Opening up the black box of marine phototroph-heterotroph interactions

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List of Abbreviations

°C – degree(s) Celsius

A – adenine

AMT23 – 23rd Atlantic Meridional Transect research cruise

AntiSMASH – Antibiotics and Secondary Metabolite Analysis Shell

Ap – ampicillin

ASW – Artificial Sea Water

b – base

BLAST – Basic Local Alignment Search Tool

blastp – protein to protein BLAST

BP – boiling point

bp – base pairs

c – cluster

C – cytosine

CA – cellulose acetate

CHS – chalcone synthase

Cn – chloramphenicol

C_T – cycle threshold value

CTD – conductivity, temperature and pressure of water (depth) probe

DAD – Diode Array Detector

DMSO – dimethyl sulfoxide

DNA – deoxyribose nucleic acid

DNAse – deoxyribonuclease

dNTPs – deoxynucleotide triphosphates

DOC – dissolved organic carbon

DOM – dissolved organic matter

DON – dissolved organic nitrogen

DOP – dissolved organic phosphorus

dsDNA – double stranded DNA

EDTA – ethylenediamine tetraacetic acid

e.g. – *exempli gratia*

ESI – electrospray ionisation

et al. – *et alii*

f – femto (SI prefix, $x10^{-15}$)

FDR – false discovery rate

FSC – forward scatter

g – gravity (when describing centrifugation)

g – gram(s) (when describing mass)

G – giga (SI prefix, $x10^{9}$)

G – guanine (when describing nucleobases)

h – hour(s)

HPLC – High Performance Liquid Chromatography

ID – identification (when describing sample name or number)

ID – internal diameter (when describing tubing)

i.e. – *id est*

k – kilo (SI prefix, $x10^{3}$)

Kn – kanamycin

l – litre

L1 – 1st liquid culture

L3 – 3rd liquid culture

LB – Lysogeny Broth

LC – liquid chromatography

LP – long pass

M – mega (SI prefix, $x10^{6}$)

M – molar (mol l^{-1})

m – milli (SI prefix, $x10^{-3}$)

m – meter(s)

MB – Marine Broth

MeOH – methanol

min – minute(s)

MS – mass spectrometry

MS^{n} – sequential (multistage) mass spectrometry

MS/MS – tandem mass spectrometry

m/z – mass to charge ratio

μ – micro (SI prefix, $x10^{-6}$)

n – nano (SI prefix, $x10^{-9}$)

n/a – not available or not applicable

NCBI – National Center for Biotechnology Information

NMR – Nuclear Magnetic Resonance

NRPS – Non-ribosomal peptide synthethase

O – original enrichment

OD – optical density

OTU – operational taxonomic unit

PCoA – Principal Coordinates Analysis

PCR – Polymerase Chain Reaction

PES – polyethersulfone

pH – potential of hydrogen

PKS – polyketide synthase

POC – particulate organic carbon

POM – particulate organic matter

PON – particulate organic nitrogen

POP – particulate organic phosphorus

PVDF – polyvinylidene difluoride

Q30 – Phred quality score of 30 (incorrect base call 1 in 1000 times)

QIIME – Quantitative Insights Into Microbial Ecology

qPCR – quantitative Polymerase Chain Reaction

RCC – Roscoff Culture Collection

RDP – Ribosomal Database Project

RNA – ribonucleic acid

rpm – revolutions per minute

rRNA – ribosomal ribonucleic acid

s – second(s)

sp. – species (singular)

spp. – species (plural)

SSC – side scatter

ST – station

T – thymine

T3 – type 3

TAE – tris(hydroxymethyl)aminomethane, acetic acid, EDTA

w – weight

v – volume

WT – wild type

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I would like to dedicate this thesis to my husband and my parents, for their unwavering support throughout many years of my tertiary education. Their patience in listening about my project and willingness to feed me and provide endless cups of tea during thesis write up were greatly appreciated. Dziękuję!

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

- Chapter 3, section 3.2.1: Seawater samples collection and clonal purification of *Synechococcus* sp. enrichments was performed by Mónica Joyce Moniz (School of Life Sciences, University of Warwick)
- Chapter 4, section 4.2.1: *Synechococcus* sp. WH7803 – heterotroph co-cultures maintenance and sample collection was performed by Despoina Sousoni (School of Life Sciences, University of Warwick).

No parts of this thesis have been published by the author.

Abstract

Although marine microorganisms drive the major biogeochemical cycles in marine ecosystems, there is a dearth of information on interactions between phototrophic and heterotrophic organisms co-occurring in oceanic waters. The aim of this project was to study these interactions using *Synechococcus* sp. as the model phototroph – a cosmopolitan and highly abundant member of the picophytoplankton.

Heterotrophic bacteria most-frequently present in non-axenic *Synechococcus* sp. cultures, were identified by PCR screening using primers targeting the 16S rRNA gene. Members of the *Nitratireductor*, *Rhodobacteraceae, Muricauda* and *Phyllobacteriacae* genera were present in more than half of all the cultures tested (Chapter 3).

Using a member of the *Rhodobacteraceae* as the model heterotroph, specific metabolites present in axenic cultures and co-cultures were analysed (Chapter 4). Much lower concentrations of these specific metabolites were present in the milieu of *Synechococcus* – *Roseobacter* co-cultures compared to axenic *Synechococcus* cultures as discovered by LC-MS. Natural product database searches suggest that these may be a group of novel compounds.

A *Synechococcus* sp. WH7803 null mutant in the gene encoding a type III polyketide synthase was constructed (Chapter 5). A targeted exometabolomic analysis showed a decreased production of the metabolites identified above in the mutant strain compared to the wild type. Growth was considerably affected in the T3 PKS mutant and T3 PKS mutant culture supernatants had a stronger negative growth effect on a range of picocyanobacteria and green algal species than the wild type extract.

Further research is required to establish the precise biological function of the observed molecules, their biosynthetic pathway and their function in the natural environment. Improving our understanding of interactions between environmentally important microorganisms not only helps us to learn more about how biogeochemical cycles in the ocean function, but can also provide new natural products for use in the pharmaceutical industry.

1. Introduction

1.1 Interactions between microorganisms in the marine environment

1.1.1 Microorganisms in the marine environment

Numerous different interactions between marine organisms have been described in the literature – for example cnidarians and foraminifera with endosymbiotic unicellular algae (Lee, 2006; Rowan, 1998), sponges with bacteria (Althoff et al., 1998; Unson et al., 1994), bryozoans with proteobacteria (Lim & Haygood, 2004), cleaner shrimps operating cleaning stations (Bunkley-Williams & Williams, 1998), anemones and anemonefish (Fautin, 1991). Many of these relationships are mutualistic and involve, if any, only one microbe. Although marine microorganisms are responsible for about 50% of global carbon fixation (Field, 1998), play a crucial role in nutrient cycling (Arrigo, 2005) and are very abundant in surface ocean waters with about 10^6 microbial cells per ml (Cole, 1982; Giovannoni & Stingl, 2005), there is a dearth of information about relationships between microbes.

The two major ways of obtaining energy in the photic zone of marine environments are photoautotrophy (fixation of carbon using photons) and heterotrophy (acquisition of carbon from organic sources). The pool of organic matter (OM) leaked or released from photo- and heterotrophic cells voluntarily or upon cell death contains dissolved and particulate carbon (DOC and POC, respectively), nitrogen (DON, PON), phosphorus (DOP, POP) and other elements which can be recycled by organisms or sink to the aphotic zone and eventually, if unused, be stored on the ocean floor (Figure 1.1.1.1) (Cho & Azam, 1988; Buchan et al., 2014; Letscher et al., 2015; McCarren et al., 2010; Strom, 2008).

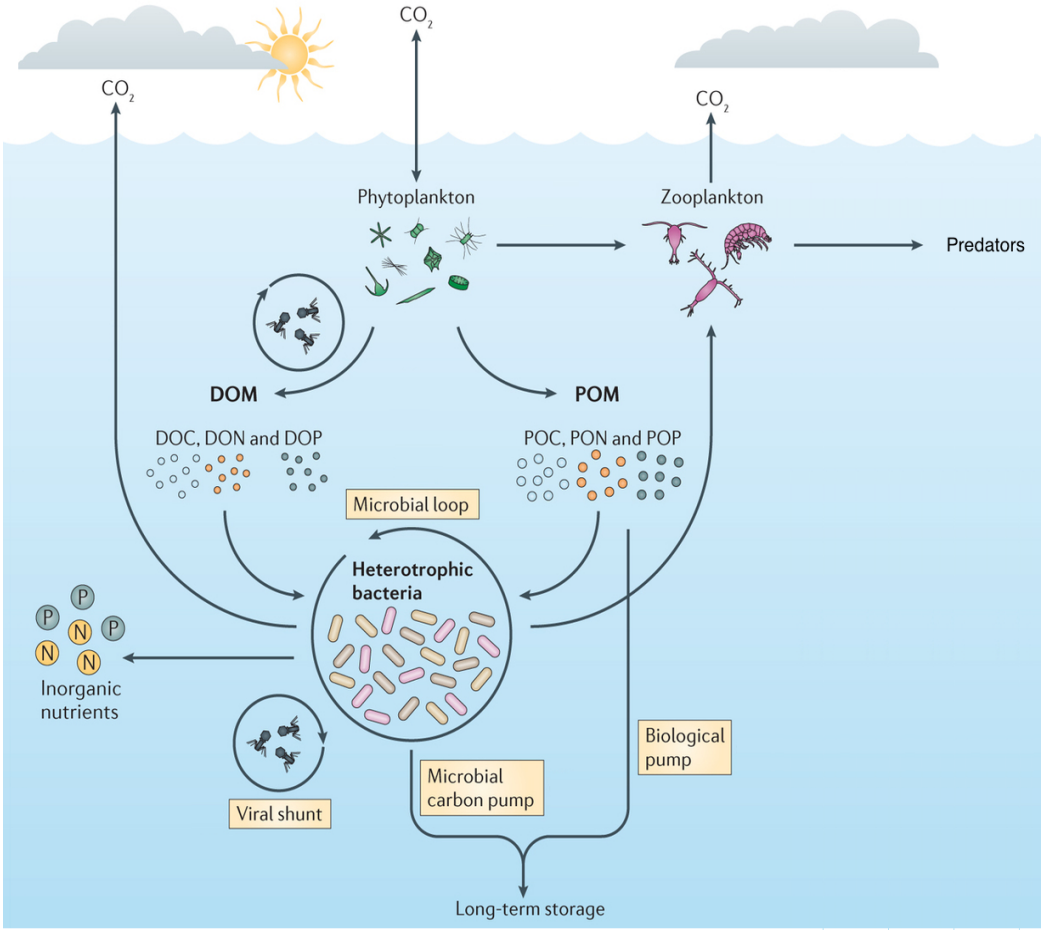


Figure 1.1.1.1 Role of microorganisms in nutrient cycling and the marine food web. Modified from: Buchan et al., 2014.

The interactions between phototrophs and heterotrophs that have been studied most extensively are between microalgae and heterotrophic bacteria. Heterotrophic bacteria provide nutrient regeneration and vitamins to algae, facilitate iron uptake, as well as modify the environmental conditions, such as oxygen concentration, in close proximity to algal cells (Amin et al., 2009; Amin et al., 2012; Bolch et al., 2011; Cole, 1982). This may enhance the growth of both organisms, as microalgae can provide a source of carbon for heterotrophs. There are also some studies indicating competition between microalgae and heterotrophic bacteria for nutrients and even parasitism, when only heterotrophic bacteria benefit from the relationship causing microalgal cell lysis (Grossart, 1999; Ramanan et al., 2016).

1.1.2 Microalgae – heterotroph interactions

Proteobacteria and *Bacteroidetes* (especially *Alteromonas, Glaciecola, Pseudoalteromonas, Hyphomonas, Ruegeria, Roseobacter, Sulfitobacter, Paracoccus, Flavobacterium, Lacinutrix, Cytophaga*) are consistently observed to be associated with diatoms (Amin et al., 2012; Schafer et al., 2002), while the *Cytophaga-Flavobacterium-Bacteroides* group, α- and γ-classes of *Proteobacteria* are usually observed with dinoflagellates (Fandino et al., 2001; Moustafa et al., 2010). A study of *Ostreococcus tauri* cultures found that *Gammaproteobacteria* dominate the green alga's phycosphere (Lupette et al., 2016), while sequencing coccolithophore cultures revealed mainly α- and γ- classes of *Proteobacteria* and *Bacteroidetes* (Green et al., 2015). Diatom-dominated biofilms have increased relative abundance of *Gammaproteobacteria* – diatoms produce large amounts of extracellular polymeric substances which provide a good pool of polysaccharides and glycoproteins for these heterotrophic bacteria (Haynes et al., 2007).

Up to 60% of diatom cells did not have heterotrophic bacteria attached to them after a 30 days long culture experiment, while the diatoms that did carry bacteria on their surface, usually displayed one to five bacterial cells (Kaczmarska et al., 2005). The absence of heterotrophic bacteria was observed to cause a reduction in cell length and morphological aberrations of frustules in diatom cultures (Windler et al., 2014), whilst the presence of bacteria in dinoflagellate cultures results in increased growth, cell volumes and photosynthesis after several months of culture (Jauzein et al., 2015). Colonization by bacteria does not impede diatom growth (Smith et al., 1995) and the communities living on microalgal cells appear to be distinct from bacteria free-living in the surrounding water (Grossart et al., 2005; Sapp et al., 2007).

There is some discussion as to whether heterotrophic bacteria growing on diatoms as epiphytes could be responsible for changes in domoic acid production, a neurotoxin, between axenic and non-axenic diatom cultures (Bates et al., 1995; Bates et al., 2004; Guannel et al., 2011; Kobayashi et al., 2009; Osada & Stewart, 1997) and whether domoic acid plays a role in

acquisition of iron and copper (Maldonado et al., 2002; Rue & Bruland, 2001; Wells et al., 2005). Another function of epiphytic bacteria may be consumption of hydrogen peroxide produced during photosynthesis by a diatom, to protect the microalgae from oxidative stress (Hunken et al., 2008). In contrast, an enhanced expression of photoprotection and oxidative stress response genes by microalgae was observed in dinoflagellate – heterotrophic bacteria co-cultures, compared to axenic cultures (Moustafa et al., 2010).

Motile heterotrophic bacteria were found to be able to track motile microalgae, which suggests that, taking into account the higher swimming speeds of bacteria compared to that of algae, they are able to enter and leave the phycosphere as needed to make use of the available nutrients (Barbara & Mitchell, 2003). Heterotrophic bacteria were also shown to facilitate photochemical redox cycling of iron, which promotes algal assimilation of iron and supports photosynthesis (Amin et al., 2007; Amin et al., 2009).

Different bacterial species or assemblages were shown to reduce the mobility of dinoflagellates (Mayali et al., 2008), stimulate their growth (Ferrier et al., 2002; Jauzein et al., 2015), affect their reproduction (Adachi et al., 2002), induce temporary cyst formation (Mayali et al., 2007) and influence toxin production (Hold et al., 2001; Uribe & Espejo, 2003). A study investigating the effect of a natural marine community and three different bacterial isolates on the growth of a marine diatom showed that transparent exopolymer particles and protein-containing particles were produced only in the presence of bacteria, but also that the effect of bacteria addition on growth of a diatom culture may vary depending on the growth media used for culturing (Grossart & Simon, 2007). There is also some evidence that bacteria-induced phosphate limitation, due to better uptake of phosphate by bacteria than diatoms, results in higher polysaccharide production in diatoms (Guerrini et al., 1998; Thingstad et al., 1993). On the other hand, diatoms are superior in nitrogen uptake and outcompete ammonia-oxidising bacteria in microcosm experiments (Risgaard-Petersen et al., 2004). A

study of a diatom – *Roseobacter* co-culture identified a compound, C_3-sulfonate, that may play an important role in carbon and sulfur cycling (Durham et al., 2015). The authors also note that chemical analysis of a seawater sample can yield several thousands of unique compounds, which poses a question of how to identify compounds which are of biogeochemical relevance from such a pool.

In a study of 41 strains of 27 species of marine microalgae, 95% of strains were shown to be auxotrophs of vitamin B_{12}, 76% - of vitamin B_1 and 29% - of vitamin B_7 (Tang et al., 2010). In the case of vitamin B_{12} supply (Haines & Guillard, 1974), these interactions may be different even between closely related species (Croft et al., 2005), as symbiosis arises through loss of the *metE* gene (encoding the vitamin B_{12}-independent methionine synthase) in response to an available environmental pool of vitamins (Helliwell et al., 2011). Although it is usually considered to be a mutualistic relationship in laboratory cultures, there are arguments that it is not a direct symbiosis in the natural environment, but indirect scavenging for available molecules present in seawater (Droop, 2007).

This loss of genes otherwise essential for survival is well-described by The Black Queen Hypothesis, which states that gene loss may provide an advantage to the organism, as long as the function is leaky in other organisms providing an available public pool of the product (Morris et al., 2012). Unlike Hamilton's rule, this is not based on altruistic action, but just a simple, selectively favoured, reduction in living costs. Examples supporting the hypothesis include: hydrogen peroxide removal (Morris et al., 2011), iron and reduced sulphur acquisition (D'Onofrio et al., 2010; Tripp et al., 2008) and algae-bacteria biofilm formation (Lubarsky et al., 2010; Roeselers et al., 2007). Helper strains are always present in the community, although in significantly smaller numbers than the organisms requiring help and thus may be considered to be keystone species (Morris et al., 2012). If removed, the organism with a reduced genome may not be able to grow due to toxicity or lack of nutrients. However, apart from explaining the hypothesis and examples supporting it, no data was presented to explain how these

relationships form and evolve, how specific they are and whether this is the reason why axenic cultures are difficult to grow. It has also been suggested that a similar relationship can be formed not through a loss of function, but through a gain of function – if a species acquires a gene, through horizontal gene transfer or a mutation that increases the production of the common good, it will become the key species in a community and will have a reduced risk of being replaced by competing species (Mas et al., 2016).

Heterotrophic bacteria also play a role in aggregation of microalgae (Grossart et al., 2006) – in some diatom species bacterial degradation of DOM prevents aggregation until the stationary phase of growth of the diatom, while in other diatom species aggregation of cells is enhanced by bacterial presence. Another study, by the same authors, also observed a difference in diatom – heterotrophic bacteria relationships depending on diatom species, growth and physiological state (Grossart et al., 2005). Axenic diatom cultures were inoculated with natural heterotrophic bacterial cultures and bacterial growth and composition was monitored by DAPI counts, FISH and 16S rRNA sequencing. Bacterial growth was found to be affected by diatom species, growth and health. Free-living bacteria were identified to be mostly *Roseobacter*, similar for all diatom cultures, while bacteria attached to diatoms belonged mostly to the *Flavobacteria-Sphingobacteria* group, but specific species were different in different diatom cultures. Within the same diatom species, there seems to be a difference in aggregation between photosynthetically active and inactive cells – with photosynthetically inactivated diatoms not aggregating regardless of the presence of bacteria and photosynthetically active diatoms aggregating in the presence of specific, cell-attaching bacteria (Gardes et al., 2011), which suggests that the relationship between diatoms and heterotrophic bacteria may be nutrient-driven rather than a constant symbiosis.

Microalgae – heterotrophic bacteria relationships can also change with time – *Phaeobacter gallaeciensis* promotes the growth of *Emiliania huxleyi* by producing antibiotics and auxins until it detects a lignin breakdown product,

which signals ageing of the algae, and then it switches to producing algicidal compounds, roseobacticides (Seyedsayamdost et al., 2011a; Seyedsayamdost et al., 2011b; Wang et al., 2016). A similar interaction, with a different *Roseobacter* strain, *Phaeobacter inhibens*, was also described, where growth of *E. huxleyi* is first promoted by a phytohormone indole-3-acetic acid, but after a while the same compound triggers algal death (Segev et al., 2016). An earlier study suggested a positive effect of indole-3-acetic acid on diatom growth, but did not describe an eventual algicidal effect (Amin et al., 2015). Another study, which manipulated nutrient concentration in algal non-axenic cultures, observed that bacterial community structure change due to a change in bacterial activity and function can cause algal lysis (Wang et al., 2010). However, although bacteria capable of algicidal activity have been described, there is no conclusive evidence whether they play a significant role in phytoplankton mortality in the natural environment (Paul & Pohnert, 2011; Furusawa et al., 2003; Lee et al., 2000; Mayali & Azam, 2004; Skerratt et al., 2002).

On the other hand, there have also been some reports of production of antibacterial compounds by marine diatoms (Desbois et al., 2008; Findlay & Patil, 1984). Polyunsaturated aldehydes, produced by diatoms as a response to grazing, have antibacterial properties, suppressing most of the 33 bacterial strains tested, but not those typically associated with diatoms isolated during a diatom bloom (Ribalet et al., 2008; Wichard et al., 2007).

Apart from the environmental importance of studying microalgae – heterotrophic bacteria interactions, there is also an interest in this topic from industry: to improve biofuel yields (Cho et al., 2015; Kim et al., 2014; Fuentes et al., 2016; Santos & Reis, 2014), sustainability and disease control in aquaculture (Natrah et al., 2011; Natrah et al., 2014), to convert biogas into bioproducts (van der Ha et al., 2012) and to control harmful algal blooms (Kim et al., 2008; Roth et al., 2008; Su et al., 2007). Microalgal – bacterial consortia are also used for wastewater treatment – where oxygen produced by the phototroph is used as an electron acceptor by the heterotroph to degrade organic pollutants and the carbon dioxide released

by the heterotroph is used as a carbon source by the phototroph (Bahr et al., 2011; Cho et al., 2015; de Bashan et al., 2004; Ferrero et al., 2012; Subashchandrabose et al., 2011); to enhance nitrification rates in wastewater treatment lagoons (McLean et al., 2000) and combining wastewater treatment with biofuel production (Hernandez et al., 2013).

1.1.3 *Cyanobacteria* – heterotrophic bacteria interactions

However, the most abundant photoautotrophic organisms in the global ocean are not eukaryotic. Marine picocyanobacteria are responsible for about 25% of ocean net primary productivity and the two main genera, *Prochlorococcus* and *Synechococcus*, have mean global abundances of 2.9×10^{27} and 7.0×10^{26} cells, respectively (Flombaum et al., 2013). At the end of the 21st century, an increase in cell number of 29% and 14% respectively is predicted due to global climate change. The two genera have small genomes (*Prochlorococcus*: 1.64-2.7 Mb with 1716-3022 genes; *Synechococcus*: 2.2-2.86 Mb with 2358-3129 genes) and carry no plasmids, which can lower nutrient intake and improve the surface-area-to-volume-ratio, contributing to the success of the genera (Scanlan et al., 2009).

Prochlorococcus are highly abundant, globally distributed phototrophic bacteria (Partensky et al., 1999), that make up 51% of phytoplankton biomass and are responsible for about 45% of carbon dioxide fixation in the northeast Atlantic (Jardillier et al., 2010). They are present between 40°N and 40°S (Johnson et al. 2006), from the surface to a depth of about 150 m (Flombaum et al., 2013) and have developed specific adaptations to different light conditions – known as high-light and low-light ecotypes (Moore & Chisholm, 1999).

Synechococcus is a phototrophic picocyanobacteria, highly abundant in the marine ecosystem (Scanlan et al., 2009). In the northeast Atlantic it forms about 20% of phytoplankton biomass and contributes about 21% to primary production (Jardillier et al., 2010). It can be found in almost all oceanic waters, including polar regions up to 82.5°N (Paulsen et al., 2016) and high nutrient waters (Martin et al., 2005), at shallower depths than

Prochlorococcus (Flombaum et al., 2013). It can reach cell densities of up to 1.2-3.7 x 10^6 cells ml^{-1} (Saito et al., 2005).

Synechococcus clades I and IV dominate in cold, coastal waters, clade II is found in warmer, subtropical areas between 30°S and 30°N, while clades III, V, VI, VII showed no latitudinal preference (Zwirglemaier et al., 2008). A later study, using a specific *petB* marker, confirmed the distribution and assigned sequences with no corresponding 16S rRNA clade to provisional names EnvA-C, clades with no cultured representatives (Mazard et al., 2012). It has been suggested that different clades have evolved independently to succeed in similar environments and can co-occur in oceanic waters increasing diversity and chances of survival of *Synechococcus* (Sohm et al., 2016).

An extensive review of ecological genomics, including nutrient acquisition, has compared and contrasted the differences between *Prochlorococcus* and *Synechococcus* (Scanlan et al., 2009). Although a lot is known about the genomics and physiology of the organisms, it is difficult to grow them axenically in laboratory conditions. To study growth of *Prochlorococcus* in axenic culture, especially on semi-solid media, a method of growing the phototrophic bacteria together with heterotrophic bacteria and subsequently removing these "helpers" to obtain a pure culture was proposed (Morris et al., 2008). The authors proposed that these "helper" bacteria removed oxidative stress, though they did not investigate the precise mechanism or whether it happens in the natural environment. Subsequently, the same authors confirmed the relationship in the open ocean – if helper species are absent, the hydrogen peroxide concentration increases to levels lethal for *Prochlorococcus* causing cell envelope damage and loss of photosynthetic activity (Morris et al., 2011). A transcriptomics study of a *Prochlorococcus* co-culture revealed that the first response to a heterotrophic partner, within 6 hours of inoculation, was reduction in stress (downregulation of DNA repair enzymes and stress-response proteins genes), followed by an increase in photosynthesis (Biller et al., 2016), which supports earlier findings.

Another study, on interactions between *Prochlorococcus* and 344 different heterotrophic bacterial isolates, revealed that the response of cells to co-culture is different between *Prochlorococcus* ecotypes adapted to low or high light conditions (Sher et al., 2011). Positive interactions, resulting in faster growth or higher final chlorophyll fluorescence, were observed only in the low-light *Prochlorococcus* ecotype and were more common than inhibitory interactions. They were possibly caused by small, diffusive molecules, not cell to cell contact, in contrast to interactions that caused a delay in growth, which were observed only when cell to cell contact was possible. In the case of *Alcanivorax* sp. HOT7G9 and *Rhodobacter* sp. HOT5F3 strains, the peak chlorophyll fluorescence was higher for co-cultures separated by a membrane than those grown together (Sher et al., 2011).

A study of *Prochlorococcus* – *Alteromonas* co-culture also showed a different response of low- and high- light ecotypes to a heterotrophic partner (Aharonovich & Sher, 2016). No effect was observed for the high-light ecotype, while the growth of the low-light ecotype was inhibited at high heterotroph concentrations, but promoted at low heterotroph concentrations. Although most genes differentially expressed in co-cultures were of unknown function, there is some indication of a modification of the membrane/cell wall (changes in expression of genes involved in biosynthesis and modification of peptidoglycan and liposaccharides), stress response (upregulated antioxidants, DNA and photosystem repair systems) as well as production or response to antimicrobials or signalling compounds (expression of transporters, prochlorosins, genes with hemolysin-like or RTX domains). There was little evidence of competition for or recycling of nutrients and genes related to production of amino acids, purines, pyrimidines, fatty acids, phospholipids and vitamin B_{12} were downregulated.

In a study using atomic force microscopy (Malfatti & Azam, 2009), 6-42% of *Synechococcus* cells were found to be conjoint with heterotrophic bacteria (Figure 1.1.3.1). Some of these were further connected to other *Synechococcus* – heterotrophic bacterial cells through fine pili or cell-

surface gel matrices, forming networks of up to 20 connections (Figure 1.1.3.2). Although the percentage of cells conjoined with heterotrophs in coastal and offshore samples was the same, more networks were observed in coastal than offshore samples (55% and 4% respectively).

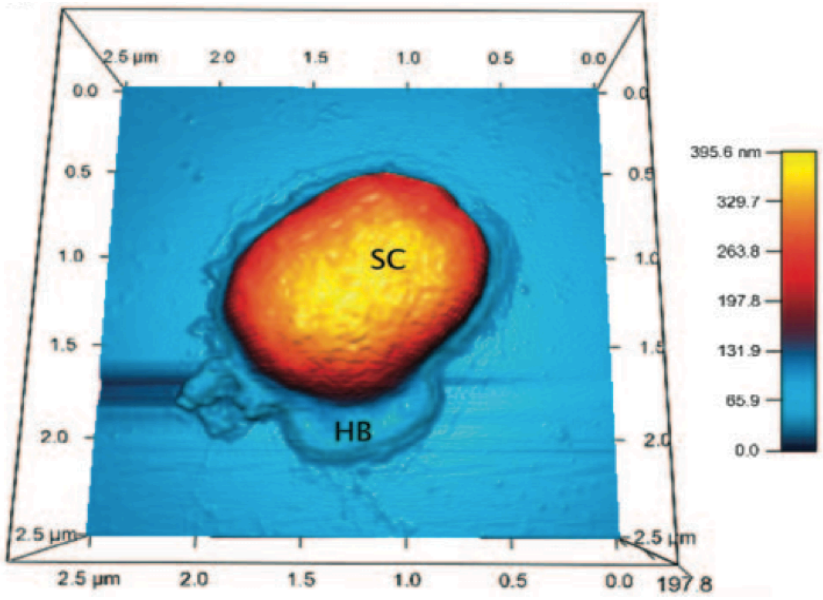


Figure 1.1.3.1 *Synechococcus* cell (SC) conjoint with heterotrophic bacterium (HB). Modified from: Malfatti et al., 2010.

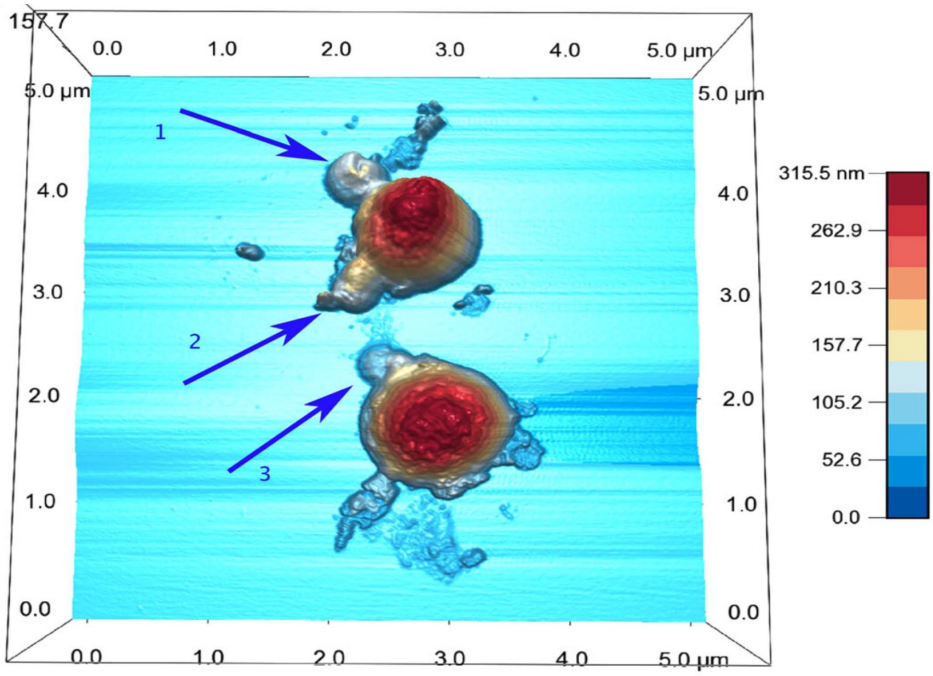


Figure 1.1.3.2 Network of two *Synechococcus* cells conjoint with three heterotrophic bacteria. Modified from: Malfatti & Azam, 2009.

A study of *Synechococcus* sp. WH8102 – *Vibrio parahaemolyticus* interactions suggests that this cyanobacterium had upregulated genes related to photosynthesis (synthesis of chlorophyll and phycobilins), nucleotide biosynthesis, cell wall synthesis, as well as experienced phosphate stress in response to its heterotrophic partner (Tai et al., 2009). Bacteria-induced phosphate limitation has previously been described in diatoms (Guerrini et al., 1998; Thingstad et al., 1993). Downregulated genes included those responsible for amino acid synthesis, proteases, DNA repair (antioxidants), as well as photosynthesis (photosystem II) and transporters (Tai et al., 2009). There is some evidence that the cyanobacterium was using ammonia generated by the heterotroph as a nitrogen source – also described in detail in a recent paper by Christie-Oleza et al (2017).

In a study examining *Synechococcus* sp. PCC 7002 – *Shewanella putrefaciens* in co-culture using transcriptomics, upregulation of carbon fixation genes (including RuBisCo) and downregulation of iron uptake and acquisition genes (suggesting increased availability of iron) were observed (Beliaev et al., 2014). The major metabolites excreted from *Synechococcus* sp. cells were formate, acetate, lactate and alanine. Surprisingly, the study suggests that *Synechococcus* sp. rather than the heterotroph was responsible for scavenging of reactive oxygen species. The heterotroph showed a downregulation of methionine and tryptophan biosynthesis and upregulation of surface attachment factors.

Although not necessarily a phototroph-heterotroph interaction, production of membrane-bound extracellular vesicles by *Prochlorococcus* and *Synechococcus* may also play a role in these types of interactions (Biller et al., 2014). These vesicles may contain proteins, toxins, DNA, RNA and other compounds and one of their hypothesised functions is stimulating heterotrophic growth by releasing food.

1.1.4 *Synechococcus – Roseobacter* interactions

Roseobacter is a diverse heterotrophic bacteria clade, estimated to form about 20% of all bacteria in coastal waters and about 15% in mixed-layer open ocean systems (Buchan et al., 2005), which has metabolically

versatile cells able to compete well with other microorganisms (Moran et al., 2007). *Roseobacter*, SAR11 and SAR86 were found to be dominant in both bloom and non-bloom surface ocean bacterial communities in 9 different studies, despite differences in methodologies (Gonzalez et al., 2000). They also play an important environmental role in regulating the generation of DMS (Zubkov et al., 2001). *Ruegeria pomeroyi*, basonym *Silicibacter pomeroyi* (Yi et al., 2007), was the first major marine heterotrophic bacterial clade to have its genome sequenced (Moran et al., 2004).

Roseobacter genes suggest frequent interactions with neighbouring cells and possibly direct capture of organic matter from eukaryotes - for example vir-related genes encoding a type IV secretion system for translocating DNA or proteins to other cells and close homologs of non-ribosomal peptide synthase genes, which may encode a novel peptide responsible for signalling or host-microbe interactions (Moran et al., 2007). Many interactions between *Roseobacter* strains and other organisms have been described – including with red and green macroalgae, diatoms, bryozoans, dinoflagellates, cephalopods and oysters (Geng & Belas, 2010; Wagner-Dobler & Biebl, 2006). Confocal laser scanning microscopy showed *Roseobacter* cells living intracellularly or as epiphytes with *Pfiesteria*-like heterotrophic dinoflagellate associated with harmful algal blooms (Alavi et al., 2001).

Based on the global importance of *Synechococcus* and *Roseobacter*, as well as indications that both organisms can interact with other microbes, but with a relative dearth of literature on phototrophic-heterotrophic bacterial interactions generally, study of their interaction was instigated.

Christie-Oleza et al. (2017) showed that the presence of *Roseobacter* dramatically increased the long-term survival of *Synechococcus* (Figure 1.1.4.1) irrespective of nutrient levels. This is a result of circulation of nutrients between the two partners in a stable interaction: the phototroph fixes carbon, while the heterotroph remineralises leaked organic matter. Proteomics analysis of *Synechococcus* sp. – *Roseobacter* co-cultures revealed a range of secreted proteins produced by the heterotroph for

hydrolyzing biopolymers (enzymes with peptidase domains or hydrolytic-related functions), transport (to be able to utilise different carbon sources, nutrients and other relevant substrates – transporters of amino acids, oligopeptides, carbohydrates, amines, putrescine, taurine, sulphate, phosphates and iron), motility (flagellar structures) and interaction purposes (Christie-Oleza et al., 2015). Unfortunately, the majority of identified enzymes had no known function. Exoproteomes of heterotrophs did not change much when grown with two different strains of *Synechococcus* sp., with many proteins detected in all 3 strains, which suggests that the relationship may be based on nutrient interactions rather than be species-specific.

Moreover, a metabolite produced by axenic *Synechococcus* cultures, which is absent from the milieu of *Synechococcus* sp. WH7803 – *Ruegeria pomeroyi* co-cultures (Figure 1.1.4.2), was discovered and awaits identification (Christie-Oleza et al., unpublished).

