; Hjelm *et al.*, 2004; Rao *et al.*, 2006, 2007; D'Alvise *et al.*, 2012). TDA has antimicrobial properties, targeting bacteria competing with *P.*

gallaeciensis, enabling the latter to chemically defend its host against pathogens (Bruhn *et al.*, 2006; Rao *et al.*, 2007).

To determine if TDA could be associated with the symbiotic part of *P. gallaeciensis* lifestyle and whether the switch between roseobacticide and TDA production is controlled by *p*CA, we quantified TDA and roseobacticides in the presence and absence of *p*CA. This can be done using spectrophotometry, as both bioactives have unique UV absorbance spectra (Bruhn *et al.*, 2005; Seyedsayamdost *et al.*, 2011b). *P. gallaeciensis* cultures were grown with and without *p*CA over 5 d and the absorbance at 600nm (growth), 430 nm (roseobacticides) and 356 nm (TDA) was measured (Figure 3-3 A & B). The amount of TDA detected is lower in the presence of *p*CA, suggesting that the latter may inhibit TDA production (Figure 3-3 A). To determine if *P. gallaeciensis*' ability to inhibit competing bacteria is influenced by *p*CA, *P. gallaeciensis* was spotted on a lawn of TDA sensitive *Vibrio celticus* J1-22 with (Figure 3-3 D) and without (Figure 3-3 C) 1mM *p*CA. There is a marked reduction in the *V. celticus* zone of inhibition in the presence of *p*CA (a 18.2% decrease, 9.87mm ± 0.15 without *p*CA compared to 8.35mm ± 0.09 with *p*CA, p value <0.001) suggesting that it inhibits TDA production.

The opposite is observed for roseobacticides, as their production is stimulated by *p*CA (Figure 3-3 B). Roseobacticide production is delayed for 3 d despite *p*CA being present throughout this time (Figure 3-3 B), suggesting that it's produced late in the *P. gallaeciensis-E. huxleyi* interaction. This suggests that *p*CA plays an important role in switching *P. gallaeciensis* between a symbiotic and pathogenic lifestyle, inhibiting the production of a symbiosis bioactive (TDA) and triggering the synthesis of pathogenicity bioactives (roseobacticides). The production of bioactives to support symbiotic and pathogenic interactions with an algal host represents a significant investment of resources by *P. gallaeciensis* and its potential dual role in both the symbiotic and pathogenic stages provides a greater compensation.

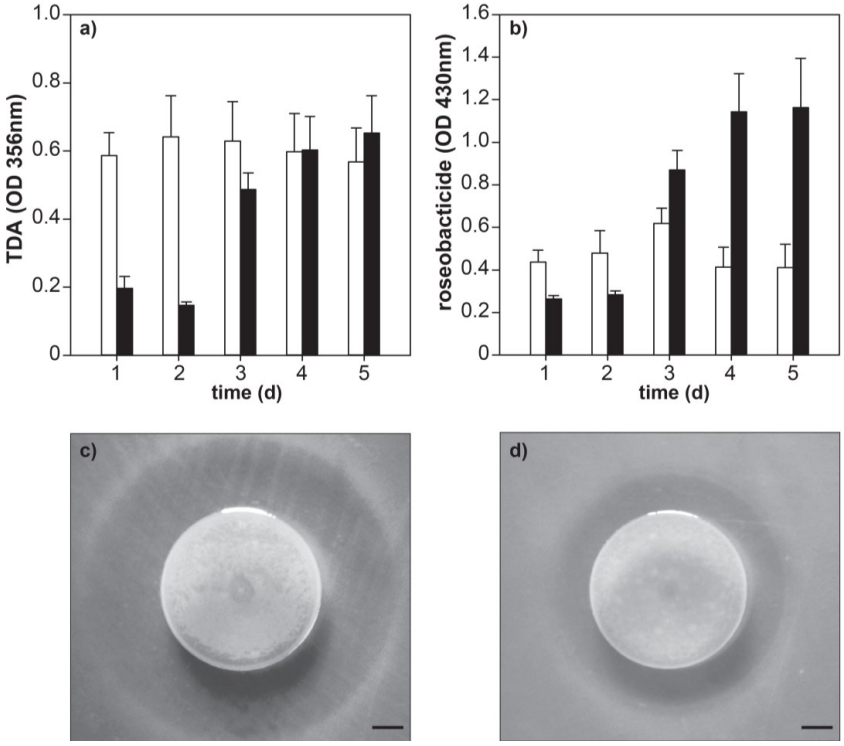

Figure 3-3: The influence of *p*-coumaric acid (pCA) on *Phaeobacter gallaeciensis* DSM 26640 production of bioactives.

The optical density (OD) of tropodithietic acid (TDA) was measured from cultures of DSM 26640 over 5 d by spectrometry (356 nm) when grown in marine minimal medium with (black bars) and without 1 mM *p*CA (white bars) (a). Roseobacticides (430 nm) were measured from the same DSM 26640 cultures with (black bars) and without 1 mM *p*CA (white bars) (b). TDA produced by DSM 26640 inhibits the growth of *Vibrio celticus* J1-22 when DSM 26640 is spotted on a lawn of J1-22 (c). *p*CA appears to have an inhibitory effect on TDA production as the zone of inhibition (ZOI) produced by DSM 26640 on J1-22 is reduced when 1 mM of *p*CA is added to the agar medium as well as DSM 26640 inoculum (d). The scale bar represents 1mm.

3.3.5 Lignin could play an important role in roseobacter metabolism and ecology

Lignin is a major structural component of terrestrial plants (Weng & Chapple, 2010). Its precursors and degradation products, such as *p*CA, are used as biomarkers for terrestrial inputs in marine systems (Opsahl & Benner, 1997; Hedges *et al.*, 1997). The acquisition of lignin is considered a key event in the adaptation of plants to a terrestrial environment and this compound should be absent from algae (Weng & Chapple, 2010). Lignin precursors and degradation products are also used by terrestrial bacterial pathogens as signals to indicate the health of their host plant (Bodini *et al.*, 2009).

Surprisingly, exposure to *p*CA, affects signals and bioactives production by roseobacters, the marine group of bacteria to which *P. gallaeciensis* belongs (Schaefer *et al.*, 2008; Seyedsayamdost *et al.*, 2011b). *p*CA could be used as a signal or a precursor for one of the bioactives they produce, as it is integrated in the structure of roseobacticides (Seyedsayamdost *et al.*, 2014). Roseobacters have also been observed to play a role in the degradation and remineralisation of lignin concentrated in coastal marine habitats from terrestrial inputs (Buchan *et al.*, 2000). Recent evidence also indicates that lignin might not be unique to terrestrial habitats. Martone *et al.* (2009) have identified lignin in a red alga and genes involved in lignin biosynthesis and intermediates in the pathway have been identified from several algal groups, including *E. huxleyi* (Seyedsayamdost *et al.*, 2011b; Labeeuw *et al.*, 2015). The growing evidence for early lignin evolution in phytoplankton, the role of roseobacters in remineralising it and the role of *p*CA in roseobacticides production by *P. gallaeciensis* is suggestive of lignin precursors or degradation products playing an important role in roseobacter metabolism and chemical ecology.

3.4 Conclusion

The ability of pathogens to selectively kill host cells that have a specific physiological state (i.e. senescent) or differentiated cell types have been demonstrated in host-pathogen models (Monack *et al.*, 1997; Weinrauch & Zychlinsky, 1999; Barth *et al.*, 2004; Häffner *et al.*, 2015). EhVs are known to display such selectivity towards their host, *E. huxleyi* (Frada *et al.*, 2008). However, this is the first example of a bacterium selectively killing algal cell types. Also, the ability of *P. gallaeciensis* to target coccolith-bearing diploid cells and not bald diploid cells is a unique niche, likely representing differentiation from EhVs, which kills both diploid cell types. Therefore, it is possible that *P. gallaeciensis* plays a role in carbon deposition (in the form of calcite) in the ocean, as it accelerates senescence and consequently coccolith loss which increases with *E. huxleyi* aging (Chow *et al.*, 2015). This observation could have important implications for the marine carbon cycle and formation of the paeleobotanical record (Coolen, 2011).

3.5 References

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Chapter 4

Indole-3-acetic acid produced by *Emiliania huxleyi*

4.1 Introduction

Small signalling molecules are important components of inter-species interactions, and have been shown to play a role in multi-cellularity, settlement, and pathogenesis (Joint *et al.*, 2002; Matsuo *et al.*, 2005; Schaefer *et al.*, 2008; Case *et al.*, 2011). While these interactions occur at the cell-to-cell interface, they can have large-scale effects at the community and ecosystem levels (Charlson *et al.*, 1987; Vardi *et al.*, 2009; Cooper & Smith, 2015). Many of these small molecules are specific in their mode of action, and can be restricted to a specific sub-population of cells in an organism. Current research indicates that signalling molecules are important components of inter-kingdom communication in bacterial-host systems (Hughes & Sperandio, 2008). One such group of small molecules, which is well-studied in plants, is the phytohormone indole-3-acetic acid (IAA). IAA is one of the most abundant and important plant auxins, and also among the first plant hormones to be discovered (Went, 1926; Teale *et al.*, 2006). Auxins are responsible for growth and development, such as in tissue differentiation, fertility, cell division, orientation, and enlargement in terrestrial plants (Lau *et al.*, 2009; Zhao, 2010; Finet & Jaillais, 2012). Although auxins were originally thought to exist only in plants, their biosynthetic pathway has since been characterized in bacteria and fungi (Spaepen *et al.*, 2007). However, much of what is known about IAA's physiological roles comes from studies of plants' responses to exogenous IAA (Sakata *et al.*, 2010). Indeed, IAA has commercial use and is used in plant horticulture to stimulate root formation (Blythe *et al.*, 1962).

At elevated levels, IAA can cause physiological damage, for instance, IAA stimulates the production of ethylene, which inhibits plant growth (Xie *et al.*, 1996). It has also been implicated in pathogenesis of

plants, as bacterial symbionts have the potential to take over the plant biosynthetic pathway (Yamada *et al.*, 1985; Yamada, 1993) and produce it in plant hosts (Patten & Glick, 2002; Gravel *et al.*, 2007). The diverse effects on the host (ranging from stimulation to pathogenesis) depends on the amount of free IAA produced, as well as the host's sensitivity to this compound (Spaepen *et al.*, 2007). Pathogenic bacteria can alter host metabolic processes for their own benefit, such as by up-regulating the production of IAA, which produces uncontrolled cellular division leading to gall disease in plants (Escobar & Dandekar, 2003). A well-studied example of this pathogenic strategy is the crown gall disease caused by *Agrobacterium tumefaciens,* which involves the formation of galls on the lower stem and roots of infected plants (Zhu *et al.*, 2000; Escobar & Dandekar, 2003). *A. tumefaciens* hijacks the infected plant cells to promote unregulated growth, resulting in the formation of galls, which act as nutrient factories producing amino acids and sugar derivatives that the pathogen uses for energy (Zhu *et al.*, 2000).

In algae, bacterially-produced auxins have been implicated in bud induction in the macroalgal rhodophyte *Gracilaria dura* (Singh *et al.*, 2011). Roseobacters, a clade of marine α-proteobacteria, have been shown to produce IAA or alter its production by a host (Fernandes *et al.*, 2011). Bacterial cells identified as roseobacters using FISH have been localised to the intercellular spaces within galls, in the red macroalga *Prionitis lanceolata* (Ashen & Goff, 2000). These galls have elevated levels of IAA; however it is not known if the bacterium or algal host produce the IAA as neither has been isolated as an axenic culture (Ashen *et al.*, 1999). Both micro- and macroalgal chlorophytes have shown sensitivity and increased growth when exposed to exogenous IAA (Jin et al., 2008; Salama et al., 2014). Amin *et al*. (2015) recently demonstrated the role IAA plays between the unicellular diatom *Pseudo-nitzschia multiseries* and its associated bacterial community, specifically looking at the roseobacter, *Sulfitobacter sp.* SA11. They showed an up-regulation of tryptophan synthesis in the alga led to a corresponding increase in IAA production by the bacterium, which in turn acted as an inter-kingdom signalling molecule

that increased growth of *Pseudo-nitzschia* (Amin *et al.*, 2015). It is perhaps serendipitous that roseobacters were first coined marine *Agrobacterium* given the striking similarity of the roseobacter – alga interaction to that of the *A. tumefaciens* – plant host system (Ahrens & Rheinheimer, 1967). Another roseobacter, *Ruegeria sp.* R11, is a known algal pathogen and produces IAA (Fernandes *et al.*, 2011; Case *et al.*, 2011; Mayers *et al.*, 2016), although the role IAA plays in this interaction (if any) is unknown.

The question of whether eukaryotic algae produce IAA remains largely unresolved (Cooke *et al.*, 2002; Lau *et al.*, 2009; Ross & Reid, 2010). Commercially, addition of algal extracts has been claimed to induce growth responses in plants characteristic of auxins (Crouch & van Staden, 1993). A vast body of studies spanning from the 1940s to the present have reported IAA in various unicellular and multicellular forms of brown, green, and red algae (Van Overbeek, 1940; Jacobs *et al.*, 1985; Sanderson *et al.*, 1987; Tarakhovskaya *et al.*, 2007; Lau *et al.*, 2009). The different lifestyles and forms of multicellular macroalgae to unicellular microalgae would suggest that IAA would play a different signalling role in these organisms, as cell growth, fertility and development occurs within a macroorganism and between microorganisms within a population. The differences of IAA signalling at the intra-and inter-organismal level will be key to understanding its role in micro-and macroalgae.

In macroalgae, IAA was detected in the brown algae *Fucus distichus* and *Ectocarpus siliculosus* (Basu *et al.*, 2002; Le Bail *et al.*, 2010), as well as in the red algae *Pyropia yezoensis* and *Bangia fuscopurpurea* (Mikami *et al.*, 2015). *Nitella*, which is part of Charophyta, the algal phylum most closely related to land plants, has also been suggested to produce IAA. This suggests that primitive auxin metabolism was present at least as early as the ancestor of land plants and charophytes (Sztein *et al.*, 2000). De Smet *et al.* (2011) found putative auxin biosynthesis and auxin transporter orthologs encoded in unicellular chlorophyte genomes. IAA has also been detected in various microalgal chlorophytes, including *Chlorella pyrenoidosa* and *Scenedesmus* spp. (Mazur *et al.*, 2001; Prieto C *et al.*, 2011).These studies have found

evidence corroborating the hypothesis that evolution of auxin biosynthesis predates the divergence of land plants and some algal taxa. However, many of these studies used non-axenic algal cultures, and as some algae-associated bacteria produce auxins (Evans & Trewavas, 1991; Fernandes *et al.*, 2011; Bagwell *et al.*, 2014; Dittami *et al.*, 2014; Amin *et al.*, 2015), it remains unknown if these studies are reporting auxin concentrations from the alga itself or its bacterial epiphytes. Furthermore, the concentration of IAA observed in some studies may not be high enough to play a role in algal development (Lau *et al.*, 2009). It is possible that multi-step IAA extractions underestimates the auxin concentration due to degradation of IAA during the procedure (Mazur *et al.*, 2001). Auxins could also be concentrated within specific algal structures or cells, making whole plant extractions an underestimation of local concentrations within the alga. Nonetheless, Ross and Reid (2010) and Lau *et al.* (2009) expressed reservations regarding evidence for IAA biosynthesis by algae described in literature, especially microalgae, which they reported to lack auxin signalling pathways homologous to those present in land plants.

Using bioinformatics and empirical experimentation, this study aims to elucidate the presence and function of IAA in a unicellular algal species, *Emiliania huxleyi*. This microalga is a small (5 μm), globally abundant haptophyte, part of a different eukaryotic supergroup than all other algae so far investigated for the presence of IAA (Archibald, 2009). It is also a major primary producer in oceans and plays a substantial role in the carbon and sulphur cycles (Holligan *et al.*, 1993). It has three distinct cell types: the non-motile diploid bald cells (type N), the coccolith producing cells (type C), and the haploid motile type cells (type S) (Klaveness & Paasche, 1971; Laguna *et al.*, 2001).

We identified homologs for the genes of several complete tryptophan dependant IAA biosynthesis pathways in the genome of *E. huxleyi.* To confirm this genotype, axenic cultures of C and N type *E. huxleyi* cell types were screened for the production of IAA after stimulation with L-tryptophan. These cell types were also exposed to exogenous IAA to look at their phenotypic response. Interestingly, only C

type cells were able to produce IAA and only N type cells had a phenotypic response to it. To verify if the known IAA producer, the roseobacter *Ruegeria sp.* R11, could also influence its host through this signalling molecule, the two organisms were co-cultured. R11 and *E. huxleyi* grown together produce less IAA than *E. huxleyi* grown alone (with stimulation by tryptophan addition in both cases). The lack of response to the bacterium able to produce IAA, combined with the differential production and response to this signal according to cell type, is unlike what has been previously observed in any other algae.

4.2 Materials and Methods

4.2.1 Genomic survey of tryptophan dependant IAA biosynthesis pathways

Sequences for enzymes in the tryptophan dependant IAA biosynthesis pathways of *Arabidopsis thaliana*, which were used to query algal and roseobacter databases for homologs, were obtained from Le Bail *et al.* (2010). Query sequences to search for the bacterial IAA biosynthesis genes were obtained from Spaepen *et al.* (2007). BLASTp searches for algal homologs were performed and up to three representative completed genomes were searched for each major algal groups (chlorophytes, rhodophytes, glaucophyte, diatoms, pelagophyte, brown algae, eustigmatophyte, chrysophyte, dinoflagellate, haptophyte and cryptophyte) (Table 4-1). Additionally, the *E. huxleyi* genome was specifically surveyed for homologs of plant signalling and transport proteins, using sequences from Le Bail *et al.* (2010) as queries. Available complete roseobacter genomes (as listed on http://www.roseobase.org) and the genome of *Ruegeria sp.* R11 (from which IAA production has been identified) (Fernandes *et al.*, 2011) were surveyed. For all homolog searches, a bi-directional best hit (BBH) BLASTp search of the resulting hits was performed against the organism(s) from which the query sequenced was obtained. An e-value of 10^{-10} or less for the BLASTp and the BBH BLASTp search was used as a cut-off for homology (Le Bail *et al.*, 2010; Kiseleva *et al.*, 2012; Lu & Xu, 2015; Mikami *et al.*, 2015).

The sequences were assigned to orthologous groups using OrthoMCL (Li *et al.*, 2003), where the hits grouping with the query sequence were considered positive. Functional prediction using the ESG package (Chitale *et al.*, 2009) was concurrently performed and only homologs which had confidence scores similar to or higher than the predicted function of the query sequences were considered positive.

4.2.2 Algal and bacterial strains

Axenic *Emiliania huxleyi* strains CCMP2090 (bald N cell type), and CCMP3266 (coccolith-producing C cell type) were obtained from the Provasoli-Guillard National Centre for Marine Algae and Microbiota (NCMA). The *E. huxleyi* strains were maintained in L1-SI medium made using natural filtered seawater (Guillard & Hargraves, 1993) at 18 °C in a diurnal incubator (12:12 hr dark-light cycle). The algal cultures and L1-SI medium were checked for bacterial contamination by microscopy 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) followed by incubation at 30 °C for 2 d. However, this screening cannot exclude the possibility of contaminating un-culturable bacteria that are not readily visualised with microscopy. The algae were grown statically for 5 d to 10^4 cells/mL (early-log) for experiments.

The bacterium *Ruegeria sp.* R11 was maintained at 30 °C on ½ marine agar plates then grown on a rotating drum to stationary phase in 5 mL ½ marine broth (18.7 g Difco Marine Broth 2216 supplemented 9 g NaCl in 1L) for 24 hr before experiments.

4.2.3 Algal growth experiments with tryptophan, IAA and *Ruegeria sp*. R11

L- and D-tryptophan (Sigma-Aldrich, St. Louis, MO, USA) were freshly prepared for each experiment in L1-SI medium and filter sterilization. The IAA (Sigma-Aldrich) stock solution was prepared in 50% ethanol-water solution and all IAA additions were made to a final 1% ethanol concentration in L1-SI medium. The compound screening and co-cultivation experiments were performed as previously

described (Bramucci *et al.*, 2015). Briefly, *E. huxleyi* strains (CCMP2090 and CCMP3266) were supplemented with L-tryptophan at 1, 0.1 and 0.01 mM and at 0.1 mM D-tryptophan in L1-SI medium, with no additional tryptophan as the controls. IAA was added to CCMP2090 and CCMP3266 at a concentration of 0.1, 0.01 and 0.001 mM in L1-SI medium with 1% ethanol and controls of CCMP2090 and CCMP3266 were in L1-SI medium with 1% ethanol. Wells were randomly assigned in 48-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with triplicate samples for each condition such that each plate contained all the samples for a single time point. *E. huxleyi* had an initial concentration of 10^4 cells/mL for all treatments.

For the bacterial-algal co-cultures, bacterial cells were washed twice by centrifugation and re-suspended in L1-SI medium, before being diluted to the initial concentration of 10^4 CFU/mL. Co-cultures were performed with and without 0.1 mM L-tryptophan. All microtiter plates were incubated in a diurnal incubator (12:12 hr dark-light cycle) at 18 °C for all experiments.

4.2.4 Microscopy

Algal cells were visualised throughout the experiment using an Axio Imager M2 microscope (Zeiss, Oberkochen, Germany) and processed using Zen 2012 (Zeiss).

4.2.5 Fluorescence measurements

To measure the chlorophyll fluorescence and photosynthetic yield, samples were taken at the mid-point of their dark cycle (5-7 hr into the dark cycle) and diluted in L1-SI medium to within the detection range using a pulse-amplitude-modulation (PAM) fluorometer (WATER-PAM, Waltz, Effeltrich, Germany). A dark adaption period of 3 min was determined, after which a saturating pulse was applied and the fluorescence readings were taken to calculate the minimal dark fluorescence (F_0) that is directly correlated to the chlorophyll content, the maximum dark fluorescence (F_m) and the Photosystem II (PSII)

potential quantum yield (F_v/F_m) ($F_v/F_m = (F_m - F_0)/F_m$) (Schreiber *et al.*, 1986; van Kooten & Snel, 1990). Three-microtiter wells were sampled (and not re-sampled) as replicates at each time point to determine the yield. Data were processed using SigmaPlot 12. Statistical significance was determined using a one-way ANOVA and Student-Newman-Keuls (SNK) test.

4.2.6 Biomass and IAA measurements

Samples were taken from each sampled well at each time point and biomass measured (OD at 680 nm) using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA). A colorimetric test was used to determine IAA production as previously described (Glickmann & Dessaux, 1995). In brief, samples were taken and centrifuged (10 min at 5000 rpm for algae and 2 min at 14,000 rpm for bacteria) then the supernatant was mixed at a 1:1 volume ratio with fresh Salkowski reagent (12g ferric chloride ($FeCl_3$) (EMB Millipore, Billerica, MA, USA) in 1 L of 7.9 M sulphuric acid (H_2S0_4) (Sigma-Aldrich)). Samples were then incubated in the dark for 30 min at room temperature. The OD (530 nm) and emission spectra for peak wavelength were measured. IAA concentrations were prepared in L1-SI medium to construct a standard curve to determine the IAA concentration from samples. For all measurements, L1-SI medium was used to zero readings. Statistical significance was determined using a one-way ANOVA and SNK test.

4.2.7 Flow cytometry

Algal samples were fixed with 0.15% glutaraldehyde (Sigma-Aldrich) for flow cytometry by incubating cells in the dark for 10 min, then flash-frozen in liquid nitrogen and stored at -80 °C until flow cytometry was performed using a FACSCalibur (Becton, Dickinson and Company). A 488 nm laser was used for excitation. Samples were first run using chlorophyll fluorescence (670 nm) for detection. Membrane integrity was evaluated using Celltox Green (Promega, Madison, WI, USA) (520 nm). Data were processed using FlowJo 9.2.

4.2.8 GC×GC-TOFMS

Replicate 100 mL cultures of CCMP2090 and CCMP3266 were grown in a 250 mL Erlenmeyer flask. Flasks were inoculated with 10^4 cells/mL *E. huxleyi* in 0.1 mM L- or D-tryptophan and no tryptophan addition for the control. Cells were harvested at 16 d by centrifuging at 5000 rpm for 10 min.