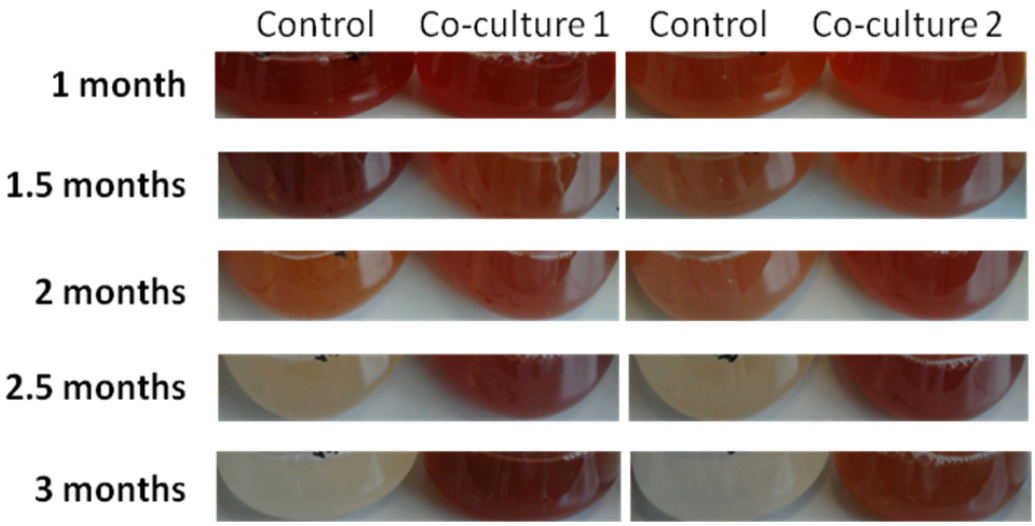


Figure 1.1.4.1 Long term growth of marine *Synechococcus* is dramatically increased in the presence of *Roseobacter*. Control: axenic *Synechococcus* sp. WH7803; Co-culture 1: *Synechococcus* sp. WH7803 – *Ruegeria pomeroyi*; Co-culture 2: *Synechococcus* sp. WH7803 – *Ruegeria lacuscaerulensis.* (Christie-Oleza et al., unpublished).

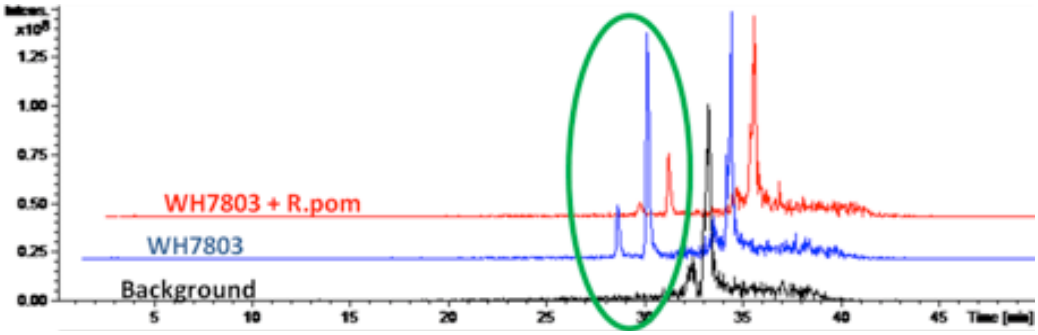


Figure 1.1.4.2 Production of a metabolite (green circle) by axenic *Synechococcus* sp. WH7803 cultures, which is absent from the milieu of *Synechococcus* sp. WH7803 – *Ruegeria pomeroyi* co-cultures. (Christie-Oleza et al., unpublished).

To summarise, despite the major role that marine bacteria play in the global ecosystem, little is known about their interactions with other organisms. Although considerable work has been undertaken to understand marine phototroph – heterotroph interactions, especially for eukaryotic phototrophs, it is still unclear how these relationships form and are maintained in the natural environment, including the molecular basis of how different members of the microbial community rely on one another. The interactions between the second most abundant phototrophic picocyanobacteria and a dominant heterotroph can be studied as a model relationship to improve our understanding of the physiology of marine microbes, interactions between them, as well as the implications these relationships may have for global biogeochemical cycles.

1.1.5 Secondary metabolites produced by marine cyanobacteria

As of 2015, over 1100 secondary metabolites from different structural classes have been isolated from 39 genera of cyanobacteria (Dittmann et al., 2015; Micallef et al., 2015; Figure 1.1.5.1), including those of interest as antimicrobials (Swain et al., 2017), anti-cancer compounds (Leao et al., 2013; Vijayakumar & Menakha, 2015), potential anti-acetylcholinesterase medicines (Carvalho et al., 2013) and agrochemical agents (Peng et al., 2003). Their biological functions include UV-protection, feeding deterrence,

signaling, storage of nitrogen and allelopathy (Leao et al., 2012). A majority (about 90%) of cyanobacterial compounds reported for antimicrobial screens come from the *Oscillatoriales*, *Nostocales* and *Chrococcales* genera (Swain et al., 2017). Most reviews of marine cyanobacterial secondary metabolites focus on filamentous cyanobacteria and pharmaceutical uses of isolated compounds (Burja et al., 2001; Ehrenreich et al., 2005; Lau et al., 2015; Mazur-Marzec et al., 2015; Tan, 2007; Tan, 2013), not on the *Prochlorococcus* and *Synechococcus* genera and the biological functions of these molecules.

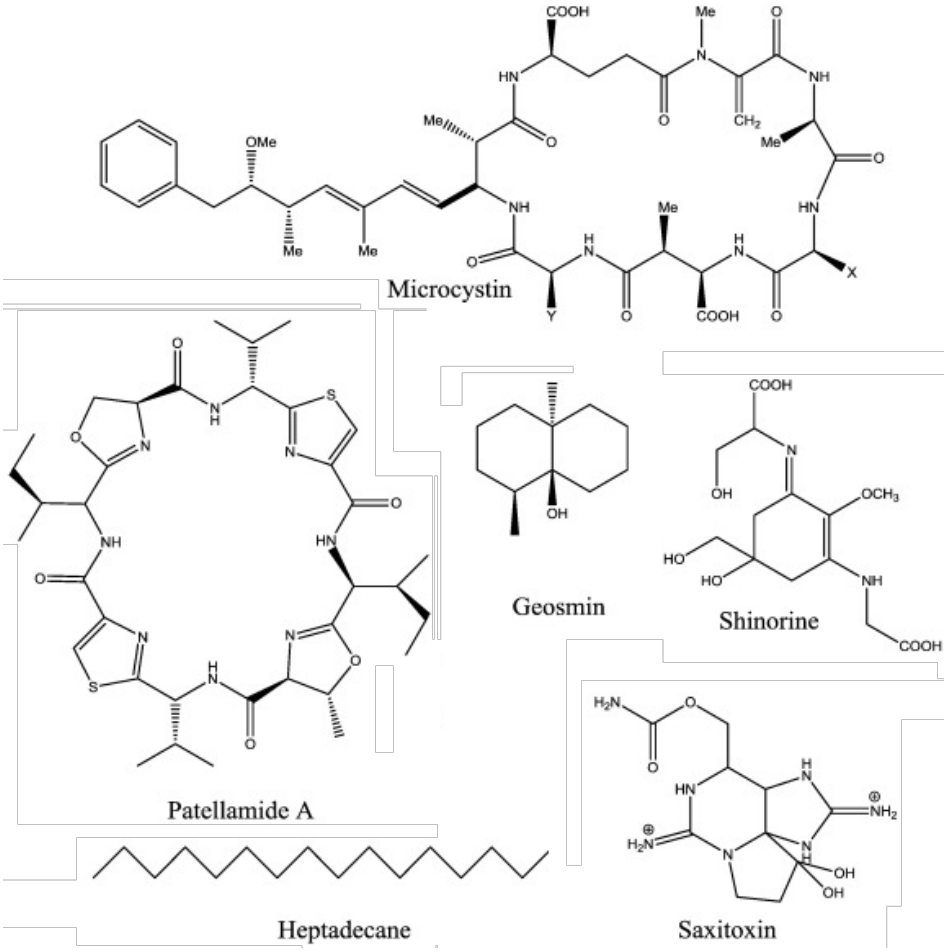


Figure 1.1.5.1 Examples of natural products belonging to different structural classes produced by cyanobacteria. NRPS/PKS compounds – microcystin; ribosomally synthesized and post translationally modified peptides – patellamide A; UV-absorbing compounds – shinorine, alkaloids – saxitoxin, terpenes – geosmin and hydrocarbons – heptadecane. Modified from Micallef et al., 2015.

There is some dispute whether bacteriocins are a true class of secondary metabolites – they are considered as such, but do not have the characteristics of a secondary metabolite, as they are produced during primary growth, are ribosomally synthesized and their antibiotic activity is not of a wide spectrum (Zacharof & Lovitt, 2012). In a study of 58 cyanobacterial genomes, 145 putative bacteriocin gene clusters were identified in 43 cyanobacteria, including *Prochlorococcus* and *Synechococcus* genera (Wang et al., 2011).

Prochlorococcus has been shown to be able to produce prochlorosins - lanthionine-containing peptides (Li et al., 2010; Tang & van der Donk, 2012; Figure 1.1.5.2), a class of extensively modified bacteriocins (Willey & van der Donk, 2007). Using a single, promiscuous biosynthetic enzyme and a range of gene-derived precursors, 29 different secondary metabolites can be produced. However, the biological function of prochlorosins remains unknown. The authors notice, however, that 0.5-5% of *Prochlorococcus* and *Synechococcus* genomes show the ability to produce lanthipeptides, which suggests that there might be thousands of cyanobacterial natural products awaiting discovery.

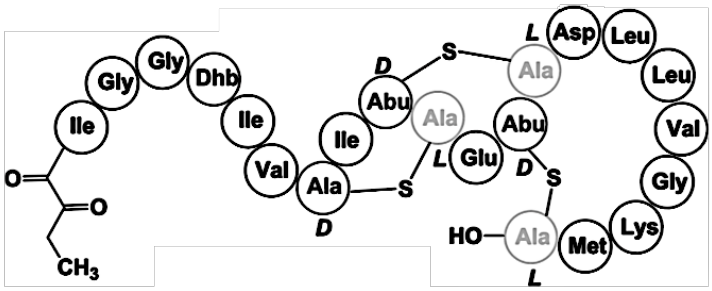


Figure 1.1.5.2 Structure of prochlorosin 1.7. Modified from Tang & van der Donk, 2012.

Some cyanobacteria are capable of producing bacteriocins that can act as antibiotics against marine picocyanobacteria – for example cyanobacterin produced by *Scytonema hofmanni* is effective against *Synechococcus* sp. (Gleason & Paulson, 1984). Over a hundred cyanobactins have been discovered (Sivonen et al., 2010) and a cyanobactin biosynthetic pathway

can be found in 24% of cyanobacterial genomes, but not in *Prochlorococcus* spp. nor *Synechococcus* spp. genomes (Leikoski et al., 2013).

Microcins are bacteriocin-like antibiotics (Baquero & Moreno, 1984). Two *Synechococcus* sp. strains have microcin C-like biosynthethic gene clusters and it has been shown that the product of the gene cluster in *Synechococcus* sp. CC9605 is involved in inhibition of *Synechococcus* sp. WH8102 and CC9311 growth (Paz-Yepes et al., 2013). It has been suggested that if *Synechococcus* sp. CC9605 forms aggregations in the natural environment, as it does in some culture conditions (Apple et al., 2011), production of antibiotics could help in keeping other microorganisms away from the aggregates (Paz-Yepes et al., 2013). A similar observation was made by Long & Azam (2001) that antagonistic interactions are more common between particle-attached than free-living bacteria.

There is also some evidence of microcystin production by *Synechococcus* sp. (Carmichael & Li, 2006) or the presence of *mcyB* gene (marker for detection of microcystins producers) in *Synechococcus* sp., but with no detectable product (Barboza et al., 2017). Microcystins are cyclic heptapeptides produced by a polyketide-peptide biosynthetic pathway (Tillett et al., 2000). Three cyanobacterial genera have been shown to produce microcystins in culture and eight are regarded as putative producers (Cires et al., 2017). There has been a lot of research on microcystins due to their toxicity to a range of organisms, including humans (Ferrao-Filho & Kozlowski-Suzuki, 2011). Although there were many suggestions for their role in the natural environment, including oxidative stress protection, facilitation of carbon dioxide uptake or cell signaling there is no consensus on their ecological and physiological roles (Paerl et al., 2016). In the natural environment, it is even more complicated to define their natural function due to the biological, chemical and physical alterations they undergo, as well as the effect a toxic bloom has on a microbial community (Schmidt et al., 2014).

Prochlorococcus and *Synechococcus* spp. have been shown to live in areas were mycosporine-like amino acids (MAAs), UV protection compounds, can

be detected (Llewellyn, et al. 2012). However, the authors suggest that picocyanobacteria do not have and do not need MAAs as their size is too small for the compounds to be effective.

Synechobactins are produced by *Synechococcus* sp. PCC7002 grown in iron-limited conditions (Ito & Butler, 2005). These are a family of photoreactive amphiphilic siderophores, the first siderophores from marine, unicellular cyanobacteria to have their structure elucidated (Figure 1.1.5.3).

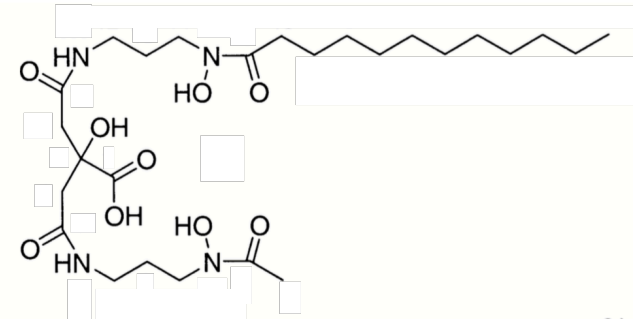


Figure 1.1.5.3 Structure of synechobactin A. Modified from Ito & Butler, 2005.

Approximately 70% of cyanobacterial genomes contain NRPS (Non-Ribosomal Peptide Synthetase) or PKS (Polyketide Synthase) gene clusters (Shih et al., 2013), most of those are filamentous strains (Brito et al., 2015). NRPSs and PKSs are two major classes of natural products with a range of activities, including antibacterials (Cortes et al., 1990), toxins (Chang et al., 2004) and siderophores (Crosa & Walsh, 2002). *Prochlorococcus* and marine *Synechococcus* genera are thought to lack NRPSs and have only Type III PKSs (Shih et al., 2013) – enzymes that catalyze the priming, extension, and cyclization of polyketides (Yu et al., 2012).

T3 PKS enzymes are found in plants, bacteria and fungi and produce a range of compounds: chalcones, pyrones, acridones, phloroglucinols, stilbenes and resorcinolic lipids (Yu et al., 2012). They are small enzymes with single active sites, where a starter molecule is loaded before it undergoes chain extension via decarboxylative condensation using extender units and then intramolecular cyclisation. They are self-containing and form homodimers. Bacterial T3 PKSs usually use malonyl-CoA as

starter and chain extender units, but were also reported to utilise long chain acyl-CoAs as starter units, to use starter units bound to acyl carrier proteins and to use ethylmalonyl-CoA and methylmalonyl-CoA as extender units (Li & Muller, 2009).

1.2 Project objectives

Focusing on the model phototroph *Synechococcus*, this PhD project aimed to identify its most frequently occurring heterotrophic partners and, based on this, a model *Synechococcus* – *Roseobacter* co-culture was used to identify and characterise molecules produced and consumed by the phototroph and the heterotroph. The project is divided into three major objectives:

I: To identify the most-frequently occurring heterotrophic bacterial partners present in non-axenic *Synechococcus* cultures. This involved PCR screening using "general" bacterial primers targeting the 16S rRNA gene of non-axenic *Synechococcus* cultures obtained from culture collections in Europe (RCC, http://www.sb-roscoff.fr/Phyto/RCC) and the US (NCMA, https://ncma.bigelow.org) and new *Synechococcus* isolates obtained during the 23rd Atlantic Meridional Transect cruise (AMT23, http://www.amt-uk.org/Cruises/AMT23).

II: To characterise the specific metabolite(s) present or absent in the milieu during *Synechococcus* – *Roseobacter* co-culture compared to growth of the axenic *Synechococcus* culture. This involved monitoring metabolite presence in different nutrient conditions, assessment of co-cultures with a range of different heterotrophs and elucidation of chemical structures.

III: To determine the biosynthetic gene cluster involved in the production of the molecules produced by *Synechococcus*. This included constructing a biosynthetic null mutant in a gene predicted to be involved in the production of the molecules and subsequent phenotypic characterisation of the mutant.

2. General materials and methods

2.1 Chemicals, equipment and organisms

All chemicals were provided by ThermoFisher Scientific, except the following: HPLC LC-MS grade water (VWR Chemicals), magnesium sulphate anhydrous (Sigma-Aldrich), formic acid eluent additive for LC-MS (Honeywell Fluka), high-resolution agarose (AGTC Bioproducts). All enzymes and other molecular biology products were supplied by ThermoFisher Scientific, unless otherwise stated.

Ultrapure water (18.2 MΩ·cm at 25 °C) was used for preparing solutions and media. For DNA and RNA applications, sterile DNase/RNase-free certified ultrapure distilled water was used. Routine sterilization of solutions, media and materials was performed by autoclaving (121°C, 15 psi, 20 min). Temperature-sensitive solutions of up to 100 ml were sterilized using 0.2 µm surfactant-free cellulose acetate (CA) syringe filters. For volumes of more than 100 ml, 0.22 µm polyethersulfone (PES) 500ml filter systems were used.

All equipment (Table 2.1.1.) was provided by and used at the School of Life Sciences (University of Warwick), except MiSeq sequencer (Warwick Medical School, University of Warwick), high resolution LC-MS (Department of Chemistry, University of Warwick), preparatory HPLC and rotary evaporator (Warwick Integrative Synthetic Biology Centre, University of Warwick).

Table 2.1.1 Details of equipment used in this study.

Type of equipment	Manufacturer	Model
15°C incubator	Panasonic	MIR-254-PE
22°C incubator	Infors HT	multitron
Autoclave	Dixon	Vario3028
Balance – analytical (d=0.0001g)	Mettler Toledo	College B204-S
Balance – toploading (d=0.01g)	Sartorius	BP 2100 S
Column – analytical	Agilent Technologies	Zorbax Eclipse Plus C18 4.6 x 150 mm 5 µm

Type of equipment	Manufacturer	Model
Column – guard	Phenomenex	SecurityGuard kit with C18 ID 3.2-8.0 cartridges
Column – preparatory	Agilent Technologies	PrepHT XDB-C18 21.2 x 100 mm 5 µm
Electrophoresis power supply	BioRad	Power Pac 300
Filtration (<100ml)	Sartorius	0.2 µm Minisart NML
Filtration (>100ml)	Corning	0.22 µm PES 500 ml filter system
Filtration (solvents)	Grace Davidson Discovery Sciences	0.2 µm PVDF centrifuge filters
Flow cytometer	Becton Dickinson	FACScan with CellQuest software
Fluorometer	ThermoFisher Scientific	Invitrogen Qubit 2.0
Gel dock	Syngene	U:Genius3
HPLC (for LC-MS)	Dionex	UltiMate 3000 with quaternary pump and thermostatted autosampler
Light meter	Skye Instruments	Display meter with Lux sensor
Lyser	Retsch	TissueLyser 85220
Magnetic stirrer	Drehzahl Electronic	IKAMAG REO S-6
Microcentrifuge	Eppendorf	5415R with F-45-24-11 rotor
MS (for LC-MS)	Bruker	amaZon SL
MS (high resolution)	Bruker	maXis
pH meter	ThermoFisher Scientific	Accumet AB150 with accuTupH Rugged Bulb pH Combination Electrode
qPCR	ThermoFisher Scientific	AP 7500 Fast Real-Time PCR system
Rotary evaporator	GeneVac	EZ-2 Elite
Safety cabinet	Envair	Bio 2+
Sequencer	Illumina	MiSeq
Spectrophotometer (1-2 ml)	Amersham Pharmacia Biotech	Ultrospec 3000 pro
Spectrophotometer (microvolume)	Thermo Fisher Scientific	NanoDrop 2000
Thermal cycler	BioRad	T100
Ultracentrifuge	Eppendorf	5810 R with A-4-62 rotor
Vortex	Scientific Industries	Vortex Genie 2
Water bath	Grant	WA11221
Water supply	Millipore	Milli-Q Plus 185

All *Synechococcus* spp. axenic cultures (Table 2.1.2) and co-cultures (Table 2.1.3) were provided by the Scanlan lab (University of Warwick). All other phototroph and heterotroph cultures (Table 2.1.2) were provided by the

Christie-Oleza lab (University of Warwick). All activities that involved handling open cultures were performed in a UV-sterilised safety cabinet to minimise the risk of contamination. All experiments were carried out at 22°C (±2°C) unless stated otherwise.

Table 2.1.2 Axenic cultures used in this study.

Organism	Culture Collection ID	Isolation location	Latitude	Longitude	Isolation depth [m]	Reference
Phototrophs						
Emiliania huxleyi AC665	RCC 1242	Pacific Ocean	-2.67	-82.72	-	Hagino et al., 2011
Micromonas commoda NOUM17	RCC 299	Pacific Ocean	-22.34	166.34	0	Fuller et al., 2006
Micromonas pusilla PLY27	RCC 834	North Sea	50.60	-4.28	-	Knight-Jones & Walne, 1951
Ostreococcus tauri OTH95	RCC 745	Mediterranean Sea	43.40	3.60	0	Chrétiennot-Dinet et al., 1995
Phaeodactylum tricornutum Pt Gen	CCMP2561	North Atlantic	54.00	-4.00	-	Lewin, 1958
Prochlorococcus marinus MED4	CCMP1986	Mediterranean Sea	-	-	5	Moore et al., 1995
Synechococcus sp. CC9311	RCC 1086	Pacific Ocean	31.90	-124.17	95	Palenik et al., 2006
Synechococcus sp. WH5701	RCC 1084	Atlantic Ocean	41.19	-73.06	-	Waterbury et al., 1986
Synechococcus sp. WH7803	RCC 752	Atlantic Ocean	33.74	-67.49	25	Waterbury et al., 1986
Synechococcus sp. WH7805	RCC 1085	Atlantic Ocean	33.74	-67.49	-	Waterbury et al., 1986
Synechococcus sp. WH8102	RCC 539	Atlantic Ocean	22.50	-65.60	-	Waterbury et al., 1986
Thalassiosira pseudonana 3H	CCMP1335	North Atlantic	40.76	-72.82	-	Armbrust et al., 2004
Heterotrophs						
Aeromicrobium marinum T2	DSM15272	Wadden Sea	-	-	0	Bruns et al., 2003
Algoriphagus machipongonensis PR1	DSM24695	Atlantic Ocean	-	-	0	Alegado et al., 2013
Alteromonas macleodii ATCC43296	DSM3776	-	-	-	-	Baumann et al., 1972
Dinoroseobacter shibae DFL12	DSM16493	Pacific Ocean	-	-	-	Biebl et al., 2005
Escherichia coli S17-1 λpir	-	-	-	-	-	Simon et al., 1983
Formosa agariphila KMM3901	DSM15362	Pacific Ocean	-	-	-	Nedashkovskaya et al., 2006
Gramella forsetii KT0803	-	North Sea	54.15	7.86	1	Eilers et al., 2001

Organism	Culture Collection ID	Isolation location	Latitude	Longitude	Isolation depth [m]	Reference
Marinobacter adhaerens HP15	DM23420	Wadden Sea	-	-	0	Kaeppel et al., 2012
Planctomyces limnophilus Mu290	DSM3776	-	-	-	-	Hirsch & Muller, 1986
Polaribacter sp. MED152	-	Mediterranean Sea	41.67	2.80	0.5	Gonzalez et al., 2008
Pseudoalteromonas citrea NCMB1889	DSM8771	Mediterranean Sea	-	-	0	Gauthier, 1977
Pseudomonas stutzeri CGMCC 1.1803	ATCC17588	-	-	-	-	Chen et al., 2011
Rhodopirellula baltica SH1	DSM10527	Baltic Sea	-	-	-	Schlesner, 1994
Roseobacter denitrificans OCh114	-	-	-	-	0	Swingley et al., 2007
Ruegeria pomeroyi DSS-3	DSM15171	Atlantic Ocean	-	-	-	Gonzalez et al., 2003
Salinispora tropica CNB-440	DSM44818	Atlantic Ocean	-	-	0	Maldonado et al., 2005
Verrucomicrobiae bacterium DG1235	-	-	-	-	-	Kielak et al., 2009

Table 2.1.3 Non-axenic *Synechococcus* sp. cultures used in this study.

***Synechococcus* sp. strain name**	**Culture collection ID**	**Isolation location**	**Latitude**	**Longitude**	**Isolation depth [m]**	**Reference**
A15-127	RCC 1100	Atlantic Ocean	-31.13	-3.93	45	Mazard et al., 2012
A15-127 Clonal	RCC 2378	Atlantic Ocean	-31.12	-3.92	45	Mazard et al., 2012
A15-130	-	Atlantic Ocean	-34.50	-1.37	20	Mazard et al., 2012
A15-30	-	Atlantic Ocean	31.25	-20.72	40	Mazard et al., 2012
A15-34	RCC 1101	Atlantic Ocean	29.12	-16.97	40	Mazard et al., 2012
A15-37	RCC 1091	Atlantic Ocean	23.56	-19.99	10	Mazard et al., 2012
A15-38	-	Atlantic Ocean	23.56	-19.99	20	Mazard et al., 2012
A15-43	-	Atlantic Ocean	21.68	-17.83	40	Mazard et al., 2012
A15-44	RCC 1104	Atlantic Ocean	21.68	-17.83	20	Mazard et al., 2012
A15-44-Clonal	RCC 2527	Atlantic Ocean	21.68	-17.83	20	Mazard et al., 2012
A15-45	RCC 1096	Atlantic Ocean	21.38	-18.84	10	Mazard et al., 2012
A15-46	RCC 1095	Atlantic Ocean	21.38	-18.84	70	Mazard et al., 2012
A15-60	RCC 1092	Atlantic Ocean	17.84	-20.89	10	Mazard et al., 2012
A15-61	-	Atlantic Ocean	17.84	-20.89	15	Mazard et al., 2012
A15-62	-	Atlantic Ocean	17.84	-20.89	30	Mazard et al., 2012
A15-72	RCC 1093	Atlantic Ocean	7.85	-23.22	15	Mazard et al., 2012
A15-74	RCC 1094	Atlantic Ocean	7.85	-23.22	25	Mazard et al., 2012
A1823	-	Atlantic Ocean	29.70	-34.83	2	Mazard et al., 2012
A1825 R12	-	Atlantic Ocean	27.77	-37.03	74	Mazard et al., 2012
A1825 R8	-	Atlantic Ocean	27.77	-37.03	2	Mazard et al., 2012
ALMO3 Syn	RCC 43	Mediterranean Sea	36.18	-1.85	0	Fuller et al., 2003
AMT23_ST10_12_2	-	Atlantic Ocean	34.95	-20.67	2	Moniz et al., in prep
AMT23_ST10_12_2_P4B2	-	Atlantic Ocean	34.95	-20.67	2	Moniz et al., in prep
AMT23_ST13_17_2	-	Atlantic Ocean	31.30	-22.67	2	Moniz et al., in prep

Synechococcus sp. strain name	Culture collection ID	Isolation location	Latitude	Longitude	Isolation depth [m]	Reference
AMT23_ST13_17_2_P1C3	-	Atlantic Ocean	31.30	-22.67	2	Moniz et al., in prep
AMT23_ST13_17_2_P1C3L	-	Atlantic Ocean	31.30	-22.67	2	Moniz et al., in prep
AMT23_ST3_5_2	-	Atlantic Ocean	45.01	-13.59	2	Moniz et al., in prep
AMT23_ST3_5_2_P4B1	-	Atlantic Ocean	45.01	-13.59	2	Moniz et al., in prep
AMT23_ST37_45_2	-	Atlantic Ocean	-10.00	-27.40	2	Moniz et al., in prep
AMT23_ST37_45_2_P4C2	-	Atlantic Ocean	-10.00	-27.40	2	Moniz et al., in prep
AMT23_ST44_54_2	-	Atlantic Ocean	-21.53	-25.16	2	Moniz et al., in prep
AMT23_ST44_54_2_P6B1	-	Atlantic Ocean	-21.53	-25.16	2	Moniz et al., in prep
AMT23_ST46_56_2	-	Atlantic Ocean	-22.57	-25.91	2	Moniz et al., in prep
AMT23_ST46_56_2_P1C5	-	Atlantic Ocean	-22.57	-25.91	2	Moniz et al., in prep
AMT23_ST46_56_2_P1C6	-	Atlantic Ocean	-22.57	-25.91	2	Moniz et al., in prep
AMT23_ST9_11_2	-	Atlantic Ocean	38.23	-20.10	2	Moniz et al., in prep
AMT23_ST9_11_2_P4A3	-	Atlantic Ocean	38.23	-20.10	2	Moniz et al., in prep
AMT23_ST9_11_2_P4C3	-	Atlantic Ocean	38.23	-20.10	2	Moniz et al., in prep
AMT23_ST9_11_20	-	Atlantic Ocean	38.23	-20.10	20	Moniz et al., in prep
AMT23_ST9_11_20_P1D6	-	Atlantic Ocean	38.23	-20.10	20	Moniz et al., in prep
AMT23_ST9_11_20_P1D6L	-	Atlantic Ocean	38.23	-20.10	20	Moniz et al., in prep
Biosope_109 C2	RCC 1022	Pacific Ocean	-30.05	-98.40	150	Le Gall et al., 2008
Biosope_112 B6	RCC 1023	Pacific Ocean	-30.78	-95.43	100	Le Gall et al., 2008
Biosope_141 D	RCC 1029	Pacific Ocean	-31.87	-91.42	40	Le Gall et al., 2008
Biosope_148 D3	RCC 1028	Pacific Ocean	-31.87	-91.42	40	Le Gall et al., 2008
Biosope_199	RCC 1026	Pacific Ocean	-34.00	-73.37	5	Le Gall et al., 2008
Biosope_199-Clonal	RCC 2533	Pacific Ocean	-34.00	-73.37	5	Le Gall et al., 2008
Biosope_211	RCC 1015	Pacific Ocean	-33.87	-73.33	30	Le Gall et al., 2008
Biosope_45 B5 463	RCC 1017	Pacific Ocean	-9.07	-136.98	100	Le Gall et al., 2008
Biosope_45 B6 465	RCC 1018	Pacific Ocean	-9.07	-136.98	100	Le Gall et al., 2008

Synechococcus sp. strain name	Culture collection ID	Isolation location	Latitude	Longitude	Isolation depth [m]	Reference
Biosope_45 C4Y	RCC 1020	Pacific Ocean	-9.07	-136.98	100	Le Gall et al., 2008
Biosope_48 A2Y	RCC 1030	Pacific Ocean	-9.07	-136.98	30	Le Gall et al., 2008
Biosope_48 B3Y	RCC 1027	Pacific Ocean	-9.07	-136.98	30	Le Gall et al., 2008
Biosope_48 B6Y	RCC 1031	Pacific Ocean	-9.07	-136.98	30	Le Gall et al., 2008
BL_36_Syn	RCC 508	Mediterranean Sea	41.67	2.80	0	Mazard et al., 2012
BL107	RCC 515	Mediterranean Sea	41.72	3.55	1800	Dufresne et al., 2008
BL123-7	-	Mediterranean Sea	41.67	2.80	-	Mazard et al., 2012
BL3	-	Mediterranean Sea	41.67	2.80	-	Mazard et al., 2012
BOUM 118-1	RCC 2379	Mediterranean Sea	33.63	32.63	5	-
CC9605	RCC 753	Pacific Ocean	30.42	-123.98	51	Dufresne et al., 2008
CC9902 (CCMP)	CCMP3074	Pacific Ocean	32.90	-117.26	5	Dufresne et al., 2008
CC9902 (RCC)	RCC 2673	Pacific Ocean	32.15	-117.42	5	Dufresne et al., 2008
EUM Syn14	RCC 37	Atlantic Ocean	21.03	-31.13	105	Fuller et al., 2003
M11.1	RCC 790	Atlantic Ocean	27.70	-91.30	275	Everroad et al., 2006
M16.1	RCC 791	Atlantic Ocean	27.70	-91.30	275	Everroad et al., 2006
MEDNS5	RCC 42	Mediterranean Sea	41.00	6.00	80	Mazard et al., 2012
MICROVIR 10CR_4-3 Clonal	RCC 2385	Atlantic Ocean	61.00	1.98	25	-
MINSyn016-15m-01-AC6A1	RCC 307	Mediterranean Sea	39.17	6.17	15	Dufresne et al., 2008
NOUM97010	RCC 66	Pacific Ocean	0.00	-180.00	30	Mazard et al., 2012
NOUM97012	RCC 67	Pacific Ocean	-22.33	166.33	80	Mazard et al., 2012
NOUM97013 Clonal	RCC 2433	Pacific Ocean	-22.33	166.33	0	-
OLI031FJ Syn	RCC 44	Pacific Ocean	-5.50	-150.00	70	Fuller et al., 2003
PROSOPE_1-9	RCC 394	Atlantic Ocean	30.13	-10.05	5	Mazard et al., 2012
PROSOPE_101	RCC 527	Mediterranean Sea	39.12	14.08	110	Mazard et al., 2012
PROSOPE_107	RCC 524	Mediterranean Sea	39.12	14.08	90	Mazard et al., 2012
PROSOPE_153-3	RCC 325	Mediterranean Sea	43.40	7.82	25	Mazard et al., 2012

Synechococcus sp. strain name	Culture collection ID	Isolation location	Latitude	Longitude	Isolation depth [m]	Reference
PROSOPE_157-13	RCC 320	Mediterranean Sea	43.40	7.82	15	Mazard et al., 2012
PROSOPE_157-21	RCC 319	Mediterranean Sea	43.40	7.82	15	Mazard et al., 2012
PROSOPE_2-16	RCC 442	Atlantic Ocean	30.13	-10.05	5	Mazard et al., 2012
PROSOPE_25-2	RCC 327	Mediterranean Sea	38.00	3.83	25	Mazard et al., 2012
PROSOPE_32-1	RCC 321	Mediterranean Sea	38.00	3.83	5	Mazard et al., 2012
PROSOPE_37-2	RCC 316	Mediterranean Sea	38.00	3.83	110	Mazard et al., 2012
RA000711-27-14	RCC 359	Atlantic Ocean	48.75	-3.95	0	Mazard et al., 2012
ROS8604 Syn	RCC 32	Atlantic Ocean	48.72	-3.98	0	Jacquet et al., 2001
RS9901	RCC 540	Red Sea	29.47	34.92	1	Fuller et al., 2003
RS9902	RCC 541	Red Sea	29.47	34.92	1	Fuller et al., 2003
RS9916	RCC 555	Red Sea	29.47	34.92	10	Fuller et al., 2003
RS9917	RCC 556	Red Sea	29.47	34.92	10	Fuller et al., 2003
Syn 20	RCC 2035	Atlantic Ocean	60.62	5.68	0	Mazard et al., 2012
TAK9802	RCC 262	Pacific Ocean	-14.50	-145.33	7	Mazard et al., 2012
TAK9802-Clonal	RCC 2528	Pacific Ocean	-14.50	-145.33	7	Mazard et al., 2012
WH8016	RCC 2032	Atlantic Ocean	41.52	-70.67	-	Waterbury et al., 1986
WH8018	RCC 2373	Atlantic Ocean	41.52	-70.67	-	Waterbury et al., 1986
WH8020	RCC 751	Atlantic Ocean	38.68	-69.32	50	Waterbury et al., 1986
WH8101 Clonal	RCC 2555	Atlantic Ocean	41.52	-70.67	-	Waterbury et al., 1986

2.2 Culturing

Synechococcus spp. liquid cultures were grown at 22°C (±1°C) in Artificial Sea Water (ASW) medium (Wyman et al., 1985) under constant light of 5-30 µmol photons m^{-1} s^{-1} and shaking at 140 rpm, unless stated otherwise. For nutrient limitation experiments, modified ASW medium was used (Table 2.2.1.). For cultures of less than 35 ml, 50 ml polystyrene vented tissue culture flasks were used (Falcon). Cultures of 35 ml or more were kept in 250 ml, narrow neck, clear, borosilicate glass Erlenmeyer flasks (Bomex) closed with sterile cotton wool and covered with two layers of aluminium foil.

Table 2.2.1 Modifications of ASW medium for nutrient limitation experiments. All values are per 1 l of media.

Component	ASW	ASW -N	ASW -P	ASW -Fe
NaCl	25 g	25 g	25 g	25 g
$NaNO_3$	0.75 g	-	0.75 g	0.75 g
$MgCl_2•6H_2O$	2 g	2 g	2 g	2 g
KCl	0.5 g	0.5 g	0.5 g	0.5 g
$CaCl_2•2H_2O$	0.5 g	0.5 g	0.5 g	0.5 g
$MgSO_4•7H_2O$	3.5 g	3.5 g	3.5 g	3.5 g
$NH_2C(CH_2OH)_3$	1.1 g	1.1 g	1.1 g	1.1 g
K_2HPO_4	0.03 g	0.03 g	-	0.03 g
H_3BO_3	2.86 mg	2.86 mg	2.86 mg	2.86 mg
$MnCl_2•4H_2O$	1.81 mg	1.81 mg	1.81 mg	1.81 mg
$ZnSO_4•7H_2O$	0.222 mg	0.222 mg	0.222 mg	0.222 mg
$Na_2MnO_4•2H_2O$	0.390 mg	0.390 mg	0.390 mg	0.390 mg
$CuSO_4•5H_2O$	0.008 mg	0.008 mg	0.008 mg	0.008 mg
$Co(NO_3)_2•6H_2O$	0.0494 mg	0.0494 mg	0.0494 mg	0.0494 mg
$FeCl•6H_2O$	3 mg	3 mg	3 mg	-
$EDTA(Na_2Mg)$	0.5 mg	0.5 mg	0.5 mg	0.5 mg

To maintain cultures, 10% (v/v) of an old culture was inoculated into fresh, sterile medium every 21 days. Axenic cultures were checked for contamination by spreading 20 µl of culture onto half of a contamination plate. Contamination plates were prepared by dissolving 0.8 g of yeast extract and 10 g of agar in 1 l of ASW, autoclaved and pouring 20 ml per sterile 92 x 16 mm transparent polystyrene Petri dish with ventilation cams (Sarstedt). The other half of the contamination plate was used as a control of sterility of the plate itself. Plates were sealed with plastic paraffin film (Parafilm M, Bemis) and kept for at least 21 days at 22°C (±1°C). In the

case of growth on a contamination plate, corresponding *Synechococcus* spp. liquid cultures were destroyed.

For growth of *Synechococcus* spp. in solid media, purified agar was prepared using the water-ethanol-acetone wash method (Millard, 2009). SN medium (Waterbury et al., 1986; Table 2.2.2) was supplemented with 3 g of purified agar per 1 l of medium and poured into sterile 50 ml clear polypropylene conical centrifuge tubes (Falcon), 35 ml per tube. The tubes were kept in a 37°C (±1°C) water bath for at least 15 min to ensure temperature stability. A volume of liquid *Synechococcus* spp. culture (calculated based on the required final colony count and assuming 1% plating efficiency) was added to the tubes and the tubes were gently inverted three times to mix the cells with the medium before pouring the contents onto a sterile 92 x 16 mm transparent polystyrene Petri dish with ventilation cams. The plates were left unsealed for at least 30 minutes for the agar to cool down and solidify. Once solid, the plates were sealed with plastic paraffin film and kept at 22°C (±1°C), 5 µmol photons m^{-1} s^{-1} for the first 48 hours and at 22°C (±1°C), 20 µmol photons m^{-1} s^{-1} after that.

Table 2.2.2 SN medium composition.

Component	Ammount per 1 l
$NaNO_3$	0.75 g
K_2HPO_4 (anhydrous)	15.86 mg
$Na_2EDTA{\cdot}2H_2O$	5.6 mg
Na_2CO_3	10.4 mg
Vitamin B_{12}	1 µg
$C_6H_8O_7{\cdot}H_2O$	6.25 mg
$(NH_4)_5[Fe(C_6H_4O_7)_2]$	6 mg
$MnCl_2{\cdot}4H_2O$	1.4 mg
$Na_2MoO_4{\cdot}2H_2O$	0.39 mg
$Co(NO_3)_2{\cdot}6H_2O$	0.025 mg
$ZnSO_4{\cdot}7H_2O$	0.222 mg

Ruegeria pomeroyi liquid cultures were grown in Marine Broth (MB) medium (37.4 g of Marine Broth dissolved in 1 l of water, boiled for 1 min, autoclaved and sterile filtered to remove any precipitate) at 29°C (±1°C), shaking at 140 rpm, in sterile 50 ml clear polypropylene conical centrifuge tubes. For growth on solid medium, a liquid culture of *R. pomeroyi* was streaked onto a

MB plate (37.4 g of Marine Broth and 10 g of agar dissolved in 1 l of water, boiled for 1 min, autoclaved, sterile filtered to remove any precipitate and poured onto Petri dishes, 20 ml per plate). Plates were kept at 29°C (±1°C).

Escherichia coli liquid cultures were grown in a shaking incubator, in Lysogeny Broth (LB) medium (Bertani, et al., 1951) at 32°C (±1°C), 140 rpm, in sterile 50 ml clear polypropylene conical centrifuge tubes. For growth on solid medium, a liquid culture was streaked onto a LB plate (10 g tryptone, 5 g yeast extract, 10 g NaCl and 10 g agar dissolved in 1 l of water, autoclaved and poured onto Petri dishes, 20 ml per plate). Plates were kept at 32°C (±1°C).

2.3 Growth assessment

Growth of *Synechococcus* sp. cultures was assessed using Optical Density (OD) at 750 nm measured on a spectrophotometer (Henley and Yin, 1998). A volume of 1-2 ml of culture was transferred to a semi-micro polystyrene 10 mm light path cuvette (ThermoFisher Scientific). All measurements were taken against a blank of sterile growth medium. In the case of $OD_{750\ nm}$ readings higher than 1, cultures were diluted appropriately.

Cells were counted using a flow cytometer with single laser excitation at 488 nm and red (650 LP) and orange (585/42 nm) fluorescence filters on a high flow rate setting. Cultures were diluted with ASW to obtain no more than 300 cells in a gate per 100 fluorescent beads (1.7-2.2 µm High Intensity Nile Red Fluorescent particles; BD Biosciences). The flow cytometer was cleaned before and after use with FACSClean and FACSRinse (BD Biosciences) and ultrapure water was ran between samples to prevent carryover.

Growth of *R. pomeroyi* and *E. coli* cultures was assessed using OD at 600 nm measurements on a spectrophotometer in a semi-micro polystyrene 10 mm light path cuvette against a blank of an appropriate sterile growth medium, with dilution for values above 1. In the case of co-cultures,

presence of *R. pomeroyi* or *E. coli* was checked by streaking a co-culture onto a MB or LB plate respectively, as described in section 2.2.

2.4 DNA extraction, amplification and sequencing

DNA extraction of *Synechococcus* sp. cultures and co-cultures was performed using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Cells were lysed mechanically using 2 ml Lysing Matrix E centrifuge tubes (MP Biomedicals) and three 30 s runs of the tissue lyser at 30 Hz. In between runs, samples were kept on ice for 1 min. Purified DNA was stored at -20°C as two aliquots until required.

Routine Polymerase Chain Reaction (PCR) was done using MyTaq Mix (Bioline), according to the manufacturer's instructions, with annealing temperature being 5°C below the lower of the melting temperatures of the primers used (Table 2.4.1). All primers were designed manually (17-21 bp, 40-60% GC content, GC clamp, no secondary structure, no more than 4 runs, no more than 4 di-nucleotide repeats, similar melting temperatures for primer pairs) and tested for specificity using BLAST (Altschul et al., 1990) against the genomes of *Synechococcus* spp. WH7803, *E. coli* and *R. pomeroyi*. Melting temperatures were calculated using the online IDT Technologies Oligo entry tool prior to ordering (25 nmol, standard desalting). All reactions were run with a no DNA template control. PCR products for sequencing were purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and sent for LightRun Standard sequencing by GATC following submission instructions.

For 16S rRNA gene sequencing of *Synechococcus* spp. and *Prochlorococcus* spp. co-cultures, samples were prepared following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, 2014) using Nextera XT Index kit with generic primers (Klindworth et al., 2013) and Kapa High Fidelity Hot Start reaction mix (Kapa Biosystems). The optional verification of size was done by running 4µl of PCR products on a 1.3% (w/v) agarose TAE (40mM Tris, 20mM acetic acid, and 1mM EDTA) gel (140V, 30 min) and comparing the size of products with a Quick-Load 100

bp DNA ladder (New England Biolabs). The library was quantified using Qubit dsDNA High Sensitivity Assay (Life Technologies) and sequenced on MiSeq M01757 (MiSeq Control 2.5.0.5; GenerateFASTQ workflow) with MiSeq Reagent Kit v2 500-cycles (Illumina). Reads were merged, quality filtered, dereplicated and clustered using USEARCH 10.2.240 (Edgar, 2013) and VSEARCH 2.4.3 (Rognes et al., 2016). Data was analysed using QIIME 1.9.1 (Caporaso et al., 2010).

Table 2.4.1 Properties and sequences of PCR primers designed for the study.

Primer name	Target organism	Sequence (5'-3')	Restriction site	Melting temperature [°C]
pgp3463_F	*E. coli* plasmid *Synechococcus* sp. mutants	GGG AAT AAG GGC GAC ACG G	-	69
pgp3533_F	*E. coli* plasmid *Synechococcus* sp. mutants	CAG GGT TAT TGT CTC ATG	-	53
cmc107_R	*E. coli* plasmid *Synechococcus* sp. mutants	GAT TCA GGT TCA TCA TGC	-	56
cmc806_F	*E. coli* plasmid *Synechococcus* sp. mutants	CTT CCC GGT ATC AAC AG	-	56
PKS7803_252_XbaF	*Synechococcus* sp. WH7803	TT**T CTA GA**T CGC GTT TCA GCG TCA TGC	***Xba*I**	75
PKS7803_779_XbaR	*Synechococcus* sp. WH7803	TT**T CTA GA**C GGT CTG CGG CAC CTG GG	***Xba*I**	78
PKS7803_118_F	*Synechococcus* sp. WH7803	CTG CAG CGC ATT CAT CAG CG	-	73
PKS7803_835_R	*Synechococcus* sp. WH7803	GTG TGA GAT CCC AAG CAC CC	-	67
WH7803_CHS_F	*Synechococcus* sp. WH7803	GAA CTA ATG CAC TGG CGA ATC	-	54
WH7803_CHS_R	*Synechococcus* sp. WH7803	CAA GCA CCC AAC CAA TCA TC	-	54
7803_454_F	*Synechococcus* sp. WH7803	ATC GAG GAA GAC GGG TAT CT	-	55
7803_454_R	*Synechococcus* sp. WH7803	AAA GGT TGC GGG TTC TGT	-	55

2.5 RNA extraction, generation of cDNA, qPCR

To extract RNA, 15 ml of cultures were centrifuged for 15 minutes at 3220 g, supernatant was discarded and cell pellets were flash frozen in liquid nitrogen. Samples were stored at -80°C until further processing. RNA extraction was performed using a RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Cells were lysed in the same way as for DNA extractions (see section 2.4). Any residual DNA was removed using TURBO DNA-free Kit (Life Technologies) according to the manufacturer's instructions. Purified RNA was stored at -20°C until required.

To generate cDNA, all purified RNA samples were quantified using a microvolume spectrophotometer. Samples were diluted with DNA/RNA/RNase free water to the concentration of the sample with lowest amount of RNA. cDNA was generated according to the SuperScript III Reverse Transcriptase manufacturer's instructions (random hexamer primer protocol), but using Ribolock RNase inhibitor instead of the recommended RNaseOUT Recombinant RNase Inhibitor. The final product was aliquoted to create an undiluted stock stored at -80°C and undiluted, 1:10 and 1:100 diluted stocks to be stored at -20°C for further use.

qPCR was performed to study the expression of the *Synechococcus* sp. WH7803 T3 PKS gene (*synWH7803_1003*) relative to a housekeeping gene (phosphoenol pyruvate carboxylase: *synWH7803_454*). The reaction was prepared using FastSYBR Green Master Mix, according to the manufacturer's instructions (polymerase activation at 95°C for 20 s and 40 cycles of denaturing at 95°C for 3 s and annealing/extending at 60°C for 30 s), in DNA/RNA/RNase free MicroAmp Fast 96-well reaction plate (ThermoFisher Scientific) sealed with MicroAmp optical adhesive film (ThermoFisher Scientific). Concentrations of DNA for forming a standard curve were determined using a fluorometer (Qubit dsDNA High Sensitivity Assay). Primers were designed in the same way as for standard PCR (section 2.4) for the T3 PKS gene synWH7803_1003 (primers WH7803_CHS_F and WH7803_CHS_R; predicted product size 116 bp;

Table 2.4.1) and the housekeeping gene *synWH7803_454* (7803_454_F and 7803_454_R; predicted product size 108 bp; Table 2.4.1). Optimal concentrations of primers were determined by testing a range of concentrations and choosing the concentration with highest efficiency and no extra products visible on the melting curve. For T3 PKS primers at 100 nM and phosphoenol pyruvate carboxylase primers at 200 nM, the efficiencies were 99.14% and 101.40% respectively with a single amplicon. All samples were ran as three technical replicates. Every run included two controls – no reverse transcriptase reaction and no template reaction. Data was analysed using the AB7500 2.0.6 software and the expression level was normalized to that of the housekeeping gene using the Livak method equation (Livak & Schmittgen, 2001):

$$2^{\Delta\Delta CT}=2^{[CT(target,untreat)-CT(ref,untreat)-(CT(target,treat)-CT(ref,treat)]}$$

where:

CT=cycle threshold value;

CT(target,untreat)=CT value of gene of interest in untreated sample;

CT(ref,untreat)=CT value of control gene in untreated sample;

CT(target,treat)=CT value of gene of interest in treated sample;

CT(ref,treat)= CT value of control gene in treated sample.

2.6 Assessment of metabolite production

Routine checks of exometabolite production were done using 200 µl of 0.2 µm filtered culture supernatant. The supernatant was combined with 200 µl of methanol and filtered using PVDF centrifuge filters for 5 min at 3700 g. The flow through was transferred into a flat bottom 31mm x 6mm clear glass insert (ThermoFisher Scientific) in a 2 ml snap-seal amber glass autosampler vial with 11 mm clear polypropylene cap with PTFE liner (Thames Restek). Samples were analysed by Liquid Chromatography coupled with Electrospray Ionisation (ESI) Mass Spectrometry (LC-MS) using methods “30 mins 5 95 cg7 1000ul” and “positive 100 1000 400 sf 200

ul" (Tables 2.6.1-2.6.2), HPLC grade water and methanol with 0.1% formic acid, injection of 10 µl, a guard and analytical columns. Every run of samples started with and ended with a blank and a medium control, with blanks also ran in between samples from different experiments and after particularly concentrated samples (e.g. extract dilutions) or samples of unknown concentration. Data was analysed using Bruker DataAnalysis 4.2 software. Baseline was subtracted (flatness 0.8) and chromatograms were smoothed (Gauss algorithm, width 2, cycles 1) before exporting.

As the structures of the molecules are not known, there are no standards available that could be used for quantification. All comparisons between samples within the same experiment, analysed in a single run, were done based on peak intensity. A positive control from the same culture of *Synechococcus* sp. WH7803 with high level of the molecules was ran with every batch of samples throughout the project to ensure consistency of the results generated. Although no inter-experiment comparisons of levels of metabolites were performed, the positive control was an indicator of consistency of the method, equipment and conditions, as well as stability of the compound.

Table 2.6.1 Major parameters of HPLC methods.

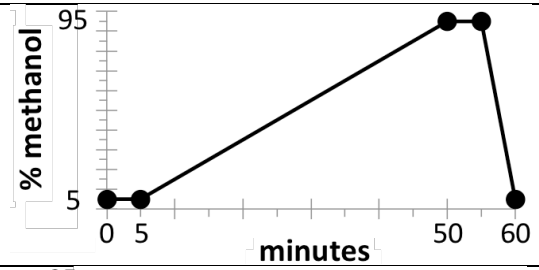
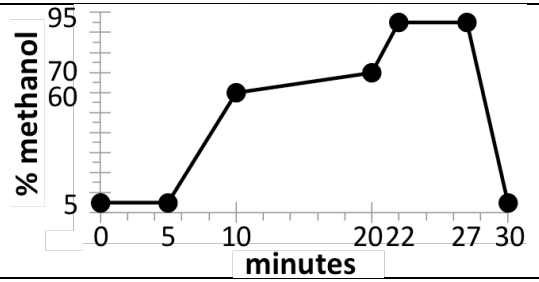
Method name	Flow rate [ml min^{-1}]	Gradient	Pressure limits [bar]	Column oven [°C]	Sampler dispense speed [µl s^{-1}]	Injection wash	Fraction collection
60 mins 5 95 1000ul	1		2-240	25	2	after draw, 100 µl s^{-1}	no
30 mins 5 cg7 95 1000ul	1		2-240	38	2	after draw, 100 µl s^{-1}	no

Table 2.6.2 Major parameters of MS methods.

Method name	Polarity	Ion Charge Control	Scan range [m/z]	Capillary [V]	End plate offset [V]	Nebulizer	SPS target [m/z]	MS(n)
positive 100 1000 400 sf 200 ul	positive	target 100000; max accumulation time 50 ms	100-1000	4500	500	15 psi; 8 l min^{-1}; 260°C	400	off
positive 15 500 300 sf 200 ul ms	positive	target 100000; max accumulation time 50 ms	15-500	4500	500	15 psi; 8 l min^{-1}; 260°C	300	auto MS^5
positive HR 50 3000	positive	n/a	50-3000	4500	500	23 psi; 8 l min^{-1}; 180°C	n/a	off

For large volume organic solvent extraction, the pH of 1.5 l of a culture supernatant was adjusted to 3 with HCl, unless stated otherwise. The supernatant was transferred to a 2 litre borosilicate glass separating funnel (Pyrex) and 500 ml of ethyl acetate was added. The funnel was vigorously shaken for 3 min and then left for 15 min on a retort stand for phases to separate. The ethyl acetate phase was collected and the aqueous phase was poured back into the separating funnel with another 500 ml of ethyl acetate added to repeat the extraction. Water was removed from the combined ethyl acetate phases using anhydrous magnesium sulphate. The extract was filtered through a cellulose filter paper (Whatman) into borosilicate glass test tubes (Pyrex) and ethyl acetate was evaporated using a rotary evaporator on a "low BP" programme with a maximum temperature of 30°C for 1 hour. Dry extract was resuspended in 2 ml of 50% methanol for further processing. A 1:10 dilution of extracts was analysed by LC-MS using methods "60 mins 5 95 1000 ul" and "positive 100 1000 400 sf 200" (Tables 2.6.1-2).

2.7 Chemical formula determination and MS^n fragmentation

The supernatant extracts of *Synechococcus* sp. WH7803 were ran on the LC-MS to identify the fragmentation pattern of the compounds of interest. Using method "30 min 5 95 cg7 1000ul" (Table 2.6.1), samples were ran in positive polarity mode ("positive 15 500 300 sf 200 ul ms", Table 2.6.2) to identify ions of the compounds and then to fragment them. Two precursor ions of the highest intensity were chosen at every step for further fragmentation.

Chemical formulae for compounds of interest were determined by running a *Synechococcus* sp. WH7803 supernatant sample on LC-MS with high resolution MS ("60 mins 5 95 1000ul " and "positive HR 50 3000" methods, see Tables 2.6.1-2.6.2) at the Department of Chemistry, University of Warwick. Possible formulae were generated using Bruker DataAnalysis 4.2 SmartFormula Manually function with tolerance of 4 ppm, M+H adduct and automatic location of monoisotopic peak. For every compound of interest, a

formula was chosen based on lowest formula error score and best isotopic pattern fit, as well as agreement with the MS^n fragmentation of the molecule data, if available.

2.8 Genetic modifications of *Synechococcus* sp.

To determine whether a specific gene plays a role in the production of the molecules of interest, *Synechococcus* sp. WH7803 can be modified genetically. One of the methods of gene inactivation is insertion of a plasmid into the gene, which will disrupt the sequence inhibiting effective transcription and translation, while at the same time introducing a marker that can be used for selection of the mutant over the wild type strain. *E. coli* strains carrying kanamycin resistance can be used for conjugation with *Synechococcus* sp. WH7803. To be able to isolate single colonies that survive kanamycin, Synechococcus sp. has to be pour plated. Given poor survival of pour plating and low efficiency of conjugation, accumulation of organic matter in the medium due to cell death can decrease the success rate of the process. To improve the chances of survival of genetically modified *Synechococcus* sp. WH7803, a helper heterotrophic bacterium, resistant to the selectable marker, such as kanamycin-resistant strain of *R. pomeroyi*, can be introduced during pour plating to provide recycling of accumulated organic matter. As an axenic *Synechococcus* sp. WH7803 genetically modified culture is required at the end of the process, the heterotroph helper has to be sensitive to another marker, such as sucrose, to enable its removal once the mutant strain is growing. After confirmation of presence of the plasmid in the disrupted gene and absence of any wild type copies of the gene by PCR, the axenic genetically modified culture can be used for further experiments.

A *Synechococcus* sp. WH7803 mutant in the gene encoding a type 3 polyketide synthase *synWH7803_1003* was constructed. A T3 PKS gene fragment of approximately 500 bp was amplified by PCR (95°C for 3 min followed by two cycles of 95°C for 30s, 60°C for 30 s and 72°C for 20 s and 30 cycles of 95°C for 30 s and 72°C for 50s and a final step of 72°C for 5

min) with primers PKS7803_252_XbaF and PKS7803_779_XbaR (Table 2.4.1). The product was purified using the Purelink PCR Purification kit (ThermoFisher Scientific) and digested with FastDigest *Xba*I restriction enzyme according to manufacturers' instructions. A part of the digest was ran on a 1% agarose TAE gel (140V, 30 min) with GeneRuler 1kb DNA ladder to confirm the presence and size of the product.

E. coli S17-1 λpir pGP704 (kanamycin, chloramphenicol and ampicillin resistant, Figure 2.8.1; Table 2.8.1) was grown in a liquid culture overnight. Plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen), digested with FastDigest *Xba*I restriction enzyme, purified using the Purelink PCR Purification kit and dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase according to manufacturers' instructions. The dephosphorylated plasmid was centrifuged (16100 g, 5 min) and part of the sample was ran on a 1% agarose TAE gel (140 V, 30 min) with GeneRuler 1kb DNA ladder to confirm the presence of a product.

Table 2.8.1 Plasmids used in the study.

Organism	Plasmid	Important features	Insert	Reference
E. coli S17-1 λpir	pGP704	*mob* gene, ampicillin resistance gene, polylinker	chloramphenicol and kanamycin resistance genes at polylinker *Sma*I site	Miller & Mekalanos, 1988
E. coli S17-1 λpir	pAD34	same as pGP704, extra gene fragment insert	middle fragment (252-779 bp) of *synWH7803_1003* gene at polylinker *Xba*I site	This study
R. pomeroyi DSS-3	pBBR1MCS-2	*mob* gene, kanamycin resistance gene, polylinker	*sacB* gene at polylinker *Sal*I site	Kovach et al., 1994; Kovach et al., 1995
R. pomeroyi DSS-3	pKNG101	*mob* gene, streptomycin resistance gene, *sacB* gene	-	Kaniga et al., 1991

The digested T3 PKS gene fragment (11 µl) and dephosphorylated digested pGP704 plasmid (3 µl) were combined and ligated using T4 DNA ligase (Promega) following buffer and enzyme concentrations recommended by the manufacturer, but with incubation at 14°C for 17 hours. Competent *E. coli* S17-1 λpir cells were received from J. Christie-Oleza (University of Warwick). Once thawed, 20 µl of the ligated plasmid was added to the cells. The cells were incubated on ice for 40 min, then heat shocked at 42°C for 45 s and again incubated on ice for 15 min. 1 ml of LB medium was added to the cells before incubation at 37°C for 60 min. 100 µl of the culture were spread on a LB plate containing ampicillin (100 µg ml^{-1}) and kanamycin (50 µg ml^{-1}). The remaining 900 µl of culture were centrifuged (16100 g, 5 min), the supernatant was discarded and the pellet spread on a second LB ampicillin kanamycin plate. The plates were incubated overnight at 30°C. 40 colonies were picked and transferred to a fresh LB ampicillin kanamycin plate and incubated for another 8 hours at 30°C. 10 colonies were picked and used to inoculate 3 ml of LB containing ampicillin (100 µg ml^{-1}) and kanamycin (50 µg ml^{-1}) each and were incubated overnight at 30°C, 140 rpm. A control of *E. coli* without the insert was also inoculated into 3 ml of LB. The following day, plasmids were extracted from the liquid cultures using a QIAprep Spin Miniprep kit according to manufacturer's instructions and parts of the samples were ran on a 1% (w/v) agarose TAE gel (140V, 30 min) with GeneRuler 1kb DNA ladder to check for the presence of the insert based on a change in length. Two plasmids demonstrating the change and were chosen for Fastdigest *Xba*I and *Sma*I digestion to confirm the presence of the insert. Together with a control of plasmid with no insert, the plasmids were digested according to restriction enzymes manufacturer's instructions and the digests were ran on a 1% (w/v) agarose TAE gel (140V, 30 min) with GeneRuler 1kb DNA ladder. Both plasmids demonstrated band patterns expected for a plasmid with the insert, one was chosen for further work and labelled pAD34 (Table 2.8.1).

The colony with confirmed insert and an insert-free control were used to inoculate 7.5 ml of LB each for overnight incubation at 30°C. 200 ml of *Synechococcus* sp. WH7803 culture were centrifuged (3220 g, 10 min) and the supernatant was discarded. The cells were resuspended in 300 µl of SN medium. *E. coli* overnight cultures were centrifuged (3220 g, 10 min), the supernatants were discarded and the pellets were resuspended in 500 µl of SN medium containing 10% (v/v) LB each. A mixture of 50 µl *Synechococcus* sp. WH7803 cells : 50 µl *E. coli* cells with the insert was spotted onto a centre of a 0.6% (w/v) purified agar SN plate (sterile SN medium with 6 g of purified agar per 1 l of medium, 20 ml per Petri dish). The process was repeated with 25 µl : 50 µl and 10 µl : 50 µl ratios (*Synechococcus* sp. : *E. coli*) and with *E. coli* cells without the insert. Plates were left to dry for 30 minutes, sealed with plastic paraffin film and incubated for 48 hours at 22°C (±1°C), 5 µmol photons m^{-1} s^{-1}.

The spots were resuspended in 1 ml of ASW and the cells were pour plated as described in section 2.2. Every tube with 0.3% purified agar SN medium was supplemented with 100 µl of *R. pomeroyi* DSS-3 pBBR1MCS-2 pKNG101 (kanamycin resistant, sucrose sensitive; Table 2.8.1) culture (10 ml overnight MB culture, centrifuged at 3220 g for 10 min, supernatant discarded and pellet resuspended in 2 ml ASW), 30 µl of kanamycin (50 mg ml^{-1} stock) and inoculated with either 100 µl or 900 µl of the resuspended cell spot. The contents were poured into Petri dishes, left to dry, sealed and incubated at low light for the first 48 hours.

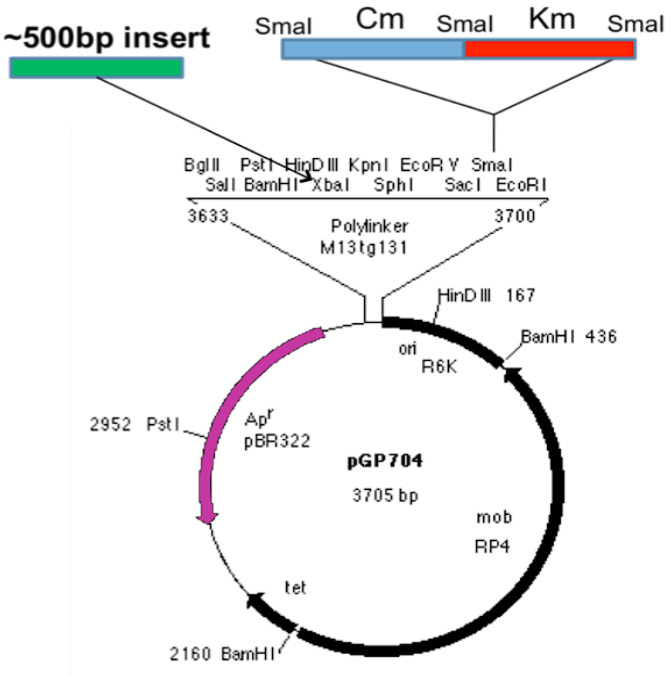


Figure 2.8.1 *E. coli* plasmid pGP704 with kanamycin (red), chloramphenicol (blue) and ampicillin (pink) resistance used for conjugation: 500bp insert (green) is the middle part of the T3 PKS gene.

Colonies which appeared after at least 21, but no more than 60 days, were transferred into 3 ml of liquid SN medium with 25 µg ml^{-1} kanamycin each. After 3 weeks, 1 ml of every culture was used for DNA extraction as described in section 2.4. Plasmid presence and the segregation of mutants was checked by PCR with pgp3533_F and PKS7803_835_R (approximately 600 bp product) and PKS7803_252_XbaF – PKS7803_835_R primers (approximately 600 bp product) respectively. Cultures confirmed as having the plasmid and segregated were pour plated (section 2.2) in 1% purified agar SN with 1% (w/v) sucrose and 100 µg ml^{-1} ampicillin to remove *R. pomeroyi*. Colonies that appeared were transferred into 3 ml of liquid SN each and tested for the presence of *R. pomeroyi* and *E. coli* by streaking the liquid culture onto MB and LB plates respectively (section 2.3). An axenic culture was chosen for further work and was routinely maintained as described in section 2.2, but with additional 50 µg ml^{-1} of kanamycin.

3. Interactions of *Synechococcus* spp. with heterotrophic bacteria

3.1 Introduction

The global ocean is inhabited by 1.2×10^{29} prokaryotes (Whitman et al., 1998), living in various environments with varying levels of resources and shaped by different pressures. Different organisms evolved to live in specific niches, and it is impossible to find any part of the ocean not colonized by microorganisms since even at great depths (Sogin et al., 2006), temperatures (Huber et al., 1986), concentrations of toxic compounds (De et al., 2008) and with no light (Nakagawa & Takai, 2008), life is possible for bacteria.

However, looking at a much smaller scale, every bacterial cell is also shaping its own environment. Apart from adapting to the environment it is living in, it is also modifying it and creating new micro-niches for other organisms to live in by changing the physical and chemical composition of its immediate surroundings. Any “leaky” metabolic process, exudation of waste products or production of compounds specifically made for excretion is a possible source of nutrients for other microorganisms or can improve their chances of survival by, for example, changing the pH or viscosity of the surrounding environment. It has been suggested that extracellular vesicles produced by marine picocyanobacteria (Biller et al., 2014) can act as “food packages” to facilitate growth of heterotrophic bacteria.

To fully understand global biogeochemistry it is extremely important to gain an understanding of microscale interactions between microorganisms and to study their biochemistry (Azam & Malfatti, 2007). As cyanobacteria are present in high numbers in most photic regions of the oceans, playing a critical role in marine carbon cycling (see section 1.1.3), it is therefore crucial to understand what bacterial genera are living in their closest proximity and with which organisms they interact.

Earlier studies, as described in Chapter 1, focused on either assessment of the character of these relationships (i.e. whether they have positive or negative effects on the partners), or on understanding the biochemistry of interactions between specific pairs of bacteria, not necessarily found to co-exist in the natural environment. This study was designed to perform an identification of the bacterial heterotrophs present in *Synechococcus* sp. non-axenic cultures isolated from the natural environment, but at the same time giving the opportunity to study any interesting relationships in greater detail as all organisms used are currently in culture and can be obtained from culture collections.

The aims of this Chapter were to:

- Identify those bacterial heterotrophs most commonly occurring in *Synechococcus* sp. co-cultures.
- Determine if any similarities and differences in the composition of the heterotrophic community in *Synechococcus* co-cultures are related to the *Synechococcus* ecotype, the composition of the growth medium, the isolation location or depth, or the length of time in culture.
- Assess how the heterotrophic bacteria 'partner community' changes during clonal purification of a *Synechococcus* strain.

3.2 Materials and methods

3.2.1 DNA extraction

Seventy five *Synechococcus* sp. non-axenic cultures (Table 3.2.1), grown for at least 90 days, were well mixed to ensure more uniform microorganism distribution and opened in sterile conditions. 2 ml of culture was then transferred to sterile microcentrifuge tubes for DNA extraction (see section 2.4).

Table 3.2.1 Details of the clonal *Synechococcus* enrichment cultures sequenced, including location on the sequencing plate and indices.