IAA standards were created by adding 1 mM (~500 ppm) IAA to control algal samples. All samples were then extracted using an equal volume of methanol, ultrasonicated, and successively vacuum filtered three times on 1.6 µm Whatman 1820-047 GF/A, 47 mm diameter, filters (Sigma-Aldrich). This was then passed through a column of sodium sulphate (Na_2SO_4) (Sigma-Aldrich). Derivatization was done according to previous literature (Birkemeyer *et al.*, 2003), whereby 5 µL of the extracted algal sample was added to 100 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (Sigma-Aldrich), then incubated at 90 °C for 30 min. To determine any possible background IAA levels in the medium used, 10mL of the L1-Si medium was also tested for the presence of IAA. However, since this was a liquid extraction, additional treatment was needed. First, the pH of the sample was lowered to ~1.5 using hydrochloric acid (Sigma-Aldrich), then the liquid sample was extracted by adding methylene chloride (Sigma-Aldrich), vortexing vigorously, and removing the methylene chloride layer. This was repeated three times. The methylene chloride was then evaporated off, after which methanol was added and the above algal extraction procedure, after filtration, was followed. In order to verify that this extraction method worked, a sample of L1-Si medium was spiked with IAA at ~5 mM, and the expected IAA peak was detected.

The samples were analyzed on a Leco Pegasus 4D GC×GC-TOFMS (Leco Instruments, St. Joseph, MI, USA). The columns used for the first- and second- dimensions were a 30 m × 0.25 µm, 1 µm film thickness Rtx-5MS (Chromatographic Specialties, Brockville, ON, Canada) and a 1.6 m × 0.25 mm, 0.25 µm film thickness Rtx-200MS (Chromatographic Specialties) respectively. Helium (5.0 grade; Praxair, Edmonton, AB, Canada) was used as the carrier gas with flow controlled at 1.5 mL/min. The analytes

were desorbed in the split/splitless injection port of the GC×GC-TOFMS using an inlet temperature set at 230 °C, operating in splitless mode. The 47 min GC method began with an initial oven temperature of 70 °C for 1 min, followed by a ramp of 6 °C/min up to 320 °C, and ending with a 5 min hold in the first oven. Relative to the primary oven, the secondary oven was programmed to have a constant offset of +5 °C and the modulator a constant offset of +15 °C. The modulation period was 2.0 s. The TOFMS had an acquisition rate of 100 Hz and acquired over a mass range of m/z 10-700. The detector voltage was -1350 V, the ion source temperature was 200 °C, and the MS transfer line temperature was 250 °C.

GC×GC-TOFMS data were processed using ChromaTOF® (v.4.43; Leco). For processing, the baseline offset was set at the middle of the noise (0.5), the minimum S/N for the base peak and the sub-peaks were set at 10, and the data were auto smoothed by the software. The first dimension peak width was set at 14 s while the second dimension peak width was set at 0.15 s. Identifications of the compounds were made based on library matches with the NISTMS 2008 Library mass spectral database and relative retention index. In this case, a minimum match factor of 75% of the library was required before a name was assigned to a compound.

4.3 Results and Discussion

4.3.1 Presence of tryptophan dependent IAA biosynthesis pathways in algae and roseobacters

Indole-3-acetic acid (IAA) biosynthesis consists of a complex set of tryptophan dependent and independent pathways that is not yet fully understood (Zhao, 2010; Tivendale *et al.*, 2014). The tryptophan independent pathway remains genetically undefined (Normanly, 2010; Nonhebel, 2015). In contrast, the tryptophan dependent pathways have been well studied in *Arabidopsis thaliana* (Sztein *et al.*, 2000; Zhao, 2010), with many of the genes encoding for its enzymes functionally described.

Biosynthesis of IAA by bacteria has also been studied, but members of this domain are thought to use pathways and enzymes different than those found in plants (Spaepen *et al.*, 2007). To investigate the presence of tryptophan dependent pathways outside of plants, public databases were searched using *A. thaliana* and known bacterial IAA biosynthesis genes as queries against algal and roseobacter genomes. Homologs of several of these genes were found in a wide array of algae (Table 4-1) and roseobacters (Appendix Table B-1). However, it should be noted that the presence of homologs does not necessarily translate to the presence of the bona-fide functional enzyme involved in this pathway, especially for those that fall under a functionally broad protein family (such as CYP79B2 and CYP79B3 that are cytochrome P450s). To determine which homologs were likely to have a conserved function with plant enzymes, functional prediction was performed with the ESG package (Chitale *et al.*, 2009) and homologs were categorized in orthologous groups using OrthoMCL (Li *et al.*, 2003). Only homologs part of the same orthologous groups as the plant enzymes and/or with identical functions predicted with ESG were included (Table 4-1 and Appendix Table B-1 and Table B-2). Patterns of co-occurring genes suggesting the presence of complete tryptophan dependent pathways were found in some algae, and genes encoding partial pathways in others (Table 4-1). In algae in which only partial pathways were found, IAA could potentially be produced as a result of the completion of essential biochemical steps by their proximal bacterial symbionts (Dittami *et al.*, 2014). We also found homologs of some bacterial IAA biosynthesis genes in algae (Appendix Table B-2), suggesting that alternate pathways for IAA biosynthesis to those found in plants might be present.

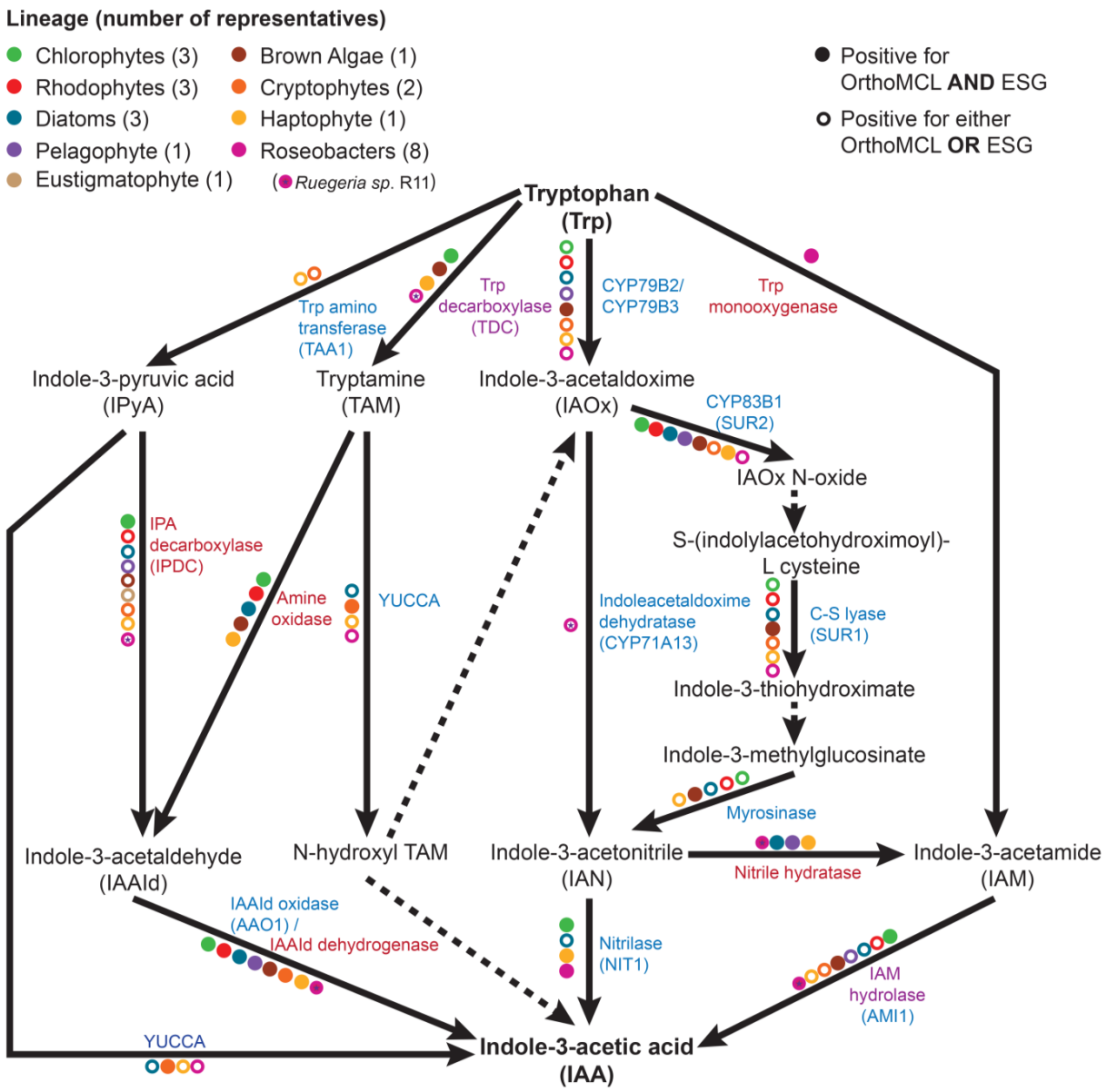

Figure 4-1: The tryptophan dependent indole-3-acetic acid (IAA) biosynthesis pathways as currently conceptualized in plants and bacteria.

Metabolic reactions catalysed by known enzymes are indicated with solid lines while dashed lines indicate a currently unknown enzyme. Listed in blue are the enzymes with a plant seed sequence, while in red are the enzymes with a bacterial seed sequence. Listed in purple are the enzymes that are found in plants, algae and bacteria. Coloured dots represent the presence of the given enzyme in at least one member of a specific taxonomic group, as indicated by bi-directional best hit (BBH) BLAST search followed by Ortho MCL and ESG functional prediction.

As shown in previous studies (Kobayashi *et al.*, 1993; Amin *et al.*, 2015), the roseobacter genomes encode for the genes known in bacteria to be involved in the synthesis of IAA through the indole-3-acetamide (IAM) pathway (Figure 4-1). There is a possibility that roseobacters are capable of synthesizing IAA through another pathway, as they contain a putative tryptophan decarboxylase that produces the IAA precursor tryptamine, a putative indole-3-pyruvate decarboxylase, as well as indole-3-acetaldehyde (IAAd) dehydrogenase, which converts the tryptamine derivative IAAd to IAA (Appendix Table B-1). However, Amin *et al.* (2015) highlighted the difficulty in determining whether the IAAd dehydrogenase found in roseobacters is specific to IAA biosynthesis or used in other pathways. Nitrile hydratase and IAM hydrolase, which can together convert indole-3-acetonitrile (IAN) to IAA, have also been identified in roseobacters (Fernandes et al., 2011) (Figure 4-1 and Appendix Table B-1). However, there is currently no known pathway in bacteria that can produce the IAN this enzyme pair use as an initial substrate. Interestingly, the putative genes to produce IAN from tryptophan (the indole-3-acetaldoximine, or IAOx, pathway) are widely distributed in algae and in roseobacters using plant genes (Figure 4-1). This complementarity could form the basis of a symbiosis between algae and roseobacters enabling the production of IAA. This symbiosis has been suggested as the basis for the presence of IAA in the brown algae *Ectocarpus siliculosus*, as the bacterial symbiont *Candidatus* Phaeomarinobacter ectocarpi contains the genes necessary to complete the synthesis of IAA in complementarity with the genes found in the alga (Dittami *et al.*, 2014).

The survey of public sequence databases also revealed that the principal tryptophan dependent IAA biosynthesis pathway in plants, the indole-3-pyruvic acid (IPyA) pathway, using tryptophan amino transferase (TAA) and YUCCA (Mashiguchi et al., 2011), is not widely distributed in algae, if at all. It has been previously noted by similar genomic surveys that some chlorophytes have the genetic potential for IAA biosynthesis, but that the tryptamine (TAM) and IAN are the most likely biosynthetic pathways through which they could synthesize this compound (De Smet et al., 2011; Kiseleva et al., 2012). More

distantly related algae (outside the supergroup Archaeplastida, which contains plants, chlorophytes, and rhodophytes) have not been thoroughly investigated, except for the brown alga *E. siliculosus*, in which the IAOx and TAM pathways had the most supporting evidence for being at least partially present (Le Bail et al., 2010), although it should be noted that combining known bacterial and plant genes, at least one complete pathway is present (Figure 4-1). Surprisingly, phylogenetically diverse microalgae such as diatoms, haptophytes, and cryptophytes, have a wide distribution of putative enzymes enabling the production of IAA from tryptophan (Figure 4-1 and Table 4-1). The haptophyte Emiliania huxleyi, for example has a complete putative pathway to yield IAA from tryptophan, combining plant and bacterial enzymes (using tryptophan decarboxylase, bacterial amine oxidase, and indole-3-acetaldehyde oxidase) (Figure 4-1 and Appendix Table B-2). The fact that this haptophyte contains homologs of all the genes for a complete putative pathway, as well as homologs of some genes involved in nearly every other tryptophan dependent pathway, makes it a good candidate for further investigation.

Table 4-1: Distribution of IAA biosynthesis genes in algal genomes[#]

	YUCCA	AMI1	TAA1	CYP79B2	CYP79B3	AAO1	CYP71A13	TDC	MYR1	SUR1	SUR2	NIT1
LAND PLANTS												
Arabidopsis thaliana	AEE86075	Q9FR37	Q9S7N2	NP_195705	NP_179820	Q7G193	O49342	Q8RY79	P37702	O65782	Q9SIV0	AEE77887
GREEN ALGAE												
Ostreococcus sp RCC809[a]	-	-/+	-	-/+	-/+	-	-	-	-/+	-/+	+	-
Coccomyxa subellipsoidea[a]	-	+	-	-/+	-/+	+	-	+	-/+	-/+	-/+	+
Chlamydomonas reinhardtii[a]	-	+	-	-/+	-/+	+	-	+	-/+	-/+	-	-
RED ALGAE												
Cyanidioschyzon merolae[b]	-	-/+	-	-	-	-	-	-	-	-	+	-
Porphyridium purpureum[c]	-	-	-	-	-	-	-	-	-	-	-	-
Chondrus crispus[d]	-	-/+	-	-/+	-/+	+	-	-	-/+	-/+	+	-
GLAUCOPHYTES												
Cyanophora paradoxa[d]	-	-	-	-	-	-	-	-	-	-	-	-
DIATOMS												
Fragilariopsis cylindrus[a]	-	-	-	-	-	-	-	-	-	-	-	-
Phaeodactylum tricornutum[a]	-	-/+	-	-/+	-/+	+	-	-	-/+	-/+	+	-/+
Pseudo-nitzschia multiseries CLN-47[a]	+/-	-/+	-	-	-/+	+	-	-	-/+	-/+	+	-
PELAGOPHYTE												
Aureococcus anophagefferens[a]	-	-/+	-	-/+	-/+	+	-	-	-	-	+	-
BROWN ALGAE												
Ectocarpus siliculosus[d]	-	+	-	+	+	+	-	+	+	+	+	-
EUSTIGMATOPHYTES												
Nannochloropsis oculata[d]	-	-	-	-	-	-	-	-	-	-	-	-
CHRYSOPHYTES												
Ochromonas danica[d]	-	-	-	-	-	-	-	-	-	-	-	-
DINOFLAGELLATES												
Symbiodinium minutum[e]	-	-	-	-	-	-	-	-	-	-	-	-
CRYPTOPHYTES												
Guillardia theta[a]	+/-	-/+	-/+	-/+	-/+	+	-	-	-	-/+	-/+	-
Hemiselmis andersenii[d]	-	-	-	-	-	-	-	-	-	-	-	-
HAPTOPHYTES												
E. huxleyi CCMP1516[d]	+/-	-/+	-/+	-/+	-/+	+	-	+	-/+	-/+	+	+

[#]Footnote = Presence is determined initially by a bi-directional best hit (BBH) BLASTP hit with an E-value < 1x10-10 or less using the characterized land plant *A. thaliana* gene as a query, as well as correct functional prediction using ESG and orthology with the plant enzyme as determined by OrthoMCL. (+) indicates that it matches all three criteria, (-/+) indicates it is confirmed by ESG but not OrthoMCL, while (+/-) indicates confirmation by OrthoMCL but not ESG. The following databases were searched: JGI (a), http://merolae.biol.s.u-tokyo.ac.jp/blast/blast.html (b), http://cyanophora.rutgers.edu/porphyridium/ (c), NCBI (d), and OIST Marine Genomics Unit (http://marinegenomics.oist.jp/genomes/gallery/) (e). The abbreviations used for the enzyme can be described as follows: Tryptamine monooxygenase (YUCCA); Indole-3-acetamide hydrolase (AMI1); Tryptophan amino transferase (TAA1); Cytochrome P450s (CYP79B2 and CYP79B3); Indole-3-acetaldehyde oxidase (AAO1); Indole-acetaldoxime dehydratase (CYP71A13); Tryptophan decarboxylase (TDC); Myrosinase (MYR1); C-S lyase (SUR1); CYP83B1 (SUR2); and Nitralase (NIT1).

Genes belonging to the IAOx pathway seem to be the most widely distributed in algae, which in plants is limited to the Brassicaceae family (Mano & Nemoto, 2012). When putative IAA biosynthesis genes are found in an alga, they are not always present in every member of the group to which it belongs. For example, Mikami *et al.* (2015) did not find any homologs for IAA biosynthesis genes in bangiophycean red algae (not included in this study), while this study found homologs in cyanidiophyceaen and florideaphycean red algae (Table 4-1). Such a patchy distribution of the genes that could be involved in IAA biosynthesis within each algal group and between algal groups can be interpreted in two main ways. It could suggest that the basic mechanisms for IAA biosynthesis were present in the ancestor of terrestrial plants and archaeplastid algae, with multiple loss events occurring, or alternatively that the pathway was distributed by lateral gene transfer (LGT). These hypotheses have been greatly debated for the IPyA pathway, which is composed of the tryptophan aminotransferase and flavin-containing monooxygenase (YUCCA) enzymes, converting tryptophan in IAA via indole-3-pyruvic acid. LGT from bacteria to an ancestor of land plants was argued to be the most parsimonious explanation for the origin of IPyA pathway (Yue *et al.*, 2014; Turnaev *et al.*, 2015). Another view is that the pathway evolved earlier in the ancestor of land plants and charophytes (Wang *et al.*, 2014b, 2016). Interestingly, IAA biosynthesis genes found in bacteria but not plants were also found widely in algae, giving additional evidence for LGT events contributing to its evolution in eukaryotes (Appendix Table B-1

and Table B-2). Further research would be needed to determine where the different tryptophan dependent IAA biosynthesis pathways originated and what role bacteria played in their evolution.

Although many algae may possess one or more tryptophan dependent IAA biosynthesis pathways (or parts of it), there has been little to no evidence to suggest that they contain the necessary receptor proteins known to facilitate IAA signalling and the polar auxin transport that is crucial in determining cell directionality in plants (Křeček *et al.*, 2009; Lau *et al.*, 2009; De Smet *et al.*, 2011). There have been recent studies suggesting a few possible homologs in the transcriptome and genome of brown and red algae (Le Bail *et al.*, 2010; Wang *et al.*, 2015). A survey of *E. huxleyi* did not identify any homologs of known response proteins, including: ARF (auxin response factors); PIN proteins (transmembrane proteins that actively regulate the transport and efflux of auxins); or the Aux/IAA transcriptional repressors. Only one hypothetical protein homologous to TIR1/AFB (transport inhibitor response / auxin signalling f-box) was found (XP_005761773, with an e-value of $3e^{-18}$). However, if IAA serves as a growth-promoting hormone in algal systems, it has been suggested that the AUX/IAA/ARF signalling pathway may not be required, and other signalling mechanisms may exist (Lau *et al.*, 2009; Zhang & van Duijn, 2014).

4.3.2 *E. huxleyi* coccolith bearing (C) cells produce IAA when stimulated by tryptophan

Since genes encoding enzymes of tryptophan dependent IAA biosynthetic pathways are present in the *E. huxleyi* genome, L-tryptophan was added to axenic cultures of coccolith bearing C type (CCMP3266) and bald N type (CCMP2090) cells at various concentrations to determine if these strains would convert it to IAA. IAA was detected using the Salkowski reagent, a rapid colorimetric assay which recognizes indolic compounds, with IAA having an optimal peak of 530 nm (Glickmann & Dessaux, 1995). The lower limit of detection of the Salkowski reagent in this experimental set-up is 0.001 mM. While 1 mM L-tryptophan was inhibitory to the growth of CCMP3266 (Appendix Figure B-1), the Salkowski

reagent indicated that the alga was converting the L-tryptophan into IAA, at concentrations of L-tryptophan as low as 0.1 mM, with the characteristic colour and optimal wavelength observed (Figure 4-2). IAA was detected soon after the addition of L-tryptophan, and its concentration stayed constant, reaching 0.1 mM when 1 mM of L-tryptophan was added, and 0.07 mM with 0.1 mM L-tryptophan. The addition of D-tryptophan, which should not stimulate IAA production (Baldi *et al.*, 1991), yielded no detectable IAA (Figure 4-2).

Unlike CCMP3266, growth of CCMP2090 was not inhibited at 1 mM of L-tryptophan, although it did cause a slight decrease in PSII health (Appendix Figure B-1). However, when CCMP2090 was supplemented with tryptophan, IAA was not produced (Figure 4-2).

The Salkowski reagent is a rapid assay demonstrating the conversion of L-tryptophan to IAA, but use of the reagent in algal samples has been criticized due to its lack of specificity for IAA, as it also binds to other indole compounds (Buggeln & Craigie, 1971). Consequently, we performed GC×GC-TOFMS analysis on the samples to confirm that the compound detected was indeed IAA. GC×GC-TOFMS was run on the harvested 16 d samples of CCMP3266 and CCMP2090 grown with 0.1 mM L- and D-tryptophan. GC×GC-TOFMS is an analysis that facilitates detection of unknown compounds using the retention times in two dimensions and uses a library search for the resulting hits. It has several distinct advantages, including better separation of components, simplified sample preparation, increased peak capacity, and high selectivity and sensitivity (Dallüge *et al.*, 2002; Adahchour *et al.*, 2008). An IAA standard was added to algal samples and compared to the algal controls as well as samples with the addition of 0.1 mM of L- or D- tryptophan. Identification was based on the first and second retention time matching the standard, as well as the similarity (how well the peak matches the library) and reverse (how well the library matches the peak) match factors. The peak retention times and peak identification based on NISTMS 2008 Library mass spectral database determined the presence of IAA in the CCMP3266 sample containing L-tryptophan (Figure 4-3), and the absence of hits in the control, D-tryptophan supplemented

sample or any of the CCMP2090 samples (Table 4-2 and Figure 4-3). When tested, the algal medium had a barely detectable peak at the same retention times (Table 4-2). However, peak area and the signal to noise ratio was very low (Table 4-2). Additionally, the volume of liquid medium used for the control extraction (10 mL) was much larger than that of the liquid left on top of the biomass sample tested for IAA (1 mL maximum), yet yielded a ~1000 fold lower IAA signal per unit volume compared to CCMP3266. This provides strong evidence that the *E. huxleyi* C cells produces IAA when stimulated with L-tryptophan, but that N cells are unable to produce this compound when grown with or without L-tryptophan under the conditions tested.

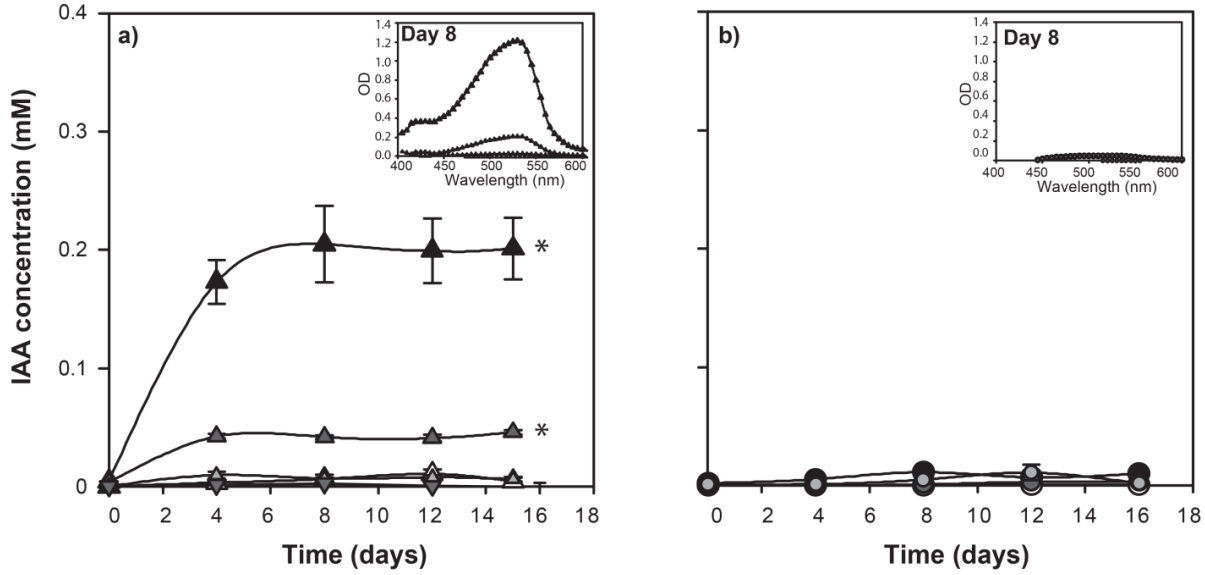

Figure 4-2: Co-culturing experiment of *E. huxleyi* with various concentrations of tryptophan, demonstrating the production of indole-3-acetic acid (IAA).

IAA concentration was derived from OD measurements of culture supernatants after addition of Salkowski reagent. The experiment was performed with coccolith bearing CCMP3266 (a) and bald CCMP2090 (b) *E. huxleyi* strains. Triangles represent CCMP3266 with various concentrations of L-tryptophan (black for 1 mM, dark grey for 0.1 mM, light grey for 0.01 mM and white for the control with no L-tryptophan added). Inverted triangles represent CCMP3266 grown with 0.1 mM D-tryptophan. Using the same colour scheme, circles represent CCMP2090 grown with various concentrations of L-tryptophan. Inset is the emission spectrum taken on 8 d to indicate the peak wavelength. Error bars represent ±1 SE. An asterisk (*) at a time point indicates that it is significantly different to the control and an asterisk at the end of the line indicates that the treatment is significantly different to the control.

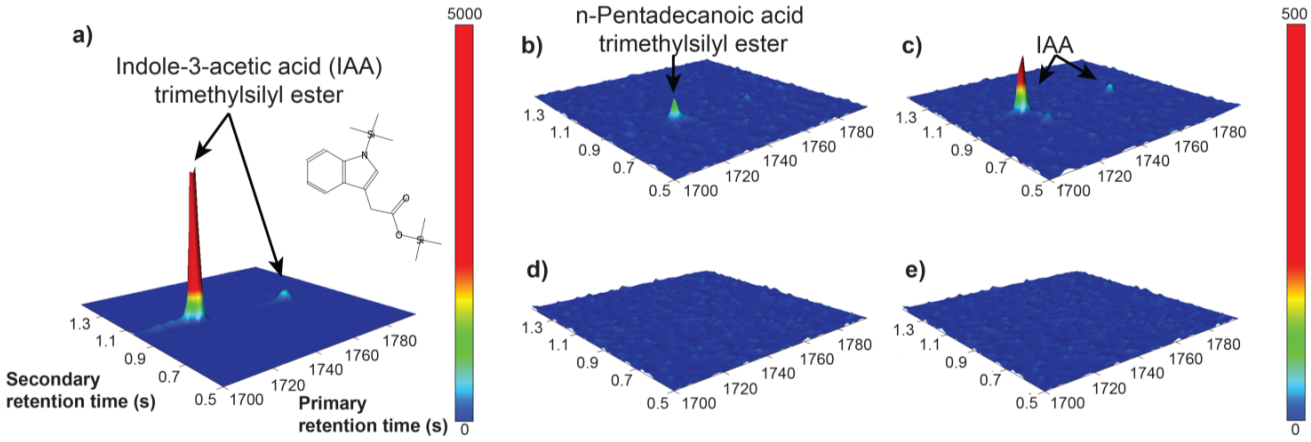

Figure 4-3: GC×GC–TOFMS surface plots of selected mass channel (*m/z*) of 130.

A standard of 1 mM IAA is shown (a), along with *E. huxleyi* C type culture (CCMP3266) control (no tryptophan added) (b), CCMP3266 grown with 0.1 mM L-tryptophan (c), *E. huxleyi* N type culture (CCMP2090) control (no tryptophan added) (d) and CCMP2090 growth with 0.1 mM L-tryptophan (e). Cells were harvested from cultures on 16 d. Identifiable peaks are labelled with the compound to which they correspond.

Table 4-2: GC×GC-TOFMS peak table

Sample ID	Name	R.T. (s) (1D*, 2D**)	Peak Area	Quant Mass	Simil-arity	Reverse	Signal to Noise (S/N) ratio
IAA standard 10^{-3} M	3-Indoleacetic acid, trimethylsilyl ester	(1728, 1.020)	23810	73	816	846	812.56
	3-Indoleacetic acid, trimethylsilyl ester	(1774, 1.000)	218651	73	862	862	2760.90
CCMP3266 10^{-4} M L-tryptophan	3-Indoleacetic acid, trimethylsilyl ester	(1728, 1.020)	1299	73	416	796	34.08
	3-Indoleacetic acid, trimethylsilyl ester	(1772, 0.990)	17418	73	765	793	639.58
CCMP3266 10^{-4} M D-tryptophan	No peak found						
CCMP3266 Control	No peak found						
CCMP2090 10^{-4} M L-tryptophan	No peak found						
CCMP2090 10^{-4} M D-tryptophan	No peak found						
CCMP2090 Control	No peak found						
L1-Si Medium (seawater)	Unknown	(1772, 0.990)	290	73	NS#	NS	10.80

*1D = 1st Dimension, **2D = 2nd Dimension, #NS = Not searchable

4.3.3 Differential effect of exogenous IAA added to bald and coccolith bearing *E. huxleyi* cell types

To determine a potential role for IAA produced by C cells of *E. huxleyi*, various concentrations of exogenous IAA were added to axenic cultures of both the C and N type strains in early log phase and then monitored for biomass (OD), cell morphology (microscopy and flow cytometry), chlorophyll and PSII health (PAM fluorometry) and membrane integrity (cell staining and flow cytometry) (Figure 4-4,

Figure 4-5, and Figure 4-6). The C strain (CCMP3266) showed a small increase in potential quantum yield (F_v/F_m) with the addition of 0.1 mM exogenous IAA, while the N strain (CCMP2090) showed a greater increase in potential quantum yield with 0.1 mM of IAA (Figure 4-4). Lower concentrations of IAA did not affect the potential quantum yield (Figure 4-4 A and B). An effect on potential quantum yield, which is an indicator of PSII health and overall photosynthetic performance, is consistent with the suggestion that IAA stimulates photosynthetic reactions in the chloroplast of plants (Tamás *et al.*, 1972). Such a stimulating effect on photosynthesis has also been demonstrated in diatoms, which harbour bacterial symbionts producing IAA (Amin *et al.*, 2015).

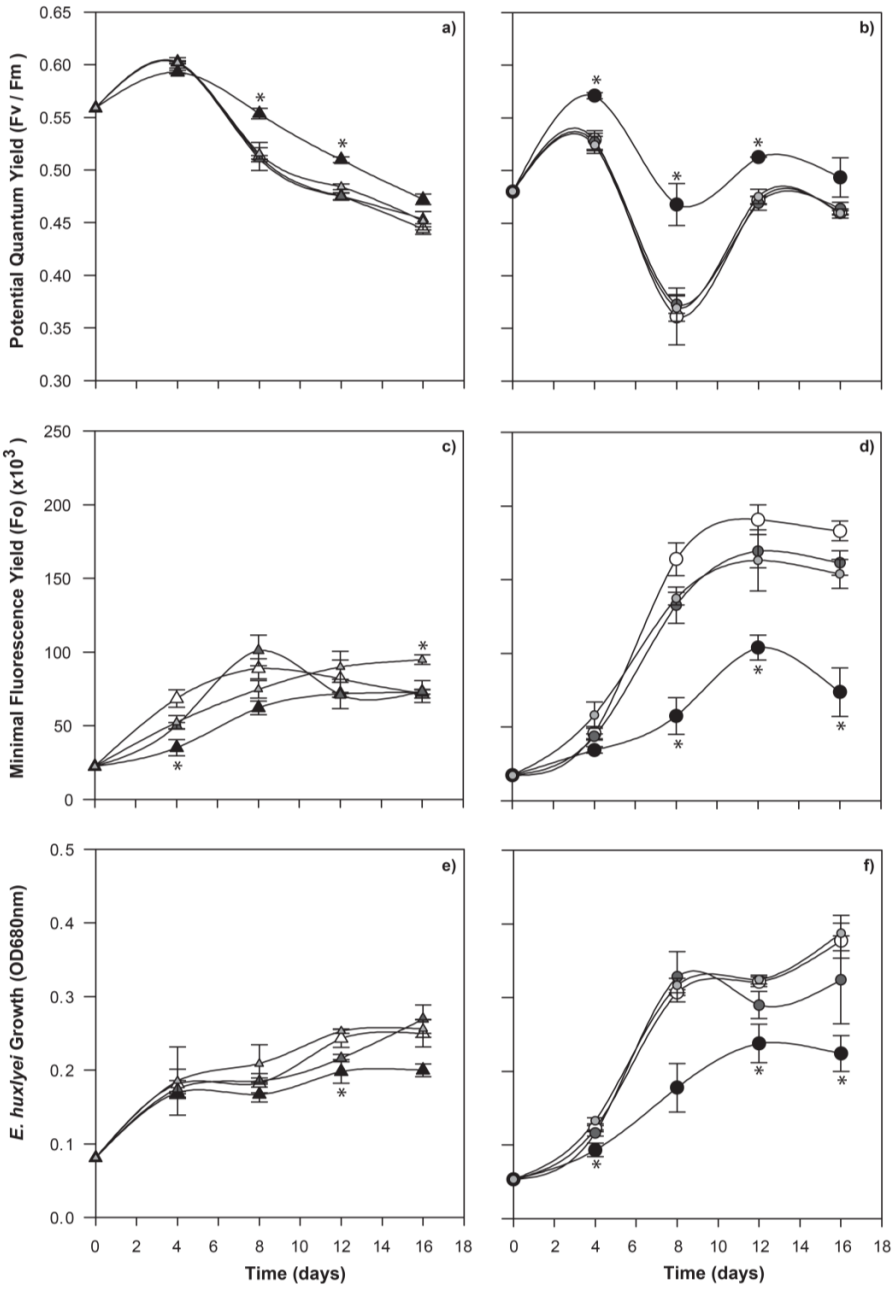

Figure 4-4: Co-culturing experiment of exogenous indole-3-acetic acid (IAA) with the bald (CCMP2090) and coccolith bearing (CCMP3266) *E. huxleyi* strains.

The algae were co-cultured with concentrations of 0.1 to 0.001 mM of IAA (black for 0.1 mM, dark grey for 0.01 mM and light grey for 0.001 mM, and white for the control (L1-SI medium with 1% ethanol)). Triangles represent CCMP3266 while circles represent CCMP2090. The potential quantum yield of CCMP3266 (a) and CCMP2090 (b) with various concentrations of IAA is shown, as well as the minimal fluorescence for the two strains, CCMP3266 (c) and CCMP2090 (d). Growth is displayed as the OD measurement at 680 nm for CCMP3266 (e) and CCMP2090 (f). Error bars represent ±1 SE. An asterisk (*) at a time point indicates that it is significantly different to the control and an asterisk at the end of the line indicates that the treatment is significantly different to the control.

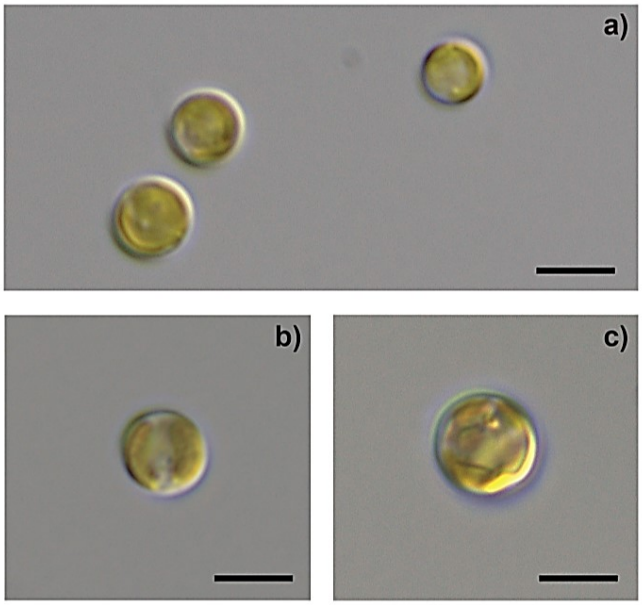

Figure 4-5: DIC microscopic observation of the bald *E. huxleyi* strain (CCMP2090) exposed to indole-3-acetic acid (IAA).

The CCMP2090 control is grown in L1-SI medium with 1% ethanol at 8 d (a), the alga with 0.01 mM IAA at 8 d (b), and the alga with 0.1 mM IAA at 12 d (c). The scale bar represents 5µm.

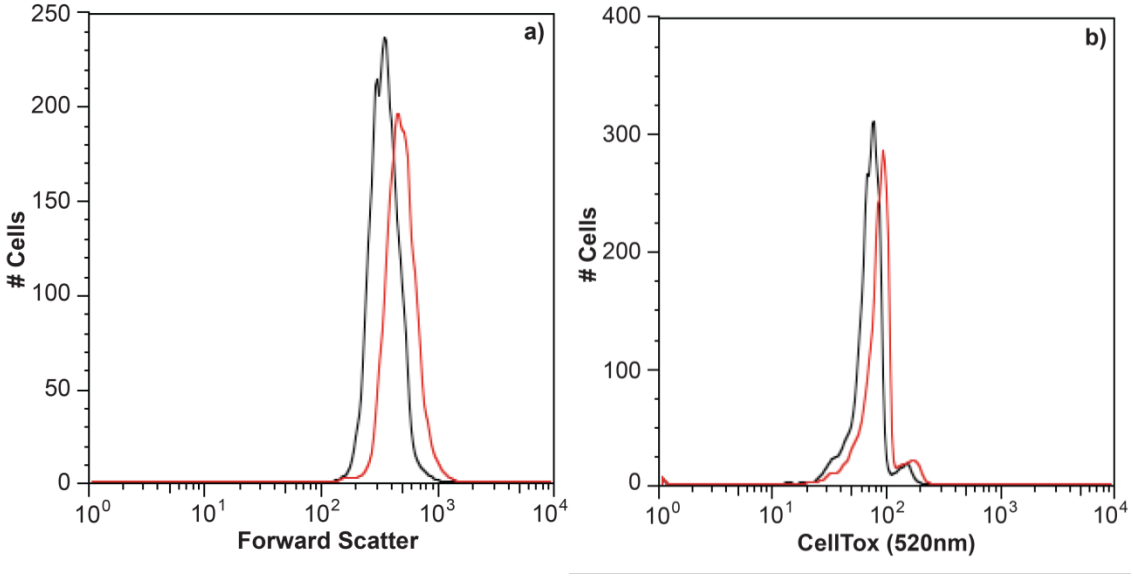

Figure 4-6: Flow cytometry analysis of the bald *E. huxleyi* strain (CCMP2090) treated with indole-3-acetic acid (IAA).

Histograms of the algal cell population with and without 0.01 mM IAA addition are shown with the forward scatter (a) and Celltox green stain (b) after 8 d. The control in L1-SI medium with 1% ethanol is in black while the culture with IAA added is in red.

CCMP3266 was relatively unaffected physiologically even by high concentrations of IAA, showing a minor decrease in growth, with F_o (chlorophyll fluorescence) unaffected (Figure 4-4). However, IAA had a notable effect on the chlorophyll content and biomass of CCMP2090 from 8-16 d of the experiment (Figure 4-4). The more noticeable effect of IAA was at the transition from log to stationary phase, which is consistent with IAA having been shown to be most effective on aged plants samples, such as old samples of maize, rather than freshly cut samples (Evans & Cleland, 1985). Auxins are important regulators of the cell cycle (De Veylder *et al.*, 2007), and have been shown to influence cell division in algae (Vance, 1987). The addition of IAA to diatoms, as well as the addition of IAA and IAA-like compounds (from kelp extracts) to unicellular green algae has been shown to increase biomass (Mazur *et al.*, 2001; Li *et al.*, 2007; Amin *et al.*, 2015). However, elevated IAA can be toxic, with the concentration range for growth promotion being quite narrow (Fässler *et al.*, 2010), which is consistent with its effect on *E. huxleyi* (Figure 4-4).

It is generally thought that auxin function in algae would most likely parallel its function in land plants (Bradley, 1991; Tarakhovskaya *et al.*, 2007), and therefore macro-algae were more likely to be responsive to auxin addition due to its role in cell differentiation (Mazur *et al.*, 2001). It has also been suggested that auxins would have no signalling role in microalgae, and that they are merely side-products of other metabolic functions in these organisms (Stirk *et al.*, 2014). While there was no visual difference between CCMP3266 cultures with or without the addition of IAA, CCMP2090 showed a morphological switch when grown in the presence of IAA. Cells were noticeably larger when grown with IAA compared to the 50% EtOH solvent control (Figure 4-5), or with the addition of the IAA precursor L-tryptophan. This morphological change was confirmed by an increase in the forward scatter of the algal population taken at 8 d (Figure 4-6 A). Cells were also stained with Celltox Green cytotoxicity assay, which binds to the DNA of cells with impaired membrane integrity, resulting in a fluorescent signal. Under this treatment, there was an increase in the fluorescence of cells treated with IAA, indicating an

increase in membrane permeability and as such, an overall loss of cell membrane integrity (Figure 4-6 B).

While auxins do not act directly on cell walls, stimulation of cell elongation and cell wall synthesis is one of its functions in plants (Evans & Cleland, 1985; Cleland, 2010), although high concentrations (~0.2 mM) of IAA can cause inhibition of cell wall synthesis (Baker & Ray, 1965). Auxins can induce acidification in cell walls and an increase in the cell membrane potential. This can be through the transport of the lipophilic form of IAA (IAAH) into the cell which affects the membrane potential as it then dissociates into the anionic form (IAA^-) (Nelles, 1977; Cleland, 2010; Zhang & van Duijn, 2014), which leads to the activation of plasma membrane H^+-ATPases and potassium channels, inducing modifications to the cell wall and hyperpolarizing the membrane (Osakabe *et al.*, 2013; Ng *et al.*, 2015; Velasquez *et al.*, 2016). This change in a cell membrane's pH gradient, and the subsequent modifications on the cell wall by the released enzymes, leads to the cleavage of load-bearing cell-wall crosslinks promoting the turgor pressure needed for cell expansion (Cleland, 2010; Velasquez *et al.*, 2016). The hyperpolarization of the membrane potential has also been demonstrated in the macroalgae *Chara corallina* (Zhang *et al.*, 2016). These changes in cell membrane structure may explain why the N cell type is more affected by IAA than the C cell type in terms of morphological differences, as coccoliths and acidic extracellular polysaccharide coat C type cells and perhaps provide protection from these effects of IAA. Although the coccoliths may not prevent the IAA from entering the cell, and the role of coccoliths has not been fully established, they have been suggested to provide additional strength due to their interweaving structure and may protect the integrity of the cell (Paasche, 2002). Alternatively, it is possible that the lack of a visual morphological effect in C cells as a result of exposure to exogenous IAA is a consequence of the capability of these cells to produce it endogenously.

4.3.4 Role of IAA in bacterial-algal interaction

IAA has been shown to be an important bioactive molecule mediating bacterial-algal interactions between a diatom *Pseudo-nitzschia* and its bacterial community (Amin *et al.*, 2015). In that system, the diatom up-regulated its tryptophan biosynthesis resulting in its symbiotic bacterium *Sulfitobacter sp.* SA11 up-regulating its IAA biosynthesis genes. This relationship, where the alga produces tryptophan for the bacterium to convert into IAA, which in turn promotes algal growth, demonstrates a cross-kingdom relationship in which there is a metabolic and signalling exchange (Amin *et al.*, 2015). The roseobacter *Ruegeria sp.* R11 has been shown to produce IAA (Fernandes *et al.*, 2011), and its genome encodes the putative enzymes of the biosynthetic pathway (Figure 4-1 and Appendix Table B-1). Therefore, we postulated a role of IAA in the relationship between R11 and *E. huxleyi,* as R11 has recently been shown to be pathogenic to *E. huxleyi* CCMP3266, but not CCMP2090 (Mayers *et al.*, 2016). However, the Salkowski reagent did not detect indolic compounds from R11 cultures grown in L1-SI medium or when co-cultured with *E. huxleyi*. Furthermore, the addition of L-tryptophan at a concentration that is not inhibitory to the growth of CCMP3266 did not stimulate the production of IAA in the bacterium (Figure 4-7). As R11 is known to make IAA, it may be that our methods are not sensitive enough to detect IAA production by R11, or that R11's biomass is not great enough to detect its IAA (since IAA was identified from 10 d cultures in ½ YTSS medium (Fernandes *et al.*, 2011) which is much more nutrient rich than L1-SI medium and supports R11 cell density 1000 times higher) or that the host, *E. huxleyi*, drives IAA production in this partnership.

There was also little difference in *E. huxleyi's* potential quantum yield when co-cultured with R11 with or without the addition of L-tryptophan for CCMP2090 or CCMP3266. The only difference is that CCMP3266 died twice as fast from R11 infection when L-tryptophan was added to the co-culture (Figure 4-7). This could implicate tryptophan or IAA produced from tryptophan in R11 virulence or CCMP3266

host susceptibility, however further experiments are needed to decipher how tryptophan addition accelerates CCMP3266 death in co-culture with R11.

IAA concentration was expected to be elevated in the R11 co-cultures. However, less IAA was present compared to the alga grown alone for CCMP3266 and no IAA was detected from the R11-CCMP2090 co-culture. The source of the IAA produced in the R11-CCMP3266 co-culture is not known but this result is suggestive that R11 may divert L-tryptophan into alternate pathways. This is unlike the symbiotic relationship found by Amin *et al.* (2015) in *Pseudo-nitzschia*, where IAA plays a crucial role in the bacterial-host interaction. While R11 has been found in the same geographic area as *E.huxleyi*, they may not have a shared natural history. Their interaction is also different, pathogenic not symbiotic, and so IAA may play differing roles in symbiotic and pathogenic marine bacteria as it does with their terrestrial counterparts (Escobar & Dandekar, 2003; Vessey, 2003).

Regardless of its origin, we propose that IAA has evolved different signalling functions in haptophytes and diatoms. The data presented here suggest that IAA plays a role in cell-cell signalling between different cell types within an *E. huxleyi* population. In terrestrial plants and the red alga *Gracilaria dura,* IAA influences cell membrane permeability so as to direct cell maturation and differentiation. Therefore, IAA could be involved in similar processes in a unicellular alga, which while not having a multicellular form, has differentiated cell types within its population.

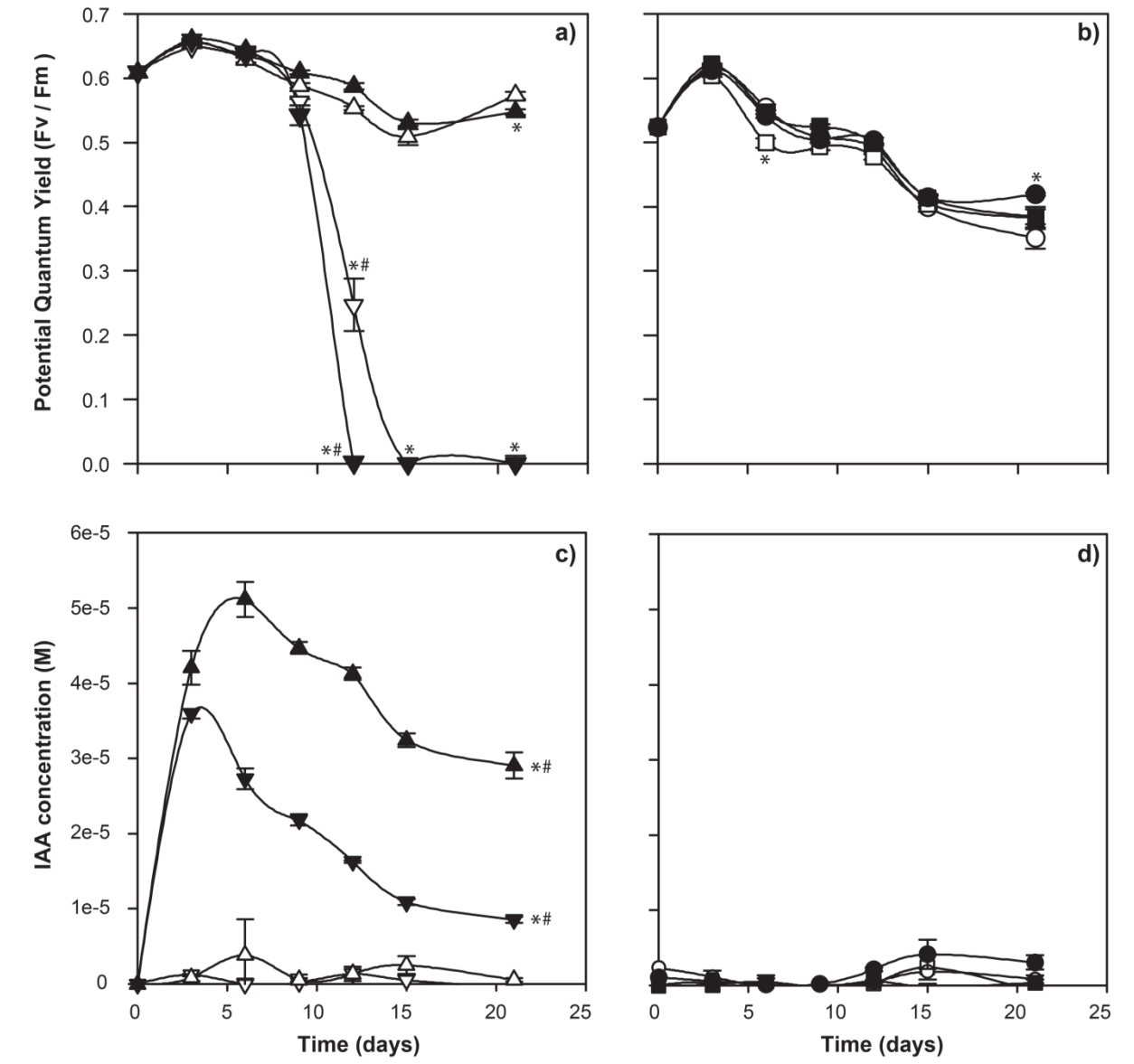

Figure 4-7: Co-culturing experiment of *Ruegeria sp.* R11 with bald (CCMP2090) and coccolith bearing (CCMP3266) *E. huxleyi* strains.