Well	i7 index	i5 index	Sample ID	*Synechococcus* sp. strain	Sub-cluster	Clade	Sub-group	Medium	Latitude	Longitude	Depth [m]	Isolator	Year
A01	N701	S517	RCC0067	Noum97012	5.1	VII	VII	ASW	-22.33	166.33	80	J. Blanchot, S. Boulben	1996
A02	N702	S517	A1537	A15-37	5.1	II	IIa	ASW NH4	23.56	-19.99	10	K. Zwirglmaier	2004
A03	N703	S517	RCC0066	Noum97010	5.1	CRD1	CRD1b	ASW	0.00	-180.00	30	J. Blanchot, S. Boulben	1996
A04	N704	S517	RCC2673	CC9902	5.1	IV	IVa	PCRS11	32.15	-117.42	5	B. Palenik	1999
A05	N705	S517	C3702C2	AMT23_ST37_45_2_P4C2	-	-	-	ASW	-10.00	-27.40	2	M. Moniz	2013
A06	N706	S517	RCC1026	Biosope_199	5.1	CRD1	CRD1a	ASW NH4	-34.00	-73.37	5	L. Garczarek, D. Marie	2004
A07	N707	S517	RCC0320	PROSOPE_157-13	5.1	VI	VIa	ASW	43.40	7.82	15	F. Partensky	1999
A08	N708	S517	A1543	A15-43	5.1	V	V	ASW	21.68	-17.83	40	K. Zwirglmaier	2004
A09	N709	S517	RCC0442	PROSOPE_2-16	5.1	II	IIh	ASW	30.13	-10.05	5	L. Garczarek	1999
A10	N710	S517	C1302O	AMT23_ST13_17_2	-	-	-	PCRS11	31.30	-22.67	2	M. Moniz	2013
A11	N711	S517	RCC2379	BOUM 118-1	5.1	III	IIIa	PCRS11	33.63	32.63	5	D. Marie, F. Le Gall	2008
A12	N712	S517	A1546	A15-46	5.1	V	V	ASW	21.38	-18.84	70	K. Zwirglmaier	2004
B01	N701	S502	RCC1104	A15-44	5.1	II	IIa	ASW	21.68	-17.83	20	K. Zwirglmaier	2004
B02	N702	S502	C0902O	AMT23_ST9_11_2	-	-	-	ASW	38.23	-20.10	2	M. Moniz	2013
B03	N703	S502	RCC1015	Biosope_211	5.1	I	Ia	ASW NH4	-33.87	-73.33	30	L. Garczarek, D. Marie	2004
B04	N704	S502	RCC0555	RS9916	5.1	IX	IX	ASW	29.47	34.92	10	N. Fuller	1999

Well	i7 index	i5 index	Sample ID	*Synechococcus* sp. strain	Sub-cluster	Clade	Sub-group	Medium	Latitude	Longitude	Depth [m]	Isolator	Year
B05	N705	S502	RCC2555	WH8101 Clonal	5.1	VIII	VIII	ASW	41.52	-70.67	-	F. Valois	1981
B06	N706	S502	RCC1027	Biosope_48 B3Y	5.1	CRD1	CRD1b	ASW	-9.07	-136.98	30	D. Vaulot, D. Marie	2004
B07	N707	S502	RCC0037	EUM Syn14	5.1	III	IIIa	ASW	21.03	-31.13	105	F. Partensky	1991
B08	N708	S502	RCC1029	Biosope_141 D	5.1	I	Ic	ASW	-31.87	-91.42	40	L. Garczarek, D. Marie	2004
B09	N709	S502	BL107	BL107	5.1	IV	IV	ASW	41.72	3.55	1800	L. Guillou	2000
B10	N710	S502	RCC1101	A15-34	5.1	III	IIIa	ASW	29.12	-16.97	40	K. Zwirglmaier	2004
B11	N711	S502	C0902A3	AMT23_ST9_11_2_P4A3	-	-	-	ASW	38.23	-20.10	2	M. Moniz	2013
B12	N712	S502	RCC0042	MEDNS5	5.1	VI	VIa	ASW	41.00	6.00	80	N. Simon	1993
C01	N701	S503	RCC0751	WH8020	5.1	I	Ia	ASW	38.68	-69.32	50	J. Waterbury	1980
C02	N702	S503	RCC0790	M11.1	5.1	II	IIh	ASW NH4	27.70	-91.30	275	C. Everroad	2004
C03	N703	S503	C0902C3	AMT23_ST9_11_2_P4C3	-	-	-	ASW	38.23	-20.10	2	M. Moniz	2013
C04	N704	S503	A1530	A15-30	5.1	III	III	ASW	31.25	-20.72	40	K. Zwirglmaier	2004
C05	N705	S503	RCC1030	Biosope_48 A2Y	5.1	CRD1	CRD1b	ASW NH4	-9.07	-136.98	30	D. Vaulot, D. Marie	2004
C06	N706	S503	RCC2378	A15-127 Clonal	5.1	WPC 1	WPC1	ASW	-31.12	-3.92	45	K. Zwirglmaier	2004
C07	N707	S503	C1302C3	AMT23_ST13_17_2_P1C3	-	-	-	PCRS11	31.30	-22.67	2	M. Moniz	2013
C08	N708	S503	A15127	A15-127	5.1	WPC 1	WPC1	ASW	-31.13	-3.93	45	K. Zwirglmaier	2004

Well	i7 index	i5 index	Sample ID	*Synechococcus* sp. strain	Sub-cluster	Clade	Sub-group	Medium	Latitude	Longitude	Depth [m]	Isolator	Year
C09	N709	S503	RCC1031	Biosope_48 B6Y	5.1	CRD1	CRD1b	ASW	-9.07	-136.98	30	D. Vaulot, D. Marie	2004
C10	N710	S503	BL3	BL3	5.3	SC5.3	SC5.3	ASW	41.67	2.80	-	L. Guillou	2000
C11	N711	S503	RCC2032	WH8016	5.1	I	Ib	ASW	41.52	-70.67	-	F. Valois	1980
C12	N712	S503	RCC2385	MICROVIR 10CR_4-3 Clonal	5.1	I	Ib	PCRS11	61.00	1.98	25	E. Foulon, S. Masquelier	2007
D01	N701	S504	A1545	A15-45	5.1	V	V	ASW	21.38	-18.84	10	K. Zwirglmaier	2004
D02	N702	S504	C0302O	AMT23_ST3_5_2	-	-	-	ASW	45.01	-13.59	2	M. Moniz	2013
D03	N703	S504	A1560	A15-60	5.1	VII	VII	ASW	17.84	-20.89	10	K. Zwirglmaier	2004
D04	N704	S504	RCC0262	TAK9802	5.1	II	IIa	ASW	-14.50	-145.33	7	F. Le Gall , S. Boulben	1998
D05	N705	S504	A1562	A15-62	5.1	II	IIb	ASW	17.84	-20.89	30	K. Zwirglmaier	2004
D06	N706	S504	RCC1017	Biosope_45 B5 463	5.1	CRD1	CRD1b	ASW NH4	-9.07	-136.98	100	D. Vaulot, D. Marie	2004
D07	N707	S504	BLANK	- (blank)	-	-	-	-	-	-	-	-	-
D08	N708	S504	RCC0394	PROSOPE_1-9	5.1	II	IIh	ASW	30.13	-10.05	5	L. Garczarek	1999
D09	N709	S504	C4602O	AMT23_ST46_56_2	-	-	-	PCRS11	-22.57	-25.91	2	M. Moniz	2013
D10	N710	S504	RCC0316	PROSOPE_37-2	5.1	XVI	XVI	ASW NH4	38.00	3.83	110	F. Partensky	1999
D11	N711	S504	A1572	A15-72	5.1	II	IIf	ASW	7.85	-23.22	15	K. Zwirglmaier	2004
D12	N712	S504	C0302B1	AMT23_ST3_5_2_P4B1	-	-	-	ASW	45.01	-13.59	2	M. Moniz	2013
E01	N701	S505	RCC0524	PROSOPE_107	5.1	I	Ia	ASW	39.12	14.08	90	F. Partensky	1999
E02	N702	S505	C1302LC3	AMT23_ST13_17_2_P1C3L	-	-	-	PCRS11	31.30	-22.67	2	M. Moniz	2013

Well	i7 index	i5 index	Sample ID	*Synechococcus* sp. strain	Sub-cluster	Clade	Sub-group	Medium	Latitude	Longitude	Depth [m]	Isolator	Year
E03	N703	S505	C4602C6	AMT23_ST46_56_2_P1C6	-	-	-	PCRS11	-22.57	-25.91	2	M. Moniz	2013
E04	N704	S505	RCC0307	MINSyn016-15m-01-AC6A1	5.3	SC5.3	SC5.3	ASW NH4	39.17	6.17	15	F. Partensky	1996
E05	N705	S505	RCC0753	CC9605	5.1	II	IIc	ASW	30.42	-123.98	51	B. Palenik	1996
E06	N706	S505	C0920O	AMT23_ST9_11_20	-	-	-	PCRS11	38.23	-20.10	20	M. Moniz	2013
E07	N707	S505	A1823	A1823	5.1	III	IIIa	ASW NH4	29.70	-34.83	2	M. Ostrowski	2008
E08	N708	S505	C3702O	AMT23_ST37_45_2	-	-	-	ASW	-10.00	-27.40	2	M. Moniz	2013
E09	N709	S505	BL123	BL123-7	5.1	VI	VIa	ASW	41.67	2.80	-	L. Guillou	2000
E10	N710	S505	RCC0556	RS9917	5.1	VIII	VIII	ASW NH4	29.47	34.92	10	N. Fuller	1999
E11	N711	S505	RCC0527	PROSOPE_101	5.1	VI	VIa	ASW NH4	39.12	14.08	110	F. Partensky	1999
E12	N712	S505	A1825A	A1825 R8	5.1	WPC 1	WPC1	ASW NH4	27.77	-37.03	2	M. Ostrowski	2008
F01	N701	S506	RCC1022	Biosope_109 C2	-	-	-	ASW NH4	-30.05	-98.40	150	L. Garczarek, D. Marie	2004
F02	N702	S506	RCC0325	PROSOPE_153-3	5.1	VI	VIa	ASW NH4	43.40	7.82	25	F. Partensky	1999
F03	N703	S506	A15130	A15-130	5.1	WPC 1	WPC1	ASW	-34.50	-1.37	20	K. Zwirglmaier	2004
F04	N704	S506	C4402O	AMT23_ST44_54_2	-	-	-	ASW	-21.53	-25.16	2	M. Moniz	2013
F05	N705	S506	A1561	A15-61	5.1	II	IIb	ASW	17.84	-20.89	15	K. Zwirglmaier	2004
F06	N706	S506	RCC1028	Biosope_148 D3	5.1	CRD1	CRD1b	ASW NH4	-31.87	-91.42	40	L. Garczarek, D. Marie	2004

Well	i7 index	i5 index	Sample ID	*Synechococcus* sp. strain	Sub-cluster	Clade	Sub-group	Medium	Latitude	Longitude	Depth [m]	Isolator	Year
F07	N707	S506	RCC0043	ALMO3 Syn	5.1	I	Ib	ASW	36.18	-1.85	0	F. Partensky	1991
F08	N708	S506	C1002B2	AMT23_ST10_12_2_P4B2	-	-	-	ASW	34.95	-20.67	2	M. Moniz	2013
F09	N709	S506	RCC0540	RS9901	5.1	IX	IX	ASW	29.47	34.92	1	N. Fuller	1999
F10	N710	S506	A1538	A15-38	5.1	II	IIa	ASW	23.56	-19.99	20	K. Zwirglmaier	2004
F11	N711	S506	RCC2035	Syn 20	5.1	I	Ib	ASW	60.62	5.68	0	R.A. Sanda	-
F12	N712	S506	RCC2528	TAK9802-Clonal	5.1	II	IIa	PCRS11	-14.50	-145.33	7	F. Le Gall, S. Boulben	1998
G01	N701	S507	RCC2373	WH8018	5.1	VIc	VIc	ASW	41.52	-70.67	-	J. Waterbury	1981
G02	N702	S507	RCC2533	Biosope_199-Clonal	5.1	CRD1	CRD1a	ASW	-34.00	-73.37	5	L. Garczarek, D. Marie	2004
G03	N703	S507	RCC0791	M16.1	5.1	II	IIa	ASW NH4	27.70	-91.30	275	C. Everroad	2004
G04	N704	S507	C1002O	AMT23_ST10_12_2	-	-	-	ASW	34.95	-20.67	2	M. Moniz	2013
G05	N705	S507	RCC1020	Biosope_45 C4Y	5.1	CRD1	CRD1b	ASW NH4	-9.07	-136.98	100	D. Vaulot, D. Marie	2004
G06	N706	S507	RCC0327	PROSOPE_25-2	-	-	-	ASW NH4	38.00	3.83	25	F. Partensky	1999
G07	N707	S507	RCC0541	RS9902	5.1	II	IIa	ASW	29.47	34.92	1	N. Fuller	1999
G08	N708	S507	C4602C5	AMT23_ST46_56_2_P1C5	-	-	-	PCRS11	-22.57	-25.91	2	M. Moniz	2013
G09	N709	S507	RCC0044	OLI031FJ Syn	5.1	III	IIIa	ASW	-5.50	-150.00	70	D. Vaulot	1994
G10	N710	S507	C4402B1	AMT23_ST44_54_2_P6B1	-	-	-	ASW	-21.53	-25.16	2	M. Moniz	2013
G11	N711	S507	RCC2433	NOUM97013 Clonal	5.1	VII	VII	ASW	-22.33	166.33	0	J. Blanchot, S. Boulben	1996

Well	i7 index	i5 index	Sample ID	*Synechococcus* sp. strain	Sub-cluster	Clade	Sub-group	Medium	Latitude	Longitude	Depth [m]	Isolator	Year
G12	N712	S507	RCC2527	A15-44-Clonal	5.1	II	IIa	PCRS11	21.68	-17.83	20	K. Zwirglmaier	2004
H01	N701	S508	C0920D6	AMT23_ST9_11_20 _P1D6	-	NA	NA	PCRS11	38.23	-20.10	20	M. Moniz	2013
H02	N702	S508	RCC0508	BL_36_Syn	5.1	VI	VIb	ASW	41.67	2.80	0	L. Guillou	2000
H03	N703	S508	RCC1023	Biosope_112 B6	5.1	CRD1	CRD1a	ASW	-30.78	-95.43	100	L. Garczarek, D. Marie	2004
H04	N704	S508	RCC0032	ROS8604 Syn	5.1	I	Ib	ASW	48.72	-3.98	0	D. Vaulot, C. Courties	1986
H05	N705	S508	A1825C	A1825 R12	5.1	III	IIIa	ASW	27.77	-37.03	74	M. Ostrowski	2008
H06	N706	S508	C0920LD6	AMT23_ST9_11_20 _P1D6L	-	-	-	PCRS11	38.23	-20.10	20	M. Moniz	2013
H07	N707	S508	RCC0359	RA000711-27-14	5.1	VI	VIb	ASW NH4	48.75	-3.95	0	F. Le Gall	2000
H08	N708	S508	RCC1018	Biosope_45 B6 465	5.1	CRD1	CRD1b	ASW NH4	-9.07	-136.98	100	D. Vaulot, D. Marie	2004
H09	N709	S508	RCC1094	A15-74	5.1	VII	VII	ASW	7.85	-23.22	25	K. Zwirglmaier	2004
H10	N710	S508	RCC0319	PROSOPE_157-21	5.1	VI	VIa	ASW NH4	43.40	7.82	15	F. Partensky	1999
H11	N711	S508	CC9902	CC9902 (CCMP)	5.1	IV	IVa	ASW	32.90	-117.26	5	B. Palenik	1999
H12	N712	S508	RCC0321	PROSOPE_32-1	5.1	II	IIe	ASW	38.00	3.83	5	F. Partensky	1999

Seawater samples were collected during the AMT23 research cruise (Atlantic Ocean transect, from the UK to the Falkland Islands; October-November 2013 see http://www.amt-uk.org/Cruises/AMT23) and enriched with PCR-S11 or ASW media (5 ml of medium per 20 ml of seawater and 1 ml of medium per 4 ml of seawater, respectively). Upon confirmation of *Synechococcus* sp. presence by flow cytometry, the enrichments were plated in 2% weight per volume (w/v) agarose in the same medium as the one used for enrichment. Four random colonies resembling *Synechococcus* sp. were picked from the plates into the same liquid medium (1st liquid), and after developing colour were plated again in 2% (w/v) agarose in the same medium. One representative colony was picked into the same liquid medium (2nd liquid) and again, after developing colour, the culture was plated in 2% (w/v) agarose in the same medium. One representative colony was picked again and transferred into the same liquid medium (3rd liquid). This process of enrichment and isolation was carried out by Mónica Joyce Moniz (School of Life Sciences, University of Warwick). Samples for DNA extraction were obtained from the original enrichments (at least 5 ml; within 2 weeks of research cruise completion), 1st liquid (if possible; 2 ml) and 3rd liquid (2 ml) and were processed on the day of collection. Out of all the samples collected, only 8 original enrichments were maintained until the step of 3rd liquid (most were lost due to very poor growth, contamination or unsuccessful DNA extraction; some were used up for other purposes), including 2 full sets (with 1st liquid step sample) (Table 3.2.2).

Every batch of DNA extraction reactions consisted of up to 23 samples and a blank (DNA-free water). Equal volumes of blanks from all DNA extraction reactions were combined to form the final combined blank sample used throughout the sequencing process to determine contamination levels due to handling, non-sterile DNA extraction kit reagents and cross-contamination between wells.

Table 3.2.2 Samples originating from the AMT23 research cruise enrichments.

Sample details [station number, CTD recording, depth, medium]	Original enrichment (O)	1st liquid (L1)	3rd liquid (L3)
ST 3, CTD 5, 2 m, ASW	yes	no	yes, P4B1
ST 9, CTD 11, 2 m, ASW	yes	no	yes, P4A3 yes, P4C3
ST 9, CTD 11, 20 m, PCR-S11	yes	yes, P1D6	yes, P1D6
ST 10, CTD 12, 2 m, ASW	yes	no	yes, P4B2
ST 13, CTD 17, 2 m, PCR-S11	yes	yes, P1C3	yes, P1C3
ST 37, CTD 45, 2 m, ASW	yes	no	yes, P4C2
ST 44, CTD 54, 2 m, ASW	yes	no	yes, P6B1
ST 46, CTD 56, 2 m, PCR-S11	yes	no	yes, P1C5 yes, P1C6

3.2.2 Sequencing

Samples of DNA from the 75 *Synechococcus* sp. cultures, 20 cruise isolates and a blank were assigned to wells of a 96-well plate (Table 3.2.1) in such a way that no samples of the same origin (cruise enrichment for cruise isolates and same ocean region for culture collection samples) were next to each other. The blank was assigned to well D4 at the centre of the plate to maximise the chances of cross contamination. Sample preparation for sequencing followed the 16S library preparation pipeline (see section 2.4), which includes two PCR steps, normalisation and pooling. The first PCR reaction (95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final step of 72°C for 5 min) was performed to generate 16S rRNA gene amplicons (primers targeting the V3 and V4 regions with overhang adapters: forward 5'-TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAGCCTACGGGNGGCWGCAG and reverse 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTAT CTAATCC, product of about 460-480 bp) with overhang adapters, the second step added a unique combination of indices to all PCR products of a given sample and Illumina sequencing adapters (95°C for 3 min, 8 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final step of 72°C for 5 min). All samples were then quantified (section 2.4) and, if needed, diluted to obtain approximately the same concentration as that of the least

concentrated sample (excluding the blank). Equal volumes of normalised samples were pooled and the library was diluted to 4nM. 5 µl of the final library was run on a MiSeq.

3.2.3 Data analysis

Fastq files for all samples were uploaded to the Vettel server (Bio-Linux 8 running on Ubuntu Server 14.04 LTS; provided by the University of Warwick, School of Life Sciences IT services). Forward and reverse reads were merged using USEARCH 10.2.240, command fastq_merge pairs with no minimum/maximum merge length, a maximum of 5 mismatches per alignment and default filtering. Primer sequences were removed using fastx_truncate command with trimming 17 and 21 letters from left and right ends respectively. Quality filtering was performed using command fastq_filter, with the maximum expected error threshold set to 1.0 (Edgar & Flyvbjerg, 2015). Reads were then dereplicated using the fastx_uniques command. OTU (Operational Taxonomic Unit) clustering was performed using the UPARSE-OTU algorithm (Edgar, 2013) at 97% with concurrent chimera filtering and discarding of single reads by the cluster_otus command. An OTU Table was created in VSEARCH 2.4.3 (Rognes et al., 2016) using the OTU list and merged reads database, at 97% identity by usearch_global. OTUs with counts of less than 0.5% of total sample reads were discarded (otutab_trim) and UNCROSS algorithm command (uncross) was executed to minimize cross-talk error (Edgar, 2016). Samples with less than 10 000 reads were discarded (outtab_trim) and remaining samples were normalized to 200 000 reads (otutab_norm).

Taxonomy was assigned using the Ribosomal Database Project (RDP) Classifier (Wang, et al., 2007) at 97% identity against the Greengenes 13.8 database by QIIME 1.9.1 script assign_taxonomy.py (Caporaso et al., 2010). The output file was modified to include a header row (OTU ID taxonomy confidence). The OTU table was converted into a biom HDF5 format using BIOM 2.1.4 convert command. Taxonomy data was added to the table by the “add-metadata” command. Area plots and bar charts were

generated in QIIME (summarize_taxa_through_plots.py). OTU heatmaps were created using the make_otu_heatmap.py script. Core microbiome at the levels of 50-90% (i.e. OTUs present in 50-90% of samples) was determined by the core_microbiome.py command.

Statistical analysis used script observation_metadata_correlation for testing for correlation between OTU abundance and continuous data (such as latitude and longitude) using the Spearman's Rho method and group_significance.py for comparing OTU frequencies across sample groups (such as sample type or medium type) using the Kruskal-Wallis test method. Principal Coordinates Analysis (PCoA) was performed using a rarefied OTU table (sampling depth 1 000) using QIIME script core_diversity_analyses.py.

3.3 Results

3.3.1 Comparison of culture collection and cruise enrichment cultures

The sequencing run generated 8.75 Gbp with an average ≥Q30 of 87.72% (i.e. the percentage of base calls with an accuracy equal to or higher than 99.9%), a cluster density of 934 kc/mm^2 and a percentage of clusters of reads passing filter at 88.33%. Out of 96 samples 95 passed quality and minimum number of reads filtering. The sample which did not pass the process was the combined blank (95 reads). The final OTU table comprised 11.9 million reads, 6555 counts, for 69 OTUs and 95 samples.

The average number of OTUs per *Synechococcus* sp. cruise enrichment sample was 9.85 (range of 4-20) and for culture collection sample 13.59 (3-22). At both the phylum and family level, *Synechococcus* sp. cruise enrichments cultures display a different composition of taxa than culture collection cultures (Figures 3.3.1.1-3.3.1.2) and some OTUs have significantly different frequencies between the two groups of samples (Table 3.3.1.1)

Table 3.3.1.1 OTUs with statistically significant different frequencies between *Synechococcus* sp. cruise enrichments and culture collection samples.

OTU	Taxonomic assignment	p value (FDR corrected)
OTU72	*Actinobacteria; Actinobacteria; Actinomycetales; Microbacteriaceae*	0.0420
OTU18	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Muricauda*	0.0047
OTU319	*Cyanobacteria; Chloroplast; Stramenopiles*	0.0177
OTU10	*Cyanobacteria; Synechococcophycideae; Synechococcales; Synechococcaceae; Synechococcus*	0.0117
OTU9	*Planctomycetes; Phycisphaerae; Phycisphaerales*	0.0070
OTU4	*Proteobacteria; Alphaproteobacteria*	0.0229
OTU28	*Proteobacteria; Alphaproteobacteria; Kiloniellales; Kiloniellaceae; Thalassospira; xiamenensis*	<0.0000
OTU79	*Proteobacteria; Alphaproteobacteria; Rhizobiales*	0.0070

OTU	Taxonomic assignment	p value (FDR corrected)
OTU11	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Cohaesibacteraceae*	0.0189
OTU12	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Parvibaculum*	0.0117
OTU7	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae*	<0.0000
OTU13	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; Nitratireductor*	<0.0000
OTU71	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae*	0.0498
OTU16	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Hyphomonas*	0.0120
OTU3	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Oceanicaulis*	0.0008
OTU162	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	<0.0000
OTU43	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	<0.0000
OTU21	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	<0.0000
OTU68	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	<0.0000
OTU15	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	0.0001
OTU35	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	0.0004
OTU2	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	0.0017
OTU87	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Loktanella*	0.0420
OTU105	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas*	<0.0000
OTU14	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter*	0.0171
OTU258	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter*	0.0177
OTU48	*Proteobacteria; Gammaproteobacteria; Thiotrichales; Piscirickettsiaceae; Methylophaga*	0.0090
OTU49	*Proteobacteria; Gammaproteobacteria; Thiotrichales; Piscirickettsiaceae; Methylophaga*	0.0117

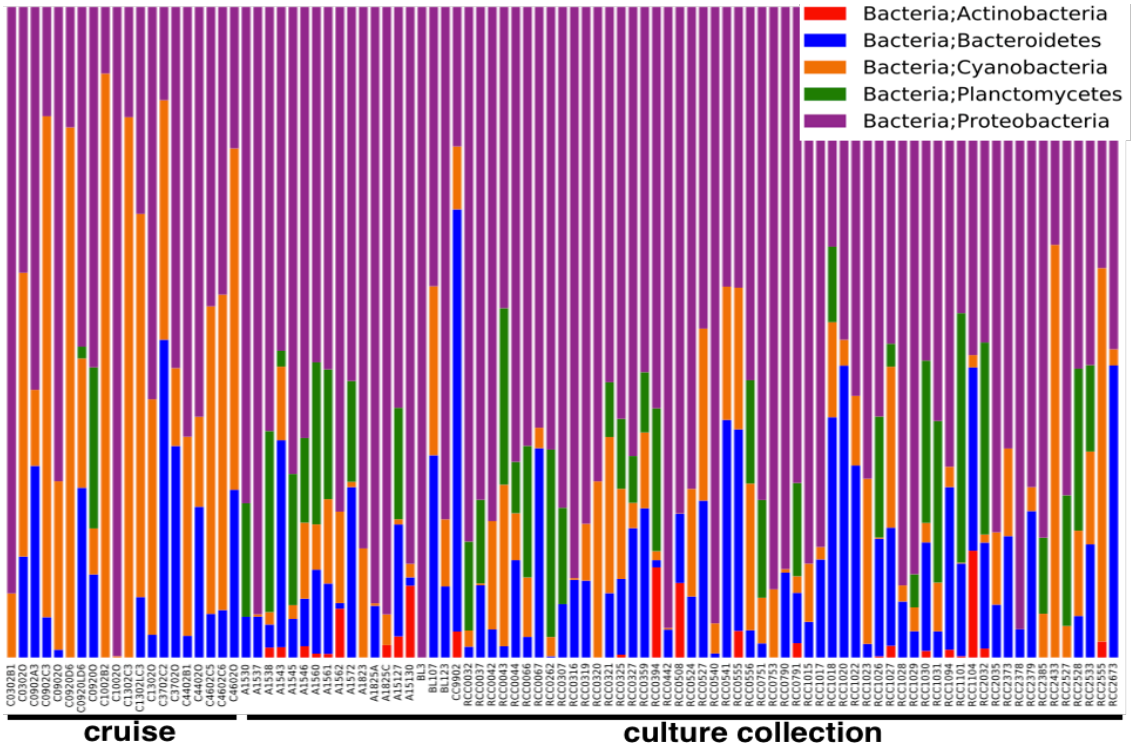


Figure 3.3.1.1 Summary of taxa present in cruise enrichment and culture collection cultures at phylum level.

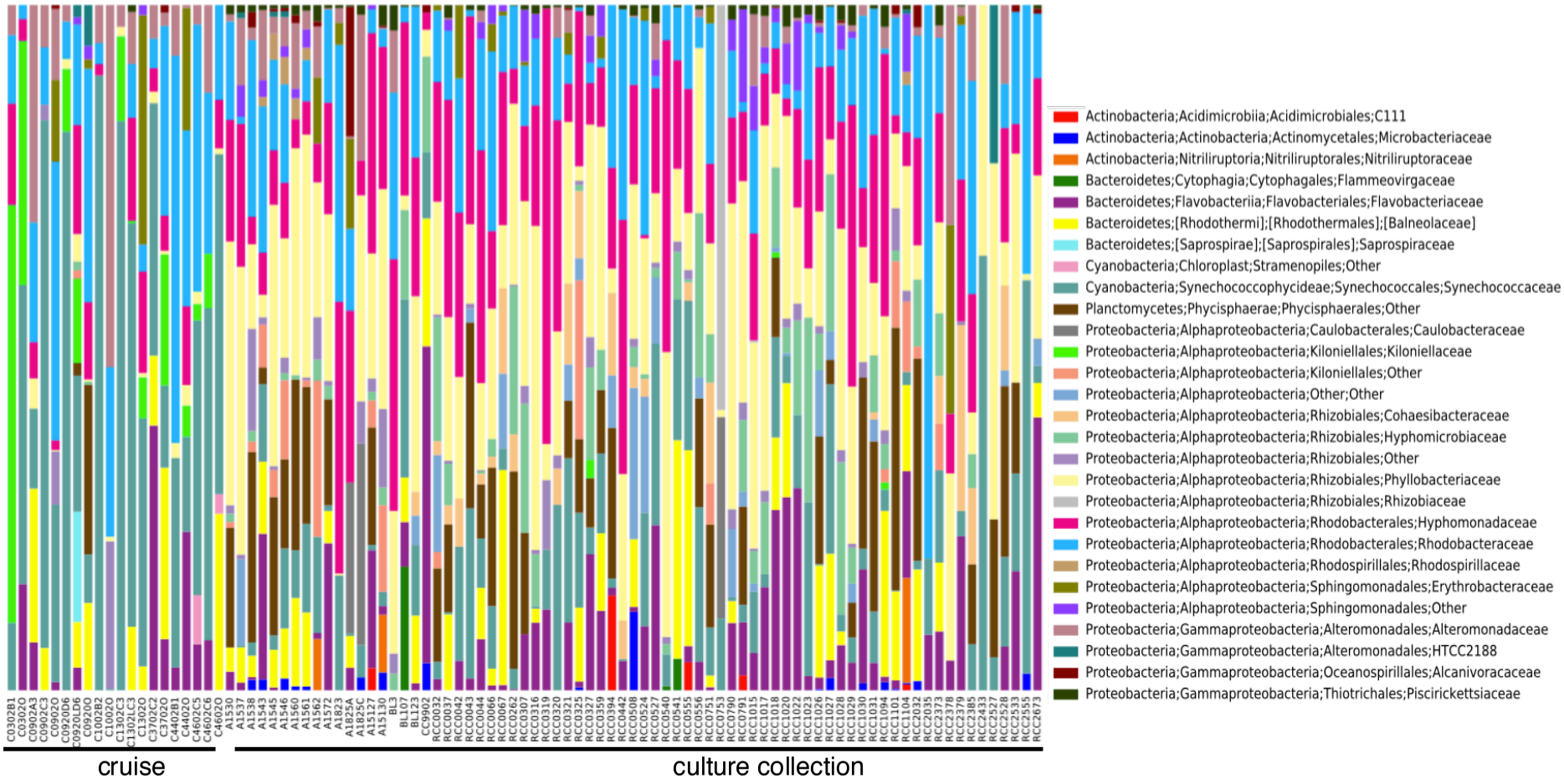


Figure 3.3.1.2 Summary of taxa present in cruise enrichment and culture collection cultures at family level.

Synechococcus sp. was detected in 91 samples, including all cruise enrichment cultures. Only two cultures, both cruise enrichment samples, were positive for reads of "*Cyanobacteria*" other than *Synechococcus* sp. (*Stramenopiles* sp.). Although the taxonomic assignment of this sequence with the Greengenes database showed a confidence value of 1 when searched with BLAST against the 16S ribosomal RNA database, no matches above 90% were found. The closest matches all included *Cyanobacteria* species: *Calothrix desertica* strain PCC 7102 (86% identity with 100% query cover), *Scytonema hofmanni* strain PCC 7110 (85% identity with 100% query cover) and *Trichocoleus desertorum* strain ATA4-8-CV2 (85% identity with 99% query cover), which suggests that the query might be more closely related to Cyanobacteria than the assigned *Stramenopiles* sp. (*Eukaryota*, *Heterokonta*). However, the USEARCH SINTAX algorithm (Edgar, 2016) with RDP training set v16 database, recognised the OTU as *Bacillariophyta* sp. (*Eukaryota*, *Heterokonta*, *Ochrophyta*; confidence value of 1), which suggests that it could be a diatom.

3.3.2 Identification of most frequently occurring heterotrophs in *Synechococcus* sp. cultures

The OTU heatmap (Figure 3.3.2.1) confirmed not only that there are OTUs present in more than one sample, but also that some species were present in a majority of samples. These most frequently occurring organisms were identified by QIIME and listed at different frequency levels (Table 3.3.2.1).

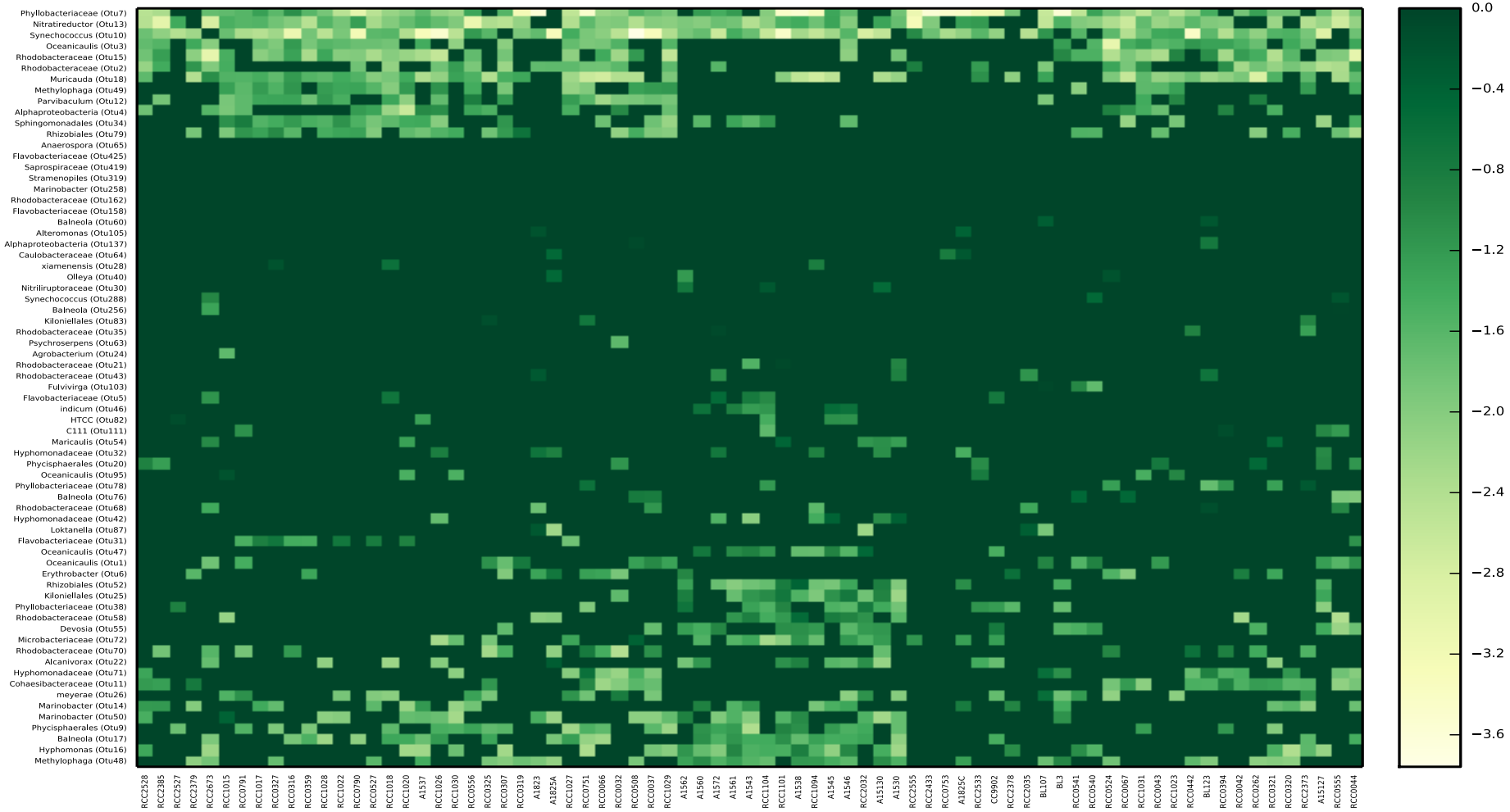


Figure 3.3.2.1 Heatmap of OTUs present in *Synechococcus* sp. cultures.

Table 3.3.2.1 Core microbiome of *Synechococcus* sp. cultures.

OTU assignment	Percentage of samples detected in:								
	50%	55%	60%	65%	70%	75%	80%	85%	90%
OTU10 *Synechococcus*	✓	✓	✓	✓	✓	✓	✓	✓	✓
OTU13 *Nitratireductor*	✓	✓	✓	✓	✓	✓	✓	✓	
OTU7 *Phyllobacteriacae*	✓	✓	✓	✓	✓	✓	✓	✓	
OTU18 *Muricauda*	✓	✓	✓	✓					
OTU15 *Rhodobacteraceae*	✓	✓							

To confirm the annotation of the core microbiome, the 5 most common OTUs were searched against the 16S rRNA database using BLAST (Table 3.3.2.2). The *Nitratireductor* and *Muricauda* annotation was confirmed, while the *Phyllobacteriacae* (OTU7) search resulted in 3 hits of *Aquamicrobium* with high identity and query coverage (97-98%/100%) which suggests that the organism may be closely related to or a member of this genus. The *Rhodobacteraceae* (OTU15) search returned three matches of high sequence identity and query coverage (98%/100%), but of different genera: *Loktanella atrilutea* IG8, *Primorskyibacter insulae* SSK3-2 and *Poseidonocella sedimentorum* KMM9023.

Table 3.3.2.2 BLAST annotation of the 4 most common heterotrophic partners.

OTU annotation	Top 3 matches (identity/query coverage)
Nitratireductor (OTU13)	*Nitratireductor aquimarinus* CL-SC21 (99%/100%) *Nitratireductor aquibiodomus* NL21 (99%/100%) *Nitratireductor pacificus* pht-3B (99%/100%)
Rhodobacteraceae (OTU15)	*Loktanella atrilutea* IG8 (98%/100%) *Primorskyibacter insulae* SSK3-2 (98%/100%) *Poseidonocella sedimentorum* KMM9023 (98%/100%)
Muricauda (OTU18)	*Muricauda lutimaris* SMK-108 (99%/100%) *Muricauda aquimarina* SW-63 (99%/100%) *Muricauda ruestringensis* DSM 13258 (98%/100%)
Phyllobacteriacae (OTU7)	*Aquamicrobium defluvii* DSM 11603 (98%/100%) *Aquamicrobium ahrensii* 905 (97%/100%) *Aquamicrobium terrae* hun6 (97%/100%)

Annotation with the RDP Classifier suggested *Rhizobium* sp. PRLIST04 (S_ab score 0.961), *Mesorhizobium* sp. DG943 (0.961) and uncultured *Phyllobacteriacae bacterium* (0.972) for OTU7 and *Ruegeria* sp. LTs-2 (0.958), uncultured *Rhodobacteraceae* bacterium (0.969) and *Alphaproteobacterium* SY190 (0.982) for OTU15 as closest matches.