The potential quantum yield of CCMP3266 (a) and CCMP2090 (b), IAA production (measured using the Salkowski reagent) for CCMP3266 (c) and CCMP2090 (d). Triangles represent CCMP3266 alone, inverted triangles represent CCMP3266 co-cultured with R11, circles represent CCMP2090 alone and squares represent CCMP2090 co-cultured with R11. White shapes represent the control grown in L1-SI medium, while black indicates the addition of 0.1 mM tryptophan. Error bars represent ±1 SE. An asterisk (*) at a time point indicates that it is significantly different to the control and an asterisk at the end of the line indicates that the treatment is significantly different to the control. A hashtag (#) indicates the treatments are significantly different to the other treatment with a hashtag.

4.4 Conclusion

IAA has been identified from a variety of photosynthetic organisms, including cyanobacteria (Sergeeva et al., 2002; Ahmed et al., 2010; Hussain et al., 2010), chlorophytes (Sztein et al., 2000; Mazur et al., 2001; Jirásková et al., 2009; Stirk et al., 2013), as well as some rhodophytes and brown algae (Le Bail et al., 2010; Mikami et al., 2015), but to our knowledge, this is the first time auxins have been identified from an axenic haptophyte culture. This raises further questions about the evolution of auxins, and their possible early role in cell-cell signalling between differentiated cell types within populations of unicellular organisms. Several lines of evidence suggest that the coccolith bearing (C) strain of E. huxleyi produces IAA, including: the putative presence of pathway genes in the genome; the positive result of the Salkowski reagent when grown with L-tryptophan as well as the correct peak when analysed with GC×GC-TOFMS; and the lack of this peak or Salkowski result when grown with D-tryptophan. The bald (N) strain did not test positive for IAA, but did show morphological changes in response to IAA. This suggests that not only does IAA play a role in the interaction between algae and their consortia of bacteria (Amin *et al.*, 2015), but could play a role in the signalling between different cell types of algae themselves. These two cell types of *E. huxleyi* co-occur in *E. huxleyi* blooms, but have their highest population density at different times, with the N cell type increasing in proportion at the end of a bloom (Frada *et al.*, 2012). It is therefore possible that IAA could play a role in cell-cell signalling in blooms, acting as a molecular signal throughout the bloom-bust cycle.

4.5 References

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Chapter 5

Lipid yield of algae cultured with bioactives and roseobacters

5.1 Introduction

Biofuels are a promising source of alternative fuel in a world increasingly aware of its carbon footprint. However, first generation biofuels from starch and sugars have not come without drawbacks including land use impacts, increased water usage, food source disruption and price increases, as well as fossil fuel energy costs (Doornbosch & Steenblik, 2007; Wang *et al.*, 2008). Microalgae are a promising source of next generation biofuels as they can accumulate a relatively high proportion of neutral lipids (which are easily trans-esterified into biofuels), mostly in the form of triacylglycerols (TAG) (Chisti, 2007; Zhang et al., 2014), with some common algal species comparable to plant biofuel feedstocks shown in Table 5-1. These are often stored in lipid droplets in order to protect the cell (Farese & Walther, 2009).

Table 5-1: Lipid content of algal and plant biofuel feedstocks*

Species	Lipid content (% dry weight biomass)	Biodiesel yield (L/ha/yr)
Dunaliella tertiolecta	16.7-71.0	-
Chlorella vulgaris	5.0-58.0	8200
Chaeteceros muelleri	18-36	-
Isochrysis sp.	7.1-33	-
Nannochloropsis oculata	22.7-29.7	23,000-34,000
Emiliania huxleyi	18.9-20	-
Tetraselmis sp.	12.6-14.7	-
Oilseed rape (UK)	40.0-44.0	1560
Jatropha	30.0	2700
Soya	20.0	544

* Sources: (Griffiths & Harrison, 2009; Mata *et al.*, 2010; Scott *et al.*, 2010; Moheimani *et al.*, 2011)

Microalgae have been subject to a lot of focus in their growth conditions and how to improve their lipid content, including the possibility of genetic engineering (Rosenberg *et al.*, 2008; Greenwell *et al.*, 2010), although TAG synthesis in algae remains poorly understood in comparison to their plant

counterparts (Merchant *et al.*, 2012). Lipid content generally increases when the cells are no longer actively growing and have entered stationary phase (Mansour *et al.*, 2003). Environmental conditions have been shown to be important drivers of lipid content, with nitrogen deprivation shown to be very effective in increasing lipid production (Liu *et al.*, 2008; Rodolfi *et al.*, 2009; Converti *et al.*, 2009). This is closely linked to lipid metabolism and functions within algae, discussed in detail in various reviews (Thompson, 1996; Guschina & Harwood, 2006; Du & Benning, 2016). However, nitrogen limitations can lead to decreased growth, and in turn to competition for the limiting nutrients with bacteria (Wang *et al.*, 2014a). Bacteria have been implicated in metabolizing the lipids produced by algae (Krohn-Molt *et al.*, 2013). However, their overall impact on algal biofuel stocks has not been well investigated (Wang *et al.*, 2014a), while the potential of adding bioactive molecules is only recently gaining interest (Franz *et al.*, 2013).

Various methods can be employed to investigate an alga's lipid content. These include lipid extraction with solvents followed by gravimetric methods for quantification. These often cannot be done *in situ* (Bligh & Dyer, 1959; Chen *et al.*, 2011a). Therefore, various rapid fluorometric methods have been developed for rapid lipid detection, including the fluorescent dyes boron-dipyrromethene (BODIPY) (Cooper *et al.*, 2010) and the more widely established Nile Red (Cooksey *et al.*, 1987). The lipid-soluble fluorescent probe Nile red (9-diethylamino-5H-benzo[α]phenoxa-phenoxazine-5-one) has been shown to be an effective method for approximating lipid content by selectively staining intracellular lipid, although it should be noted that certain species with thicker cell walls have proven less susceptible to the Nile red staining methods (Greenspan & Fowler, 1985; Cooksey *et al.*, 1987; Chen *et al.*, 2011a). Despite this, Nile red is an effective rapid method for initial screening co-cultures of algae with bioactives or bacteria that may regulate the algal lipid content and to determine those that warrant further with more detailed, analysis.

Using Nile Red, various bioactive molecules produced by bacteria, as well as a marine bacterium itself, was co-cultured against various algal species in order to assess their impact on lipid content. The bacterium, *Ruegeria sp.* R11, is commonly associated with algae (Goecke *et al.*, 2013), while the plant hormone IAA has shown some promise as lipid enhancing molecules (Jusoh *et al.*, 2015).

5.2 Methods

5.2.1 Algal and bacterial strains

All algal strains were obtained from the Provasoli-Guillard National Centre for Marine Algae and Microbiota (NCMA). These included: the haptophyte *Emiliania huxleyi* strains CCMP2090 (bald N cell type), and CCMP3266 (coccolith-producing C cell type), the haptophyte *Isochrysis sp.* CCMP1324, and the chlorophyte *Dunaliella tertiolecta* CCMP1320. The *E. huxleyi* strains were maintained in L1-SI medium made using natural filtered seawater (Guillard & Hargraves, 1993), while CCMP1320 and CCMP1324 were maintained in L1-SI medium using artificial seawater (35g/L of Instant Ocean, Blacksburg, VA, USA), and all were maintained at 18 °C in a diurnal incubator (12:12 hour dark-light cycle). The algal cultures and medium were checked for bacterial contamination by microscopy 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) followed by incubation at 30 °C for 2 d. The algae were grown statically for 5 d to 10^4 cells/mL (early-log) for experiments.

The bacteria *Ruegeria sp.* R11, *Phaeobacter gallaeciensis* DSM 26640, and *Vibrio cholerae* 11EO7 were maintained at 30 °C on ½ marine agar plates then grown on a rotating drum to stationary phase in 5 mL ½ marine broth (18.7 g Difco Marine Broth 2216 in 1L) for 24 hr before experiments.

5.2.2 Co-culture experiments

Algal co-culture experiments were set up as previously described (Bramucci *et al.*, 2015), and in the same manner as Chapter 3.2 and Chapter 4.2. The only variation was that 10^4 CFU/mL was used as the initial starting concentrations of bacteria. Wells for the controls and each experimental group were randomly assigned in 48-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (triplicate for each group at each time point, each plate had same assignations per well), and one mL was aliquoted into each.

In addition, five replicates of 150-250 µL of *Dunaliella tertiolecta* co-cultured with various bacteria were aliquoted to randomly assigned wells in 96-well plates (Becton, Dickinson and Company). All microtiter plates were incubated in a diurnal incubator (12:12 hour dark-light cycle) at 18 °C for all experiments. Statistical significance was determined using a one-way ANOVA and Tukey test.

5.2.3 Biomass and lipid measurement

Samples were taken from three independent wells for each time point samples, and the biomass measured (OD at 680nm) using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA). A fluorometric test was adapted from previous literature to determine approximate lipid content (Franz *et al.*, 2013). The optimal conditions were determined to be 25 µL of Nile red (Sigma-Aldrich, St. Louis, MO, USA) (5 mg/100 mL of acetone (Sigma-Aldrich)) added to 250 µL algal culture and mixed well. The samples were incubated in the dark at room temperature for 20 min, and then a reading was taken using 530 nm as the excitation wavelength and 590 nm as the emission. L1-SI medium was used to determine a baseline reading. Standards of glyceryl trioleate (triolein) (Sigma-Aldrich) in the solvent isopropanol (Fisher Scientific, Waltham, MA, USA) (10 mg/mL) were added to L1-SI medium to create a standard curve to determine the approximate triolein lipid equivalent concentration of the OD reading (Bertozzini *et al.*, 2011).

5.2.4 Flow cytometry

The same method used in Chapter 4.2 was applied for flow cytometry, with samples stained with Nile Red and measured using 488 nm laser for excitation and 530 nm emission on a FACSCalibur (Becton, Dickinson and Company).

5.3 Results and Discussion

5.3.1 Determination of optimal methodology

Franz *et al.* (2013) reported a methodology for screening for lipids in algae treated with various compounds using 96-well plates. However, when this same methodology was performed with *Dunaliella tertiolecta*, there was too much variability between replicate wells. The inability to achieve reproducibility among replicates throughout the plate was due to evaporative effects across the plate (data not shown). As such, it was determined that 48-well plates were the most effective method for rapid screening. Despite this leading to a lower throughput in what could be screened simultaneously, the increased replicability and lack of evaporative effects means it is an overall more reliable methodology. All lipid values are normalized to the lipid per biomass (OD at 680nm) in order to better compare between treatments. The lipid content per cell reaches a maximum at the stationary phase (12 d) (Figure 5-1). In commercialized systems, this is the point at which they would be harvested. As such, only 12 d readings have been considered for their lipid content in subsequent experiments.

5.3.2 The lipid content of algae co-cultured with *Ruegeria sp.* R11

D. tertiolecta CCMP1320 co-cultured with the bacteria *Ruegeria sp.* R11 showed an increase in the lipid content per biomass unit (Figure 5-1). This was achieved without a difference in biomass concentration, as measured by the OD at 680 nm, indicating the lipid content per cell was higher. The

lipid content of the bacteria cultured alone were negligible. However, as with DSM 26640 and *E. huxleyi* (Figure 3-2), R11 grew better with *D. tertiolecta*, and lived longer than when cultured alone (data not shown). R11 was the only one of the tested bacteria to demonstrate this lipid stimulation. Co-culturing with *Phaeobacter gallaeciensis* DSM 26640 and *Vibrio cholerae* 11EO7 did not show any significant change in lipid content (data not shown). No other algae tested with R11 demonstrated this statistically significant boost in lipid content (Figure 5-2).

There has previously been some success in using bacteria to increase lipid production (de-Bashan *et al.*, 2002; Cho *et al.*, 2015). The mechanism by which this occurs is currently not known. One possibility is that there is increased competition for nutrients, thereby leading to the algae being deprived of certain compounds or becoming stressed, which are known factors to achieve higher lipid content (Hu *et al.*, 2008; Wang *et al.*, 2009). There is the possibility that bioactive molecules cause this change, but if they are, they are not currently well known. Determining which bacteria have this stimulatory effect, without detriment to the biomass and overall growth, could lead to the production of an artificial ecosystem which is robust and leads to increased yield of lipids.

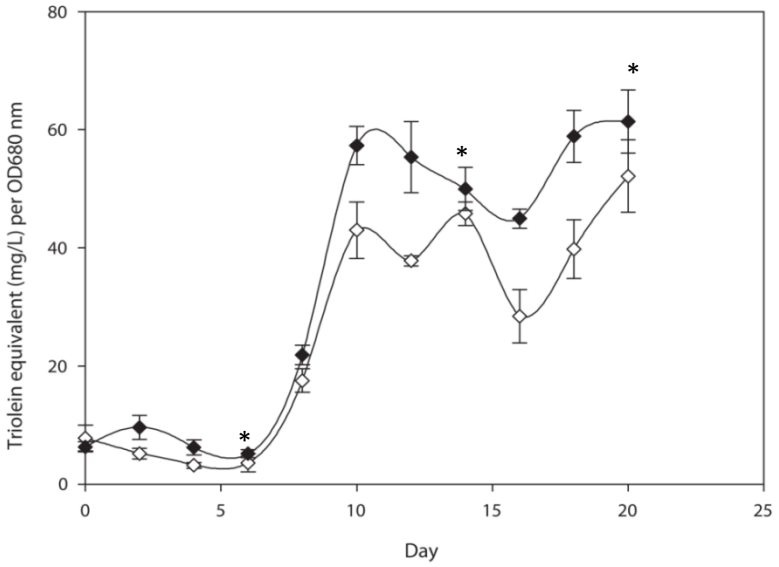

Figure 5-1: Co-culturing experiment of *Ruegeria sp.* R11 with *Dunaliella tertiolecta* (1320), indicating the lipid content.

The lipid concentration in equivalent triolein concentration, normalized per OD_{680} measured, is given for control (white), and R11 co-cultures (black). Error bars are ±1 SE. The two treatments were significantly different from each other ($p<0.05$) with the exception of the points marked with an *.

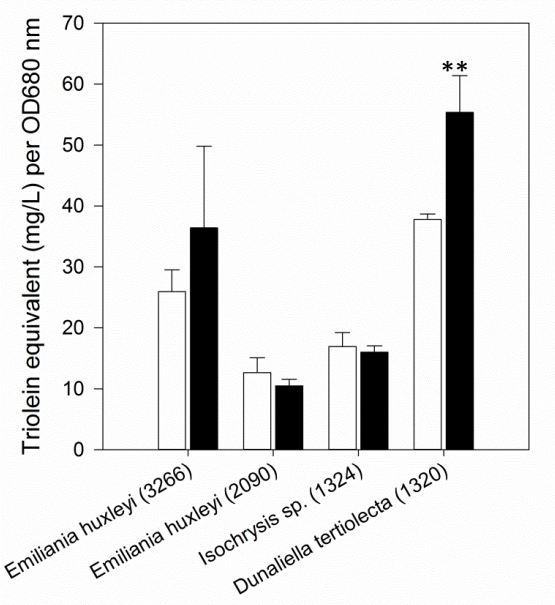

Figure 5-2: Co-culturing experiment *Ruegeria sp.* R11 with algae at 12 d, indicating stimulation of algal lipid content.

The lipid concentration on d 12 in equivalent triolein concentration (mg/L), normalized per OD_{680}, is given for controls (white), and R11 co-cultures (black) for various algae. Error bars are ±1 SE. ** indicates that the R11 treatment is significantly different ($p<0.05$) from the control.

5.3.3 The lipid content of algae co-cultured with tryptophan

Tryptophan is an important amino acid that is the precursor for many bioactive molecules. Tryptophan has not been shown to have growth promoting effects previously on algae (Davidson, 1950; Ahmad & Winter, 1969), although low concentrations have been reported to improve plant growth (Frankenberger *et al.*, 1990). However, tryptophan supplemented media has been shown to have a lipid enhancing effect on *Chlorella sorokiniana* (Ngangkham *et al.*, 2012). In our tested algal species, the addition of various concentrations of tryptophan was not shown to significantly alter lipid content (Figure 2-1). One exception to this was *Emiliania huxleyi* CCMP2090 co-cultured with 10^{-3} M tryptophan, where there was a higher lipid/biomass ratio. This was because the raw lipid measurement was similar to other treatments, but there was a lower level of biomass (Appendix Figure B-1). The Nile Red could be staining burst or dying cells, hence the high lipid reading. However, since the overall levels of lipids were not changed (4.02 ± 0.44 mg/mL triolein equivalent compared to 3.88 ± 0.17 mg/mL triolein equivalent for the control samples), this would not appear to be a viable option for treatment to increase overall lipid production.

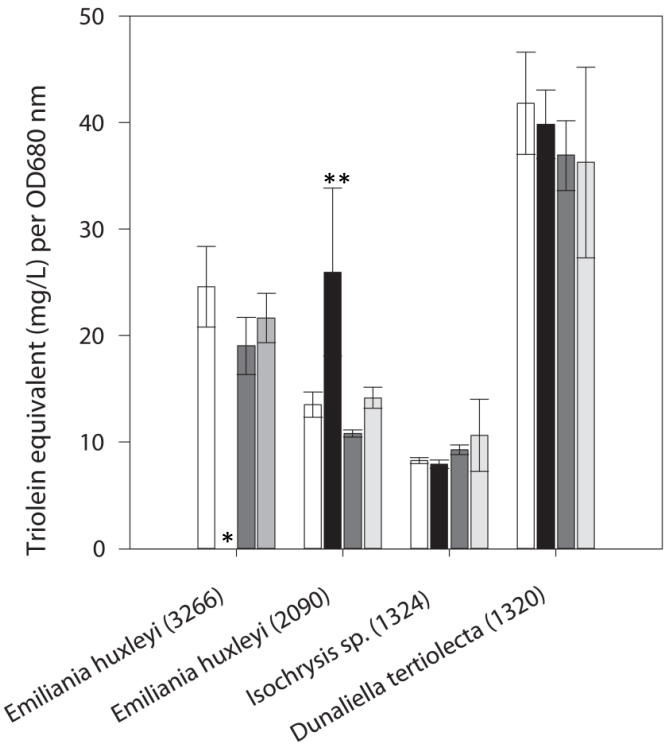

Figure 5-3: Co-culturing experiment of various concentrations tryptophan with algae at 12 d, indicating the lipid content.

The lipid concentration in equivalent triolein concentration, normalized per OD_{680} measured, is given for control (white), 10^{-3} M (black), 10^{-4} M (dark grey), and 10^{-5} M (light grey) of tryptophan. * indicates that for that point the OD was below 0.08 and the sample was dead. Error bars are ±1 SE. ** indicates that the treatment is significantly different ($p<0.05$) from the control.

Higher levels of tryptophan (1-5mM) were inhibitory for the haptophytes (*E. huxleyi* and *Isochrysis sp.*), but it was not inhibitory to *Dunaliella tertiolecta* CCMP1320. This level of tryptophan (5mM) did lead to an increase in potential quantum yield (0.290 ± 0.007 without 5mM tryptophan compared to 0.535 ± 0.001 with 5mM tryptophan on 12 d), no change to biomass, but an overall depression of lipid (12.71 ± 2.14 mg/mL triolein equivalent per OD_{680} compared to the control sample which had 41.81 ± 4.80 triolein equivalent per OD_{680} on 12 d). Higher concentrations of tryptophan have been shown to be inhibitory on leaf and shoot length in *Zea mays* (Sarwar & Frankenberger, 1994), but the effect on lipids has not been widely reported. While this high level of tryptophan may not be biologically relevant in natural systems, it does show that excess essential amino acids will impact lipid production.

5.3.4 The lipid content of algae co-cultured with indole-3-acetic acid (IAA)

IAA has been shown to be an important component of algae growth promoting bacteria (Gonzalez & Bashan, 2000; de-Bashan *et al.*, 2008), and has been correlated with an increase in lipid content in *Chlorella vulgaris* (Jusoh *et al.*, 2015). There was no significant difference in our tested algal species between samples cultured with IAA compared to without (Figure 5-4). However, it should be noted that there was an observed increase in size in *E. huxleyi* CCMP2090 cells cultured with IAA (Figure 4-5 and Figure 4-6). As such, while the lipid content per cell remains the same, the lipid per surface area or cell biovolume would be lower in CCMP2090 samples cultured with 10^{-4} M or 10^{-5} M of IAA.

Increased cell size has been linked to lower growth rates (Finkel *et al.*, 2010), but the relation to lipids is less well known. Nitrogen limitation has been shown to increase cell size in certain algal species caused by increased lipid accumulation, although increases in cell size of other algal species did not correlate to an increase in total lipids (Dean *et al.*, 2010). This, along with the microscopic observations (Figure 4-5), is suggestive of IAA increasing cell size of CCMP2090 by another method (e.g. water vacuoles) rather than by increased lipid accumulation. The decreased lipid per cell would however be in line with the increased membrane permeability (less lipid per surface area) (Figure 4-6).

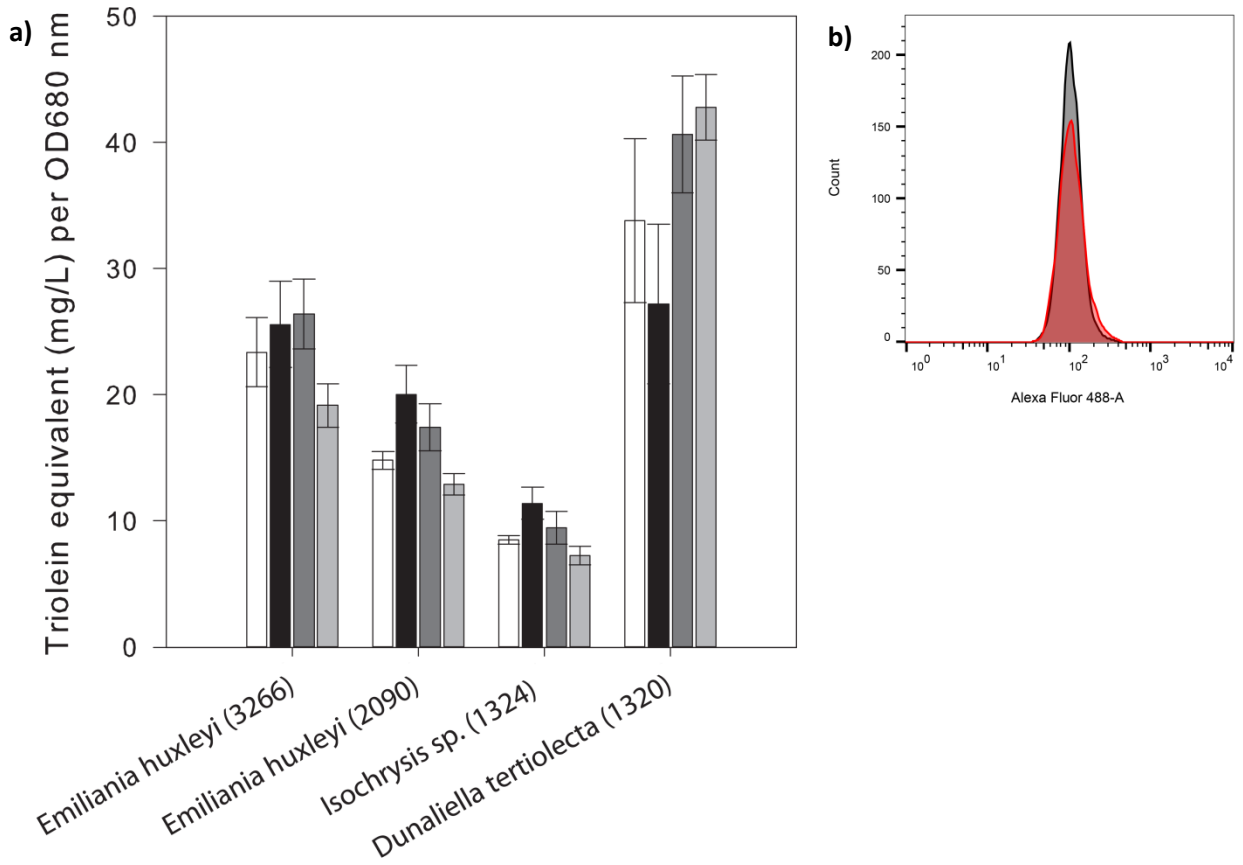

Figure 5-4: Co-culturing experiment of various concentrations indole-3-acetic acid (IAA) with algae at 12 d, indicating the lipid content.

(a) The lipid concentration in equivalent triolein concentration, normalized per OD_{680} measured, is given for control (white), 10^{-4} M (black), 10^{-5} M (dark grey), and 10^{-6} M (light grey) of IAA. Error bars are ±1 SE. (b) The lipid concentration of *Emiliania huxleyi* CCMP2090 as measured by flow cytometry is shown with the control (black) and 10^{-5} M (red).

5.4 Conclusion

There are many factors to consider when optimizing algal systems for biofuel growth. These include growth characteristics, lipid production, and overall stability. While environmental conditions such as nutrients or salt concentration (Takagi *et al.*, 2006; Liu *et al.*, 2008; Chen *et al.*, 2011b) have been shown to be important factors for lipid production, other factors such as the surrounding biome have not been well studied. The methodology outlined here shows a rapid, replicable method for initial screening to

determine the effects on lipid yield of co-culturing algae with bacteria or the addition of bioactive molecules to algal cultures. There appears to be potential for some roseobacters to increase lipid content in certain strains of algae. However, the bioactive compounds produced by the bacteria that are linked to growth stimulation do not seem to be useful for biofuel production using the algae tested.

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Chapter 6

General Discussion

6.1 Synthesis of Findings

The complex exchange of small molecules occurring between algae and the microbial consortia within their phycospheres and the surrounding area is a unique system that represents a hitherto unexplored potential source of novel bioactive molecules (Ramanan *et al.*, 2016). Less well known than bacterial-algal interactions are the hypothetical alga-to-alga communication processes (Demuez *et al.*, 2015). Our ability to investigate these systems has been revolutionized with the increasing availability of sequencing data. This body of publicly available information allows us to search within the genomes of any sequenced organism for any known gene or pathway, determining their potential for producing the compound in question. This ability is invaluable in both the search for novel pathways and the search for known pathways in species to which they are not traditionally attributed to. The results of these inquiries can then inform laboratory experiments which confirm the presence of and investigate the role of these pathways and the metabolites involved, further revealing the role of the metabolites under specific conditions. My research has identified several well-known pathways, historically thought to exist only in plants, in microalgae, and investigated parallels between terrestrial bacteria-plant interactions and bacteria-alga interactions in the marine ecosystem.

6.1.1 Presence of h-monolignol in algae

One of the key features of land plants is their ability to make lignin, which aids in water transport and structural support (Vanholme *et al.*, 2010), but the recently discovered presence of lignin in some red algae has cast doubt on the traditionally held views on the evolution of the lignin biosynthesis pathway

(Martone *et al.*, 2009). My investigation of publically available genomes of algae revealed that genes encoding enzymes of the lignin biosynthetic pathway from *p*-coumaric acid to *p*-coumaryl alcohol (H monolignol) are widespread among algal species (Labeeuw *et al.*, 2015) (Chapter 2). Ongoing work based on these results has used antibody labelling to detect these monolignols in algal species, and preliminary results indicate that H lignin is present in *E. huxleyi*, but absent in *Dunaliella tertiolecta* (Rosana, unpublished data), supporting the bioinformatics findings of this thesis and providing further evidence that this pathway is present in algae. This raises further questions about the possible roles for this pathway in algae, as the benefits derived by land plants (e.g. structure and water transport) are clearly not shared by a marine mircroalga. While *p*-coumaric acid is itself a stable molecule that may be important in signalling, and is also an intermediate to various other phenylpropanoids (e.g. flavonoids), the presence of the three core genes to produce h-monolignol is intriguing. One possible role is that of an antimicrobial defence system (Cowan, 1999). This hypothesis is supported by the results of this thesis which demonstrate that some lignin pathway intermediates (i.e. *p*CA, cinnamic and ferulic acid) have been shown to have antimicrobial properties against selected marine bacteria (Chapter 2) in addition to some terrestrial bacteria and yeast (Herald & Davidson, 1983; Barber *et al.*, 2000). It should be noted that the concentrations required for inhibition of marine bacteria was within the 4-8 mM range, which may not occur naturally in a diffuse marine system. For example, DMSP diffusion around a phytoplankton has been modelled, and predicted to be in the nano- to milli-molar range at 5 µm distance from an algal cell (depending on the rate of exudation from the cell) (Fredrickson & Strom, 2009; Breckels et al., 2010). However, the lignin intermediates are hydrophobic and as such may have a higher concentration immediately around the algal cell. One possibility is that the antibiotic function is secondary, and at the lower concentrations found in natural systems, the bioactive small molecules have a signalling purpose (Yim et al., 2007), as with *p*CA, and its role in signalling between algae and the surrounding bacteria.

6.1.2 Interactions between *Emiliania huxleyi* and *Phaeobacter gallaeciensis*

The presence of one of the lignin intermediates, *p*CA, has previously been found in the haptophyte *Emiliania huxleyi* (where it was identified in the senescent algae) and *Diacronema lutheri*, as well as a diatom (*Phaedactylum tricornutum*) and several green algae (*Tetraselmis suecica, Chlorella vulgaris* and *Haematococcus pluviaris*) (Seyedsayamdost *et al.*, 2011b; Goiris *et al.*, 2014). Additionally, *p*CA was found to play a unique role in signalling the roseobacter *Phaeobacter gallaeciensis* DSM 26640 to produce potent algaecides known as roseobacticides (Seyedsayamdost *et al.*, 2011b). The work in this thesis demonstrated that the addition of *p*CA, a molecule linked to algal senescence, has previously unknown effects on the bacterium. *p*CA caused a switch in DSM 26640. Initially, it causes a depression in the production of the antibiotic tropodithietic acid (TDA), which DSM 26640 normally produces to protect healthy algal cells. This decrease in TDA production leads to the bacterium no longer providing the algae with protection against potential pathogenic bacteria. In addition, after 3 days of co-culture with *p*CA, the production of roseobacticides is stimulated, which kill the algal host and release internal algal nutrients, thereby boosting the bacterial population. However, the work of this thesis has shown that the bacterium is only pathogenic towards one strain of *E. huxleyi*, the coccolith bearing strain, and was continually beneficial to the bald strain (Chapter 3). Another roseobacter, *Ruegeria sp.* R11 has also recently been shown to mirror this selectivity in its inability to kill the bald strain (Mayers *et al.*, 2016). This is especially intriguing since coccolith bearing cells dominate *E. huxleyi* blooms in nature, but the bald cells have been shown to increase towards the end of a bloom (Frada *et al.*, 2012), suggesting a possible role of roseobacters in skewing algal bloom composition.

6.1.3 Presence and role of indole-3-acetic acid in *Emiliania huxleyi*

R11 has previously been found to produce indole-3-acetic acid (IAA), a phytohormone important for plant growth as well as bacterial-plant interactions (Fernandes *et al.*, 2011). Recently, IAA has been

shown to play an important role in diatom-bacteria interactions as well – production by a symbiotic bacterium increases algal growth (Amin *et al.*, 2015). The role of this hormone in different algal-bacterial relationships was further investigated for this thesis using *E. huxleyi* – R11 as a model system. Surprisingly, the results of this experiment indicated that one strain of the unicellular algae, the coccolith bearing strain, is capable of synthesizing IAA themselves from the precursor L-tryptophan. However, only the bald strain showed a morphological switch in response to addition of exogenous IAA at levels found in natural plant and algal systems (10^{-4}M or lower), including increased cell size and membrane permeability, but was not able to synthesize IAA itself. Although exogenous tryptophan was found to speed up the killing effect of R11 on *E. huxleyi*, the levels of IAA produced by the *E. huxleyi* – R11 co-culture in the presence of L-tryptophan were lower than those produced by the alga alone in the presence of L-tryptophan (Chapter 4). Preliminary experiments of exogenous IAA added to R11 – bald *E. huxleyi* co-cultures, with the increased membrane permeability in the presence of IAA, did not display increased susceptibility to the bacteria (data not shown). However, this role of IAA in this interaction needs further examination, as the timing of IAA addition or other factors may be important in controlling cell death. Alternatively, R11 may be interfering with the signalling process between the two algal cell types by diverting the tryptophan away from IAA biosynthesis. The release of IAA by one cell type of *E. huxleyi* which affects another cell type would seem to indicate that it plays a role in algal cell-cell signalling, although the nuances of this interaction still need to be resolved with co-cultures of both cell types in the presence of IAA.

6.1.4 Novel biosynthetic pathways in algal genomes

The production of IAA by *E. huxleyi* prompted a subsequent investigation into the presence of the IAA biosynthetic pathway in representatives of each algal group and roseobacters. Algae, especially *E. huxleyi*, were found to have many of the genes necessary for synthesis, with *E. huxleyi* in particular

having a putative homolog for nearly every gene in the pathway. However, the only complete IAA biosynthesis pathway detected in *E. huxleyi* includes a gene normally associated with the synthesis of this compound in bacteria (Chapter 4). Further phylogenetic analysis is therefore called for to determine the origin of this path. There are two competing theories for why a haptophyte which is so distantly related to the Archaeplastida would be able to produce IAA: either IAA biosynthesis was present in the last common ancestor of algae and land plants, or the pathway was transferred through lateral gene transfer (LGT). Preliminary results indicate that *E. huxleyi* clusters with the roseobacter homologues for at least one of the genes (data not shown), providing evidence for LGT. However, one of the main plant IAA pathways, the IPyA pathway, has been suggested to have originated in charophytes before their divergence with land plants (Wang *et al.*, 2015), so it is also a possibility that certain pathways for IAA biosynthesis originated in a common ancestor to land plants and algae.

The presence of two biosynthetic pathways normally associated with terrestrial plants (IAA and h-monolignol) in marine algae brings up further questions as to their evolution. Haptophytes are particularly complicated as their exact position in the phylogenetic tree has not been resolved yet (Burki *et al.*, 2012), although haptophytes, along with other algae (excluding red, green and glaucophytes) are thought to have diverged from archaeplastids 1500 million years ago (Yoon *et al.*, 2004). One possible explanation is that these two pathways were present in algae for antimicrobial or intra-species signalling, before being appropriated by multicellular plants for another purpose. There is also the possibility that these pathways were spread by LGT that will need further research.

6.1.5 Commercial applications of the bioactives and roseobacters

The evolutionary puzzle of these pathways in algae is certainly intriguing, but so is their potential role in commercial systems. Bacteria are currently sorely underrepresented in research of commercial algal systems (Wang *et al.*, 2014a). Increasing the study into understanding the complexities of bacterial-algal

interactions provides the opportunity of discovering novel beneficial roles that can be adapted for commercialization. For instance, bacterial symbionts could make an algal system more robust and able to handle contaminating species, certain bacteria might be used as probiotics to protect algae from potential grazers or pathogens, while other symbionts might increase the biomass or lipid yield of the overall system (de-Bashan *et al.*, 2002; Kazamia *et al.*, 2012). The use of bioactive compounds has been less studied, with only limited research into which could be beneficial for commercial use (Franz *et al.*, 2013). IAA has been implicated in the growth promoting effects of some of these bacteria (Li *et al.*, 2007; de-Bashan *et al.*, 2008), but my research did not show that they significantly increased lipid concentration in the three algal species tested, *E. huxleyi, Isochrysis sp.* and *D. tertiolecta.* However, R11 did show some promise by boosting lipid content of *D. tertiolecta*, which would warrant further research (Chapter 5). Often, research on growth-promoting bacteria that affect algae has focused on terrestrial bacteria or more common species such as *Pseudomonas* spp. (Oh *et al.*, 2001; Rivas *et al.*, 2010; Lenneman *et al.*, 2014). Roseobacters and their bioactives represent a new area in which to explore, and exploiting natural systems for commercial applications would offer a cheap way to improve processing.

6.1.6 Revised model

The various components of this thesis have highlighted how complex the relationship is between algae and bacteria, and even between different algal cell types of the same species. In light of this, the model from Figure 1-2 is revised in Figure 6-1. In the original model, algae were shown to interact with the bacteria by releasing *p*CA, which may form part of the hypothesized lignin biosynthesis pathway within the alga, which caused the bacteria to produce algaecides which in turn targeted the algae. In addition, roseobacters were hypothesized to have a growth promoting effect on the alga by providing phytohormones and preventing competing bacteria from colonising through the production of TDA.

The model now has to be updated to take into account the alga-alga interactions specific to *E. huxleyi* observed in my research. Roseobacters were shown to be highly specific in their killing effect, targeting only specific cell types, as well as being very responsive to algal signals and aging, indicating a wider role the bacteria might play as ecosystem drivers. Production of TDA is shown to be influenced by *p*CA, indicating a stronger role for this molecule. While auxins have been shown to be intermediates between other algae and their bacterial consortia (Amin *et al.*, 2015), in my model system of *E. huxleyi* – *Ruegeria sp.*, the alga seemed to be the main producer of IAA, affecting other *E. huxleyi* cell types, with a role of bacteria in IAA mediated interactions currently unknown.

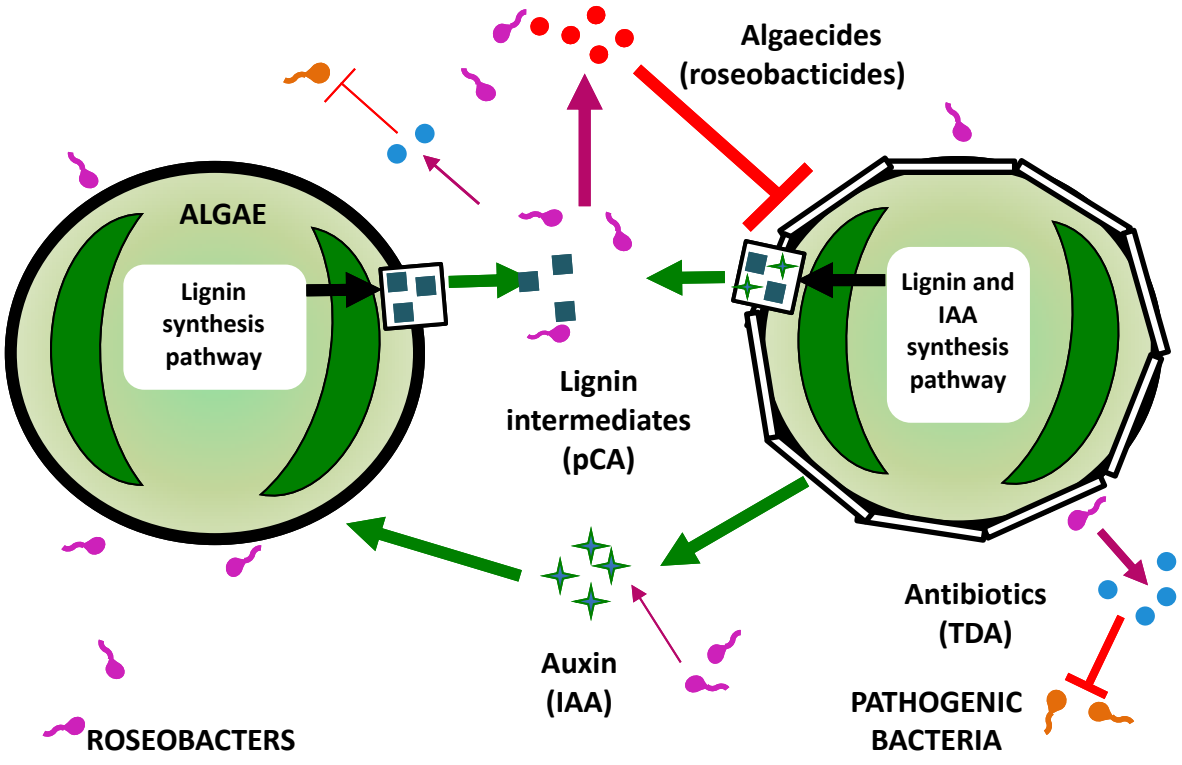

Figure 6-1: Refined model of proposed interactions between roseobacters and *Emiliania huxleyi*.

Central is the algae (green), surrounded by roseobacters (pink) and other potential bacteria (orange). Various small molecules are shown mediating the interactions where black arrows indicated that internal algal production, green arrows indicate it is produced by the algae, pink arrows produced by the bacteria, and red means it has a negative effect towards the target. Thickness of the lines indicates level produced (i.e. thinner lines indicate less is made).

6.2 Future research

Many new questions have been raised by the research in this thesis. My research into the lignin pathway in algae is being carried on by other members of the Case lab, who hope to clarify the role and presence of lignin or the monolignols in algae (specifically *E. huxleyi*), the natural next step after determining the presence of the lignin biosynthetic pathway.

Timing of E. huxleyi *death by DSM 26640:* One interesting feature noted in the DSM 26640 – *E. huxleyi* co-cultures was that the timing of death seemed to be dependent on the level of domestication or the original level of coccolith bearing cells in the inoculum. Cultures obtained recently from the NCMA seemed to have a mechanism to recover before the precipitous death event took place at 27 d (Figure 3-2), however, cultures which had been cultivated in lab conditions for over 6 months to a year did not seem to have this recovery period and died much quicker (14 d), losing whatever defence mechanism they may have initially had. This effect would be interesting to study to determine what is causing this change in susceptibility, but also to demonstrate that domestication is an important feature to keep in mind when looking for new algal strains (Formighieri *et al.*, 2012), as some promising algae species are acquired directly from natural systems with little thought to their long-term domestication when researching biofuels. A co-culture of DSM 26640 with both the bald and coccolith bearing strains should further clarify whether the bacterium has the potential to control the dynamics between the different cell types. In addition, the potential of *p*CA in controlling the death of *E. huxleyi* warrants further investigation. Preliminary tests adding in *p*CA to early log co-cultures of the algae with DSM 26640 did not accelerate death of the coccolith bearing strain, nor did it cause the bald strain to die (data not shown). However, addition may be required at specific moments in the life cycle of the algae.

Timing of addition of IAA to algae: Preliminary tests of IAA added at early, mid and late log on *D. tertiolecta* revealed that adding at early log was the most likely to cause any effect (data not shown).

The timing, as well as the possible continuous addition of IAA to cultures, needs further investigation in *E. huxleyi* which will further aid in understanding when IAA might be released in natural systems. In addition, screening of more roseobacters for IAA production will lead to identification of bacteria that do produce it in conditions conducive for both the algae and the bacteria, and then further co-cultures of those bacteria with algae would be useful in revealing the role of IAA in the algal-bacterial interactions. The bioinformatics survey of the pathway revealed good bacterial candidates (i.e. *Ruegeria pomeroyi* DSS-3 or *Silicibacter sp.* TM1040), and the methodology outlined in Chapter 4 is a rapid way of screening multiple bacteria. I have already begun screening additional roseobacters, and found that DSM 26640 did not produce IAA, while *Silicibacter sp.* TM1040 did produce IAA in the presence of L-tryptophan (data not shown).

Transcriptomics: Transcriptomics has been shown to be a powerful tool in elucidating the response in bacteria and algae in co-cultures or in response to specific compounds (Amin *et al.*, 2015). While the methods employed in this thesis demonstrate the phenotypic responses in individual cultures, they cannot directly determine the source of the IAA or the potential pathway in real time in the species. Using a transcriptomics approach on the specific interactions of interest determined from this thesis, such as why IAA is depressed in R11-*E. huxleyi* co-cultures or the role of IAA in alga-to-alga interactions will allow for better resolution of what exactly is occurring within each species in a complex co-culture.

Scale up: The small scale, 48 well plate, methodology created for this thesis (Bramucci *et al.*, 2015) is a rapid way to screen the effect of bacteria/bioactives on algal species. It can be adapted to multiple purposes, and reveal effects previously unknown. However, the next step would be to scale up these systems and to determine how relevant they are in a heterogeneous environment instead of a well-mixed, homogenous environment present at such small-scales. The addition of L-tryptophan was shown to stimulate IAA production even in a larger scale (Chapter 4), while other work done in the Case lab has shown the bacteria have the same effect in 40 mL cultures instead of 1 mL, which demonstrates the

ability of this small scale system for investigating the model is relevant at larger scales. However, in order to draw wider conclusions, a less homogenous environment has to be tested at a larger scale.

6.3 Conclusion

The models and proposed relationships drawn from this thesis are good starting points in looking at larger scales and natural systems, but need the iterative feedback from those systems in order to further improve the model and launch new investigations. Understanding the small molecules harvested from natural systems, including those shown in this thesis, can help understand the evolution of algae, further their exploitation in commercial systems, and create better models to understand the natural world.

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Appendix A

Lignin intermediates inhibitory mechanism

A.1 Introduction

Some of the lignin intermediates were shown to have an inhibitory effect against marine bacteria (Chapter 2) and terrestrial bacteria and yeast (Herald & Davidson, 1983; Barber *et al.*, 2000), but the mechanism by which they do so are unknown. One recent theory is that there is a common mechanism of killing induced antibiotics which targets the Fenton reaction (Kohanski *et al.*, 2007, 2010). Briefly, this mechanism proposes that the antibiotic stimulates the oxidation of NADH in the electron transport chain, and this hyper activation stimulates superoxide (O_2^-) formation. This in turn damages iron-sulphur (Fe-S) clusters, making ferrous iron (Fe^{2+}) available for the Fenton reaction, which leads to the formation of hydroxyl radicals (·OH) which then leads to damage of DNA, lipids, proteins, and eventual cell death (Touati, 2000; Kohanski *et al.*, 2007).

A.2 Methods

Wild type *Vibrio cholerae* WT C6707 was maintained on LB5 agar plates (10g Bacto tryptone, 5g yeast extract, 5g NaCL, and 15g acgar in 1L, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with 100 µg/mL streptomycin (Sigma-Aldrich). Three mutant strains were obtained from Dr. Stefan Putkatzki to remove the enzyme superoxide dismutase which can inhibit the superoxide driven Fenton reaction (Gutteridge, 1985). Each of these strains had one of the identified superoxide dismutase genes knocked out (*Vibrio cholerae* C6707 strains 1583, 2048, and 2694) and was maintained on LB5 with 100 µg/mL streptomycin and 50 µg/mL kanamycin (Sigma-Aldrich). The WT and 3 mutant strains were grown in LB5

broth with the appropriate antibiotic for 24 hours on a rolling drum. The procedure from Chapter 2.2.5 was then followed to determine the MIC using 0, 0.5, 1, 2, 4, 8, and 10 mM of *p*-coumaric acid (*p*CA).

The addition of iron chelator 2,2'-dipyridyl would effectively prevent the Fenton reaction from occurring by mopping up excess ferrous iron. Likewise, thiourea is a hydroxyl radical scavenger, so if there is increased formation of these by the anti-microbial, addition of thiourea would be expected to protect against the harmful effects. As such, stock solutions of dipyridyl (Sigma-Aldrich) and thiourea (Sigma-Aldrich) were created in bacterial media and filter sterilized.

Vibrio cholerae WT was grown with 0mM, 0.001 mM, 0.01 mM, 0.025 mM, 0.05 mM, and 0.1 mM of dipyridyl. Dipyrdidyl did not have a negative effect on growth (OD_{600}) of the bacterium at these various concentrations, but impeded growth at higher concentrations. The method from Chapter 2.2.5 was then followed with the addition of 0mM, 2mM, 4mM, and 8mM *p*CA at the mid-point of the exponential growth of the bacterium, with the modification that the initial inoculum at t=0hr contained the varying concentrations of dipyridyl.