3.3.3 Factors explaining the variation in community composition

Rarefaction of the OTU Table showed that 1000 sequences per sample formed a reliable representation of the full dataset (Figure 3.3.3.1). Principal Coordinates Analysis (PCoA) was performed for *Synechococcus* sp. culture collection samples for a general overview of sample diversity and whether any of the metadata collected for the cultures (*Synechococcus* sp. clades and subgroups, depth and location of isolation, the person isolating, year of isolation, medium) could explain the patterns seen (Figures 3.3.3.2-3.3.3.5).

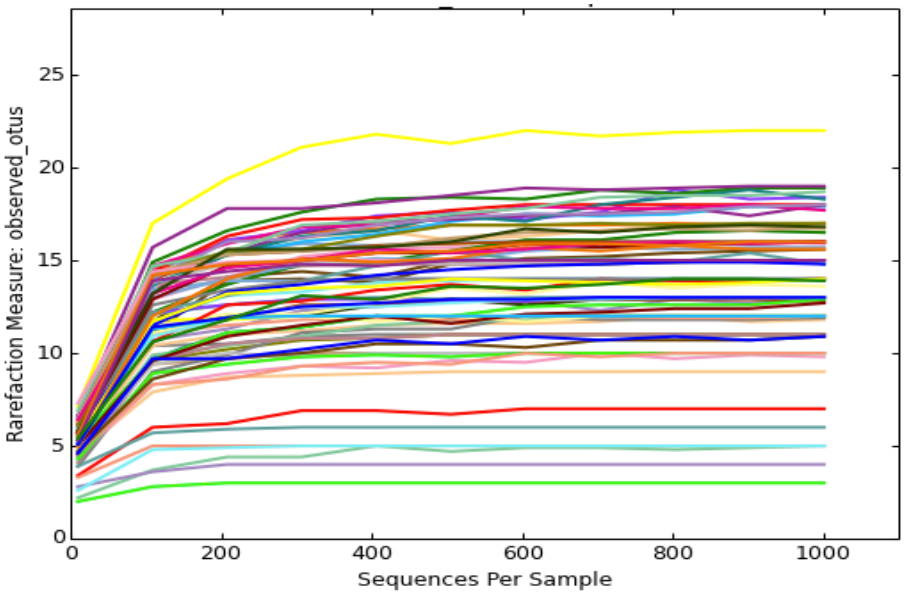


Figure 3.3.3.1 Number of OTUs observed in all *Synechococcus* sp. culture collection samples when a given number of sequences is analysed.

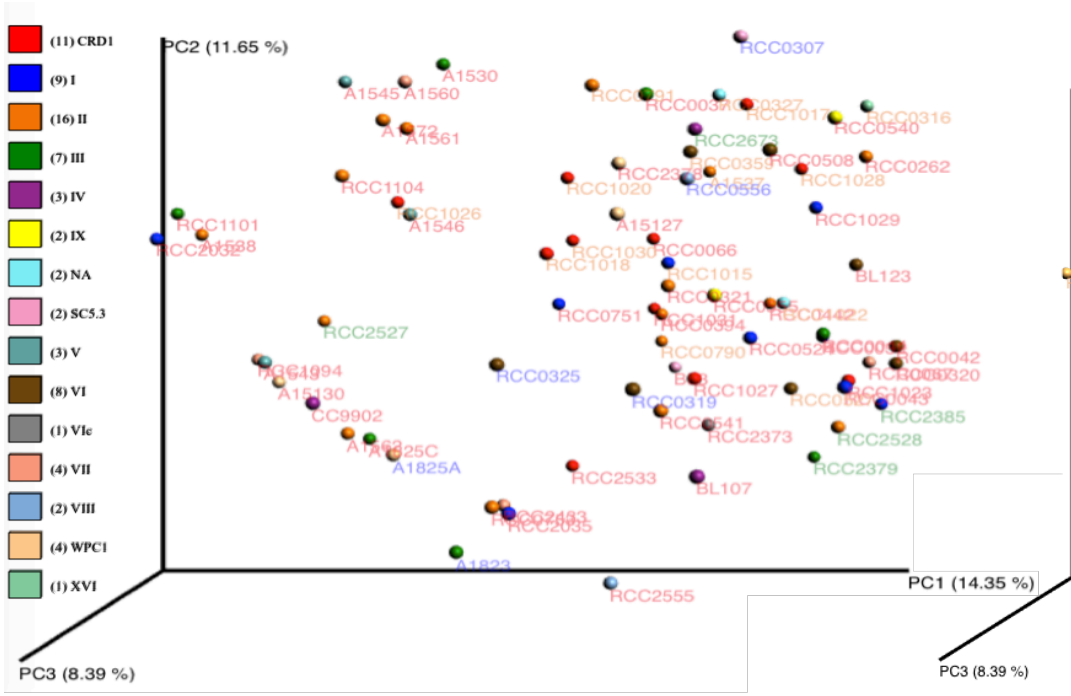


Figure 3.3.3.2 PCoA analysis of *Synechococcus* sp. culture collection samples, based on Bray Curtis beta diversity – medium type and *Synechococcus* sp. clades. Label colours represent types of media used (ASW –red, ASW NH4 – orange and blue, PCR-S11 – green) and data points colours represent *Synechococcus* sp. clades.

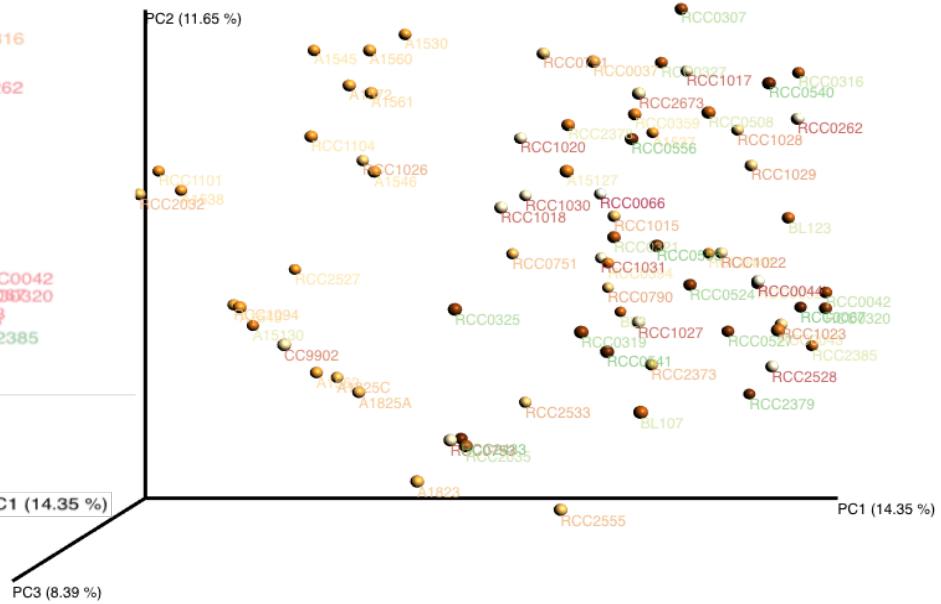


Figure 3.3.3.3 PCoA analysis of *Synechococcus* sp. culture collection samples, based on Bray Curtis beta diversity – isolation location. Label colours represent longitude (red to green scale representing far West to far East) and data points colours represent latitude (yellow to brown scale representing far South to far North).

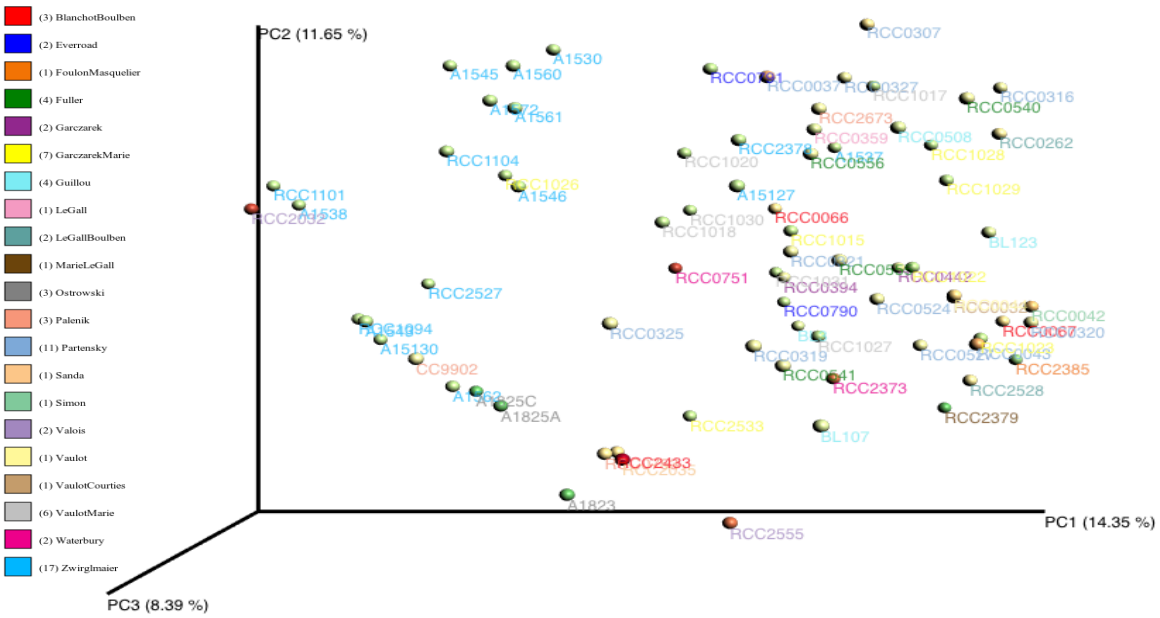


Figure 3.3.3.4 PCoA analysis of *Synechococcus* sp. culture collection samples, based on Bray Curtis beta diversity – isolator and year of isolation. Label colours represent the person(s) who isolated the culture and data points colours represent years (red to green scale representing 1980-2008).

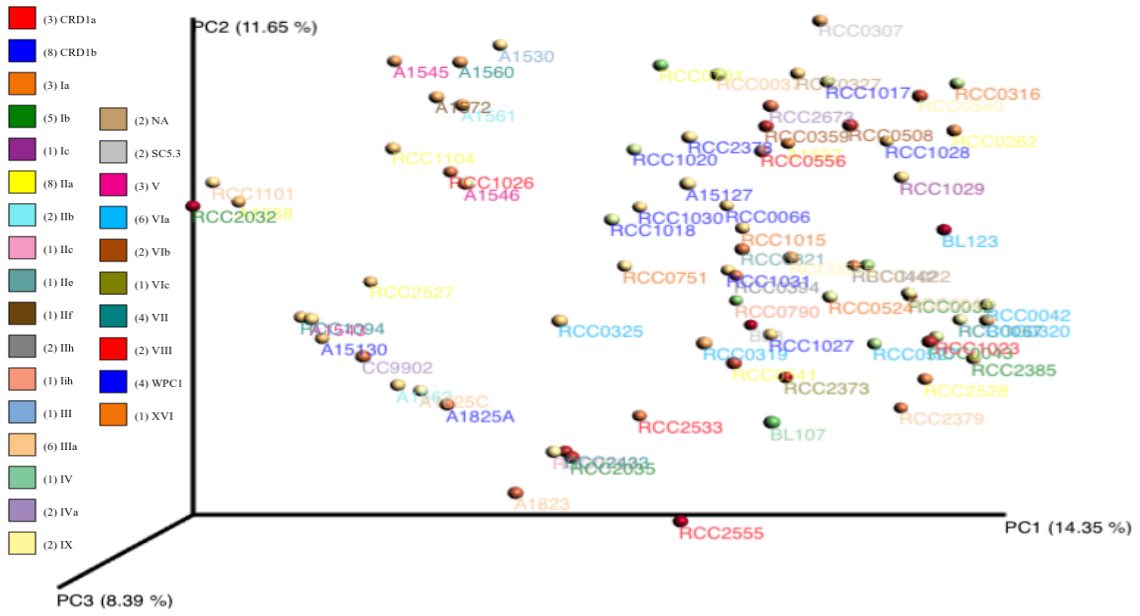


Figure 3.3.3.5 PCoA analysis of *Synechococcus* sp. culture collection samples, based on Bray Curtis beta diversity – depth of isolation and *Synechococcus* sp. subgroup. Label colours represent *Synechococcus* sp. subgroups and data points colours represent depth of isolation (red to green scale representing 0-1800 m).

Analysing the graphs in Figs 3.3.3.1 – 3.3.3.5 showed no clear patterns of sample clustering. There is a slight indication that high eastern and western latitudes may be different than mid-longitudes (Figure 3.3.3.3). Analysing specific OTUs instead of all OTUs per sample, a list of bacteria significantly different between samples was created (Table 3.3.3.1).

OTU frequencies were different for some samples grouped by *Synechococcus* sp. clade, isolation depth and medium groups and two OTUs (OTU 13 and OTU95) showed a correlation with latitude, but no correlation was found for any OTU with longitude. Two of the OTUs with frequency varying in different media (OTU13 and OTU18; Table 3.3.3.1 in bold) and one of the OTUs correlated with latitude (OTU13) are part of the core microbiome of *Synechococcus* sp. cultures.

Table 3.3.3.1 OTUs present at different frequencies between samples of different clade, depth and medium and showing correlation between latitude and counts.

OTU	**Taxonomic assignment**	**p value (FDR corrected)**			
		Latitude	Clade	Depth	Medium
OTU13	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; Nitratireductor*	0.0346			0.0256
OTU95	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Oceanicaulis*	0.0380			
OTU46	*Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; Oceanibaculum; indicum*		0.0053		
OTU103	*Bacteroidetes; Cytophagia; Cytophagales; Flammeovirgaceae; Fulvivirga*			<0.0000	
OTU64	*Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae*			0.0015	
OTU105	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas*			0.0084	

OTU	Taxonomic assignment	p value (FDR corrected)			
		Latitude	Clade	Depth	Medium
OTU87	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Loktanella*			0.0435	
OTU34	*Proteobacteria; Alphaproteobacteria; Sphingomonadales*				<0.0000
OTU31	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae*				0.0002
OTU79	*Proteobacteria; Alphaproteobacteria; Rhizobiales*				0.0002
OTU49	*Proteobacteria; Gammaproteobacteria; Thiotrichales; Piscirickettsiaceae; Methylophaga*				0.0011
OTU18	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Muricauda*				0.0020
OTU158	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae*				0.0264
OTU12	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Parvibaculum*				0.0264

3.3.4 Identification of heterotrophic partners lost and retained in the process of *Synechococcus* sp. isolation from AMT23 cruise enrichments

An OTU heatmap generated for the *Synechococcus* sp. cruise enrichments shows that there is no clear pattern of heterotroph loss (Figure 3.3.4.1). Some heterotrophs are no longer detected in 1st liquid and 3rd liquid samples, but there are also bacteria undetected in the original enrichment, yet present in the liquid samples (OTU105 in 4 samples, OTUs 50, 28, 7, 87 and 68 in 2 samples and OTUs 16, 18, 40 and 65 in 1 sample). The core microbiome of cruise isolate samples had only one OTU in common with culture collection samples (OTU7 *Phyllobacteriacae* in more than 50% of samples; Table 3.3.4.1), but 3 out of 4 could be detected in some samples (Table 3.3.4.2). The average number of OTUs detected in the original enrichment was 11.5 (range of 8-17), while for the 3rd liquid it was 7.5 (4-12).

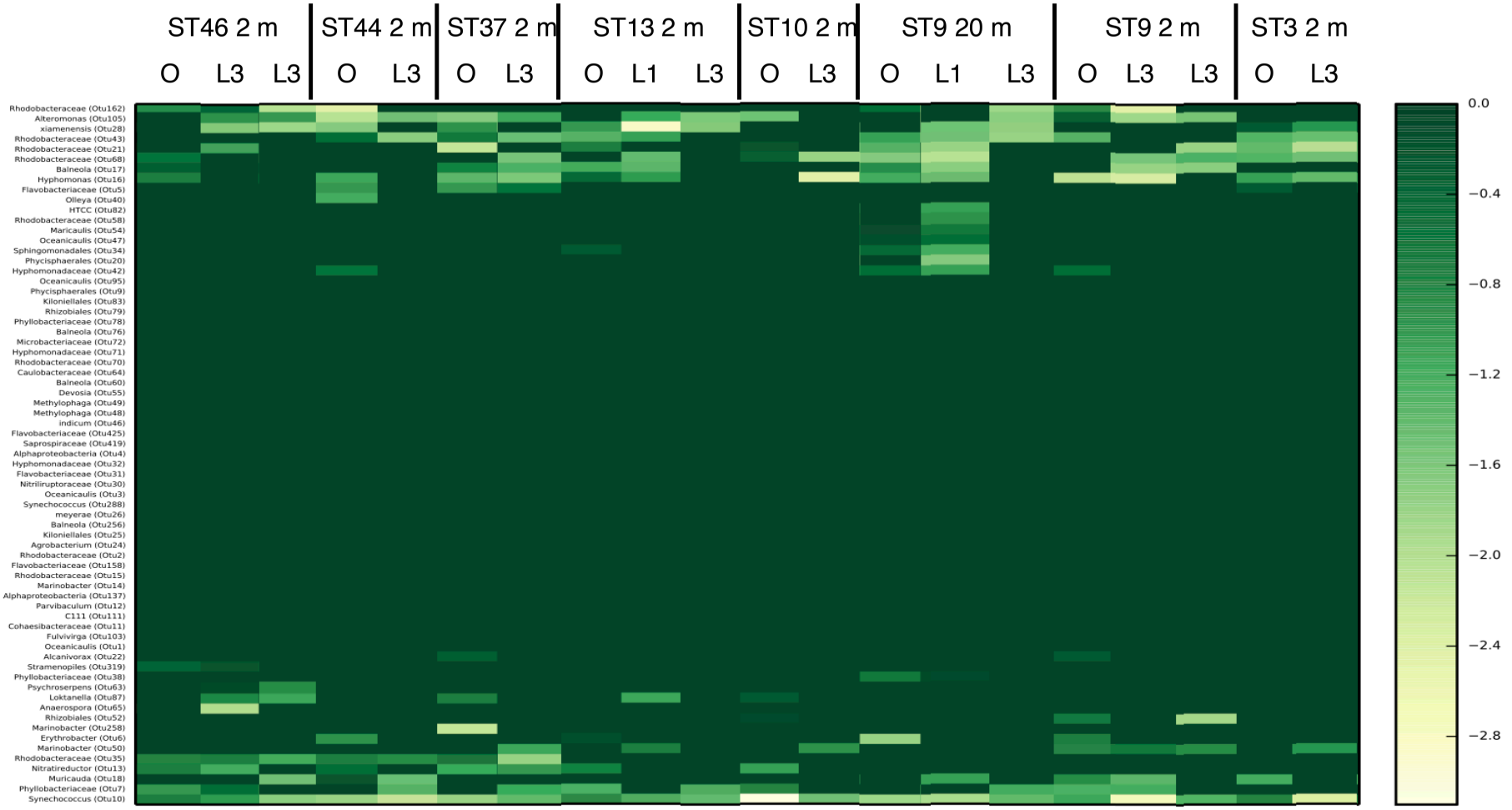


Figure 3.3.4.1 Heatmap of OTUs present in *Synechococcus* sp. cruise enrichment cultures – original enrichments (O), 1st liquid (L1) and 3rd liquid (L3) cultures.

Table 3.3.4.1 Core microbiome of *Synechococcus* sp. cruise enrichment cultures.

OTU assignment	Percentage of samples detected in:										
	50%	55%	60%	65%	70%	75%	80%	85%	90%	95%	100%
OTU10 *Synechococcus*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
OTU105 *Alteromonas*	✓	✓	✓	✓							
OTU16 *Hyphomonas*	✓	✓	✓	✓							
OTU43 *Rhodobacteraceae*	✓	✓	✓								
OTU28 *Thalassospira xiamenensis*	✓	✓									
OTU68 *Rhodobacteraceae*	✓	✓									
OTU7 *Phyllobacteriacae*	✓										

Table 3.3.4.2 OTUs maintained, gained and lost from the original enrichment to the 3rd liquid sample.

OTU	Taxonomy	Maintained	Gained	Lost
OTU10	*Cyanobacteria; Synechococcophycideae; Synechococcales; Synechococcaceae; Synechococcus*	10		
OTU43	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	4		2
OTU35	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	4		
OTU105	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas;*	4	4	1
OTU162	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	4		2
OTU16	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Hyphomonas*	3	1	6
OTU50	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter*	2	3	
OTU18	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Muricauda*	2	1	2
OTU28	*Proteobacteria; Alphaproteobacteria; Kiloniellales; Kiloniellaceae; Thalassospira; xiamenensis*	2	3	2
OTU52	*Proteobacteria; Alphaproteobacteria; Rhizobiales*	2		2
OTU7	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae*	2	3	3
OTU13	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; Nitratireductor*	2		4

OTU	Taxonomy	Maintained	Gained	Lost
OTU68	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	2	3	3
OTU319	*Cyanobacteria; Chloroplast; Stramenopiles*	1		1
OTU17	*Bacteroidetes; [Rhodothermi]; [Rhodothermales]; [Balneolaceae]; Balneola*	1	2	4
OTU5	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae*	1		2
OTU21	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*	1	2	4
OTU40	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Olleya*		1	1
OTU63	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Psychroserpens*		2	
OTU20	*Planctomycetes; Phycisphaerae; Phycisphaerales*			1
OTU55	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Devosia*			1
OTU38	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae*			1
OTU42	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae*			4
OTU54	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Maricaulis*			1
OTU47	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Hyphomonadaceae; Oceanicaulis*			1
OTU58	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae*			1
OTU65	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Anaerospora*		1	2
OTU82	*Proteobacteria; Gammaproteobacteria; Alteromonadales; HTCC2188; HTCC*			1
OTU87	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Loktanella*		2	2
OTU34	*Proteobacteria; Alphaproteobacteria; Sphingomonadales*			2
OTU6	*Proteobacteria; Alphaproteobacteria; Sphingomonadales; Erythrobacteraceae; Erythrobacter*			5
OTU258	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter*			2
OTU22	*Proteobacteria; Gammaproteobacteria; Oceanospirillales; Alcanivoracaceae; Alcanivorax*			3

3.4 Discussion and conclusions

The process of DNA extraction, sample preparation and sequencing generated an expected amount of data of good quality. This, together with the type of research question asked, allowed stringent quality filtering, which resulted in a dataset that can help in developing an understanding of heterotrophic partners present in *Synechococcus* sp. non-axenic cultures.

It is important to note that the absence of reads in a given sample is not necessarily indicative of the absence of a given species in a culture. The process of sample collection, DNA extraction, sequence of 16S rRNA gene primers used, read similarity above 97% and quality filtering steps may all contribute to false negative results. In a similar manner, the presence of a species may be a false positive result due to contamination of a culture or DNA sample, undetected chimeras, unrecognized sequencing errors or cross-talk. All reasonable measures were taken to minimize these errors whilst trying to capture the widest community possible during the extraction and sample preparation process, but focusing on the reads of the highest quality during the data analysis stages. This approach, although not eliminating the risk of errors mentioned above, provided a dataset suitable for analysis to answer the specific questions asked, rather than to describe the complete community composition.

There is some preliminary evidence that the relationship between the number of reads and abundance of a given species in a culture may not be of high correlation due to varying gene copy numbers (1-16 copies), primer mismatches, sequence composition (e.g. GC content) and amplification of biases (Edgar, 2017). Moreover, it is unclear how traditional measures such as alpha and beta diversity can be applied in the case of amplicon sequencing, taking into account both the process of sample preparation and data analysis, especially steps such as discarding singletons or cross-talk. However, in the case of this study, the main focus is to find which organisms are present over a number of samples, rather than their abundances.

The heterotrophic bacteria found co-occurring in *Synechococcus* sp. non-axenic cultures have been observed in previous studies of the marine environment and in association with other phototrophs in cultures. Both *Alphaproteobacteria* and *Bacteroidetes* (see Figure 3.3.1.1) were previously found to be associated with diatoms, dinoflagellates and cocolithophores (Amin et al., 2012; Fandino et al., 2001; Green et al., 2015; Moustafa et al., 2010; Shafer et al., 2002). *Rhodobacters,* members of the *Rhodobacteraceae,* were also found to coexist with *Prochlorococcus* (Sher et al., 2011). In freshwater cyanobacteria communities, *Alphaproteobacteria* and *Bacteroidetes* were also found to dominate cultures and samples from the natural environment (Zhu et al., 2016). The authors also note low abundance of *Actinobacteria*, which was also seen in the case of this study (Figure 3.3.1.2).

However, the main question of this study involved evaluating which heterotrophic partners are most frequently present in *Synechococcus* sp. non-axenic cultures. Four heterotrophs were identified as detected in more than 50% of cultures and two in more than 85% cultures. Given that the cultures are subsampled together and media for growth are prepared in batches for all cultures at the same time, it could be possible that these bacteria were introduced as contaminants. However, as these genera are associated with seawater and the risk of cross-contamination between cultures is very low (one culture open in a flow hood at a time, transferred into prepoured medium), this is unlikely. Moreover, three out of four of these heterotrophs were later detected in the *Synechococcus* sp. cruise enrichment samples that are not maintained together with culture collection samples, which further suggests that the presence of these genera is not due to cross-contamination.

Nitratireductor spp. have been isolated from beach sand (Kim et al., 2009), deep sea water of the Indian Ocean (Lai et al., 2011a), dried seaweed (Kang et al., 2009), Pacific Ocean sediments (Lai et al., 2011b), methanol-fed denitrification reactor treating seawater (Labbe et al., 2003) and a

diatom culture (Jang et al., 2011). The three BLAST hits with highest identities (99%) *Nitratireductor aquimarinus* CL-SC21, *Nitratireductor aquibiodomus* NL21 and *Nitratireductor pacificus* pht-3B are all nitrate reducers (Jang et al., 2011; Lai et al., 2011b), which suggests that the organism detected in over 85% of *Synechococcus* sp. culture collection samples may also have this ability. The *Synechococcus* genus comprises organisms that can and cannot grow on nitrate and nitrite as sole nitrogen sources, even two strains isolated from the same water sample and belonging to the same clade can be different in this respect (Fuller et al., 2003). There is also some evidence of a strain that could not grow on nitrate as a sole source of nitrogen, but could grow on nitrite (Moore et al., 2002). The presence of bacteria that reduce nitrate to nitrite could be advantageous for a cyanobacterium incapable of performing the process itself. Unfortunately, there is not enough data on utilization of different sources of nitrogen by all the strains used in this study to try to correlate these datasets. Moreover, out of 75 cultures, 49 are growing in a medium with nitrate as the main source of nitrogen (ASW), which suggests that the presence of these bacteria may simply be an effect of the type of media used for enrichment during the isolation process and for routine maintenance of cultures.

For OTUs classified as *Rhodobacteraceae*, it is difficult to find a close match. The BLAST algorithm predicted 98% identity with three marine bacteria: *Loktanella atrilutea* IG8 (Hosoya & Yokota, 2007), *Primorskyibacter insulae* SSK3-2 (Park et al., 2015) and *Poseidonocella sedimentorum* (Romanenko et al., 2012) while the RDP Seqmatch suggested *Rugeria* sp. TCg9 (S_ab score 0.982). A strain with high similarity (99.4%) to *Rugeria* sp. TCg9 was found to be associated with the dinoflagellate *Alexandrium tamarense* (Jasti et al., 2005). It is difficult to try to predict the function of the organism in cultures with information only to the level of family, especially a family as extensive and diverse as the

Rhodobacteraceae. More research is required to isolate, sequence and establish the role of this bacterium in *Synechococcus* sp. cultures.

Muricauda spp. have been isolated from Antarctic seawater (Wu et al., 2013), mangrove sediments (Yang et al., 2013), crude-oil contaminated seawater (Hwang et al., 2009), coastal hot springs (Arun et al., 2009), a seawater sediment suspension from an intertidal sediment off the German North Sea coast (Bruns et al., 2001), tidal flats (Yoon et al., 2008; Lee et al., 2012; Kim et al., 2013) as well as open ocean South Pacific Gyre waters (Zhang et al., 2015), which suggests that this genus of Gram negative bacteria is widely present in marine ecosystems. The first representative of this genus, *Muricauda ruestringensis*, was isolated from a bacterial community growing on hexadecane as the sole carbon source, although it was not able to degrade hexadecane itself (Bruns et al., 2001), which suggests that it was living on the chemical compounds produced or released by the community. It was also established that the bacterium could not grow on glucose, acetate, pyruvate, serine, ethanol or mannitol as sole carbon sources, but was able to grow on a wide spectrum of amino acids, mannose, fructose, lactose, sucrose, cellobiose and raffinose. Genome sequencing of the type species revealed a reduced number of motility-related genes (Huntemann et al., 2012) and no motility was observed in live cultures (Bruns et al., 2001). Instead, the authors noted that the polar located appendages, a continuum of the outer membrane, could be used for cell-to-cell connections or adhesion to a substratum. If a *Synechococcus* sp. or other organisms it lives in a community with, could be a source of carbon and energy for *Muricauda* sp., it could potentially stick to the cyanobacterial cell and be very difficult to remove or be lost from a culture, which could explain its high frequency in *Synechococcus* sp. culture collection samples.

Phyllobacteriacae was found to have its sequence most similar to *Aquamicrobium* sp. (isolated from an activated sewage sludge – Bambauer et al., 1998; biofilters – Lipski & Kampfer et al., 2012; polluted soil – Wu et al., 2014) by BLAST, but using the RDP Seqmatch, its closest matches

were *Mesorhizobium* sp. DG943 and *Rhizobium* sp. PRLIST04 (both isolated from dinoflagellate cultures; Green et al., 2004 and Prokic et al., 1998 respectively). As in the case of *Rhodobacteraceae*, it is difficult to infer the closest relatives and the function of a bacterium when only the family is know. Again, more work needs to be done to learn more about how members of this genera may be interacting with *Synechococcus*.

Synechococcus sp. was not detected in 4 culture collection samples: A15-30, BL3, RCC0307 and RCC2378. This may be due to several reasons – poor susceptibility to the DNA extraction process, mismatched primer sequences, too stringent quality filtering (especially cross-talk filtering) or even absence of the cyanobacteria from the culture. The presence of the cyanobacterium could be confirmed by PCR with genus specific primers.

Metadata related to the culture collection samples (*Synechococcus* sp. clade, subgroup, isolation latitude, longitude, depth, person isolating the stain and year of isolation) did not explain the differences in sample composition. There may be several reasons for this: there could actually be no correlation, the sample size could be too small, the composition could be dependent on more than one factor at the same time, the process of heterotroph loss or enrichment from very diverse natural communities could be stochastic to some extent or the process of heterotroph enrichment could depend largely on the metabolic functions of community members rather than their phylogeny. To answer these questions a wider study, with more samples per group and a larger number of replicates, should be designed. Taking into account that for most of the OTUs no reliable database matches at the genus level could be found, perhaps it would be more useful to look into community metagenomics instead of 16S rRNA gene amplicons only. This would provide not only a similar level of taxonomic assignment, but also shed some light on the pool of genes of the community which could help determine the metabolic abilities of its members. Transcriptomics and proteomics work on the same samples would provide invaluable information on the metabolic activities within the culture, which would help in forming an

understanding of the community structure, member interactions and flow of nutrients between *Synechococcus* sp. and heterotrophs.

As less OTUs were detected on average in *Synechococcus* sp. cruise enrichment cultures than culture collection samples and the original enrichments had on average more OTUs than 3rd liquid samples, it is important to stress that the absence of reads does not necessarily imply an absence of an organism in a culture. Thus, it is not possible to draw conclusions that culture collection samples are more or less diverse than cruise enrichments. The samples from our culture collection were collected after at least 90 days of growth to capture as wide a community as possible, including more slowly growing members, while cruise enrichment cultures were collected within a few weeks of collection or inoculation, which means that any organism with low abundance due to, for example, slow growth could be missed while sampling or discarded due to low, cross-talk level, read numbers. The same applies to the 13 OTUs which appear to be gained at some point between the original enrichment and 3rd liquid steps (Table 3.4.2). Moreover, when other OTUs (Table 3.3.4.2) were lost from the samples, these organisms, until then kept at low numbers due to competition, could gain new resources and improve their abundance up to a level above the detection threshold. At this point, with many of the OTUs not annotated to genus or species level (Table 3.3.4.2), it is not possible to predict why the abundance of certain members changed, as no information about their physiology is available.

Identification of members of a community by 16S rRNA gene amplicon sequencing is only as good as the databases used for comparing the new reads with already known and well annotated sequences. Unfortunately, many OTUs were classified to the level of family only due to lack of similar sequences in the databases used, especially in the case of the *Rhodobacteraceae* family. To improve our understanding of the composition of natural and laboratory culture communities, there is a strong need for a reliable database of 16S rRNA gene sequences from environmental

samples. A good starting point for such a database would be sequences from organisms we currently have in cultures with other organisms.

This study is not only useful in terms of improving our understanding of *Synechococcus* sp. – heterotroph interactions, but could also be useful for improving cultivation methods, especially the efforts to make cultures axenic. If a community composition is known, it may be easier to remove the unwanted species by understanding and exploiting, for example, its susceptibility to antibiotics or inability to use certain carbon or nutrient sources.

To conclude, the heterotrophic bacteria found to be most frequently co-occurring with *Synechococcus* sp. in cultures are representatives of the groups of heterotrophic bacteria most commonly associated with other phototrophs in the marine environment. A member of *Rhodobacteraceae* was found to be associated with more than 50% of all *Synechococcus* sp. cultures. Given this result and the fact that members of *Rhodobacteraceae* are consistently found to be associated with a range of phototrophs and to be dominant in both bloom and non-bloom surface ocean communities (Gonzalez et al., 2000), it seems that members of this group of marine bacteria could be utilised as model organisms for studying interactions between phototrophs and heterotrophs.

4. Exometabolites of *Synechococcus* spp. in axenic culture and during co-culture with heterotrophic bacteria

4.1 Introduction

A number of different types of interaction between members of a bacterial community can occur – both intra-species (Paz-Yepes et al., 2013) and inter-species (Sher et al., 2011); ranging from positive, such as improved iron assimilation (Amin et al., 2007; Amin et al., 2009), to negative, such as reduced growth (Ribalet et al., 2008). These interactions, even though are between microscopic organisms, can affect nutrient cycling (Letscher et al., 2015), shape phytoplankton communities (Bolch et al., 2017) and change the physio-chemical properties of the surrounding environment (Cole, 1982; Roeselers et al., 2007).

Some of these interactions can be facilitated by molecules – as a means of interacting with their environment, such as siderophores (Ito & Butler, 2005), and with other members of the community, such as antimicrobial compounds (Paz-Yepes et al., 2013). Compounds released into the environment can be produced with the purpose of excretion, come from leaky metabolic pathways or be released upon cell death. A metabolomics study of *Synechococcus elongatus* CCMP 1631 revealed that the most abundant extracellular compounds during growth of a culture are: adenosine, thymidine, n-acetylglutamic acid, 4-hydroxybenzoic acid, phenylalanine, tryptophan, succinic acid, inosine, indole 3-acetic acid, n-acetyltaurine, 3-mercaptopropionate, cyanocobalamin and taurocholate (Fiore et al., 2015). Although the study has shown that *Synechococcus* can contribute nucleosides, amino acids and organosulfur compounds to the DOM pool, it did not differentiate between molecules released intentionally and those that leak passively from the cell.

As there is a dearth of information on natural communities in the ocean – the members, interactions between them, pools of resources and metabolic dependencies – it is crucial to simplify the system to gain a better

understanding of the very basic principles governing the interactions between microorganisms. Using a cyanobacterium and a member of the *Rhodobacteraceae*, the group 'associated' with the vast majority of *Synechococcus* sp. cultures (see section 3.3), a simple, yet robust model of interactions was developed. The system involved co-culture between *Synechococcus* sp. WH7803 and *Ruegeria pomeroyi* DSS-3 which has recently been shown to stabilize the growth of *Synechococcus* over the long term (Christie-Oleza et al., 2017), while an axenic *Synechococcus* sp. WH7803 culture in the same medium can survive no more than 90 days. Previous studies of this model system (Christie-Oleza et al., 2017 & see section 1.1.4) did not involve small molecules or metabolites excreted from cells into the environment.