Vibrio cholerae WT was grown with 0mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM, 10 mM, 25 mM, 50 mM, 100mM, and 150 mM of thiourea. There was no noted detrimental effect of thiourea on the growth (OD_{600}) of the bacterium. The method from Chapter 2.2.5 was then followed with the addition of 0mM and 10mM *p*CA at the mid-point of the exponential growth of the bacterium, with the modification that the initial inoculum at t=0hr contained the varying concentrations of thiourea.

A.3 Results and Discussion

The results for the superoxide dismutase shown in Figure A-1 and Figure A-2 show that there is no increased inhibition in the mutant strains 2045 and 2694 compared to the WT, as would be expected by loss of the protective enzyme superoxide dismutase. However, the results for the last mutant, 1583, are

inconclusive. There would appear to be increased inhibition at the lower concentration of 8mM *p*CA, while the bacterial counts may be affected. However, further replication and investigation is needed for this, especially for the differing bacterial counts (Figure A-2) before any conclusions can be drawn. Additionally, it may be necessary to make a triple mutant knockout of all superoxide dismutases in order to see the full loss of protection.

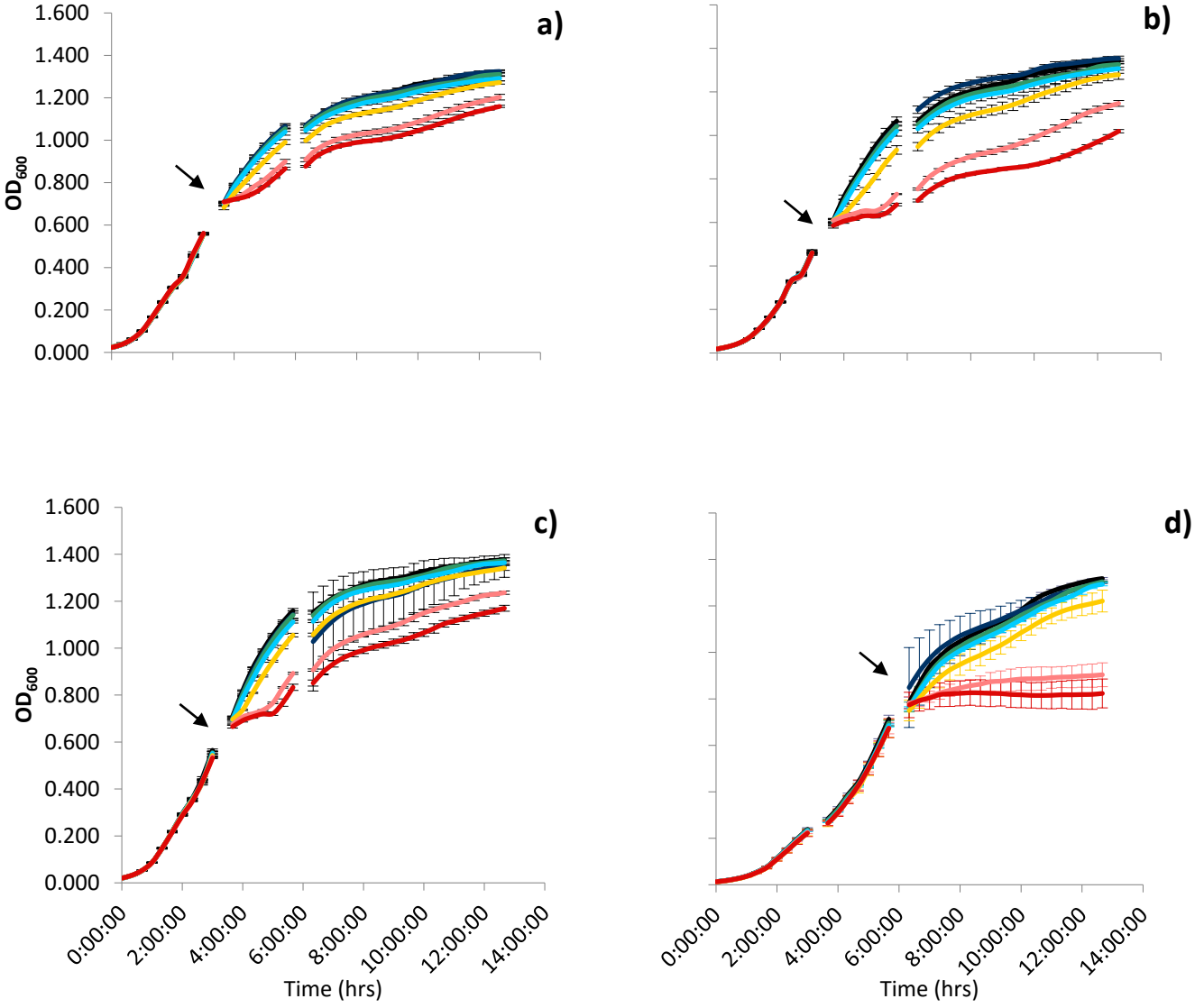

Figure A-1: MIC of *p*CA against *Vibrio cholerae* WT and superoxide dismutase knockout mutants.

The MIC determined by measuring the OD at 600nm over 14 hrs at various concentrations of *p*CA against various strains of *Vibrio cholerae* WT (a), mutant strains 1583 (b), 2694 (c) and 2045 (d). In all graphs, the concentrations of *p*CA are: 0mM (black), 0.5mM (purple), 1mM (green), 2mM (blue), 4mM (yellow), 8mM (pink), and 10mM (red). The arrow points to when the *p*CA was added.

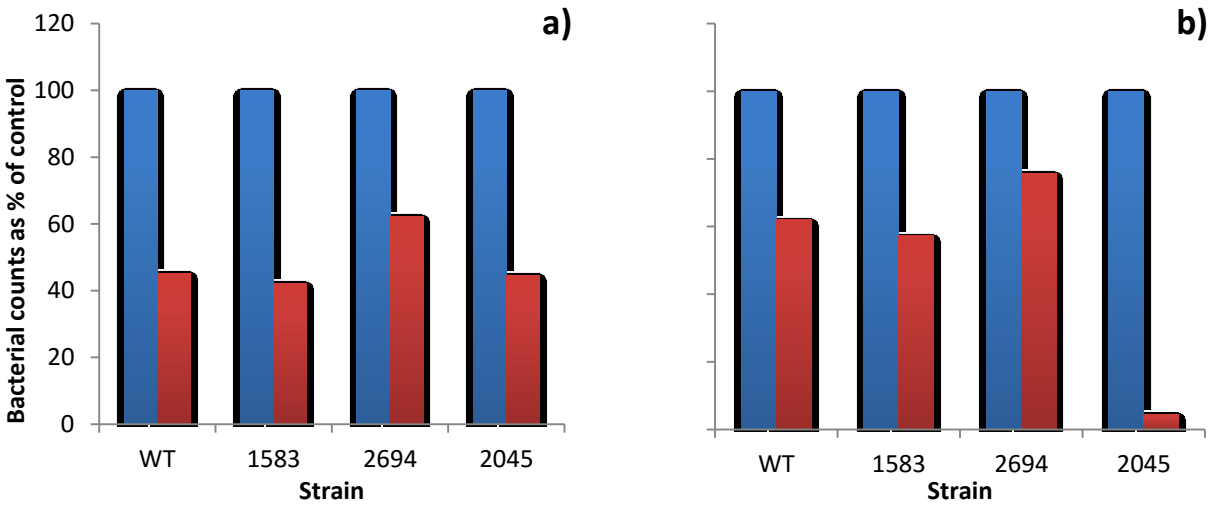

Figure A-2: Bacterial counts of *p*CA against *Vibrio cholerae* WT and superoxide dismutase knockout mutants.

The bacterial counts at the end point of the MIC experiment as a percentage of the control for the given strain, given in blue for the control of 0mM *p*CA and red for 10 mM. Conflicting results were given in separate trials, so the two replicate experiments are shown in (a) and (b).

Thiourea was not shown to protect the bacterium against the inhibitory concentration of *p*CA (Figure A-3). Lower concentrations of thiourea (data not shown) demonstrated this same trend, indicating that hydroxyl radicals were either not produced or not scavenged effectively.

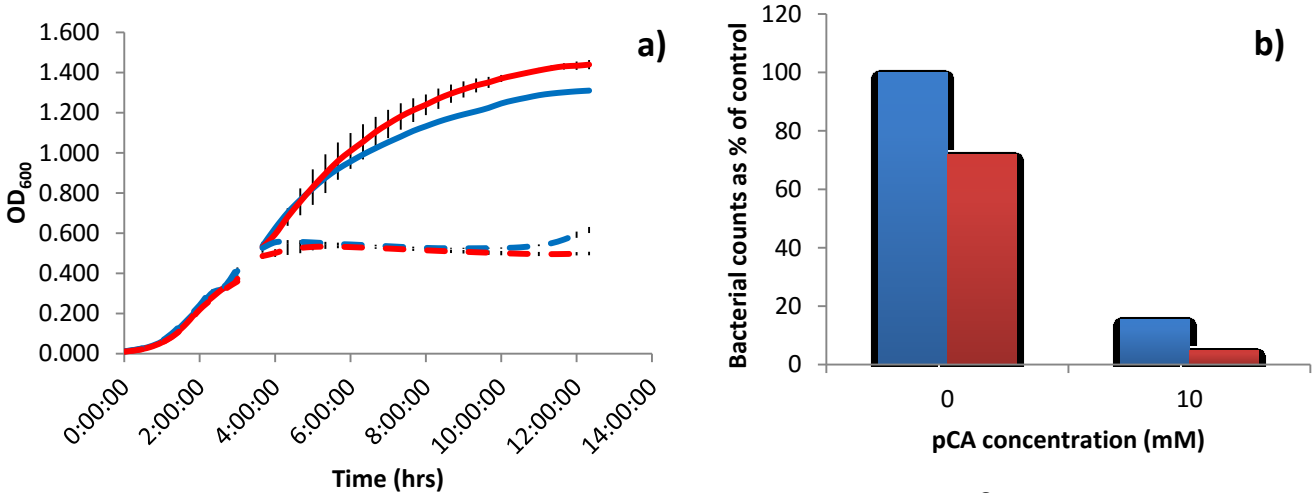

Figure A-3: MIC and bacterial counts of *p*CA against *Vibrio cholerae* WT grown with thiourea.

The MIC determined by measuring the OD at 600nm over 14 hrs at 0mM (whole line) and 10 mM (dashed line) *p*CA against various strains of *Vibrio cholerae* WT, with differing concentration of thiourea

(a). The bacterial counts at the end point of the MIC experiment as a percentage of the control for the given strain. For all graphs, blue corresponds to 0mM thiourea, while red is 150mM thiourea.

Dipyridyl had some inconclusive results, as it seemed to protect *Vibrio cholerae* WT at 4 mM when looking at bacterial counts, but this did not match the OD data (Figure A-4). In addition, this effect was not present at 8mM *p*CA.

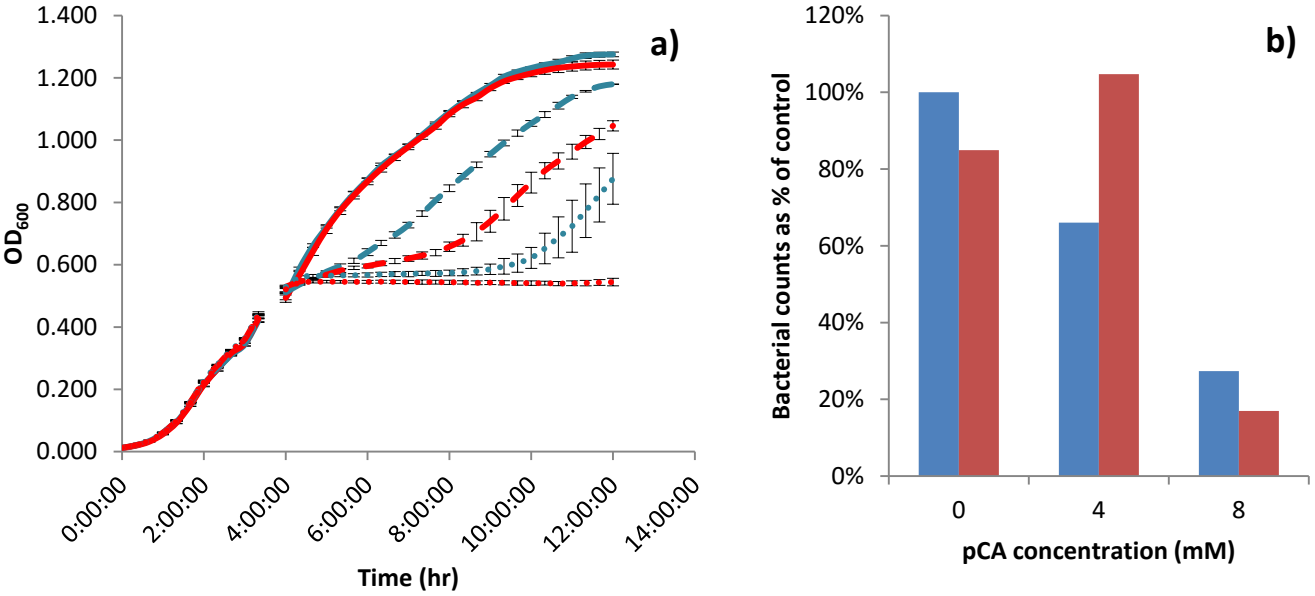

Figure A-4: MIC and bacterial counts of *p*CA against *Vibrio cholerae* WT grown with thiourea.

The MIC determined by measuring the OD at 600nm over 14 hrs at 0mM (whole line), 4 mM (dashed line), and 8 mM (dotted line) *p*CA against various strains of *Vibrio cholerae* WT, with differing concentration of dipyridyl (a). The bacterial counts at the end point of the MIC experiment as a percentage of the control for the given strain. For all graphs, blue corresponds to 0mM thiourea, while red is 0.1 mM dipyridyl.

The results from the various tests to determine if the Fenton reaction is the mode of action by which the lignin intermediate *p*CA inhibits growth are inconclusive. While one of the superoxide dismutases knockout mutants showed increased susceptibility, and dipryidyl seemed to protect the bacterium at 4mM, further tests are needed. However, the Fenton reaction and reactive oxygen species (ROS) linkage as a universal mode of action has been criticized (Hassett & Imlay, 2007; Fang, 2013). Screening of *Escherichia coli* single gene knockout strains for antibiotic susceptibility did not reveal ROS targets

(Tamae *et al.*, 2008). TCA cycle mutations were not shown to affect bacterial susceptibility or promote drug resistance (Ricci *et al.*, 2012). More recently, the hypothesis was directly tested, and antibiotics were shown to remain lethal in anaerobic conditions, while not elevating the quantity of free iron within a cell as would be expected in this model (Liu & Imlay, 2013). As such, it is likely that inhibition occurs through another, as yet unknown, mechanism.

A.4 References

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Tamae C, Liu A, Kim K, Sitz D, Hong J, Becket E, Bui A, Solaimani P, Tran KP, Yang H, Miller JH (2008). Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*. *Journal of Bacteriology* **190**: 5981–5988.

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Appendix B

Supplementary for Chapter 4

Note about the supplementary tables:

Presence is determined initially by a bi-directional best hit (BBH) BLASTP hit with an E-value < $1x10^{-10}$ or less, with the correct functional prediction, using the characterized bacterial genes as queries for Suppl. Table 1 (p2), and plant *A. thaliana* gene as a query for Suppl. Table 2 (p10), followed by ESG functional prediction and OrthoMCL orthology.

(+) indicates that it is matches all three criteria, (-/+) indicates it is absent in OrthoMCL, present in ESG, while (+/-) indicates presence in OrthoMCL, absence in ESG. Presence in OrthoMCL was determined if it had the same group as the seed sequence. Presence in ESG was determined if it fell in the same confidence interval as the seed sequence, where >70% indicates very high confidence, >40% indicates high confidence, >=30% indicates moderate confidence, and below 30% indicates below moderate confidence.

The following databases were searched: JGI (http://genome.jgi.doe.gov/) ([a]), http://merolae.biol.s.u-tokyo.ac.jp/blast/blast.html ([b]), http://cyanophora.rutgers.edu/porphyridium/ ([c]), NCBI (http://www.ncbi.nlm.nih.gov/) ([d]), and OIST Marine Genomics Unit (http://marinegenomics.oist.jp/genomes/gallery/) ([e]). The abbreviations used for the enzyme can be described as follows: Tryptamine monooxygenase (YUCCA); Indole-3-acetamide hydrolase (AMI1); Tryptophan amino transferase (TAA1); Cytochrome P450s (CYP79B2 and CYP79B3); Indole-3-acetaldehyde oxidase (AAO1); Indole-acetaldoxime dehydratase (CYP71A13); Tyrosine decarboxy lase (TDC); Myrosinase (MYR1); C-S lyase (SUR1); CYP83B1 (SUR2); and Nitralase (NIT1).

Table B-1: Distribution of bacterial IAA biosynthesis genes in roseobacter and algal genomes

	Enzyme	Nitrile Hydratase		Tryptophan mono-oxygenase	Indole-3-acetamide hydrolase	Indole-3-pyruvate decarboxylase	Indole-3-acetaldehyde dehydrogenase	Tryptophan decarboxylase	Copper amine/ tyramine oxidase
TERRESTRIAL									
Agrobacterium tumefaciens C58 [d]				AAD30489	AAD30488				
Agrobacterium radiobacter K84 [d]		ACM26968	ACM26967						
Pseudomonas aeruginoasa PA7 [d]				ABR84797					
Enterobacter cloacae [d]	**Accession #**					P23234			
Paenibacillus polymyxa [d]						ABV24338			
Ustilago maydis FB1 [d]							AAC49575		
Catharanthus roseu s [d]								CAA47898	
Enterobacter aerogenes [d]									P49250
Klebsiella pneumoniae [d]		AIE03299	AIE03300						
	Ortho MCL	OG5_ 194743	NO_ GROUP	OG5_130946	OG5_ 175845	OG5_129158, OG5_126899	OG5_126638	OG5_127644	OG5_ 127171
	ESG Molecular Function terms	indole-3-acetonitrile nitrile hydratase activity	indole-3-acetonitrile nitrile hydratase activity	L-amino-acid oxidase activity	glutaminyl-tRNA synthase (glutamine-hydrolyzing) activity,	pyruvate decarboxylase activity	oxidoreductase activity,	aromatic-L-amino-acid decarboxylase activity	copper ion binding
	ESG Confidence	89.60%	90.40%	100.00%	93%	53.80%	38.40%	43.50%	50.60%
ROSEOBACTER									
Ruegeria sp. R11 [d]	**Accession #**	EEB72184	EEB70836	-	WP_008560 074	EEB71015	EEB70673	WP_0085588 90	-
	E-value	2.00E-86	2.00E-47	-	8.00E-33	3.00E-51	7.00E-113	4.00E-105	-
	Reciprocal e-value	3.00E-84	7.00E-42	-	0.00E+00	2.00E-57	2.00E-112	1.00E-100	-
	OrthoMCL	OG5_ 194743	NO_GROU P		OG5_12746 6	OG5_126899	OG5_131868	OG5_129065	
	ESG Confidence	89.40%	90.10%		92.00%	0.00%	48.10%	47.70%	-
	Presence	+	+	-	-/+	+/-	-/+	-/+	-

Dinoroseobacter shibae DFL 12 [d]	**Accession #**	WP_012178521	WP_012178520	-	WP_012179446	WP_012178451	WP_012177768	-	-
	E-value	3.00E-59	2.00E-10	-	8.00E-34	0.00E+00	2.00E-122	-	-
	Reciprocal e-value	5.00E-57	5.00E-09	-	1.00E-41	0.00E+00	2.00E-132	-	-
	OrthoMCL	OG5_194743	NO_GROUP	OG5_130946	OG5_127466	OG5_126899	OG5_126638		
	ESG Confidence	88.80%	87.50%	-	88.00%	0.00%	32.60%		-
	Presence	+	+	-	-/+	+/-	+	-	-
Jannaschia sp. CCS1 [d]	**Accession #**	-	-	-	ABD56894	WP_044006611	WP_011456626	WP_011456620	-
	E-value	-	-	-	2.00E-54	0.00E+00	1.00E-119	5.00E-88	-
	Reciprocal e-value	-	-	-	4.00E-58	0.00E+00	2.00E-121	2.00E-90	-
	OrthoMCL				OG5_175845	OG5_126899	OG5_131868	OG5_129065	
	ESG Confidence				100.00%	0.00%	44.60%	46.90%	
	Presence	+	+	-	+	+/-	-/+	-/+	-
Phaeobacter gallaeciensis 2.10 [d]	**Accession #**	AFO88179	AFO88178	-	WP_014875196	WP_014875766	WP_014873197	WP_014873282	-
	E-value	5.00E-91	3.00E-42	-	1.00E-31	1.00E-50	3.00E-118	3.00E-108	-
	Reciprocal e-value	1.00E-88	3.00E-37	-	0.00E+00	1.00E-56	5.00E-122	1.00E-103	-
	OrthoMCL	OG5_194743	NO_GROUP		OG5_127466	OG5_126899	OG5_131868	OG5_129065	
	ESG Confidence	89.40%	89.90%		93.50%	0.00%	43.50%	47.10%	-
	Presence	+	+	-	-/+	+/-	-/+	-/+	-
Phaeobacter gallaeciensis DSM17395 (BS107) [d]	**Accession #**	AFO92073	WP_014880670	-	WP_014880496	WP_014880356	WP_014881318	WP_014881252	-
	E-value	1.00E-89	5.00E-57	-	3.00E-32	0.00E+00	2.00E-119	1.00E-107	-
	Reciprocal e-value	3.00E-87	3.00E-38	-	0.00E+00	0.00E+00	4.00E-123	4.00E-103	-
	OrthoMCL	OG5_194743	NO_GROUP		OG5_127466	OG5_126899	OG5_131868	OG5_129065	
	ESG Confidence	89.40%	89.40%		94.00%	0.00%	42.00%	40.70%	-
	Presence	+	+	-	-/+	+/-	-/+	-/+	-

Roseobacter denitrificans OCh 114 [d]	**Accession #**	WP_011568135	WP_011568136	WP_011567748	WP_011568221	WP_011568956	WP_011568243	-	-
	E-value	1.00E-91	2.00E-53	6.00E-94	5.00E-34	0.00E+00	4.00E-115	-	-
	Reciprocal e-value	3.00E-89	1.00E-39	1.00E-47	4.00E-42	0.00E+00	2.00E-126	-	-
	OrthoMCL	OG5_194743	NO_GROUP	OG5_130946	OG5_127466	OG5_126899	OG5_131437		
	ESG Confidence	89.30%	89.30%	100.00%	93.00%	0.00%	36_0%		-
	Presence	+	+	+	-/+	+/-	-/+	-	-
Ruegeria pomeroyi DSS-3 [d]	**Accession #**	WP_011047053	WP_011047054	-	WP_011047226	WP_011048283	WP_011241895	WP_011049365	-
	E-value	1.00E-91	1.00E-48	-	2.00E-100	0.00E+00	7.00E-139	3.00E-112	-
	Reciprocal e-value	3.00E-89	4.00E-41	-	3.00E-104	0.00E+00	7.00E-141	7.00E-107	-
	OrthoMCL	OG5_194743	NO_GROUP		OG5_175845	OG5_126899	OG5_168711	OG5_129065	
	ESG Confidence	89.40%	89.40%		96.60%	0.00%	36.00%	41.30%	-
	Presence	+	+	-	+	+/-	-/+	-/+	-
Ruegeria sp. TM1040 [d]	**Accession #**	WP_011539297	WP_011539296	-	WP_011539245	WP_011538220	WP_011537227	WP_011537081	-
	E-value	1.00E-91	1.00E-48	-	2.00E-100	0.00E+00	7.00E-139	3.00E-112	-
	Reciprocal e-value	2.00E-83	6.00E-36	-	0.00E+00	0.00E+00	6.00E-131	4.00E-99	-
	OrthoMCL	OG5_194743	NO_GROUP		OG5_127466	OG5_126899	OG5_126638	OG5_129065	
	ESG Confidence	89.40%	89.40%		94.00%	0.00%	32.10%	47.10%	-
	Presence	+	+	-	-/+	+/-	+	-/+	-
LAND PLANT									
Arabidopsis thaliana [d]	**Accession #**	none	none	none	AAF73891_	NP_200307	NP_190383	NP_849999	NP_176469
	E-value	-	-	-	7.00E-31	6.00E-81	5.00E-180	0	1.00E-96
	Reciprocal e-value	-	-	-	8.00E-33	5.00E-81	4.00E-82	0	2.00E-97
	OrthoMCL	-	-	-	OG5_132924	OG5_129158	OG5_126638	OG5_129065	OG5_127171
	ESG Confidence	-	-	-	96.00%	87.50%	25.50%	52.70%	62.40%
	Presence	-	-	-	-/+	+	+/-	-/+	+