The aims of this Chapter were to:

- Identify and characterise the exometabolite(s) present or absent in *Synechococcus* sp. WH7803 – *Ruegeria pomeroyi* DSS-3 co-cultures compared to an axenic *Synechococcus* sp. WH7803 control culture;
- Determine if this molecule(s) is found in other axenic *Synechococcus* sp. cultures and in *Synechococcus* sp. WH7803 co-cultures with a range of different heterotrophs representing different taxonomic groups;
- Elucidate the effect of the presence of *Ruegeria pomeroyi* DSS-3 on the production and stability of this molecule(s).

4.2 Materials and methods

4.2.1 Screening of *Synechococcus* sp. strains

Three axenic *Synechococcus* sp. WH7803 cultures (each 100ml volume) and three *Synechococcus* sp. WH7803 co-cultures with *Ruegeria pomeroyi* DSS-3 (each 100 ml volume) were grown under standard conditions (see section 2.2) and sampled after 45 days of growth. Samples (2 ml) of all cultures were assessed in terms of growth via spectrophotometry ($OD_{750\ nm}$) (see section 2.3), filter-sterilized to remove cells and frozen at -20°C until they were thawed and ran on a LC-MS in a single run following the routine protocol described in section 2.6 for metabolite level assessment.

Five axenic *Synechococcus* strains, *Synechococcus* spp. WH5701, WH7803, WH7805, WH8102 and CCMP9311 were grown under standard conditions (see section 2.2) for 45 days, in 15 flasks each containing 100 ml volume. The cultures were subjected to centrifugation (15 minutes, 3220 g) and the supernatants were filtered through a 0.22 μm pore size PES filter. The flow through was used for extraction with ethyl acetate (see section 2.6).

The extraction process was performed on 1500 ml of *Synechococcus* sp. WH7803 culture supernatant three times, at pH 3, 7 and 11. Also, 50 ml of a *Synechococcus* sp. WH7803 cell pellet (obtained by centrifugation at 3220 g for 15 minutes) was washed 3 times with 10 ml ASW and then sonicated 3 times (highest intensity, 15 s) in ethyl acetate. Then the broken cells underwent a standard ethyl acetate extraction process (see section 2.6), with volumes of solvents appropriately adjusted for 50 ml instead of 1500 ml.

Samples from three axenic *Synechococcus* sp. WH7803 cultures and three co-cultures with every heterotroph strain (Table 4.2.1.1; strain information in Table 2.1.2) collected after 40 days of growth in standard conditions (see section 2.2) were obtained from Despoina Sousoni (School of Life Sciences, University of Warwick). The filtered supernatants were prepared for a LC-

MS run and ran following the routine protocol (see section 2.6) in a single run.

Table 4.2.1.1 Heterotrophic organisms grown in co-culture with *Synechococcus* sp. WH7803 for exometabolomic analysis.

Group	Organism
Actinobacteria	*Salinispora tropica*
	Aeromicrobium marinum
Bacteroidetes	*Polaribacter* sp.
	Algoriphagus machipongonensis
	Gramella forsetii
	Formosa agariphila
α-proteobacteria	*Ruegeria pomeroyi*
	Roseobacter denitrificans
	Dinoroseobacter shibae
γ-proteobacteria	*Pseudoalteromonas citrea*
	Alteromonas macleodii
	Marinobacter adhaerens
	Pseudomonas stutgeri
Planctomycetes	*Planctomyces limnophilus*
	Rhodopirellula baltica
Verrucomicrobia	*Verrucomicrobiae bacterium*

4.2.2 Monitoring metabolites throughout the growth curve

Three *Synechococcus* sp. WH7803 cultures of 100 ml volume were grown under standard conditions (see section 2.2) and sampled every 5 days for the first 60 days and every 10 days thereafter, until 100 days. Each time, 2 ml of culture was removed, an OD reading at 750nm was taken (see section 2.3) and then the samples were filter-sterilized and frozen at -20°C until further analysis. Once all the samples were frozen, they were thawed and ran on a LC-MS following the routine protocol (see section 2.6) for metabolite level assessment in a single run.

For nutrient limitation studies, three *Synechococcus* sp. WH7803 cultures of 100 ml volume were grown in standard ASW medium as well as -N, -P and -Fe media (see Table 2.2.1). Before inoculation from a standard ASW culture, cells were pelleted (10 ml centrifuged at 15 min, 8050 g) and washed three times with nutrient limited medium and resuspended in 10 ml of the nutrient limited medium, except for –N medium (10 ml of unwashed culture used for inoculation). 2 ml of culture was removed every 5 days for

70 days (or until death of the culture) for monitoring growth (OD reading at 750 nm) and LC-MS analysis (routine protocol, single run; see section 2.6).

4.2.3 Assessing the effect of adding a bacterial heterotroph on *Synechococcus* metabolite production

Nine *Synechococcus* sp. WH7803 cultures each of 100 ml volume were grown under standard conditions (see section 2.2) and sampled (2 ml) every 5 days until day 65, and then every 10 days until day 120. *Ruegeria pomeroyi* DSS-3 was added to separate cultures at day 40 and 65 in triplicate (i.e. 3 axenic cultures and 6 co-cultures at the end of the experiment). Samples for LC-MS were prepared from filter-sterilised supernatant of three axenic cultures and all co-cultures on days 0, 15, 25, 40, 50, 60, 75 and 85 and processed in a single run (routine protocol, see section 2.6).

The *Ruegeria pomeroyi* DSS-3 culture was washed 3 times in ASW (10 ml of 36 h culture, centrifuged at 15 min, 8050 g, and resuspended in 1.2 ml ASW) and 100 µl added to 20 ml of ASW and 20 ml of filter-sterilised *Synechococcus* sp. WH7803 supernatant (from a 45 day old culture, grown in standard conditions – see section 2.2). Together with axenic ASW and axenic filter-sterilised *Synechococcus* sp. WH7803 supernatant, the flasks were kept at 22°C (±1°C) under constant light of 5-30 µmol photons m^{-1} s^{-1} and shaking at 140 rpm. Samples for LC-MS (2 ml) were taken every 10 days for 50 days, prepared and ran following the routine protocol (see section 2.6).

4.2.4 Chemical formulae of the molecules

Synechococcus sp. WH7803 supernatant was ran on high resolution LC-MS at the Department of Chemistry, University of Warwick. The m/z values and isotopic patterns of the compounds were used to generate possible chemical formulae of the molecules (see section 2.7).

Synechococcus sp. WH7803 supernatant (1.5 l) underwent ethyl acetate extraction (see section 2.6). After resuspension in 50% methanol, MS

fragmentation patterns were obtained by LC-MS^n, depending on the intensity of the peaks, up to MS^5 (see section 2.7).

4.3 Results

4.3.1 Assessing the effect of heterotroph addition on *Synechococcus* sp. WH7803 metabolites and metabolite screening of five axenic *Synechococcus* spp. strains and a range of heterotrophs

Synechococcus sp. WH7803 – *Ruegeria pomeroyi* DSS-3 co-cultures contained lower levels of six molecules, in the m/z range of 364 to 449, than axenic *Synechococcus* sp. WH7803 cultures (Figure 4.3.1.1). These six compounds were not detected in other axenic *Synechococcus* cultures i.e. *Synechococcus* spp. WH7805, WH5701, WH8102 and CC9311 nor in ASW medium (Figure 4.3.1.2). Extracts of *Synechococcus* sp. WH7803 prepared at pH 3, 7 and 11 (Figure 4.3.1.3) showed that highest levels of the six molecules were detected at pH 3. Thus, this pH was used for all subsequent extractions. Four compounds were identified on base peak chromatograms that were present in the pellet, but not in ASW medium nor the supernatant (Figure 4.3.1.4) and six compounds (the same as detected previously) were present in the supernatant and not in the pellet nor ASW. High resolution MS allowed prediction of chemical formulae of the compounds present in the culture supernatant only (Table 4.3.1.1, Figure 4.3.1.5).

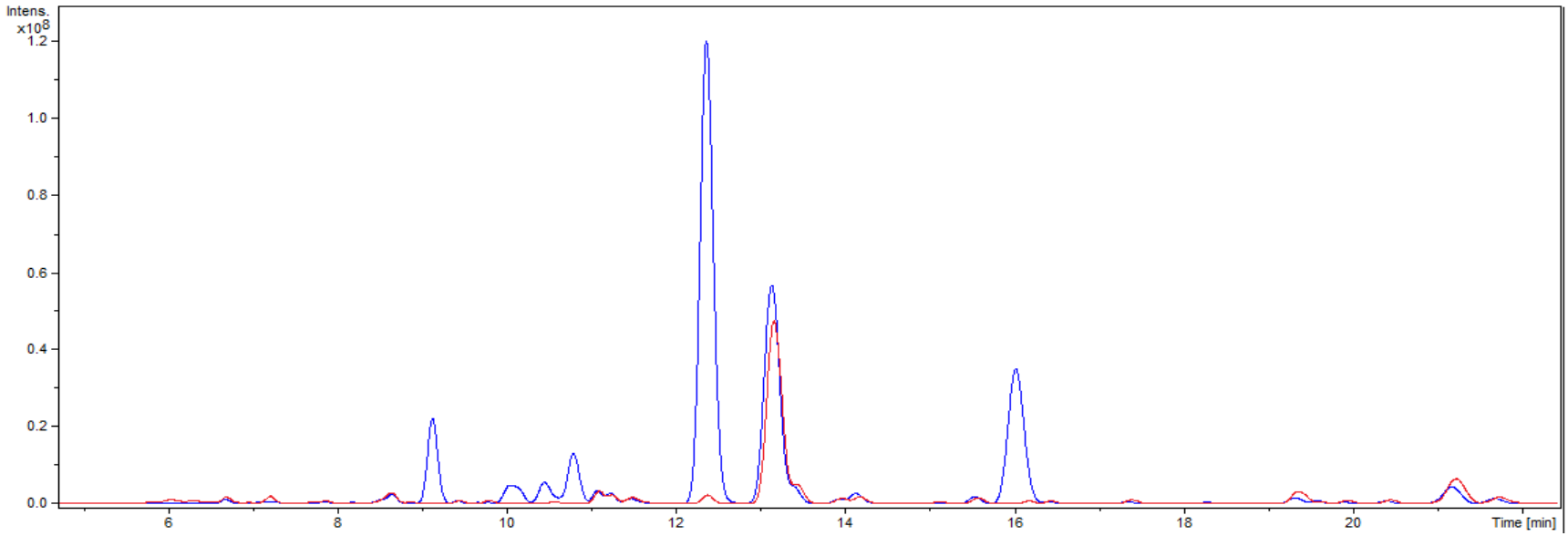


Figure 4.3.1.1 Comparison of axenic *Synechococcus* sp. WH7803 culture (blue) and *Synechococcus* sp. WH7803 – *Ruegeria pomeroyi* DSS-3 co-culture (red) metabolite profiles (base peak chromatograms).

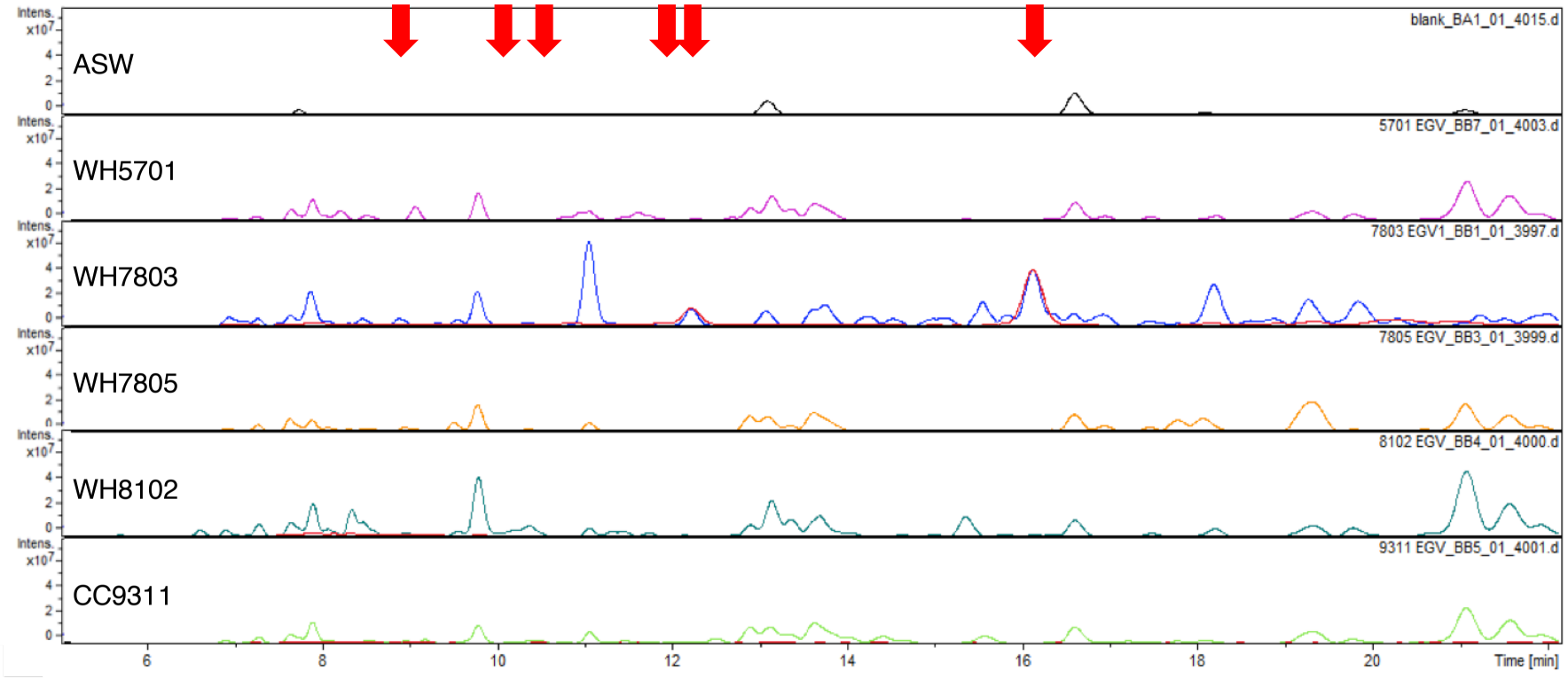


Figure 4.3.1.2 Comparison of *Synechococcus* spp. CC9311, WH5701, WH7803, WH7805 and WH8102 extracts (base peak chromatograms; in red: extracted ion chromatograms for m/z=421.72; 465.25; 364.21; 449.26; 408.22; 392.24).

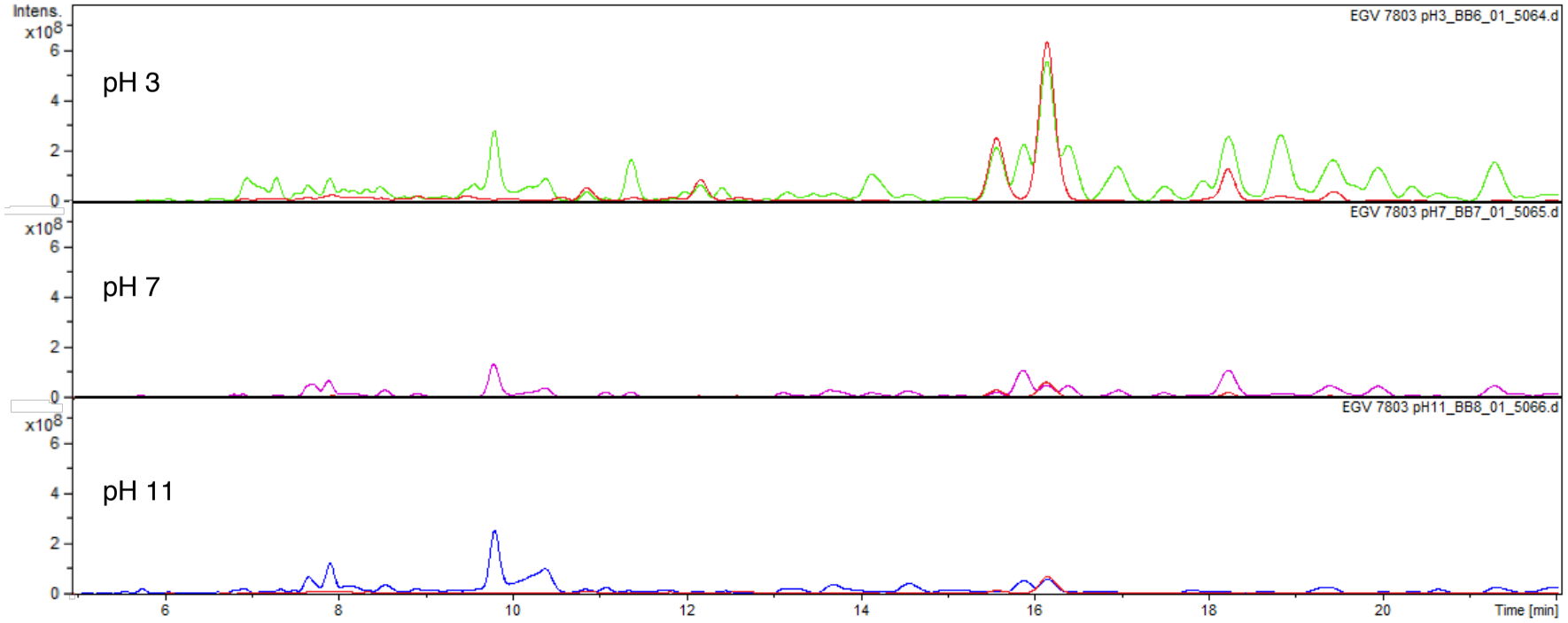


Figure 4.3.1.3 Comparison of *Synechococcus* sp. WH7803 supernatant extracted at pH 3 (green), 7 (pink) and 11 (blue) – base peak and extracted ion chromatograms for m/z=421.72; 465.25; 364.21; 449.26; 408.22; 392.24 (red).

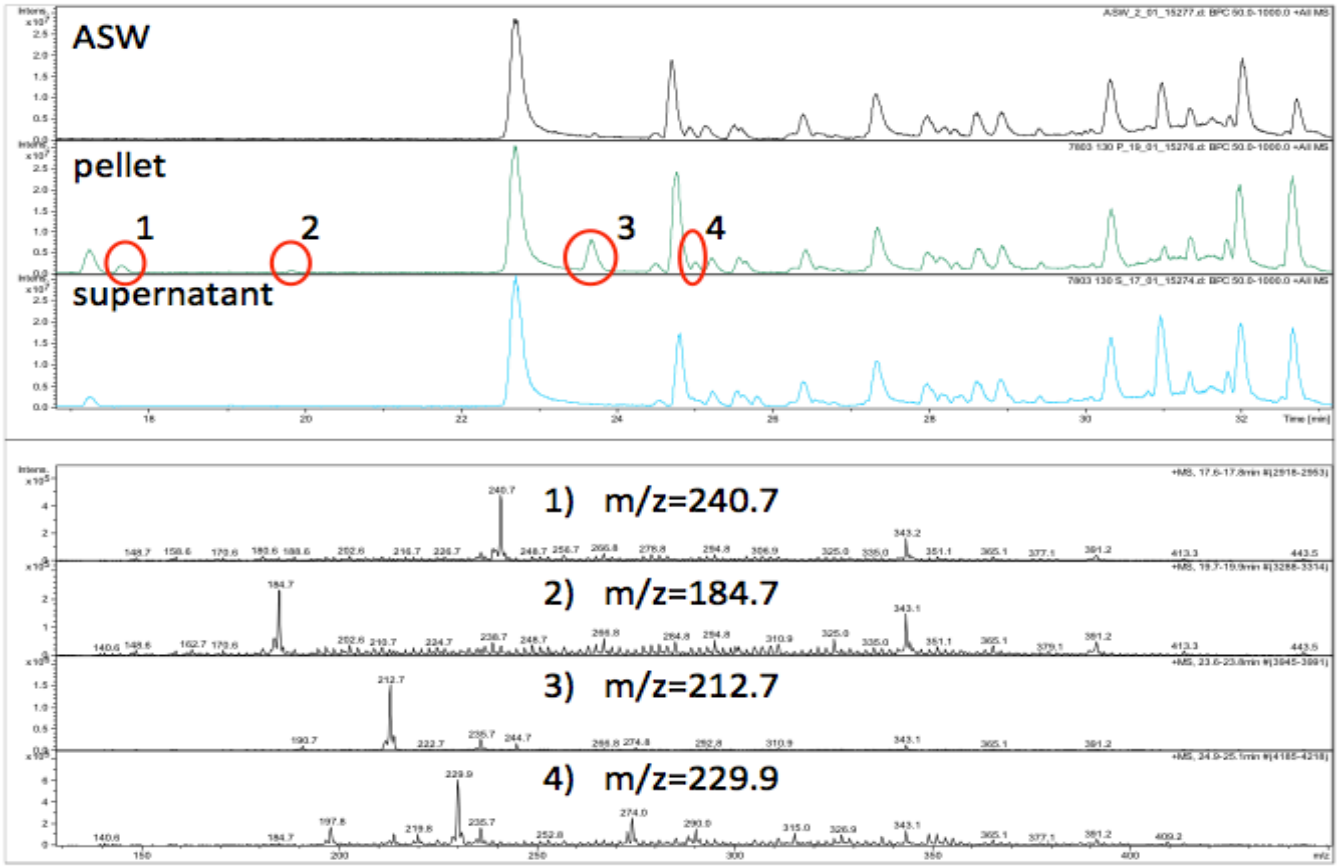


Figure 4.3.1.4 Compounds found in the cell pellet, but not in the supernatant extract of *Synechococcus* sp. WH7803.

Table 4.3.1.1 Predicted chemical formulae of ionised *Synechococcus* sp. WH7803 compounds.

Measured m/z	Retention time [min]	Predicted chemical formula	Theoretical m/z	Formula error [ppm]	Isotopic pattern fit [miliSigma]
392.2463	15.8-16.2	$[C_{19}H_{38}NO_5S]^+$	392.2465	0.7	57.0
408.2424	12.5-12.7	$[C_{19}H_{38}NO_6S]^+$	408.2414	-2.5	44.6
364.2156	10.7-10.9	$[C_{17}H_{34}NO_5S]^+$	364.2152	-1.1	40.6
449.2696	12.3-12.4	$[C_{21}H_{41}N_2O_6S]^+$	499.2680	-3.6	n/a
465.2668	9.9-10.3	$[C_{21}H_{41}N_2O_7S]^+$	465.2629	-8.4	n/a
421.23	9.0-9.2	$[C_{19}H_{37}N_2O_6S]^+$	421.2367	n/a	n/a

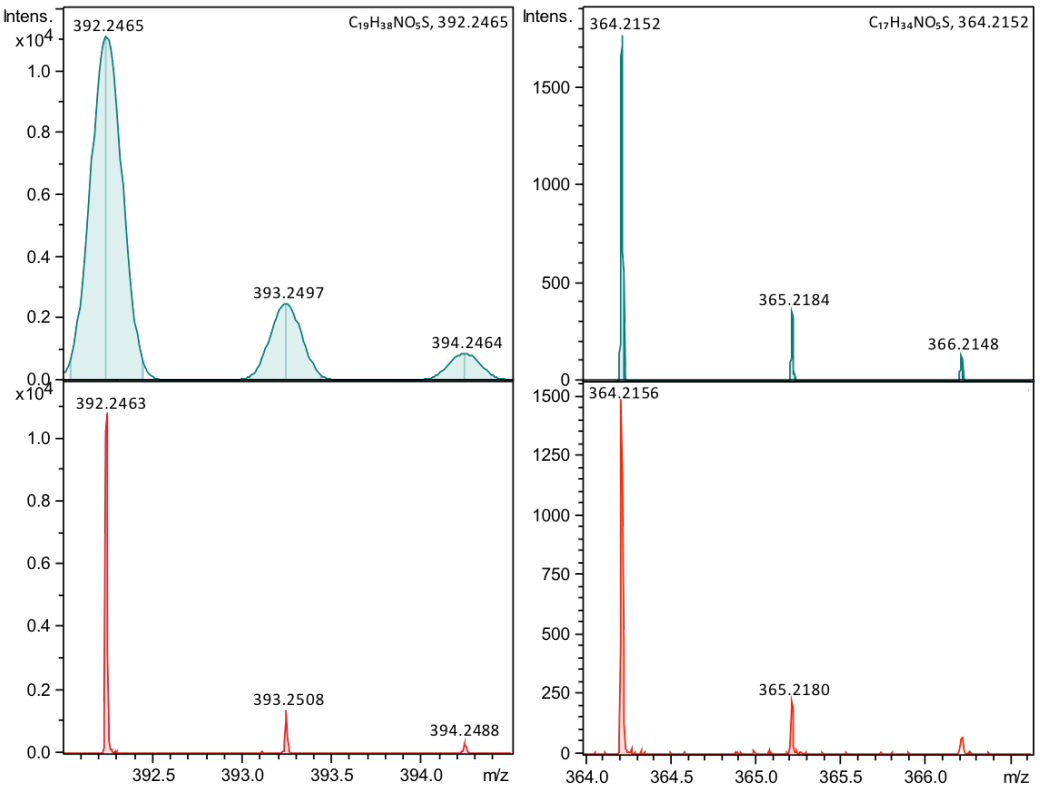


Figure 4.3.1.5 Comparison of isotopic patterns generated for predicted chemical formulae (green) of m/z=392 (left) and m/z=364 (right) compounds with patterns obtained experimentally (red).

In supernatant collected from co-cultures of *Synechococcus* sp. WH7803 with a number of different heterotrophs belonging to six major taxonomic groups (Table 4.2.1), no or very low levels of the six molecules were detected for most of the co-cultures. Two exemptions from this rule were observed – i.e. when the production of the molecules did not seem to be

affected by the presence of a heterotrophic organism. These were *Synechococcus* sp. WH7803 co-culture with *Rhodopirellula baltica* and *Verrucomicrobiae bacterium* (see Figure 4.3.1.6).

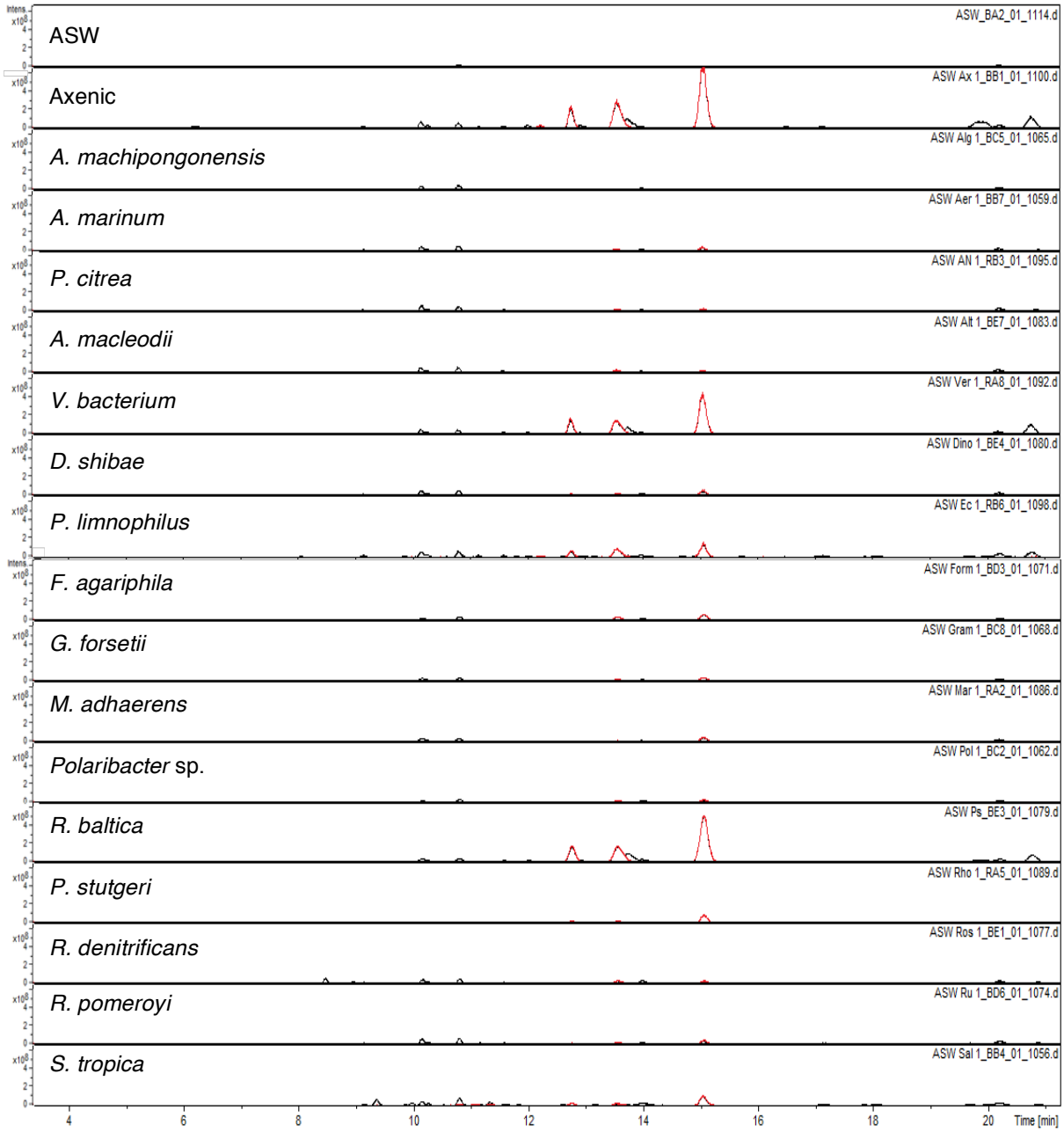


Figure 4.3.1.6 Comparison of co-cultures of *Synechococcus* sp. WH7803 with a range of heterotrophs (base peak chromatograms – black; extracted ion chromatograms for m/z=421.72; 465.25; 364.21; 449.26; 408.22; 392.24 – red).

4.3.2 Assessment of the production of the *Synechococcus* sp. WH7803 exometabolites under different growth conditions

An axenic *Synechococcus* sp. WH7803 culture was monitored for 100 days and the intensity of the m/z=392 peak assessed throughout growth. The m/z=392 compound was observed to increase slowly during the exponential phase, quickly once stationary phase was reached, peaking on day 40 (when the optical density of the culture was already declining) before then starting to decrease (Figure 4.3.2.1). The peak for the m/z=364 molecule followed the same pattern, while the intensity of the peak representing compound with m/z=449 was highest in early and late exponential phase, with a drop in mid exponential phase.

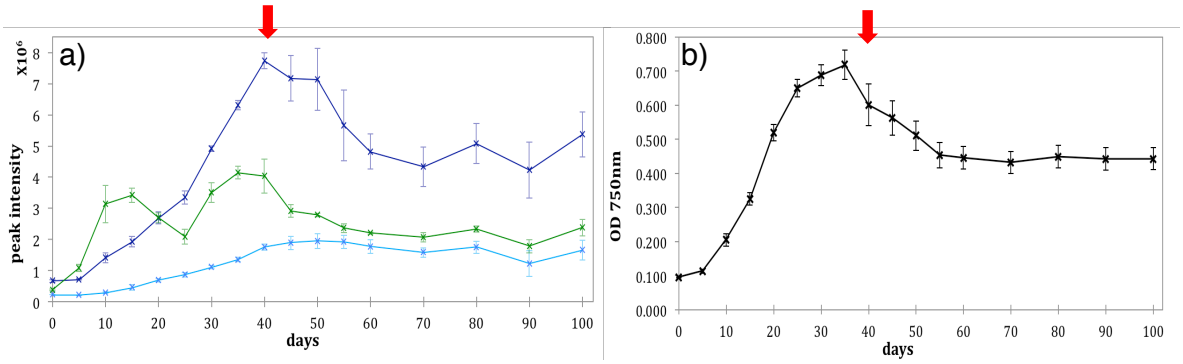


Figure 4.3.2.1 (a) Peak intensity for the m/z=364 (light blue); 449 (green) and 392 (dark blue) molecules measured by LC-MS throughout (b) growth of axenic *Synechococcus* sp. WH7803 cultures measured as optical density at 750 nm.

The same three molecules, which have the highest concentration out of all six compounds, can also be observed in *Synechococcus* sp. WH7803 cultures grown in natural seawater and natural seawater with additional nutrients (Figure 4.3.2.2). Moreover, the effect of *Ruegeria pomeroyi* DSS-3 addition on the molecules was the same as in nutrient-rich seawater (Figure 4.3.2.3). This suggests that this molecule is likely also produced under natural environmental conditions.

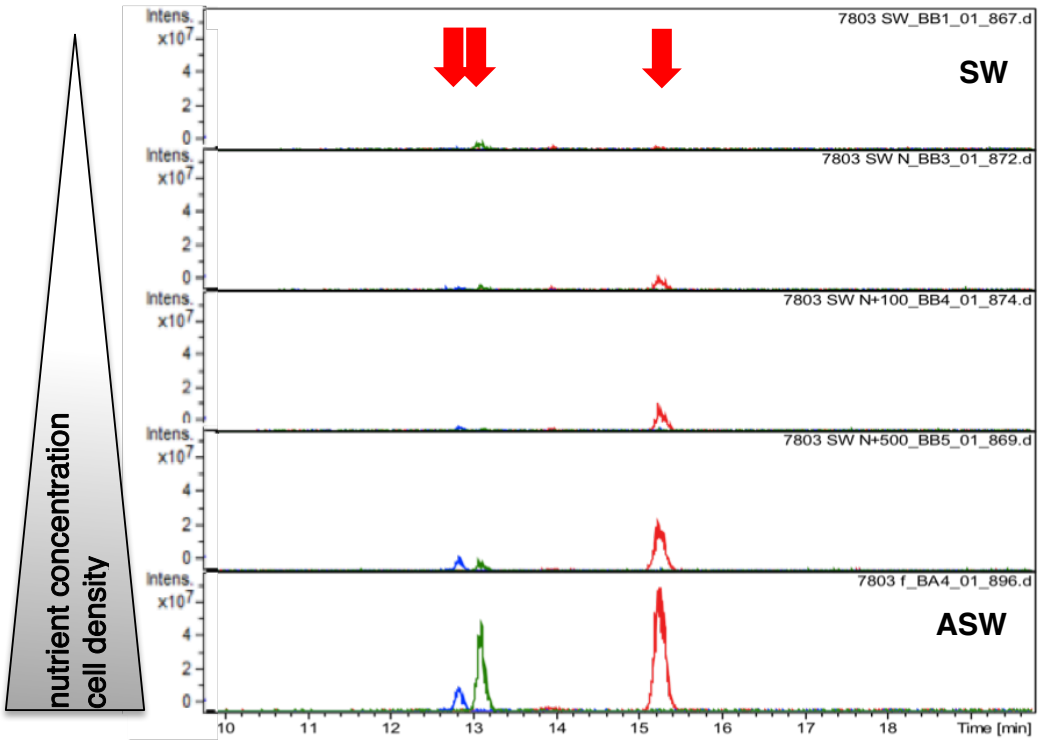


Figure 4.3.2.2 Assessing the presence of the compounds produced by axenic *Synechococcus* sp. WH7803 in different growth media – natural seawater (SW), seawater with increasing amount of extra nutrients and artificial seawater (ASW): m/z=392 (red), 449 (green) and 364 (blue).

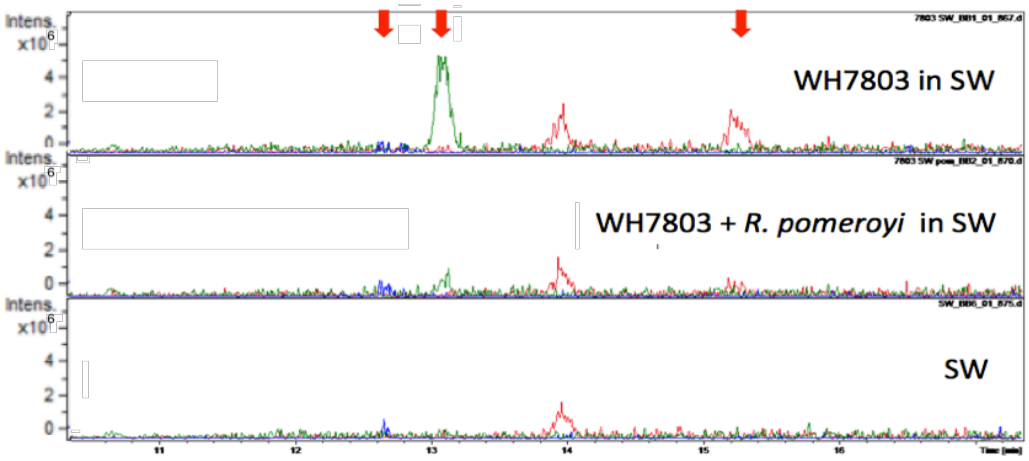


Figure 4.3.2.3 Comparison of the peak intensity of the m/z=392 (red), 449 (green) and 364 (blue) molecules produced by axenic *Synechococcus* sp. WH7803 in seawater and when grown in a co-culture with *Ruegeria pomeroyi* DSS-3, compared to a seawater control (SW).

In nutrient-limited cultures, despite poor culture growth (see Fig. 4.3.2.4 b), no major difference could be observed between cultures grown in standard ASW medium and its nutrient limited variations – the m/z=392, 364 and 449 molecules were produced in all conditions (Figure 4.3.2.4 a).

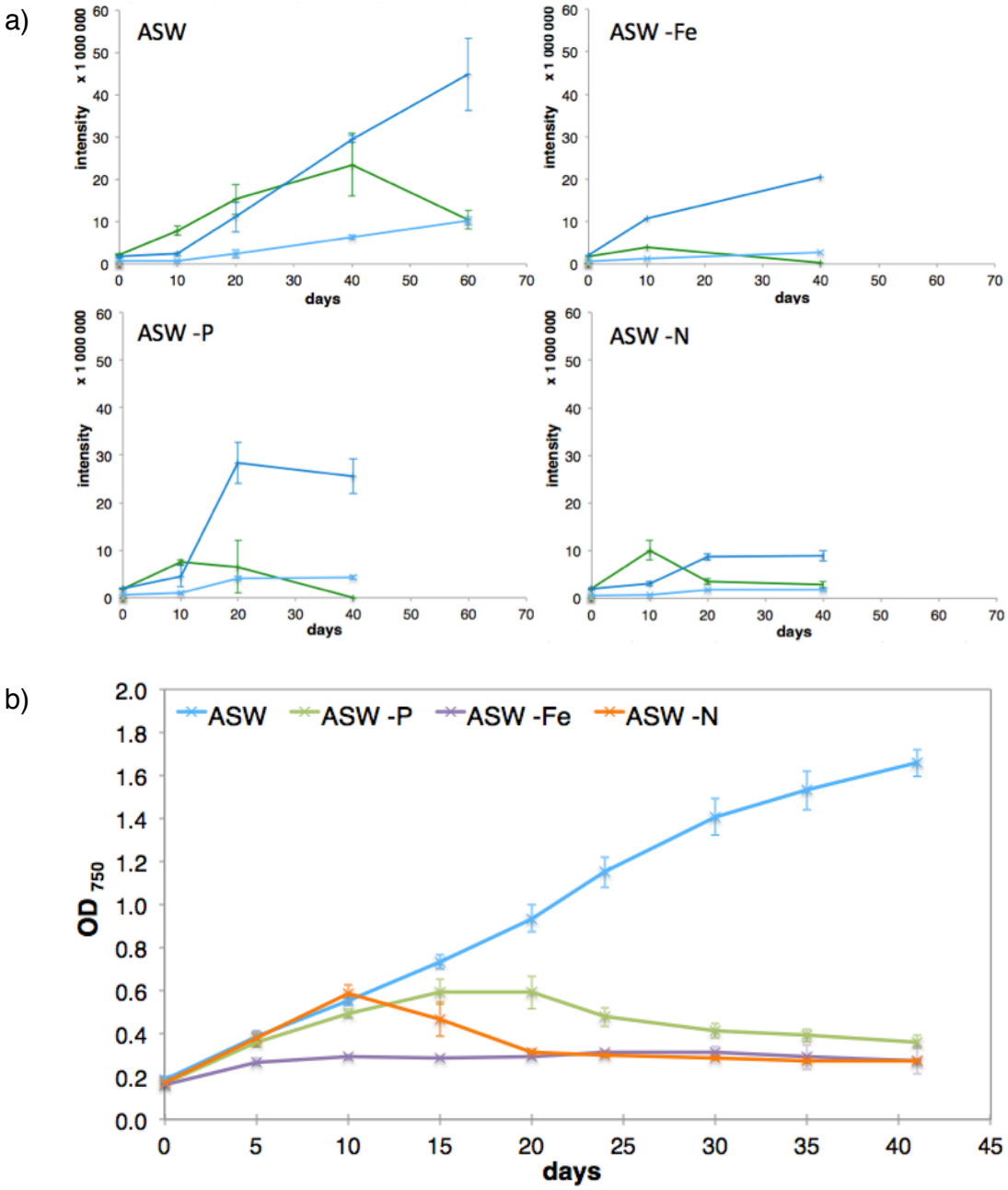


Figure 4.3.2.4 (a) Peak intensity for the m/z=364 (light blue); 449 (green) and 392 (dark blue) molecules measured by LC-MS throughout (b) growth of axenic *Synechococcus* sp. WH7803 cultures measured as optical density at 750nm in standard, no iron, no nitrogen and no phosphorus ASW media.

4.3.3 Assessing the effect of heterotroph addition at different stages of *Synechococcus* sp. WH7803 growth on the production of the exometabolites

Once *Ruegeria pomeroyi* DSS-3 was added to *Synechococcus* sp. WH7803 cultures the peak intensity for the m/z=449 compound decreased rapidly, with no molecule detected after 10-20 days (Figure 4.3.3.1). As for the molecule with m/z=392, the response depended on the growth phase *Synechococcus* sp. WH7803 was in at the time of heterotroph addition – in exponential phase there was no further increase in the peak intensity with time, but in stationary phase peak intensity followed the same pattern as that of an axenic culture, despite increased long-term survival due to the presence of the heterotroph.

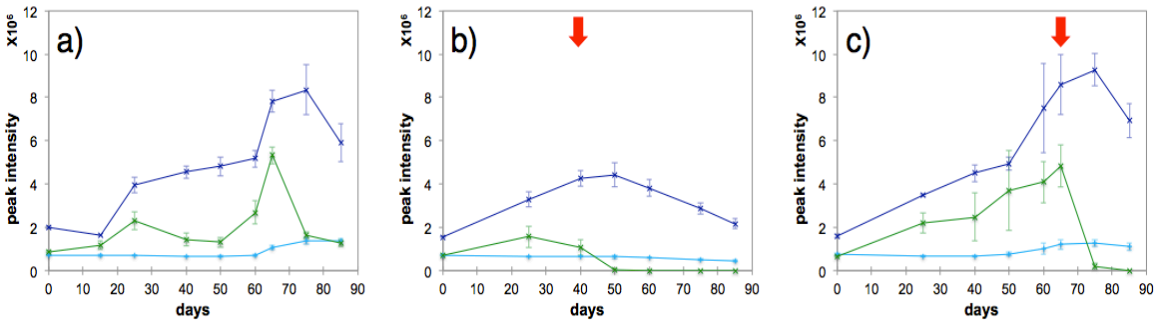


Figure 4.3.3.1 (a) Peak intensity for the m/z=364 (light blue); 449 (green) and 392 (dark blue) molecules in axenic *Synechococcus* sp. WH7803 cultures and (b) co-cultures in which *Ruegeria pomeroyi* DSS-3 was added during exponential growth of *Synechococcus* sp. WH7803 (40 days) and (c) stationary phase (65 days).

4.3.4 Assessing the effect of heterotroph addition to cell-free *Synechococcus* sp. WH7803 culture supernatant on exometabolite concentration

The intensity of the m/z=392 and 364 compounds produced from *Synechococcus* sp. WH7803 supernatants with and without *Ruegeria pomeroyi* DSS-3 did not decrease with time. This suggests that the presence of *Ruegeria pomeroyi* DSS-3 does not affect the level of these

compounds (see Figure 4.3.4.1). However, the m/z=449 compound decreases only when *Ruegeria pomeroyi* DSS-3 is present in the supernatant which suggests that the heterotroph is responsible for this decrease. The same effect was also observed in the experiment where *Ruegeria pomeroyi* DSS-3 was added at different stages of *Synechococcus* sp. WH7803 growth (see section 4.3.3).

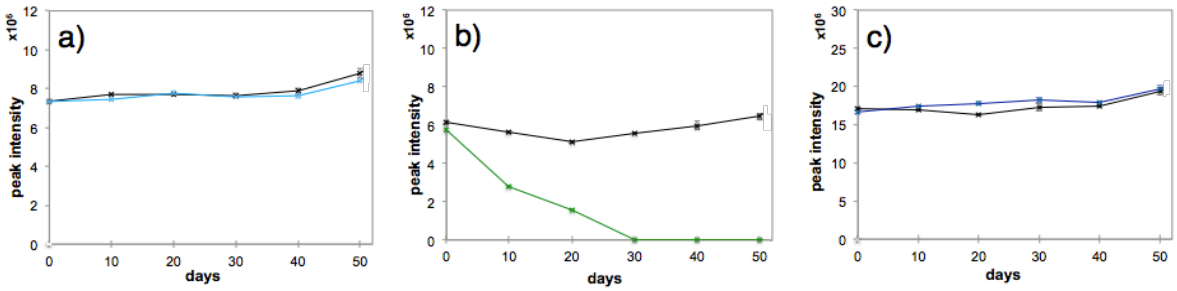


Figure 4.3.4.1 (a) Peak intensity for the m/z=364; (b) 449 and (c) 392 molecules following the addition of *Ruegeria pomeroyi* DSS-3 to cell-free supernatants from axenic *Synechococcus* sp. WH7803 cultures. Black lines represent peak intensities for the supernatants not inoculated with the heterotroph.

4.3.5 MS^n fragmentation patterns of the molecules

MS^n fragmentation data was successfully obtained for three compounds: m/z=392, m/z=364 and m/z=408 (Figures 4.3.5.1-4.3.5.3). The first two compounds have similar fragmentation patterns and are different by a C_2H_4 group. MS^2 of both m/z=392 and m/z=364 molecules results in a loss of a m/z=18 fragment (corresponding to H_2O; see Figures 4.3.5.1-4.3.5.2). The same happens during MS^3 of both compounds. MS^4 of the m/z=392 molecule results in a loss of a m/z=28 fragment (corresponding to C_2H_4) detecting a fragment ion of m/z=328, which is the same mass as that of a MS^3 fragment ion of the m/z=364 compound. Further fragmentation of fragment ion m/z=328 obtained from both precursor ions, results in exactly the same fragment ions: m/z=198, m/z=180 and m/z=154. No similarities were observed between the m/z=408 and m/z=392 molecules nor the m/z=408 and m/z=364 molecules in positive ion mode (Figure 4.3.5.3).

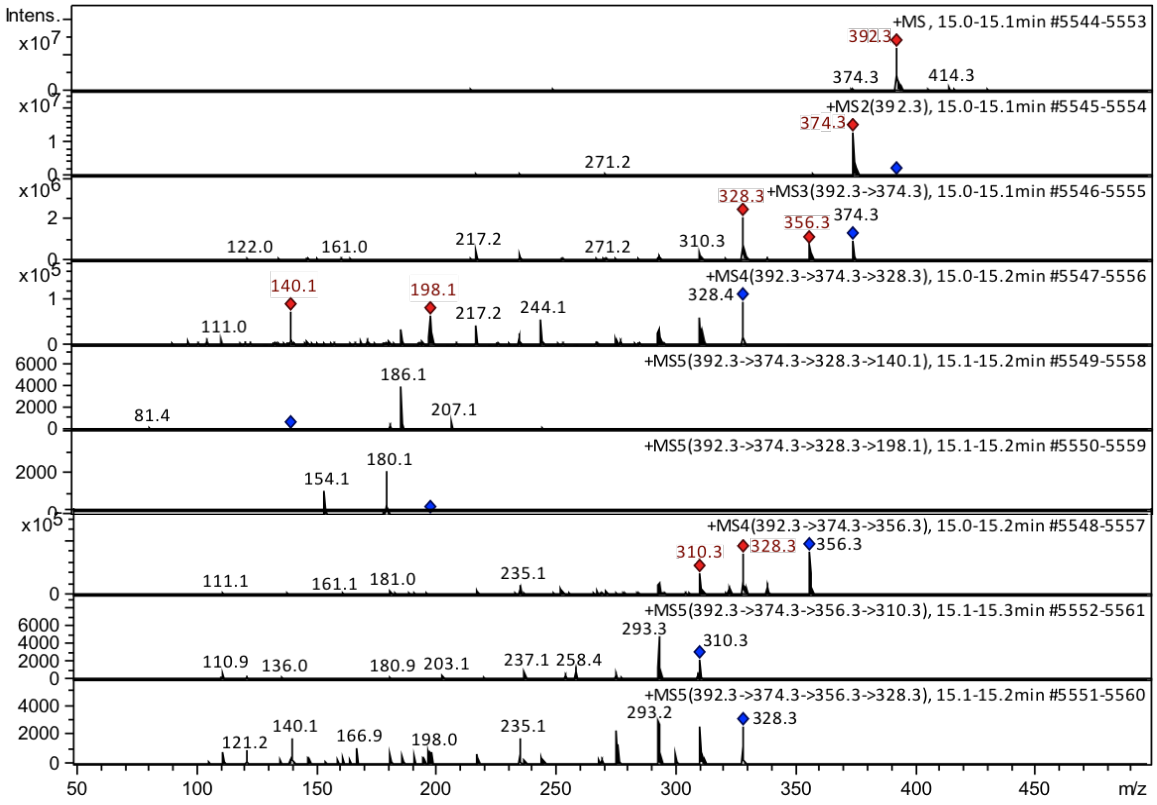


Figure 4.3.5.1 MS^n fragmentation of the m/z=392 molecule.

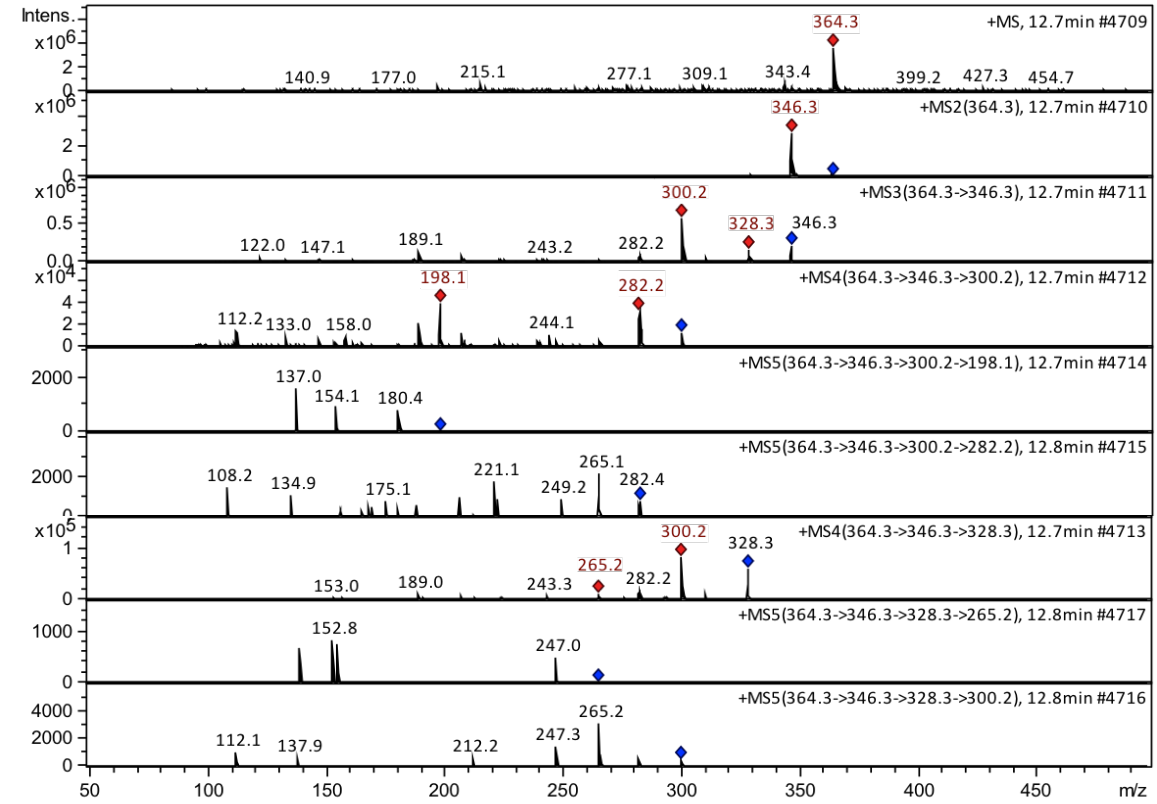


Figure 4.3.5.2 MS^n fragmentation of the m/z=364 molecule.

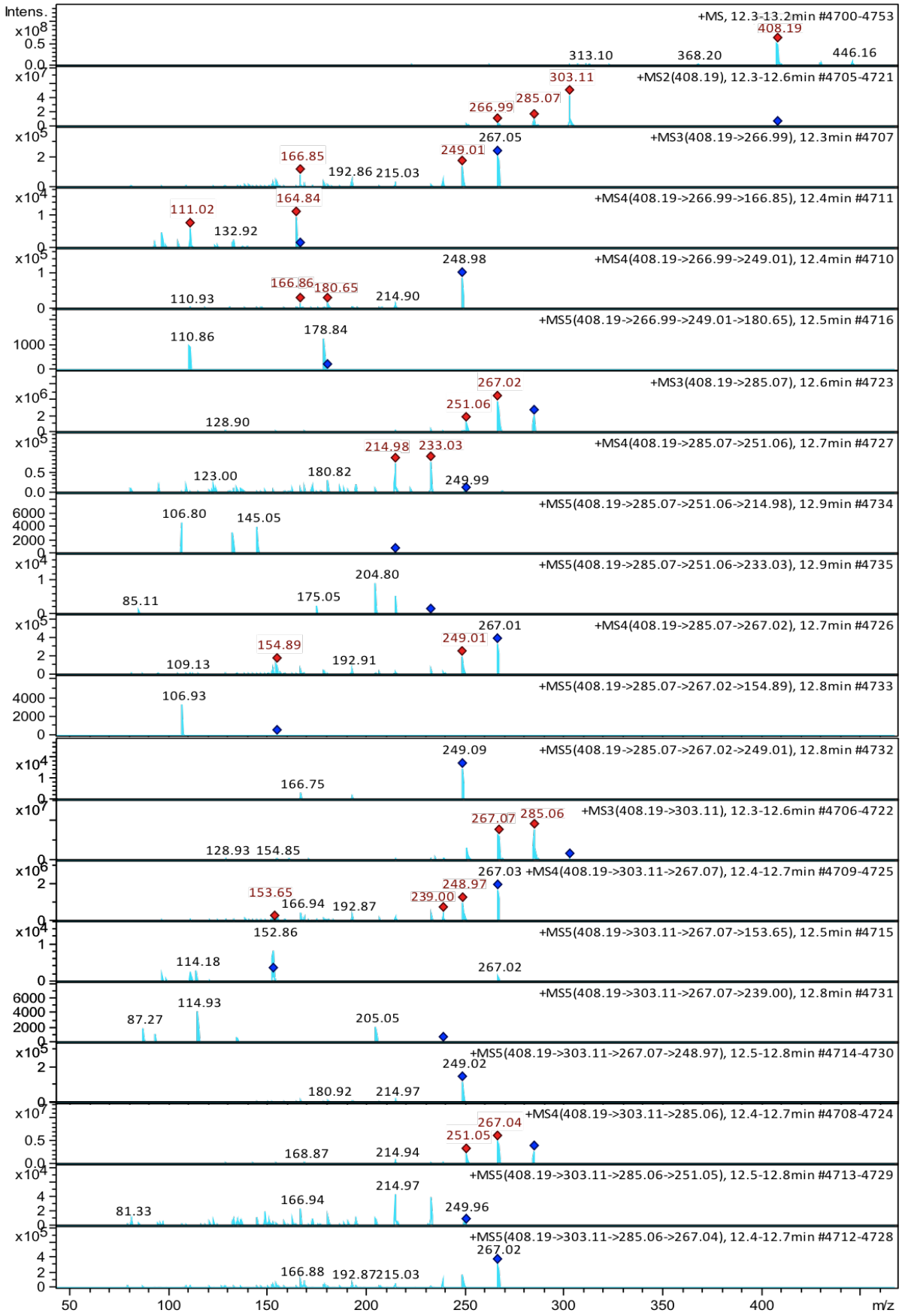


Figure 4.3.5.3 MS^n fragmentation of the m/z=408 molecule.

4.4 Discussion and conclusions

Synechococcus sp. WH7803 produces six exometabolites not present in other axenic *Synechococcus* spp. strains, even including a strain obtained in the same year and from the same location (*Synechococcus* sp. WH7805; Table 2.1.2). It is important to note that the absence of a molecule on a chromatogram could be due to not only a given strain not producing the compound, but also due to differences in expression between strains, production being below the detection level, or because of a high background signal, or to problems with sample preparation or ionization. With the LC-MS sensitivity in the low fg range, the amount of compounds present in the medium does not have to be high to give at least a weak signal, which, if not visible on a base peak chromatogram, can be noticed by generating an extracted ion chromatogram for a given m/z value. Although the same growth medium, sample preparation technique and MS settings were used for samples of all strains and the results were reproducible on more than one MS instrument, the search for the six molecules, and any related compounds, should be extended to extracts from larger culture volumes, at several different pH values and using more than one ionization technique and settings. Also, once the genes involved in the biosynthetic pathway are known, a study of similar gene clusters in other strains could shed some light on whether similar molecules can be produced by organisms related to *Synechococcus* sp. WH7803 and if so, suggest what modifications of the compounds may be possible.

Although ESI-MS is not regarded as a quantitative technique, the detected signal is proportional to analyte concentration from the limit of detection to around 10 µmol/L of analyte (Ho et al., 2003) and largely independent of flow rate and injection volume (Bruins, 1998). However, biological samples often contain non-volatile materials (e.g. salts), which can affect ionization of the compounds of interest (King et al., 2000). The ethyl acetate extraction process minimizes this risk, but is not possible for samples of low volume. The best way to quantify these molecules would be to spike samples with a

stable-isotope labelled synthesized molecule of a similar structure. However, as structures of the molecules are not yet known, this was not possible for this study.

The absence of the six *Synechococcus* sp. WH7803 molecules from the cell pellet extract suggests that the molecules are excreted from the cell – actively or passively – into the surrounding medium. This could suggest a possible toxic effect of the molecules for the cell itself – the compounds may have to be bound to carriers when inside the cell, to be isolated in a vesicle or to be modified before being released – or could be produced only outside of the cell. There is also the possibility of unsuccessful extraction – the cells may not have not been broken down properly by the solvent and sonication, causing the molecules to remain attached to cell debris or be degraded during the process.

Three of the molecules analysed (m/z 364, 392, 449) are produced throughout the growth curve, with highest levels observed in late exponential and stationary phase. The molecules seem to accumulate and not be lost from culture upon the death of *Synechococcus* sp. WH7803. It is difficult to judge whether the increase is due to upregulated production of the molecules or just an effect of the increase in cell population – a study involving direct cell counts by flow cytometry or microscopy would be required to normalize the production of the compound to cell numbers. The mid-exponential drop in the m/z=449 molecule (see Figure 4.3.2.1) was also observed in axenic cultures during the heterotroph addition experiment (see Figure 4.3.3.1). This could suggest that the molecule is produced in higher amounts during a change in growth conditions (transition to exponential growth, transition to stationary phase), or that it is broken down or converted to another compound during the most intensive growth or other compounds present during the mid-exponential phase affect the ionization of this compound.

Interestingly, levels of the six molecules were much lower, or absent, in co-cultures with heterotrophic bacteria compared to those from axenic

Synechococcus sp. WH7803 cultures. This effect was observed in all but two of the co-cultures tested. In the case of *R. baltica*, the reason for this was probably very poor growth of the heterotroph (D. Sousoni, personal communication), possibly due to very specific nutrient needs and different salt and temperature optima of this heterotroph compared to the phototroph (Schlesner, 1994). However, this was not the case for the no change in the production of the metabolite in *Synechococcus* sp. WH7803 – *V. bacterium* co-cultures compared to the axenic control and thus this interaction should be studied further to find the cause of such an effect.

The decrease, or disappearance, in the level of these molecules from co-cultures compared to axenic cultures suggests that the expression of these metabolites is directly or indirectly controlled by the presence of the heterotroph. However, these molecules do not seem to be essential for growth and division since *Synechococcus* growth continues in co-culture, unless *R. pomeroyi* can provide similar, yet undetectable with LC-MS, compounds or services to *Synechococcus* sp. WH7803 that fulfil the same function as that of the six molecules.

In co-cultures of *Synechococcus* sp. WH7803 with heterotrophic bacteria, the molecule with m/z=449 disappears within 10-20 days which suggests that the heterotroph is responsible for the decrease – either by using the molecule as a nutrient source or degrading it indirectly by producing a substance that breaks it down or binds to the compound and affects ionisation. As the molecule is stable in spent *Synechococcus* sp. WH7803 media (Figure 4.3.2.1), it is also possible that a change in pH or another physio-chemical property of the medium caused by *R. pomeroyi* affects its stability and is the cause of the disappearance. However, molecules with m/z=392 and m/z=364 were not affected by the change which suggests that these molecules have different chemical properties to the m/z=449 compound and react in a different way with molecules produced by *R. pomeroyi* or are not available to the heterotroph as a source of nutrients.

Growth of axenic *Synechococcus* sp. WH7803 cultures under iron, nitrogen, and phosphorus limitation showed no obvious differences in terms of production of the compounds. The three most abundant molecules could be detected throughout the growth curve, despite nutrient-limited growth. Again, a study with normalization to cell numbers would be useful to estimate the production of the compounds per cell. The presence of the molecules during nutrient-limited growth suggests that the molecules are either a part of an essential pathway which cannot be shut down to conserve nutrients or are essential themselves and their production cannot be stopped.

Given the similarity in the lower (or lack of) production of the six compounds in co-culture compared to axenic *Synechococcus* sp. WH7803 cultures might suggest these molecules are related i.e. be different products and intermediates of the same pathway. Indeed, there is a difference of only one C_2H_4 group between $[C_{19}H_{38}NO_5S]^+$ and $[C_{17}H_{34}NO_5S]^+$, as well as $[C_{21}H_{41}N_2O_6S]^+$ and $[C_{19}H_{37}N_2O_6S]^+$; There is also an extra oxygen in $[C_{19}H_{38}NO_6S]^+$ compared with $[C_{19}H_{38}NO_5S]^+$ and $[C_{21}H_{41}N_2O_7S]^+$ compared with $[C_{21}H_{41}N_2O_6S]^+$. Full structure determination, together with identification of the genes involved in the production of these compounds could verify this hypothesis. The fragmentation patterns of the m/z=392 and m/z=364 compounds suggest that they may contain a chain of carbons and be related to a group of secondary metabolites – polyketides. The fatty-acid-like chain of carbons, formed by repetitive decarboxylative condensation reactions with functional group modifications, is characteristic of polyketides (Hertweck, 2009).

A search of natural product databases MarinLit (http://pubs.rsc.org/marinlit/) and Reaxys (https://new.reaxys.com/) based on chemical formula of the m/z=392 compound revealed a possible similarity to spongiacysteine – a molecule isolated from marine sponge *Spongia* sp. in Japan showing antimicrobial activity against rice blast fungus *Pyricularia oryzae* (Kobayashi et al., 2004). However, the fragmentation pattern of the m/z=392 compound

indicates that the molecules are different, suggesting that the group of *Synechococcus* sp. WH7803 compounds discovered may in fact be novel.

Although marine cyanobacteria are known to produce hundreds of metabolites (see section 1.1.5), *Prochlorococcus* and *Synechococcus* genera have not been well characterised. They do not have non-ribosomal peptide synthetase gene clusters in their genomes, but some strains have type 3 polyketide synthase gene clusters (Shih et al., 2013). Some freshwater stains of *Synechococcus* sp. have been found to produce type I polyketide synthase-like compounds (Mendez-Perez et al., 2011) or were engineered to contain type III polyketide synthase genes for production of natural products with pharmacological activities (Ni et al., 2016).

To conclude, a group of possibly novel compounds was discovered in *Synechococcus* sp. WH7803. The molecules are produced in higher amounts in axenic cultures than in co-cultures with heterotrophic bacteria, but the biological function of the compounds remains unknown. Given that there is not much literature about molecules produced by marine picocyanobacteria in co-cultures and the molecules do not correspond to any known natural products, is it difficult to hypothesise about the function of these molecules. There is some indication that the molecules might belong to the polyketide synthase class of natural products. Thus, further work should include looking at putative biosynthetic gene clusters of *Synechococcus* sp. WH7803 to determine which genes may be involved in the production of the compounds. This would give an opportunity to generate a knockout mutant to confirm the role of the gene(s) in the production of the molecules, but could also provide some suggestions on the function of the compounds if a phenotypic difference is observed between a wild type and a mutant culture.

5. Characterisation of a type III polyketide synthase gene cluster in *Synechococcus* spp. WH7803

5.1 Introduction

Marine cyanobacteria offer an unparalleled opportunity for natural products research – more than 95% of discovered compounds are novel (Tan, 2007), often representing unusual and versatile chemistry (Li et al., 2010). There is also a considerable interest in cyanobacterial natural products from the pharmaceutical industry – especially in compounds displaying antimicrobial activity (Swain et al., 2017).

The two major classes of natural products – non-ribosomal peptide synthetases and polyketide synthases – have their representatives in oceanic waters as well. As mentioned in the introduction (see section 1.1.5), over 1100 secondary metabolites have been isolated from 39 genera of cyanobacteria (Dittmann et al., 2015), but unfortunately not much is known about their biological function in the open ocean.

The first T3 PKS discovered in bacteria was chalcone synthase of *Streptomyces griseus* (Funa et al., 1999). It is involved in the production of melanin, but also forms intermediates of various secondary metabolites containing a naphthoquinone ring pathways. Disruption of the *rppA* gene, encoding chalcone synthase, results in an albino-type mutant, but does not affect growth nor spore formation.

To try to shed some light on the biosynthetic basis of the metabolites identified in chapter 4 we searched the *Synechococcus* sp. WH7803 genome for secondary metabolite gene clusters (see section 5.2.1) and this identified a potential Type III Polyketide Synthase (T3 PKS) which is functionally characterized in this chapter. Using a combination of growth measurements and targeted exometabolomics, possible products of the T3 PKS gene cluster were identified and characterised to gain some insights into their possible functions in the natural environment.

The aims of this chapter were thus to:

- Predict biosynthetic gene clusters in *Synechococcus* sp. WH7803, check their prevalence in other picocyanobacteria and, in the case of T3 PKS gene clusters, annotate potential functions of the genes present in the cluster.
- Generate a *Synechococcus* sp. WH7803 T3 PKS gene mutant and characterise the mutant in terms of growth and metabolite production under different growth conditions
- Compare the effect that molecules produced by *Synechococcus* sp. WH7803 wild type and the T3 PKS mutant have on the growth of a range of marine phototrophs

5.2 Materials and methods

5.2.1 Identifying secondary metabolite gene clusters and predicting possible gene functions

Using the AntiSMASH 4.0.0 software (with ClusterFinder on and default settings and all extra features on), the *Synechococcus* sp. WH7803 genome (NCBI ID: NC_009481.1; GenBank ID: CT971583.1) was screened for gene clusters responsible for production of secondary metabolites. Amino acid sequences of the products of the genes present in the polyketide synthase gene cluster were searched against the NCBI Protein Reference Sequences with protein to protein BLAST (BLASTp). A tree of distances between the matches and the query sequence of T3 PKS gene (*synWH7803_1003*) was generated using BLASTp, at 0.9 maximum sequence difference (maximum allowed fraction of mismatched bases in the aligned region), using the Fast Minimum Evolution method (Desper & Gascuel, 2004).

Expression of the *Synechococcus* sp. WH7803 T3 PKS (*synWH7803_1003*) relative to a housekeeping gene (phosphoenol pyruvate carboxylase: *synWH7803_454*) was assessed by qPCR (see section 2.5). Samples for RNA extraction were taken from three *Synechococcus* sp. WH7803 cultures grown under standard growth conditions (see section 2.2) during exponential ($OD_{750\ nm}$=0.802) and stationary ($OD_{750\ nm}$=1.598) phases of growth.

5.2.2 Construction and characterisation of a T3 PKS mutant in *Synechococcus* sp. WH7803

A *Synechococcus* sp. WH7803 single crossover mutant in the gene encoding a T3 PKS (*synWH7803_1003*) was constructed by conjugation with *E. coli* S17-1 λpir pGP704 using a ~500 bp internal gene fragment (see section 2.8). Axenic mutant cultures were passaged every 21 days for three months (in 100 ml ASW containing 50 μg ml^{-1} kanamycin) under standard conditions (see section 2.2) before the experiment to give the culture time to adjust to the growth conditions. Six *Synechococcus* sp. WH7803 wild type

and fifteen T3 PKS mutant cultures were inoculated and grown under standard conditions (see section 2.2). Their growth was monitored in terms of growth by spectrophotometry ($OD_{750\ nm}$)

Twelve cultures of the *Synechococcus* sp. WH7803 T3 PKS mutant (three axenic in ASW containing 50 µg ml^{-1} kanamycin, three with *R. pomeroyi* in ASW with 50 µg ml^{-1} kanamycin, three axenic in ASW without kanamycin, three with *R. pomeroyi* in ASW without kanamycin) and six cultures of *Synechococcus* sp. WH7803 wild type (three axenic in ASW, three with *R. pomeroyi* in ASW) were grown in standard conditions (see section 2.2) and assessed in terms of growth by spectrophotometry ($OD_{750\ nm}$) and flow cytometry (see section 2.3) and exometabolite production by LC-MS (see section 2.6; routine protocol) on the day of inoculation, after 7 days and then every 5 days until day 72.

For nutrient limitation studies, three *Synechococcus* sp. WH7803 wild type and three T3 PKS mutant cultures (30 ml volume) were grown in standard ASW medium as well as -N, -P, and -Fe ASW media (see Table 2.2.1). For inoculation, a mid-exponential phase standard ASW culture was pelleted (30 ml centrifuged at 15 min, 8050 g), washed three times with nutrient limited medium and resuspended in 30 ml of the nutrient limited medium, except for –N medium (30 ml of pelleted, unwashed culture used for inoculation). 2 ml of culture was removed every 5 days for 40 days (or until death of the culture, i.e. no cells detected by flow cytometry) for monitoring growth by spectrophotometry ($OD_{750\ nm}$) and flow cytometry (see section 2.3). Samples for LC-MS analysis (routine protocol; see section 2.6) were collected every 10 days for 40 days or until death of the culture.

Three *Synechococcus* sp. WH7803 wild type and three T3 PKS mutant cultures were grown under standard conditions (see section 2.2) until the exponential phase of growth ($OD_{750\ nm}$=0.48). Absorption spectra were generated in the 200-1100 nm range (50 nm intervals) using a spectrophotometer. During routine flow cytometry, Side and Forward Scatter

histograms were generated for 1200 cell counts of *Synechococcus* sp. WH7803 wild type and T3 PKS cultures.

5.2.3 Toxicity assay of *Synechococcus* sp. WH7803 wild type and T3 PKS mutant exometabolites on the growth of axenic phototrophs

Axenic *Synechococcus* sp. WH7803 wild type and T3 PKS mutant were grown under standard conditions (see section 2.2) for 45 days, in 15 flasks each containing 100 ml volume. The cultures were centrifuged (15 minutes, 3220 g) and the supernatants were filter-sterilized using a 0.22 µm pore size PES filter. Sterile ASW medium (1500 ml) was kept for 45 days in the same conditions as cultures and then processed (centrifugation, filtration) in the same way. Supernatants of all three samples were used for extraction with ethyl acetate (see section 2.6).