GREEN ALGAE									
Ostreococcus sp RCC809 [a]	**Accession #**	none	none	none	29365	41388	58690	28940	none
	E-value	-	-	-	3.48E-26	8.28E-145	9.97E-61	4.68E-20	-
	Reciprocal e-value	-	-	-	5.00E-42	6.00E-20	2.00E-71	3.00E-31	-
	OrthoMCL	-	-	-	OG5_127466	OG5_126899	OG5_126638	OG5_127644	-
	ESG Confidence	-	-	-	88.50%	0.00%	43.50%	0.00%	-
	Presence	-	-	-	-/+	+/-	+	+/-	-
Coccomyxa subellipsoidea [a]	**Accession #**	none	none	none	XP_005651884	XP_005645798	XP_005642981	XP_005646768	XP_005649283
	E-value	-	-	-	7.00E-59	3.00E-175	0	4.00E-158	4.00E-83
	Reciprocal e-value	-	-	-	6.00E-61	2.00E-98	2.00E-85	4.00E-155	7.00E-84
	OrthoMCL	-	-	-	OG5_175845	OG5_129158	OG5_126638	OG5_129065	OG5_127171
	ESG Confidence	-	-	-	100.00%	59.40%	46.60%	52.70%	89.20%
	Presence	-	-	-	+	+	+	-/+	+
Chlamydomonas reinhardtii [a]	**Accession #**	none	none	none	XP_001690552	AAB88292	XP_001690955	XP_001690025	XP_001697595
	E-value	-	-	-	6.00E-31	5.00E-174	3.00E-180	8.00E-133	6.00E-70
	Reciprocal e-value	-	-	-	1.00E-40	4.00E-92	8.00E-97	9.00E-140	8.00E-81
	OrthoMCL	-	-	-	OG5_127466	OG5_129158	OG5_126638	OG5_129065	OG5_127171
	ESG Confidence	-	-	-	87.00%	59.20%	27.10%	40.10%	80.90%
	Presence	-	-	-	-/+	+	+/-	-/+	+
RED ALGAE									
Cyanidioschyzon merolae [b]	**Accession #**	none	none	none	XP_005537175	NP_849033	XP_005538376	none	XP_005538693
	E-value	-	-	-	7.00E-27	1.00E-166	1.00E-94	-	9.00E-89
	Reciprocal e-value	-	-	-	5.00E-38	1.00E-25	8.00E-92	-	1.00E-89
	OrthoMCL	-	-	-	OG5_127466	OG5_126899	OG5_127004	-	OG5_127171
	ESG Confidence	-	-	-	96.50%	0.00%	30.50%	-	67.60%
	Presence	-	-	-	-/+	+/-	-/+	-	+

Porphyridium purpureum [c]	**Accession #**	none	none	none	none	YP_008965627	none	none	none
	E-value	-	-	-	-	6.00E-175	-	-	-
	Reciprocal e-value	-	-	-	-	2.00E-28	-	-	-
	OrthoMCL	-	-	-	-	OG5_126899	-	-	-
	ESG Confidence	-	-	-	-	0.00%	-	-	-
	Presence	-	-	-	-	+/-	-	-	-
Chondrus crispus [d]	**Accession #**	none	none	none	XP_005710130	YP_007627349	XP_005718776	none	none
	E-value	-	-	-	3.00E-30	5.00E-178	4.00E-63	-	-
	Reciprocal e-value	-	-	-	7.00E-40	2.00E-22	1.00E-62	-	-
	OrthoMCL	-	-	-	OG5_127466	OG5_126899	OG5_127004	-	-
	ESG Confidence	-	-	-	93.50%	0.00%	40.40%	-	-
	Presence	-	-	-	-/+	+/-	-/+	-	-
GLAUCOPHYTE									
Cyanophora paradoxa [d]	**Accession #**	none	none	none	- none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-
DIATOMS									
Fragilariopsis cylindrus [a]	**Accession #**	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-

Phaeodactylum tricornutum [a]	**Accession #**	none	none	none	XP_002180426	XP_002181443	XP_002185692	none	XP_002180619
	E-value	-	-	-	1.00E-16	5.00E-59	1.00E-72	-	9.00E-60
	Reciprocal e-value	-	-	-	1.00E-18	1.00E-05	5.00E-66	-	9.00E-61
	OrthoMCL	-	-	-	OG5_127783	OG5_126899	OG5_127004	-	OG5_127171
	ESG Confidence	-	-	-	100.00%	0.00%	32.60%	-	80.90%
	Presence	-	-	-	-/+	+/-	-/+	-	+
Pseudo-nitzschia multiseries CLN-47 [a]	**Accession #**	55399	none	none	228151	237689	298461	none	243692
	E-value	3.98E-41	-	-	1.76E-12	1.84E-67	2.07E-68	-	4.59E-45
	Reciprocal e-value	2.00E-43	-	-	2.00E-11	1.00E-09	1.00E-57	-	2.00E-62
	OrthoMCL	OG5_194743	-	-	OG5_127466	OG5_126899	OG5_137356	-	OG5_127171
	ESG Confidence	90.20%	-	-	100.00%	0.00%	39.60%	-	84.00%
	Presence	+	-	-	-/+	+/-	-/+	-	+
PELAGOPHYTE									
Aureococcus anophagefferens [a]	**Accession #**	XP_009036233	none	none	XP_009032301	YP_003002081	XP_009041380	XP_009042616	none
	E-value	9.00E-41	-	-	6.00E-31	4.00E-20	4.00E-145	1.00E-13	-
	Reciprocal e-value	7.00E-43	-	-	7.00E-39	3.00E-20	4.00E-75	5.00E-14	-
	OrthoMCL	OG5_194743	-	-	OG5_175845	OG5_126899	OG5_126638	OG5_127644	-
	ESG Confidence	89.30%	-	-	98.00%	0.00%	34.60%	0.00%	-
	Presence	+	-	-	+	+/-	+	+/-	-
BROWN ALGAE									
Ectocarpus siliculosus [d]	**Accession #**	none	none	none	CBN78043	YP_003289152	CBJ33263	CBN75443	CBN75262
	E-value	-	-	-	3.00E-24	6.00E-19	6.00E-95	4.00E-97	2.00E-84
	Reciprocal e-value	-	-	-	5.00E-36	9.00E-19	2.00E-59	8.00E-101	3.00E-85
	OrthoMCL	-	-	-	OG5_127466	OG5_126899	OG5_137356	OG5_129065	OG5_127171
	ESG Confidence	-	-	-	100.00%	0.00%	37.00%	46.80%	80.60%
	Presence	-	-	-	-/+	+/-	-/+	-/+	+

EUSTIGMATOPHYTE									
Nannochloropsis oculata [d]	**Accession #**	none	none	none	none	AHX25385	none	none	none
	E-value	-	-	-	-	5.00E-22	-	-	-
	Reciprocal e-value	-	-	-	-	4.00E-22	-	-	-
	OrthoMCL	-	-	-	-	OG5_126899	-	-	-
	ESG Confidence	-	-	-	-	0.00%	-	-	-
	Presence	-	-	-	-	+/-	-	-	-
CHRYSOPHYTE									
Ochromonas danica [d]	**Accession #**	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-
DINOFLAGELLATES									
Symbiodinium minutum [e]	**Accession #**	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-
CRYPTOPHYTE									
Guillardia theta [a]	**Accession #**	none	none	none	XP_005837094	NP_050806	XP_005835269	XP_005821099	none
	E-value	-	-	-	1.00E-15	3.00E-20	4.00E-175	1.00E-31	-
	Reciprocal e-value	-	-	-	6.00E-18	2.00E-20	7.00E-84	3.00E-30	-
	OrthoMCL	-	-	-	OG5_132214	OG5_126899	OG5_126638	OG5_127644	-
	ESG Confidence	-	-	-	100.00%	0.00%	44.60%	0.00%	-
	Presence	-	-	-	-/+	+/-	+	+/-	-

Hemiselmis andersenii [d]	**Accession #**	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-
HAPTOPHYTE									
Emiliania huxleyi CCMP1516 [d]	**Accession #**	XP_005756629	XP_005756628	none	XP_005537175	XP_005756827	XP_005779034	XP_005776490	XP_005767244
	E-value	2.00E-27	1.00E-11	-	7.00E-27	8.00E-99	3.00E-123	2.00E-32	2.00E-19
	Reciprocal e-value	2.00E-25	-	-	2.00E-30	3.00E-25	5.00E-76	1.00E-32	4.00E-20
	OrthoMCL	OG5_194743	OG5_194743	-	OG5_127466	OG5_126899	OG5_126638	OG5_129065	OG5_127171
	ESG Confidence	88.00%	90.40%	-	95.50%	0.00%	29.00%	47.10%	76.80%
	Presence	+	-/+	-	-/+	+/-	+/-	-/+	+

Table B-2: Distribution of IAA plant biosynthesis genes in algal and roseobacter genomes

	Code	YUCCA	AMI1	TAA1	CYP79B2	CYP79B3	AAO1	CYP71A13	TDC	MYR1	SUR1	SUR2	NIT1
LAND PLANT													
Arabidopsis thaliana [d]	**Accession #**	AEE86075	Q9FR37	Q9S7N2	NP_195705	NP_179820	Q7G193	O49342	Q8RY79	P37702	O65782	Q9SIV0	AEE77887
	OrthoMCL group	OG5_129359	OG5_149946	OG5_189781	OG5_140471	OG5_140471	OG5_127252	OG5_146283	OG5_129065	OG5_164273	OG5_126582	OG5_128985	OG5_131865
	ESG Molecular Function terms	N,N-dimethylaniline monooxygenase activity,	glutaminyl-tRNA synthase (glutamine-hydro-lyzing) activity	L-phenylalanine:2-oxo-glutarate amino-transferase activity	Mono-oxygenase activity	Mono-oxygenase activity	Oxido-reductase activity	Oxido-reductase activity	aromatic-L-amino-acid decarboxy-lase activity	hydrolase activity, hydro-lyzing O-glyco-syl compounds	Oxido-reductase activity	L-phenylalanine: 2-oxoglutarate amino-transferase activity	nitrilase activity
	ESG Confidence	0.00%	100.00%	48.00%	42.70%	53.20%	100.00%	0.00%	52.70%	71.40%	30.50%	33.00%,	31.80%
GREEN ALGAE													
Ostreococcus sp RCC809 [a]	**Accession #**	39261	38981	none	40254	40254	none	58872	28940	38969	40254	27915	none
	E-value	4.54E-06	2.03E-61	-	1.08E-14	1.01E-16	-	1_0E-16	8.93E-24	1.53E-43	7.80E-25	1_7E-72	-
	Reciprocal e-value	4.00E-14	5.00E-85	-	0	0	-	4.00E-18	2.00E-37	2.00E-109	0.00E+00	2.00E-75	-
	OrthoMCL	-	OG5_150132	-	OG5_126554	OG5_126554	-	OG5_134261	OG5_127644	OG5_126690	OG5_126554	OG5_128985	-
	ESG Confidence	-	96.50%	-	49.60%	49.60%	-	36.10%	0.00%	59.50%	47.60%	55.50%	-
	Presence	-	-/+	-	-/+	-/+	-	-	-	-/+	-/+	+	-
Coccomyxa subellipsoidea [a]	**Accession #**	XP_005652161	XP_005647194	none	XP_005648231	XP_005648231	XP_005646284	XP_005652354	XP_005646768	XP_005643327	XP_005648231	XP_005650249	XP_005646017
	E-value	1.00E-36	2.00E-100	-	8.00E-19	2.00E-19	9.00E-164	2.00E-25	2.00E-170	3.00E-101	4.00E-30	9.00E-19	3.00E-45
	Reciprocal e-value	2.00E-37	7.00E-101	-	0	0	0	0	3.00E-171	3.00E-152	0	1.00E-19	4.00E-46
	OrthoMCL	OG5_126653	OG5_149946	-	OG5_134261	OG5_134261	OG5_127252	OG5_126554	OG5_129065	OG5_126690	OG5_134261	OG5_135328	OG5_131865
	ESG Confidence	73.40%	100.00%	-	53.00%	53.00%	100.00%	43.80%	47.10%	70.00%	44.40%	100.00%	30.10%
	Presence	-	+	-	-/+	-/+	+	-	+	-/+	-/+	-/+	+

Chlamydomonas reinhardtii [a]	**Accession #**	XP_005779538	XP_001698766	none	XP_001698892	ABQ59243	XP_001694090	XP_001700492	XP_00169005	XP_001700848	XP_001698892	AAB01685	XP_001692986
	E-value	1.00E-14	2.00E-50	-	2.00E-16	2.00E-17	8.00E-164	1.00E-28	2.00E-150	1.00E-18	2.00E-23	8.00E-15	5.00E-07
	Reciprocal e-value	3.00E-15	8.00E-61	-	2.00E-163	0.00E+00	2.00E-151	0.00E+00	9.00E-163	3.00E-33	2.00E-163	2.00E-15	2.00E-09
	OrthoMCL	OG5_126653	OG5_149946	-	OG5_126554	OG5_134261	OG5_127252	OG5_126554	OG5_129065	OG5_126690	OG5_126554	OG5_127112	n/a
	ESG Confidence	77.90%	95.50%	-	45.00%	44.80%	100.00%	38.30%	40_0%	64.60%	40_0%	0.00%	n/a
	Presence	-	+	-	-/+	-/+	+	-	+	-/+	-/+	-	-
RED ALGAE													
Cyanidioschyzon merolae [b]	**Accession #**	none	XP_005537175	none	none	XP_005534988	none	XP_005535456	none	none	XP_005535456	XP_005539487	XP_005538174
	E-value	-	4.00E-21	-	-	1.00E-09	-	1.00E-08	-	-	8.00E-09	2.00E-65	8.00E-08
	Reciprocal e-value	-	1.00E-126	-	-	4.00E-123	-	1.00E-16	-	-	1.00E-16	7.00E-66	1.00E-86
	OrthoMCL	-	OG5_127466	-	-	n/a	-	n/a	-	-	n/a	OG5_128985	n/a
	ESG Confidence	-	96.50%	-	-	n/a	-	n/a	-	-	n/a	68.30%	n/a
	Presence	-	-/+	-	-	-	-	-	-	-	-	+	-
Porphyridium purpureum [c]	**Accession #**	none	none	none	none	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-	-	-	-	-
Chondrus crispus [d]	**Accession #**	none	XP_005710130	none	XP_005713400	XP_005713400	XP_005718689	XP_005715170	none	XP_005715104	XP_005715216	XP_005713817	none
	E-value	-	7.00E-21	-	2.00E-19	5.00E-18	5.00E-168	1.00E-21	-	4.00E-94	9.00E-23	3.00E-70	-
	Reciprocal e-value	-	6.00E-139	-	1.00E-46	1.00E-46	0.00E+00	2.00E-39	-	1.00E-132	0.00E+00	1.00E-72	-
	OrthoMCL	-	OG5_127466	-	OG5_126554	OG5_126554	OG5_127252	OG5_126554	-	OG5_126690	OG5_126554	OG5_128985	-
	ESG Confidence	-	93.50%	-	60.10%	60.10%	99.50%	30.80%	-	44.50%	32.90%	50.60%	-
	Presence	-	-/+	-	-/+	-/+	+	-	-	-/+	-/+	+	-

GLAUCOPHYTE													
Cyanophora paradoxa [d]	**Accession #**	none	none	none	none	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-	-	-	-	-
DIATOMS													
Fragilariopsis cylindrus [a]	**Accession #**	none	none	none	none	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-	-	-	-	-
Phaeodactylum tricornutum [a]	**Accession #**	none	XP_ 002180426	none	XP_ 002185035	XP_ 002185035	XP_ 002184143	XP_ 002178044	none	XP_ 002185317	XP_ 002185035	XP_ 002186145	XP_ 002183613
	E-value	-	4.00E-13	-	2.00E-11	9.00E-11	7.00E-132	6.00E-08	-	6.00E-104	5.00E-22	1.00E-51	4.00E-17
	Reciprocal e-value	-	5.00E-21	-	0	0	8.00E-138	2.00E-17	-	1.00E-140	0	9.00E-54	2.00E-97
	OrthoMCL	-	OG5_ 127783	-	OG5_ 126554	OG5_ 126554	OG5_ 127252	n/a	-	OG5_ 126690	OG5_ 126554	OG5_ 128985	OG5_ 126651
	ESG Confidence	-	100.00%	-	44.00%	44.00%	100.00%	n/a	-	74_0%	39_0%	99.00%	0.00%
	Presence	-	-/+	-	-/+	-/+	+	-	-	-/+	-/+	+	-/+
Pseudo-nitzschia multiseries CLN-47 [a]	**Accession #**	26050	54595	none	7819	8596	36829	1342	none	41676	7819	13947	289105
	E-value	1.47E-25	2.64E-13	-	8.47E-08	5.72E-11	1.92E-84	5_2E-12	-	2.56E-90	1.74E-18	1.30E-37	1.73E-06
	Reciprocal e-value	1.00E-25	7.00E-46	-	1.00E-36	4.00E-21	5.00E-120	6.00E-165	-	2.00E-135	1.00E-36	1.00E-55	1.00E-12
	OrthoMCL	OG5_ 129359	OG5_ 127466	-	n/a	OG5_ 127298	OG5_ 127252	OG5_126554	-	OG5_ 126690	OG5_ 169987	OG5_ 128985	n/a
	ESG Confidence	69.40%	100.00%	-	n/a	51.50%	100.00%	31.30%	-	48.90%	80.00%	64.10%	n/a
	Presence	+/-	-/+	-	-	-/+	+	-	-	-/+	-/+	+	-

PELAGOPHYTE													
Aureococcus anophagefferens [a]	**Accession #**	XP_009036936	XP_009039682	none	XP_009039697	XP_009039697	XP_009034984	XP_009032102	none	XP_009035359	XP_009032928	XP_009033544	XP_009039155
	E-value	3.00E-16	2.00E-18	-	2.00E-12	1.00E-12	5.00E-126	3.00E-15	-	2.00E-78	2.00E-07	3.00E-30	3.00E-11
	Reciprocal e-value	8.00E-17	7.00E-47	-	1.00E-28	1.00E-28	4.00E-148	4.00E-24	-	4.00E-129	2.00E-164	1.00E-31	7.00E-65
	OrthoMCL	OG5_126653	OG5_132924	-	OG5_126554	OG5_126554	OG5_127252	OG5_126554	-	OG5_126690	OG5_126554	OG5_128985	OG5_126651
	ESG Confidence	83.50%	100.00%	-	47.50%	47.50%	100.00%	33.40%	-	52.50%	n/a	100.00%	0.00%
	Presence	-	-/+	-	-/+	-/+	+	-	-	-	-	+	-
BROWN ALGAE													
Ectocarpus siliculosus [d]	**Accession #**	CBJ32825	CBN78043	none	CBJ27249	CBJ27249	CBJ27195	CBN74955	CBN75443	CBN79091	CBJ27248	CBJ25743	CBN74568
	E-value	3.00E-15	2.00E-14	-	1.00E-13	4.00E-13	9.00E-119	8.00E-06	2.00E-109	7.00E-92	1.00E-24	6.00E-21	1.00E-10
	Reciprocal e-value	5.00E-17	5.00E-93	-	0.00E+00	0.00E+00	8.00E-121	1.00E-07	2.00E-117	1.00E-130	0.00E+00	3.00E-22	6.00E-10
	OrthoMCL	OG5_152674	OG5_127466	-	OG5_126554	OG5_126554	OG5_127252	n/a	OG5_129065	OG5_126690	OG5_126554	OG5_127112	n/a
	ESG Confidence	72.10%	100.00%	-	65.20%	65.10%	99.70%	n/a	46.80%	54.00%	53.80%	35.30%	n/a
	Presence	-	-/+	-	-/+	-/+	+	-	+	-/+	-/+	-/+	-
EUSTIGMATO-PHYTE													
Nannochloropsis oculata [d]	**Accession #**	none	none	none	none	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-	-	-	-	-
CHRYSOPHYTE													
Ochromonas danica [d]	**Accession #**	none	none	none	none	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-	-	-	-	-

DINO-FLAGELLATES													
Symbiodinium minutum [e]	**Accession #**	none	none	none	none	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-	-	-	-	-	-	-	-	-
	ESG Confidence	-	-	-	-	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-	-	-	-	-
CRYPTO-PHYTES													
Guillardia theta [a]	**Accession #**	XP_005831091	XP_005837094	XP_005825847	XP_005822842	XP_005822842	XP_005827081	XP_005827896	XP_005821090	none	XP_005822842	XP_005840936	XP_005837230
	E-value	3.00E-21	1.00E-15	4.00E-42	2.00E-21	8.00E-23	0.00E+00	7.00E-21	6.00E-37	-	2.00E-19	2.00E-25	3.00E-08
	Reciprocal e-value	2.00E-27	1.00E-15	4.00E-59	2.00E-50	2.00E-50	0.00E+00	0.00E+00	1.00E-37	-	2.00E-50	1.00E-24	2.00E-92
	OrthoMCL	OG5_129359	OG5_132214	OG5_146412	OG5_126554	OG5_126554	OG5_127252	OG5_126554	OG5_127644	-	OG5_126554	OG5_127112	n/a
	ESG Confidence	0.00%	100.00%	100.00%	62.40%	62.40%	99.80%	40.00%	0.00%	-	54.40%	36.10%	n/a
	Presence	+	-/+	-/+	-/+	-/+	+	-	-	-	-/+	-/+	-
Hemiselmis andersenii [d]	**Accession #**	none	none	none	none	none	none	none	none	none	none	none	none
	E-value	-	-	-	-	-	-	-	-	-	-	-	-
	Reciprocal e-value	-	-	-	-	-	-	-	-	-	-	-	-
	OrthoMCL	-	-	-	-								
	ESG Confidence	-	-	-	-	-	-	-	-	-	-	-	-
	Presence	-	-	-	-	-	-	-	-	-	-	-	-
HAPTOPHYTE													
Emiliania huxleyi CCMP1516 [d]	**Accession #**	XP_005764353	XP_005770598	XP_005759060	XP_005782001	XP_005793926	XP_005757560	XP_005793926	XP_005776402	XP_005758786	XP_005793926	XP_005780581	XP_005785520
	E-value	1.00E-37	2.00E-17	1.00E-48	3.00E-10	2.00E-11	7.00E-87	7.00E-30	7.00E-39	4.00E-91	2.00E-25	2.00E-64	2.00E-39
	Reciprocal e-value	1.00E-38	3.00E-26	8.00E-66	3.00E-24	4.00E-36	5.00E-137	5.00E-35	1.00E-39	5.00E-132	4.00E-36	6.00E-69	2.00E-41
	OrthoMCL	OG5_129359	OG5_132924	OG5_146412	OG5_127298	OG5_141359	OG5_127252	OG5_141359	OG5_129065	OG5_126690	OG5_141359	OG5_128985	OG5_131865
	ESG Confidence	58.50%	95.50%	50.30%	60.60%	73.90%	100.00%	72.40%	47.10%	62.60%	72.40%	67.40%	31.30%
	Presence	+/-	-/+	-/+	-/+	-/+	+	-	+	-/+	-/+	+	+

ROSEOBACTER													
Ruegeria sp. R11 [d]	**Accession #**	none	WP_008560074	none	none	none	WP_008561606	none	WP_008558800	none	none	WP_008563355	none
	E-value	-	5.00E-13	-	-	-	1E-95	-	3E-109	-	-	4.00E-27	-
	Reciprocal e-value	-	7.00E-19	-	-	-	2E-95	-	7E-109	-	-	7.00E-27	-
	OrthoMCL	-	OG5_127466	-	-	-	OG5_127252	-	OG5_129065	-	-	OG5_135328	-
	ESG Confidence	-	92.00%	-	-	-	100.00%	-	47.70%	-	-	99.50%	-
	Presence	-	-/+	-	-	-	+	-	+	-	-	-/+	-
Dinoroseobacter shibae DFL 12 [d]	**Accession #**	WP_012187434	WP_012177719	none	none	none	WP_012179620	WP_012179004	none	WP_012178326	WP_012179004	WP_012178466	none
	E-value	4E-23	1.00E-14	-	-	-	2E-104	4.00E-21	-	1E-54	4.00E-12	1.00E-16	-
	Reciprocal e-value	8E-27	2.00E-20	-	-	-	1E-103	1.00E-37	-	2E-58	9.00E-40	4.00E-16	-
	OrthoMCL	OG5_129359	OG5_127466	-	-	-	OG5_127252	OG5_126554	-	OG5_126690	OG5_126554	OG5_140291	-
	ESG Confidence	72.30%	100.00%	-	-	-	100.00%	52.40%	-	52.00%	52.40%	0.00%	-
	Presence	+/-	-/+	-	-	-	+	-/+	-	-	-/+	-	-
Jannaschia sp. CCS1 [d]	**Accession #**	WP_011456827	WP_011453552	none	none	none	WP_011454061	WP_011455517	WP_01145660	WP_011455066	none	WP_011457232	WP_011456849
	E-value	8E-38	9.00E-25	-	-	-	8E-100	7.00E-12	4E-95	4E-56	-	1.00E-27	2.00E-46
	Reciprocal e-value	2E-37	2.00E-27	-	-	-	2E-96	7.00E-40	5E-100	2E-64	-	2.00E-27	1.00E-45
	OrthoMCL	OG5_129359	OG5_132924	-	-	-	OG5_127252	OG5_126554	OG5_129065	OG5_126690	-	OG5_126697	OG5_131865
	ESG Confidence	39.70%	94.00%	-	-	-	100.00%	59.20%	46.90%	55_0%	-	37.30%	36.50%
	Presence	+/-	-/+	-	-	-	+	-/+	+	-	-	-/+	+
Phaeobacter gallaeciensis 2.10 [d]	**Accession #**	WP_014889329	WP_014881395	none	AFO87348	AFO87348	WP_014875707	AFO87348	WP_01487322	WP_014873899	none	WP_014889373	none
	E-value	8E-16	5.00E-19	-	3E-12	3.00E-12	2E-94	5.00E-15	2E-111	1E-60	-	3.00E-16	-
	Reciprocal e-value	8E-17	9.00E-19	-	5E-43	5.00E-43	4E-99	5.00E-43	4E-111	4E-64	-	1.00E-17	-
	OrthoMCL	OG5_126653	OG5_132924	-	OG5_126554	OG5_126554	OG5_127252	OG5_126554	OG5_129065	OG5_126690	-	OG5_126697	-
	ESG Confidence	63.30%	97.50%	-	47.30%	47.30%	100.00%	39.90%	47.10%	53.00%	-	47.00%	-
	Presence	-	-/+	-	-/+	-/+	+	-/+	+	-	-	-/+	-

Phaeobacter gallaeciensis DSM17395 (BS107) [d]	**Accession #**	WP_014881761	WP_014880496	none	AFO91064	AFO91064	WP_014880995	AFO91064	WP_01488122	WP_014879386	none	WP_014881859	none
	E-value	2E-15	3.00E-13	-	3E-12	3.00E-12	2E-94	2.00E-14	2E-110	2E-57	-	5.00E-16	-
	Reciprocal e-value	2E-16	5.00E-19	-	3E-40	3.00E-40	8E-99	1.00E-43	4E-110	2E-61	-	2.00E-17	-
	OrthoMCL	OG5_126653	OG5_127466	-	OG5_126554	OG5_126554	OG5_127252	OG5_126554	OG5_129065	OG5_126690	-	OG5_126697	-
	ESG Confidence	71.30%	94.00%	-	47.80%	47.80%	100.00%	37.90%	40.70%	64.00%	-	38.60%	-
	Presence	-	-/+	-	-/+	-/+	+	-/+	+	-	-	-/+	-
Roseobacter denitrificans OCh 114 [d]	**Accession #**	WP_011569789	WP_011566397	none	none	none	WP_011570123	WP_011567654	none	WP_011568514	WP011567654	WP_011566437	none
	E-value	2E-23	5.00E-15	-	-	-	1E-113	1.00E-14	-	1E-62	1.00E-15	4.00E-13	-
	Reciprocal e-value	2E-28	1.00E-19	-	-	-	6E-114	9.00E-48	-	2E-62	9.00E-48	8.00E-13	-
	OrthoMCL	OG5_129359	OG5_132924	-	-	-	OG5_127252	OG5_126554	-	OG5_126690	OG5_126554	OG5_135328	-
	ESG Confidence	48.30%	98.50%	-	-	-	99.80%	43.60%	-	53.00%	43.60%	100.00%	-
	Presence	+/-	-/+	-	-	-	+	-/+	-	-	-/+	-/+	-
Ruegeria pomeroyi DSS-3 [d]	**Accession #**	WP_011046547	WP_011047226	none	WP_011047630	WP_011047630	WP_011046404	WP_011047630	WP_01104935	none	WP_011047630	WP_030003254	WP_011241897
	E-value	4E-26	5.00E-20	-	1E-10	1.00E-10	1E-88	2.00E-16	3E-109	-	1.00E-12	6.00E-15	3.00E-43
	Reciprocal e-value	1E-25	7.00E-24	-	5E-46	5.00E-46	6E-95	5.00E-46	6E-109	-	5.00E-46	1.00E-14	7.00E-43
	OrthoMCL	OG5_126653	OG5_175845	-	OG5_126554	OG5_126554	OG5_127252	OG5_126554	OG5_129065	-	OG5_126554	OG5_135774	OG5_131865
	ESG Confidence	72.70%	96.50%	-	48.80%	48.80%	100.00%	39.90%	41.30%	-	39.90%	84.20%	35.90%
	Presence	-	-/+	-	-/+	-/+	+	-/+	+	-	-/+	-/+	+
Ruegeria sp. TM1040 [d]	**Accession #**	none	WP_011537675	none	none	none	WP_011540019	WP_011538791	WP_01153701	WP_011536924	WP_011538791	WP_011537211	none
	E-value	-	4.00E-22	-	-	-	4E-98	1.00E-18	1E-112	8E-57	1.00E-11	3.00E-17	-
	Reciprocal e-value	-	3.00E-25	-	-	-	4E-95	2.00E-43	3E-112	2E-58	2.00E-43	7.00E-17	-
	OrthoMCL	-	OG5_132924	-	-	-	OG5_127252	OG5_126554	OG5_129065	OG5_126690	OG5_126554	OG5_126697	-
	ESG Confidence	-	97.50%	-	-	-	98.20%	56.60%	47.10%	65.60%	56.60%	47.20%	-
	Presence	-	-/+	-	-	-	+	-/+	+	-	-/+	-/+	-

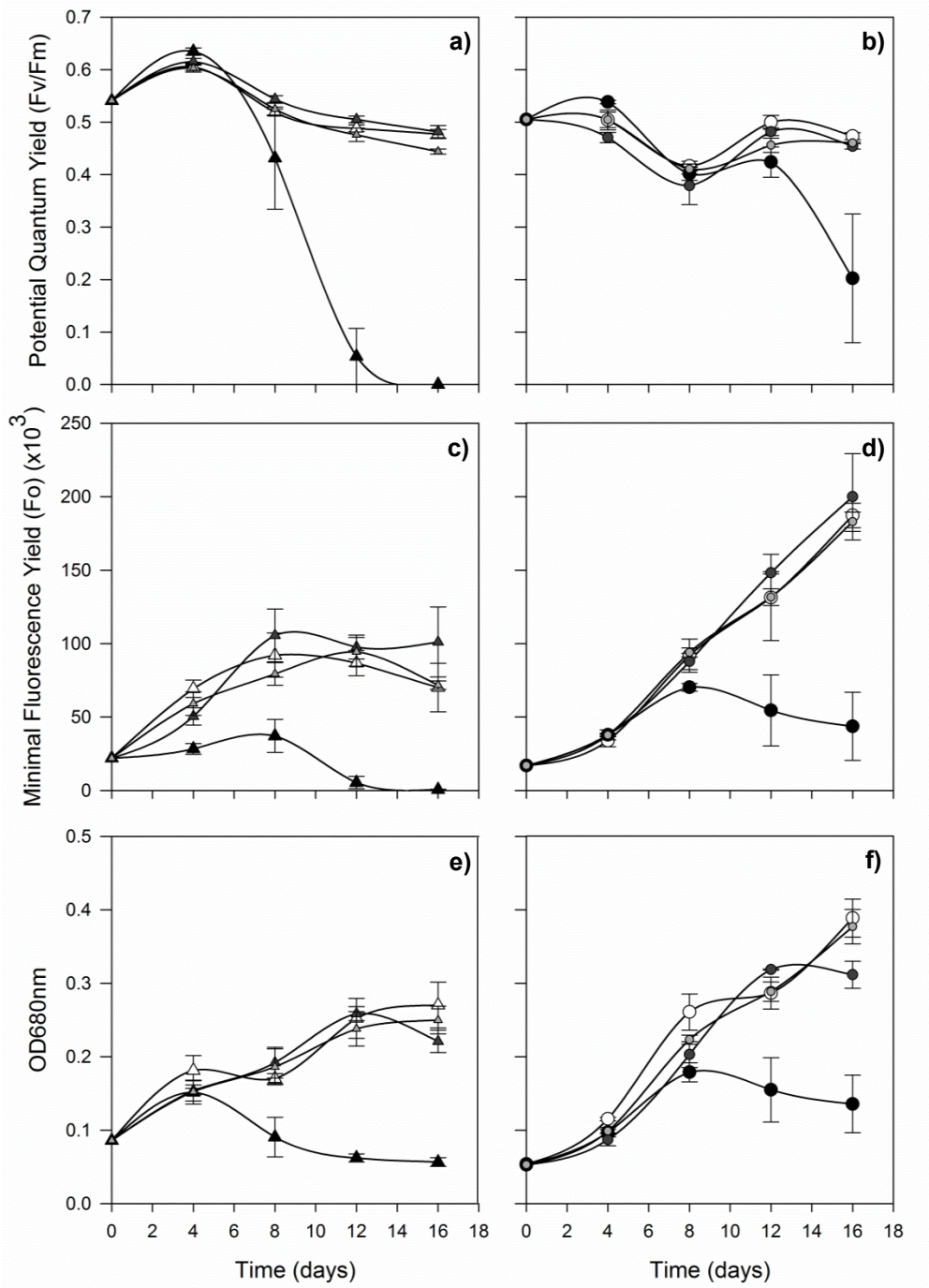

Figure B-1: Co-culturing experiment of exogenous L-tryptophan on axenic bald CCMP 2090 and coccolith bearing CCMP3266 *E.huxleyi.*

The algae were co-cultured with concentrations of 10^{-3} to 10^{-5} M of L-tryptophan (white for control samples with no additional tryptophan, black for 10^{-3} M, dark grey for 10^{-4} M, and light grey for 10^{-5} M additional tryptophan). Triangles represent CCMP3266 while circles represent CCMP2090. The potential quantum yield of CCMP3266 (a) and CCMP2090 (b) with various concentrations of tryptophan is shown, as well as the minimal fluorescence for CCMP3266 (c) and CCMP2090 (d), and the OD_{680} for CCMP3266 (e) and CCMP2090 (f). Error bars represent ±1 SE.

Appendix C

Effect of tryptophan and IAA on other algae

C.1 Results and Discussion

The effect of tryptophan and IAA on the haptophyte *Isochrysis sp.* (CCMP1324) and the chlorophyte *Duniella tertiolecta* (CCMP1320) was tested using the same methodology as Chapter 4. The only variation was that the L1-SI medium was prepared using artificial sea water (35g/L of Instant Ocean, Blacksburg, VA, USA) instead of natural filtered sea water.

High concentrations of tryptophan were inhibitory to the haptophyte, but stimulated the photosystem health of the chlorophyte (Figure A-1). The results show that *Isochrysis sp.*, an axenic haptophyte, produces indole-3-acetic acid (IAA) in response to the precursor, L-tryptophan, when using the colorimetric assay (Figure C-2). D-tryptophan did not produce any IAA (data not shown). This further supports the claim that the axenic haptophytes can produce IAA. *Isochrysis sp.* did not have a morphological response to IAA however, nor did the photosystem health or OD alter in response, although 10^{-3} M IAA was inhibitory to the algae (Figure C-3).

Despite having an apparent production of IAA (Figure C-2), the chlorophyte *D. tertiolecta* did not produce IAA in response to L-tryptophan as the peak of the Salkowski reagent was 520-525 nm, which is the reaction more typical for tryptophan (Glickmann & Dessaux, 1995), rather than the 530 nm peak typical for IAA (Glickmann & Dessaux, 1995). D-tryptophan did not produce any IAA (data not shown). Some micro green algae have been demonstrated to produce IAA, especially under stressful conditions, including D. tertiolecta (Stirk *et al.*, 2014). This was not observed in these conditions grown here. The highest concentration of IAA (10^{-3} M) was toxic to the algae (Figure C-3). For the chlorophyte however, 10^{-4}M IAA did have a small, but still statistically significant, boosting effect on the photosystem II health

(Figure C-3). At concentrations of 10^{-4} M, IAA has been demonstrated increase the cell number of this and other algal species (Li *et al.*, 2007; Piotrowska-Niczyporuk & Bajguz, 2013).

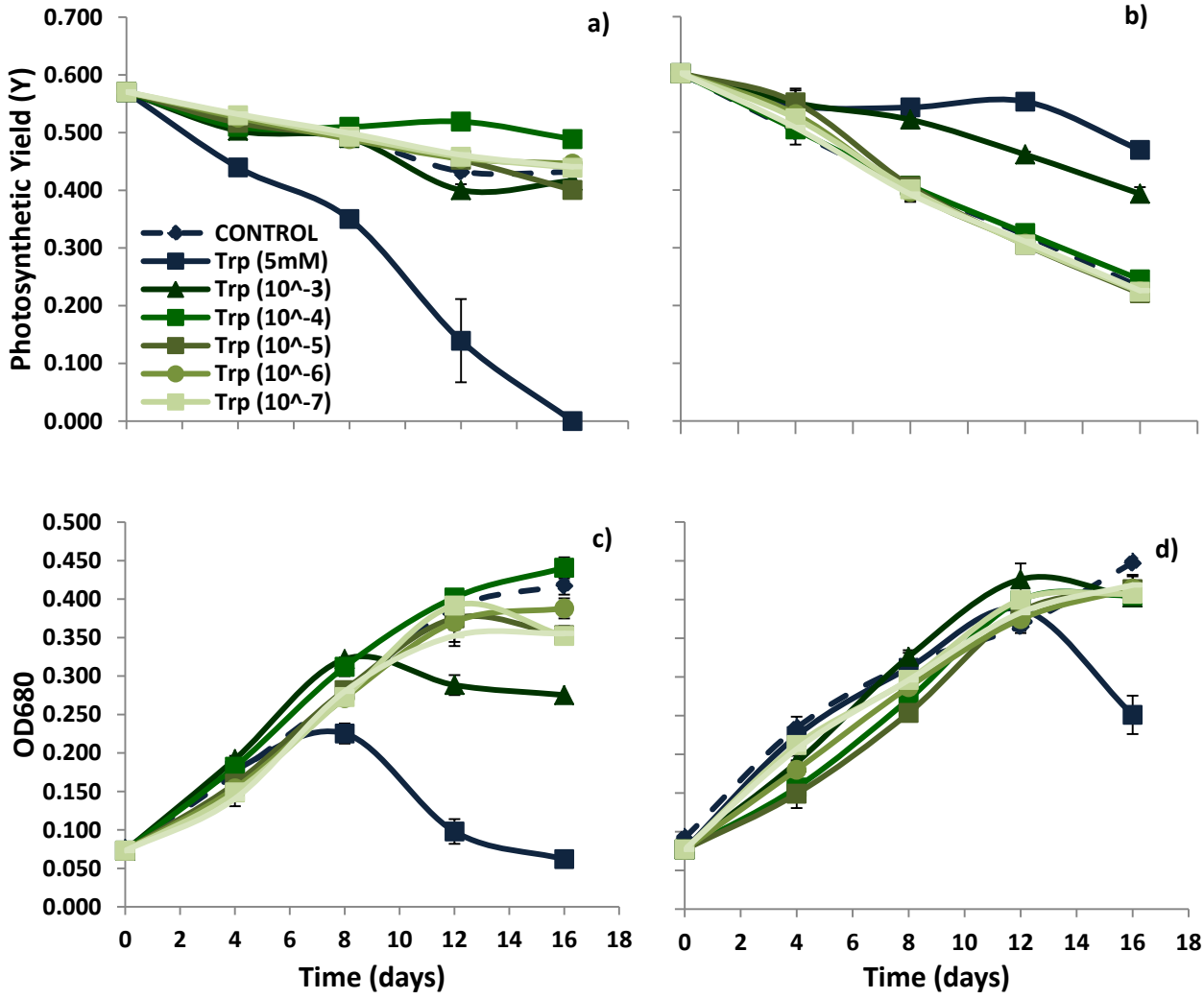

Figure C-1: Co-culturing experiment of various concentrations of L-tryptophan against *Isochrysis sp*. and *Dunaliella tertiolecta*.

The algae were co-cultured with concentrations of tryptophan or no tryptophan as the control (L1-SI medium with 1% ethanol). The potential quantum yield of *Isochrysis sp.* (CCMP1324) (a) and *D. tertiolecta* (CCMP1320) (b) with various concentrations of tryptophan is shown, as well as the OD at 680nm for the two strains, CCMP1324 (c) and CCMP1320 (d). Error bars represent ±1 SE.

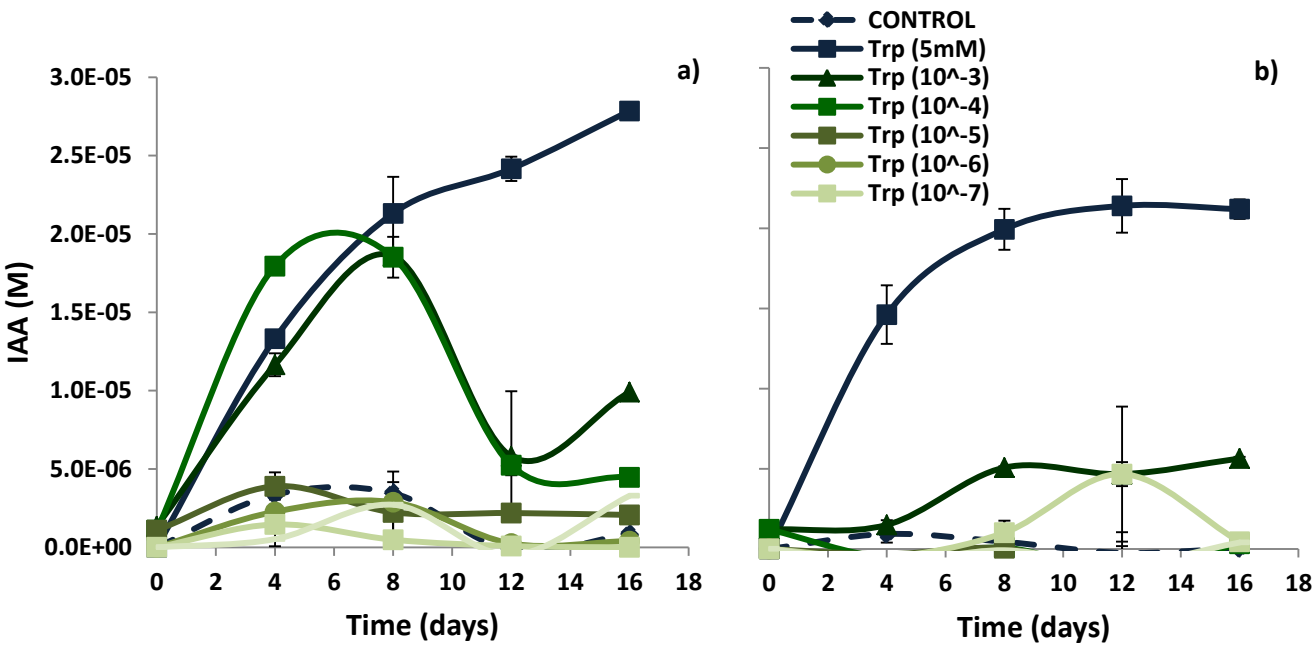

Figure C-2: Co-culturing experiment of various concentrations of L-tryptophan against *Isochrysis sp*. and *Dunaliella tertiolecta*, demonstrating the production of indole-3-acetic acid (IAA).

IAA concentration was derived from OD measurements of culture supernatants after addition of Salkowski reagent. The algae were co-cultured with concentrations of tryptophan or no tryptophan as the control (L1-SI medium with 1% ethanol), where the algae were *Isochrysis sp.* (CCMP1324) (a) and *D. tertiolecta* (CCMP1320) (b). Error bars represent ±1 SE.

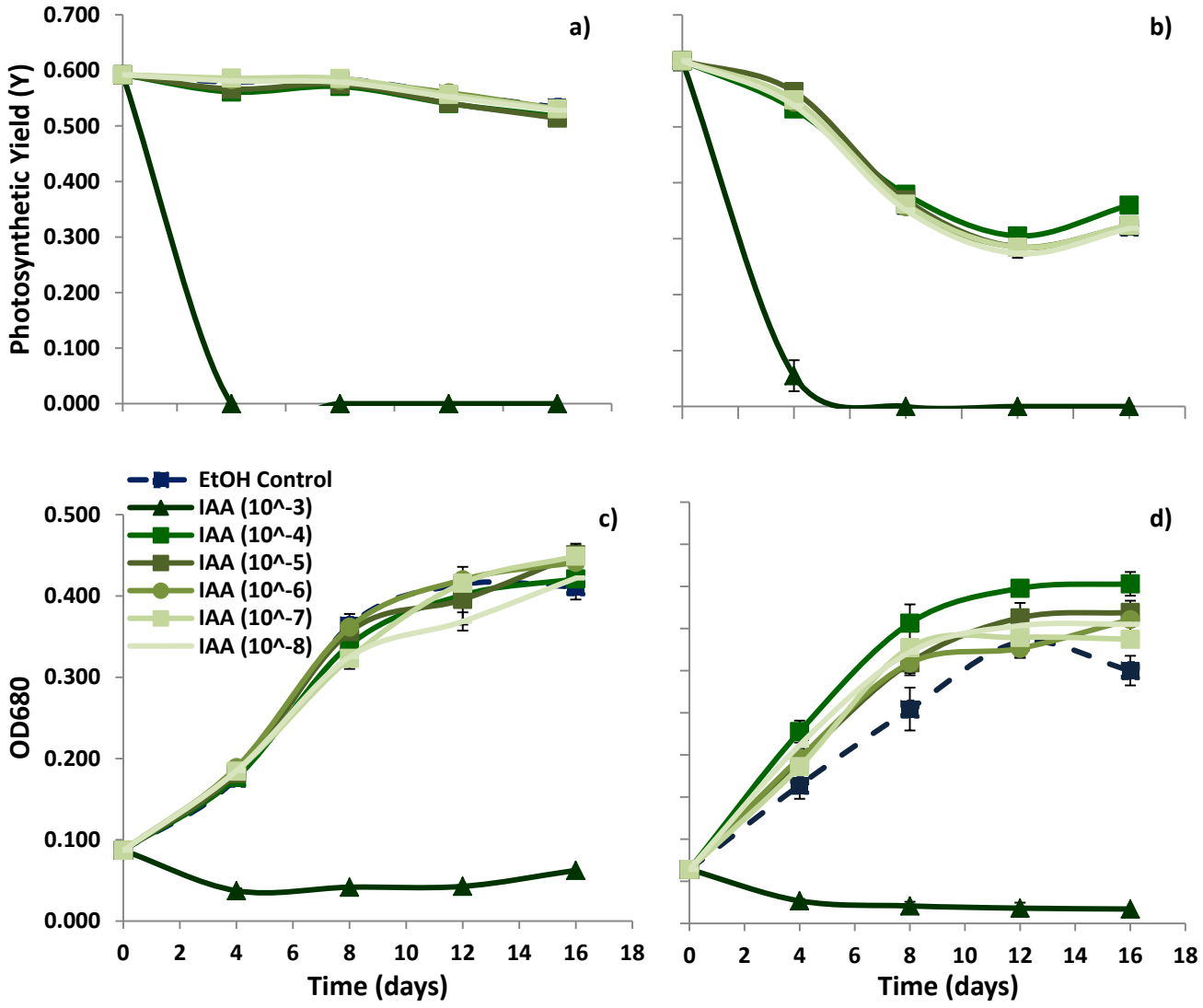

Figure C-3: Co-culturing experiment of various concentrations of of indole-3-acetic acid (IAA) against *Isochrysis sp*. and *Dunaliella tertiolecta*.

The algae were co-cultured with concentrations of IAA or L1-SI medium with 1% ethanol as the control. The potential quantum yield of *Isochrysis sp.* (CCMP1324) (a) and *D. tertiolecta* (CCMP1320) (b) with various concentrations of IAA is shown, as well as the OD at 680nm for the two strains, CCMP1324 (c) and CCMP1320 (d). Error bars represent ±1 SE.

C.1 References

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Li T, Wang C, Miao J (2007). Identification and quantification of indole-3-acetic acid in the kelp *Laminaria*

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