After LC-MS analysis, the extracts were dried (rotary evaporator "HPLC" programme, 8 hours) and resuspended in 500 µl seawater (SW; Sigma Aldrich). Seven day old axenic phototroph cultures (Table 5.2.3.1; see also Table 2.1.2) were transferred into 96 well plates (12 wells of 200 µl each), two cultures per plate, separated from each other and from the borders of the plate by at least one row of wells filled with sterile water (to minimise the risk of evaporation and cross-contamination). Three wells per strain were supplemented with 5 µl SW each, three with 5 µl ASW extract in SW each, three with 5 µl *Synechococcus* sp. WH7803 T3 PKS mutant extract in SW each and three with 5 µl *Synechococcus* sp. WH7803 wild type extract in SW each. Plates were put on two layers of white paper tissue (dampened with sterile water), sealed in transparent ziplock bags and kept at 22°C (±1°C) under constant light of 20-30 µmol photons m^{-1} s^{-1}. Cells were counted on the day of extract addition and after 1, 3 and 5 days by flow cytometry (see section 2.3).

Table 5.2.3.1. Axenic phototroph cultures used in *Synechococcus* sp. WH7803 extract toxicity assays.

Group	Organism	Medium	Temperature [°C]
Cyanobacteria	*Prochlorococcus marinus* MED4	Pro99	22
	Synechococcus sp. 5701	ASW	22
	Synechococcus sp. 7803 T3 PKS mutant	ASW	22
	Synechococcus sp. 7803 wild type	ASW	22
	Synechococcus sp. 7805	ASW	22
	Synechococcus sp. 8102	ASW	22
	Synechococcus sp. 9311	ASW	22
Chlorophyta	*Ostreococcus tauri* OTH95	K	22
	Micromonas commoda NOUM17	K	22
	Micromonas pusilla PLY27	K	22
Prymnesiophyceae	*Emiliania huxleyi* AC665	K	22
Ochrophyta	*Phaeodactylum tricornutum* Pt Gen	f/2+Si	15
	Thalassiosira pseudonana 3H	f/2+Si	15

5.3 Results

5.3.1 Gene annotation and comparison of the T3 PKS gene cluster with other cyanobacterial strains

Twenty three putative biosynthetic gene clusters were identified by AntiSMASH (Table 5.3.1.1). Based on the chemical formulae and MS^n fragmentation patterns generated for the molecules described in the previous chapter (see sections 4.3.1 and 4.3.5), a T3 PKS cluster was predicted to be involved in the production of the molecules and one T3 PKS gene cluster was found in the *Synechococcus* sp. WH7803 genome. Similar T3 PKS gene clusters were detected in 5 *Synechococcus* spp. and 4 *Prochlorococcus* spp. (AntiSMASH, search against publically available genomes; strains with gene clusters in which ≥25% genes show similarity; Figure 5.3.1.1). Seven genes present in *Synechococcus* sp. WH7803 T3 PKS gene cluster have their counterparts in all of these *Synechococcus* spp. and *Prochlorococcus* spp. strains: three annotated as hypothetical proteins (*synWH7803_1001*, *synWH7803_1018*, *synWH7803_1019*), a putative dehydrogenase (*synWH7803_1002*), a type 3 polyketide synthase (*synWH7803_1003*), a S26 family signal peptidase (*synWH7803_1020*) and a 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (*synWH7803_1021*). Unfortunately, a large portion of the products of the genes in the cluster were annotated as hypothetical proteins and did not contain any conserved domains that could aid in predicting their function (Table 5.3.1.2).

Table 5.3.1.1. Types and locations of secondary metabolite gene clusters in the *Synechococcus* sp. WH7803 genome as predicted by the AntiSMASH software.

Cluster	Type	From	To
Cluster 1	Bacteriocin / putative saccharide biosynthetic cluster	42 818	76 303
Cluster 2	Putative biosynthetic cluster	91 897	106 653
Cluster 3	Putative saccharide biosynthetic cluster	110 544	164 812
Cluster 4	Putative fatty acid biosynthetic cluster	203 954	225 201
Cluster 5	Putative saccharide biosynthetic cluster	228 528	257 255
Cluster 6	Putative biosynthetic cluster	266 336	273 467
Cluster 7	Bacteriocin	359 679	370 002
Cluster 8	Putative biosynthetic cluster	451 996	460 908
Cluster 9	Bacteriocin	497 385	507 975
Cluster 10	Bacteriocin / putative saccharide biosynthetic cluster	546 259	589 183
Cluster 11	Putative biosynthetic cluster	655 060	659 483
Cluster 12	Bacteriocin	710 512	740 547
Cluster 13	Putative biosynthetic cluster	794 525	797 632
Cluster 14	Putative biosynthetic cluster	819 528	831 907
Cluster 15	Bacteriocin	873 198	883 473
Cluster 16	Type III polyketide synthase	902 671	943 765
Cluster 17	Bacteriocin	993 365	1 003 733
Cluster 18	Bacteriocin	1 199 409	1 210 296
Cluster 19	Bacteriocin-terpene	1 446 011	1 472 589
Cluster 20	Bacteriocin	1 500 098	1 511 295
Cluster 21	Putative biosynthetic cluster	1 867 735	1 873 067
Cluster 22	Putative biosynthetic cluster	2 045 610	2 054 514
Cluster 23	Terpene	2 065 609	2 102 561

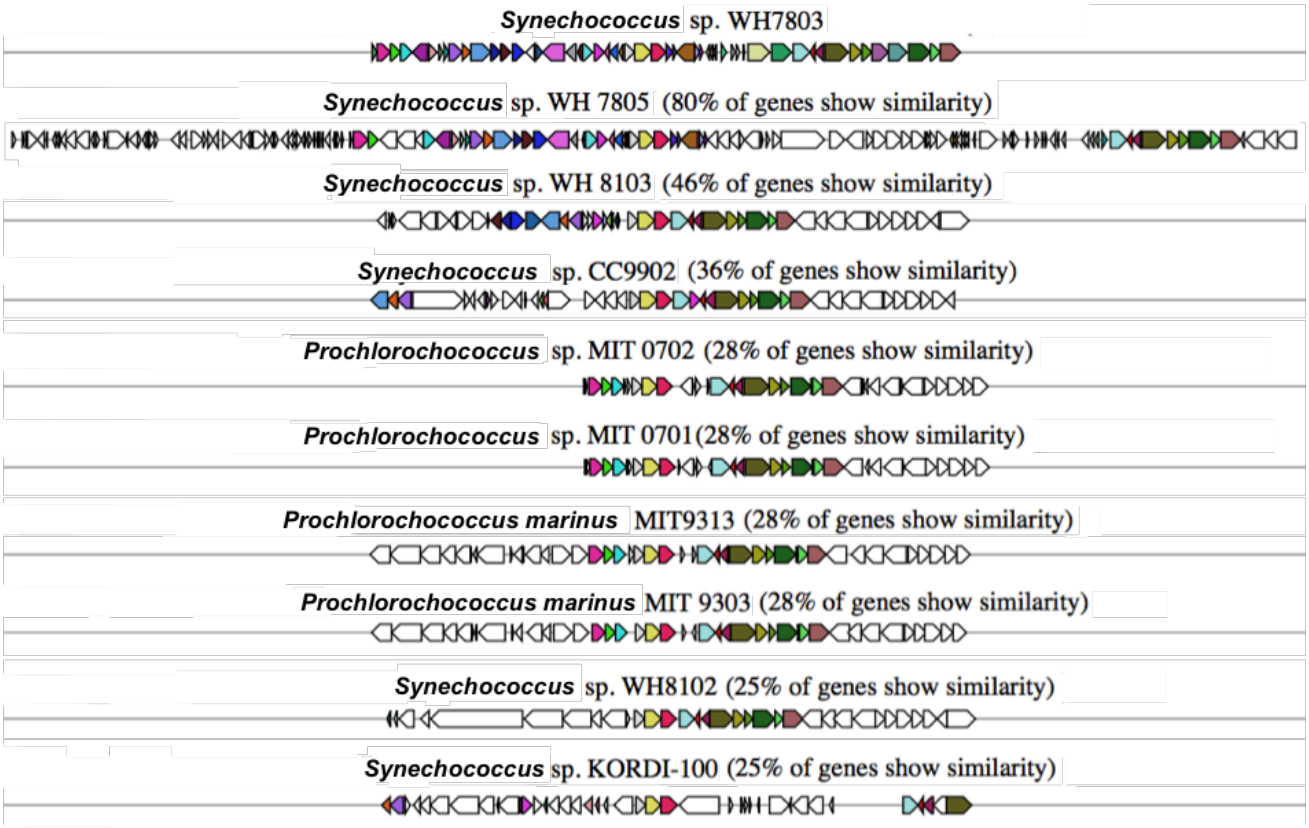


Figure 5.3.1.1 Type 3 polyketide synthase clusters in *Synechococcus* spp. and *Prochlorococcus* spp.

Table 5.3.1.2. Genes present in the T3 PKS cluster in *Synechococcus* sp. WH7803, their annotations and possible functions based on motifs found within gene sequences and BLASTp searches for proteins with high identities.

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_0977	STAS/SEC14 domain-containing protein	SpoIIAA-like	membrane associated carriers of non-polar compounds	*Synechococcus* sp. MIT S9504 (69%/100%) *Synechococcus* sp. MIT S9509 (68%/100%) *Synechococcus* sp. RS9917 (62%/84%)
synWH7803_0978	Metal ABC transporter substrate binding protein	PsaA, ZnuA, anch_rpt_subst, TroA-like	periplasmic component of ABC transporters in metal ion (e.g. Zn^{2+}, Mn^{2+}) uptake	*Synechococcus* sp. WH7805 (90%/100%) *Synechococcus* sp. MIT S9508 (75%/95%) *Synechococcus* sp. MIT S9504 (83%/87%)
synWH7803_0979	Manganese transporter	ZnuC, ABC metallic cations, PRK15056, anch_rpt_ABC, ABC_tran, P-loop_NTPase, ABC_ATPase, AAA	ATPase component of a metal ion transport system	*Synechococcus* sp. WH7805 (98%/99%) *Synechococcus* sp. CC9605 (89%/100%) *Synechococcus* sp. KORDI-52 (89%/100%)
synWH7803_0980	Metal ABC transporter permease	ABC-3, ZnuB, anch_rpt_perm, TM_ABC_iron-siderophores_like, TM_PBP1_branched-chain-AA like	permease component of a metal ion transport system	*Synechococcus* sp. WH7805 (98%/100%) *Synechococcus* sp. CC9902 (88%/98%) *Synechococcus* sp. BL107 (88%/98%)
synWH7803_0981	EamA/RhaT family transporter	RhaT, EamA, 2A78	permease of the drug/metabolite transporter superfamily	*Synechococcus* sp. WH7805 (90%/100%) *Synechococcus* sp. RCC307 (63%/98%) *Synechococcus* sp. SynAce01 (54%/96%)
synWH7803_0982	hypothetical protein	COG4337	unknown	*Synechococcus* sp. WH7805 (92%/100%) *Synechococcus* sp. MIT S9508 (75%/85%) *Synechococcus* sp. WH8016 (63%/86%)

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_0983	hypothetical protein	DUF3721	unknown	*Synechococcus* sp. WH7805 (91%/67%) *Synechococcus* sp. MIT S9508 (65%/96%) *Synechococcus* sp. RS9916 (56%/95%)
synWH7803_0984	cytochrome C biogenesis protein CcsB	Cytochrome CBB3, CccA, PRK13697, ccoP, Cytochrom C, FixP_N	cytochrome c oxidase	*Synechococcus* sp. WH7805 (93%/100%) *Synechococcus* sp. CC9311 (73%/100%) *Synechococcus* sp. WH8020 (73%/100%)
synWH7803_0985	permease	YraQ, ArsP_1	permease of unknown specificity	*Synechococcus* sp. WH7805 (95%/100%) *Synechococcus* sp. WH8016 (82%/100%) *Synechococcus* sp. MIT S9508 (86%/100%)
synWH7803_0986	TIGR03943 family protein	TIGR03943, YcgQ, DUF1980	uncharacterized conserved membrane protein	*Synechococcus* sp. WH7805 (91%/100%) *Synechococcus* sp. WH8016 (70%/100%) *Synechococcus* sp. CC9311 (68%/100%)
synWH7803_0987	hypothetical protein	-	unknown	*Synechococcus* sp. TMED90 (88%/100%) *Synechococcus* sp. WH7805 (90%/100%) *Synechococcus* sp. MIT S9508 (67%/100%)
synWH7803_0988	ABC transporter ATP-binding protein	ABC metallic cations, ZnuC, ABC_tran, AAA, hmuV, anch_rpt_ABC, P-loop NTPase, ABC ATPase	ATPase component of a metal ion transport system	*Synechococcus* sp. WH7805 (97%/100%) *Synechococcus* sp. TMED90 (96%/100%) *Synechococcus* sp. MIT S9508 (73%/100%)
synWH7803_0989	metal ABC transporter permease	ZnuB, ABC-3, TM_ABC_iron-siderophores_like, anch_rpt_perm, TM_PBP1_branched chain AA like	permease component of a metal ion transport system	*Synechococcus* sp. MIT S9508 (90%/99%) *Synechococcus* sp. WH7805 (96%/99%) *Synechococcus* sp. TMED90 (93%/99%)
synWH7803_0990	ABC transporter substrate-binding protein	ZnuA, PsaA, anch_rpt_subst, TroA-like	periplasmic component of ABC transporters in metal ion uptake	*Synechococcus* sp. TMED90 (94%/99%) *Synechococcus* sp. WH7805 (92%/100%) *Synechococcus* sp. MIT S9508 (77%/99%)

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_0991	hypothetical protein with transmembrane domains	MMPL	integral membrane protein, may be involved in lipid transport	*Folsomia candida* (35%/64%)
synWH7803_0992	hypothetical protein	-	unknown	*Synechococcus* sp. TMED90 (98%/100%) *Synechococcus* sp. RS9917 (88%/100%) *Synechococcus* sp. CB0101 (75%/100%)
synWH7803_0993	porin	SLH, OprB	carbohydrate-selective porin	*Synechococcus* sp. WH7805 (85%/99%) *Synechococcus* sp. RS9917 (72%/99%) *Synechococcus* sp. RS9916 (66%/99%)
synWH7803_0994	hydantoin utilization protein	HupE, HupE_UreJ	hydrogenase/urease accessory protein	*Synechococcus* sp. WH7805 (91%/100%) *Synechococcus* sp. TMED90 (94%/100%) *Synechococcus* sp. TMED187 (83%/92%)
synWH7803_0995	hypothetical protein	-	unknown	*Synechococcus* sp. WH7805 (97%/100%) *Synechococcus* sp. MIT S9504 (77%/100%) *Synechococcus* sp. CC9311 (69%/100%)
synWH7803_0996	hypothetical protein	-	unknown	*Synechococcus* sp. WH7805 (93%/100%) *Synechococcus* sp. TMED90 (dehydrogenase; 95%/100%) *Synechococcus* sp. MIT S9504 (77%/99%)
synWH7803_0997	16S rRNA (uracil(1498)-N(3))-methyltransferase	PRK11713, Methyltrans_RNA, RsmE, TIGR00046	modification of nucleotides during rRNA maturation (U1498 methylation)	*Synechococcus* sp. WH7805 (85%/100%) *Synechococcus* sp. TMED90 (86%/100%) Cyanobacteria bacterium TMED188 (66%/99%)
synWH7803_0998	redox protein	OsmC, YhfA, TIGR03549, YcaO	osmotically inducible protein C, may be involved in oxidative stress regulation	*Synechococcus* sp. WH7805 (hypothetical protein; 92%/100%) *Synechococcus* sp. TMED90 (osmotically inducible protein OsmC; 87%/100%) *Synechococcus* sp. MIT S9508 (osmotically inducible protein OsmC; 68%/100%)

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_0999	DUF1295 domain-containing protein	STE14, DUF1295, PEMT	methyltransferase	*Synechococcus* sp. WH7805 (88%/100%) *Synechococcus* sp. TMED66 (steroid 5-alpha reductase; 67%/100%) *Synechococcus* sp. WH8016 (67%/100%)
synWH7803_1000	DUF2973 domain-containing protein	DUF2973	unknown	*Synechococcus* sp. WH7805 (86%/98%) *Synechococcus* sp. RS9917 (58%/96%) *Synechococcus* sp. TMED 66 (hypothetical protein; 59%/96%)
synWH7803_1001	hypothetical protein	PRK06202, Methyltransf_25, UbiG, AdoMet_MTases,	S-adenosylmethionine-dependent methyltransferase	*Synechococcus* sp. WH7805 (nucleotide-binding protein; 87%/100%) *Synechococcus* sp. TMED90 (nucleotide-binding protein; 88%/100%) *Synechococcus* sp. RS9917 (73%/99%)
synWH7803_1002	dehydrogenase	GG-red-SF, FixC	dehydrogenase or geranylgeranyl reductase	*Synechococcus* sp. WH7805 (NAD-binding site; 88%/99%) *Synechococcus* sp. TMED90 (NAD-binding protein; 85%/99%) *Synechococcus* sp. RS9917 (FAD-binding protein; 74%/98%)
synWH7803_1003	type 3 polyketide synthase	CHS_like, BH0617, PLN03169, Chal_sti_synt_C, fabH, cond_enzymes, Ketoacyl-synt_C	chalcone or stilbene synthase	*Synechococcus* sp. WH7805 (90%/99%) *Synechococcus* sp. RS9917 (74%/99%) *Synechococcus* sp. TMED90 (88%/85%)
synWH7803_1004	hypothetical protein	-	unknown	*Synechococcus* sp. WH7805 (88%/100%) *Synechococcus* sp. CC9311 (52%/96%) *Synechococcus* sp. WH8016 (46%/98%)
synWH7803_1005	hypothetical protein	-	unknown	*Synechococcus* sp. WH7805 (89%/100%) *Synechococcus* sp. WH5701 (67%/96%) *Synechococcus* sp. CB0101 (72%/94%)

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_1006	cytochrome P450	CypX, PLN02302, p450, P450_cycloAA_1	haem-thiolate proteins involved in the oxidative degradation of various compounds	*Synechococcus* sp. WH7805 (91%/97%) *Synechococcus* sp. RCC307 (72%/97%) *Synechococcus* sp. MIT S9508 (77%/88%)
synWH7803_1007	hypothetical protein	-	unknown	*Synechococcus* sp. WH7805 (94%/100%) *Synechococcus* sp. WH8109 (75%/95%) *Synechococcus* sp. WH8016 (67%/97%)
synWH7803_1008	hypothetical protein	-	unknown	*Synechococcus* sp. WH7805 (96%/98%) *Synechococcus* sp. RS9916 (72%/97%) *Synechococcus* sp. MIT S9508 (68%/97%)
synWH7803_1009	hypothetical protein	-	unknown	*Synechococcus* sp. WH8016 (81%/100%) *Synechococcus* sp. RS9916 (82%/100%) *Synechococcus* sp. WH7805 (79%/100%)
synWH7803_1010	hypothetical protein	DUF3721	unknown	*Synechococcus* sp. KORDI-52 (gibberellin regulated protein; 64%/92%) *Synechococcus* sp. WH8016 (gibberellin regulated protein; 63%/85%) *Synechococcus* sp. CC9605 (59%/92%)
synWH7803_1011	hypothetical protein	-	unknown	*Synechococcus* sp. RCC307 (72%/95%) *Synechococcus* sp. WH8109 (57%/98%) *Synechococcus* sp. KORDI-49 (54%/100%)
synWH7803_1012	hypothetical protein	-	unknown	-
synWH7803_1013	hypothetical protein	-	unknown	-
synWH7803_1014	hypothetical protein	-	unknown	*Punctularia strigosozonata* HHB-11173 SS5 (43%/41%) *Kluyveromyces lactis* NRRL Y-1140 (46%/38%) *Tetrapisispora blattae* CBS 6284 (43%/41%)

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_1015	hypothetical protein	DUF1651	unknown	*Synechococcus* sp. CC9311 (72%/59%) *Synechococcus* sp. WH8016 (67%/59%) *Synechococcus* sp. WH8020 (64%/59%)
synWH7803_1016	hypothetical protein	COG5361, DUF1214, DUF1254	unknown	*Synechococcus* sp. MIT S9504 (92%/100%) *Synechococcus* sp. WH8016 (92%/100%) *Synechococcus* sp. MIT S9509 (92%/100%)
synWH7803_1017	hypothetical protein	COG5361, DUF1214, DUF1254	unknown	*Synechococcus* sp. WH8016 (93%/100%) *Synechococcus* sp. MIT S9509 (91%/100%) *Synechococcus* sp. MIT S9504 (91%/100%)
synWH7803_1018	hypothetical protein	DUF4336	unknown	*Synechococcus* sp. WH7805 (87%/100%) *Synechococcus* sp. WH8109 (75%/98%) *Synechococcus* sp. WH8102 (74%/99%)
synWH7803_1019	hypothetical protein	DUF760	unknown	*Synechococcus* sp. WH7805 (95%/100%) *Synechococcus* sp. WH8016 (91%/100%) *Synechococcus* sp. WH8020 (91%/100%)
synWH7803_1020	S26 family signal peptidase	Sigpep_I_bact, S26_SPase_I, LepB, PRK10861, Peptidase_S26, Peptidase_S24_S26	membrane-bound serine proteases that cleave the amino-terminal signal peptide extension from proteins that are translocated across biological membranes	*Synechococcus* sp. WH7805 (93%/100%) *Synechococcus* sp. MIT S9508 (signal peptidase I; 74%/98%) *Synechococcus* sp. RS9917 (72%/86%)

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_1021	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase	MenD, PRK07449, TPP_enzyme_PYR	catalyzes the thiamine diphosphate-dependent decarboxylation of 2-oxoglutarate and the subsequent addition of the resulting succinic semialdehyde-thiamine pyrophosphate anion to isochorismate to yield 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate	*Synechococcus* sp. WH7805 (82%/99%) *Synechococcus* sp. MIT S9508 (67%/99%) *Synechococcus* sp. MIT S9509 (68%/99%)
synWH7803_1022	1,4-dihydroxy-2-naphthoyl-CoA synthase	PRK07396, menB, ECH_1, crotonase-like	converts 2-succinylbenzoate into 1,4-di-hydroxy-2-naphthoate	*Synechococcus* sp. WH7805 (98%/100%) *Synechococcus* sp. MIT S9508 (89%/100%) *Synechococcus* sp. WH8020 (88%/100%)
synWH7803_1023	L,D-transpeptidase	ErfK, YkuD, PRK10260	catalyzes the formation of peptidoglycan cross-links of the cell wall and gives resistance to beta-lactams	*Synechococcus* sp. WH7805 (89%/100%) *Synechococcus* sp. MIT S9508 (89%/81%) *Synechococcus* sp. WH8016 (79%/80%)
synWH7803_1024	hypothetical protein	Periplasmic_Binding_Pro, PBP2_LTTR_substrate, PBP2_phosphate_like_2, PstS	periplasmic component of putative ABC-type phosphate transporter	*Bacillus* sp. NC2-31 (phosphate binding protein; 24%/75%) *Gloeobacter violaceus* (protein sphX; 25%/73%) *Bacillus azotoformans* (phosphate ABC transporter substrate-binding protein; 24%/55%)
synWH7803_1025	hypothetical protein	MprF, tRNA-synt_2_TM	lysyl-tRNA synthetase	-

Gene	Annotation	Conserved domains	Possible function	Highest matches (Identity/Query cover)
synWH7803_1026	glycogen synthase	glgA, GT1_Glycogen_synthase_DULL1_like, Glyco_transf_5, Glycosyltransferase_GTB_type	glycogen or starch synthase that uses ADP-glucose	*Synechococcus* sp. WH7805 (starch synthase; 94%/100%) *Synechococcus* sp. RS9916 (starch synthase; 86%/100%) *Synechococcus* sp. WH8016 (starch synthase; 86%/100%)
synWH7803_1027	hypothetical protein	PRK03427, ZipA	membrane-anchored protein necessary for assembly of the septal ring	*Synechococcus* sp. WH7805 (77%/99%) *Synechococcus* sp. MIT S9508 (62%/98%) *Synechococcus* sp. MIT S9509 (60%/98%)
synWH7803_1028	UDP-N-acetylmuramyl peptide synthase	MurF, Mur_ligase_M, PRK11929	peptidoglycan biosynthesis	*Synechococcus* sp. WH7805 (UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase; 87%/100%) *Synechococcus* sp. MIT S9504 (72%/98%) *Synechococcus* sp. MIT S9509 (72%/98%)

The T3 PKS gene is present not only in a range of picocyanobacteria – including 16 *Synechococcus* spp. strains and 6 *Prochlorococcus* spp. strains – but also in heterotrophs (Table 5.3.1.3). Cyanobacterial T3 PKS genes are most similar to a wide range of *Planctomycetes* (a phylum of aquatic bacteria; Lage & Bondoso, 2012) and two *Halomonas* strains (a genus of halophilic proteobacteria; Wang et al., 2008; Guan et al., 2010) (Figure 5.3.1.2).

Table 5.3.1.3. Genes with more than 40% identity (query coverage above 80%) to the *Synechococcus* sp. WH7803 T3 PKS gene.

Gene annotation	Organism	Query coverage	Identity
type III polyketide synthase	*Synechococcus* sp. WH 7805	99%	90%
type III polyketide synthase	*Synechococcus* sp. RS9917	99%	74%
chalcone synthase	*Synechococcus* sp. TMED90	85%	88%
chalcone synthase (CHS)	*Synechococcus* sp. RS9917	97%	73%
type III polyketide synthase	*Synechococcus* sp. CC9902	98%	60%
type III polyketide synthase	*Synechococcus* sp. KORDI-100	99%	60%
type III polyketide synthase	*Prochlorococcus marinus*	99%	61%
chalcone synthase (CHS)	*Prochlorococcus marinus* MIT 9303	99%	61%
chalcone synthase	*Prochlorococcus* sp. TMED223	99%	61%
alpha-pyrone synthesis polyketide synthase-like Pks18	*Prochlorococcus marinus* MIT 1312	99%	61%
type III polyketide synthase	*Prochlorococcus* sp. MIT 1306	99%	61%
type III polyketide synthase	*Prochlorococcus* sp. MIT 1303	99%	61%
chalcone synthase	*Synechococcus* sp. TMED20	99%	60%
type III polyketide synthase	*Synechococcus* sp. CC9616	99%	60%
type III polyketide synthase	*Synechococcus* sp. BL107	98%	61%
type III polyketide synthase	*Synechococcus* sp. CC9311	99%	57%
chalcone synthase	*Synechococcus* sp. TMED66	99%	57%
naringenin chalcone synthase	uncultured marine bacterium MedDCM-OCT-S04-C72	98%	60%
type III polyketide synthase	*Synechococcus* sp. WH 8016	99%	57%
3-Oxoacyl-[acyl-carrier-(ACP)] synthase III C terminal family protein	*Synechococcus* sp. WH 8103	92%	61%
type III polyketide synthase	*Synechococcus* sp. WH 8020	99%	57%
type III polyketide synthase	*Synechococcus* sp. WH 5701	98%	56%
type III polyketide synthase	*Cyanobium gracile*	98%	51%
type III polyketide synthase	*Gimesia maris*	98%	48%
chalcone synthase	*cyanobacterium* BACL30 MAG-120619-bin27	99%	53%

Gene annotation	Organism	Query coverage	Identity
type III polyketide synthase	*Cyanobium* sp. NIES-981	99%	52%
type III polyketide synthase	*Cyanobium* sp. PCC 7001	99%	54%
hypothetical protein BGO49_30805	*Planctomycetales bacterium* 71-10	98%	51%
type III polyketide synthase	*Gemmata obscuriglobus*	98%	50%
type III polyketide synthase	*Halomonas lutea*	98%	46%
hypothetical protein AYO47_08860	*Planctomyces* sp. SCGC AG-212-M04	98%	48%
hypothetical protein CBB71_18925	*Rhodopirellula* sp. TMED11	94%	47%
type III polyketide synthase	*Fimbriiglobus ruber*	98%	49%
hypothetical protein CBC98_04740	*Planctomycetaceae bacterium* TMED138	98%	45%
hypothetical protein CBE00_06715	*Planctomycetaceae bacterium* TMED240	97%	44%
type III polyketide synthase	*Roseimaritima ulvae*	98%	46%
hypothetical protein AY599_09790	*Leptolyngbya valderiana* BDU 20041	98%	43%
type III polyketide synthase	*Prosthecobacter debontii*	99%	41%
type III polyketide synthase	*Rubellimicrobium mesophilum*	98%	44%
type III polyketide synthase	*Aureimonas* sp. AU4	97%	42%
type III polyketide synthase	*Nesterenkonia* sp. AN1	96%	43%
hypothetical protein BGO14_03350	*Chlamydiales bacterium 38-26*	98%	40%
type III polyketide synthase	*Rhodococcus kroppenstedtii*	97%	41%
alpha-pyrone synthesis polyketide synthase-like Pks18	*Rhodococcus* sp. PBTS 1	97%	41%
type III polyketide synthase	*Rhizobiales bacterium* MIMtkB18	98%	42%
type III polyketide synthase	*Rhodococcus* sp. PBTS 1	97%	41%
type III polyketide synthase	*Curtobacterium* sp. MMLR14_010	98%	42%
type III polyketide synthase	*Plantibacter* sp. MMLR14_011	99%	42%
chalcone synthase	*Corynebacterium xerosis*	98%	42%
type III polyketide synthase	*Plantibacter cousiniae*	99%	41%
type III polyketide synthase	*Plantibacter* sp. H53	99%	42%
type III polyketide synthase	*Plantibacter elymi*	99%	42%
type III polyketide synthase	*Verrucomicrobium spinosum*	98%	40%
type III polyketide synthase	*Plantibacter flavus*	99%	42%
predicted naringenin-chalcone synthase	*Aureimonas jatrophae*	97%	40%
hypothetical protein	*Geothermobacter* sp. EPR-M	98%	40%
type III polyketide synthase	*Plantibacter flavus*	99%	41%
type III polyketide synthase	*Curtobacterium* sp. MCBA15_001	98%	44%
type III polyketide synthase	*Curtobacterium* sp. Leaf261	99%	41%
type III polyketide synthase	*Brachybacterium* sp. VR2415	98%	40%

Gene annotation	Organism	Query coverage	Identity
type III polyketide synthase	*Mesorhizobium alhagi*	97%	40%
predicted naringenin-chalcone synthase	*Microbacterium* sp. cl140	91%	42%
alpha-pyrone synthesis polyketide synthase-like Pks18	*Microbacterium azadirachtae*	91%	42%
type III polyketide synthase	*Nesterenkonia jeotgali*	98%	44%
predicted naringenin-chalcone synthase	*Microbacterium* sp. cl127	98%	40%
predicted naringenin-chalcone synthase	*Friedmanniella luteola*	98%	42%
type III polyketide synthase	*Agrococcus lahaulensis*	98%	41%
type III polyketide synthase	*Curtobacterium pusillum*	98%	42%
type III polyketide synthase	*Agrococcus pavilionensis*	98%	40%
stilbene synthase	*Altererythrobacter namhicola*	97%	42%
predicted naringenin-chalcone synthase	*Sanguibacter gelidistatuariae*	98%	40%
type III polyketide synthase	*Nesterenkonia massiliensis*	97%	41%
type III polyketide synthase	*Cnuibacter physcomitrellae*	98%	40%
type III polyketide synthase	*Tersicoccus phoenicis*	99%	42%
type III polyketide synthase	*Kocuria indica*	99%	41%
naringenin-chalcone synthase	*Kocuria* sp. ICS0012	99%	41%

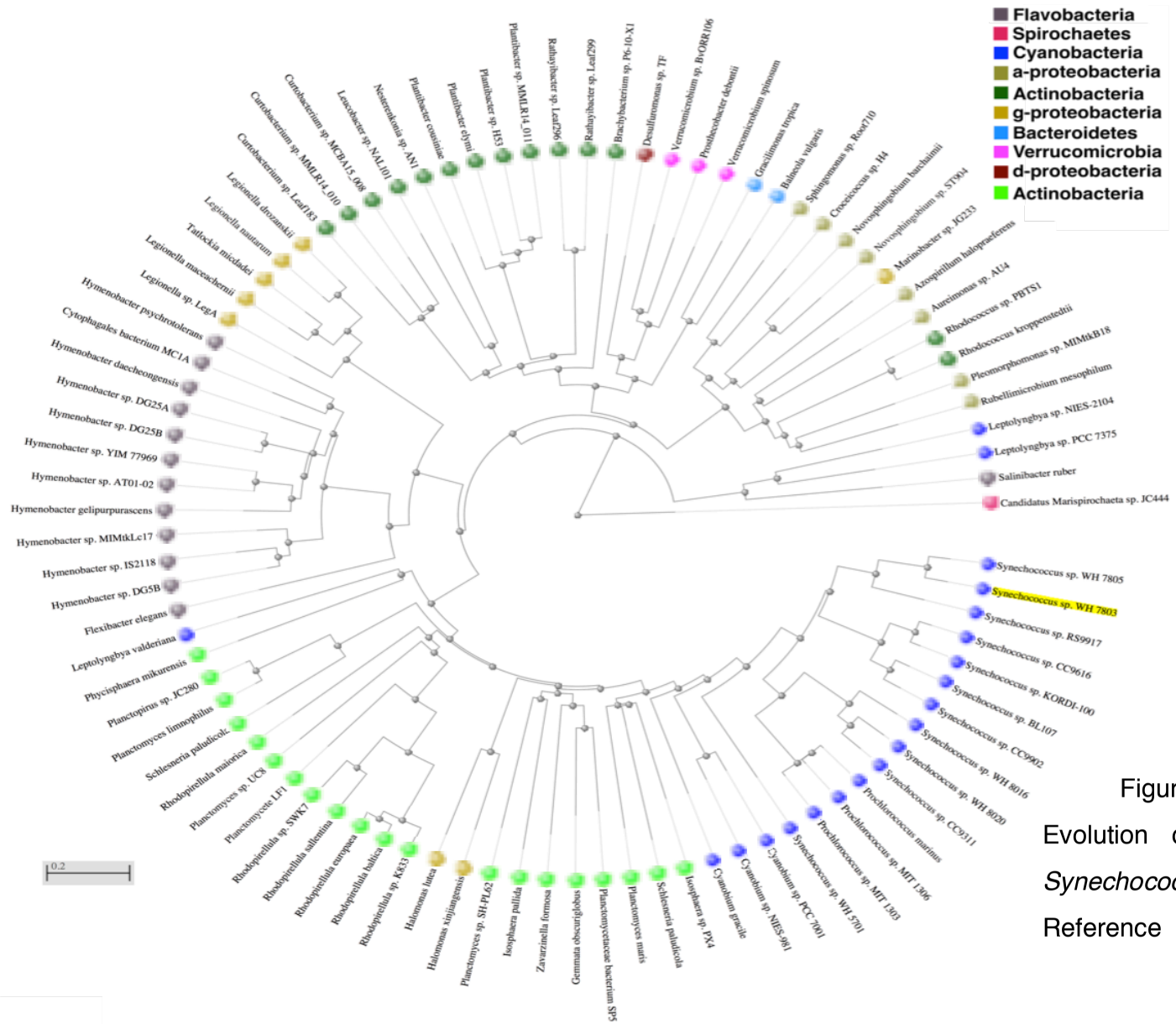


Figure 5.3.1.2 Fast Minimum Evolution distance between T3 PKS *Synechococcus* sp. matches from BLAST Reference sequences database.

5.3.2 Expression of the T3 PKS in *Synechococus* sp. WH7803

Quantitative PCR analysis of RNA extracted from both exponential and stationary phases of growth of axenic *Synechococcus* sp. WH7803 cultures, showed expression of the T3 PKS gene as well as the phosphoenol pyruvate carboxylase housekeeping gene control (see Figures 5.3.2.1-5.3.2.2, Table 5.3.2.1). When normalized to the housekeeping gene expression level (see section 2.5), expression of T3 PKS in the exponential phase of growth was only 1.27-fold higher compared to the stationary phase of growth, which does not suggest differential expression of the T3 PKS gene during growth.

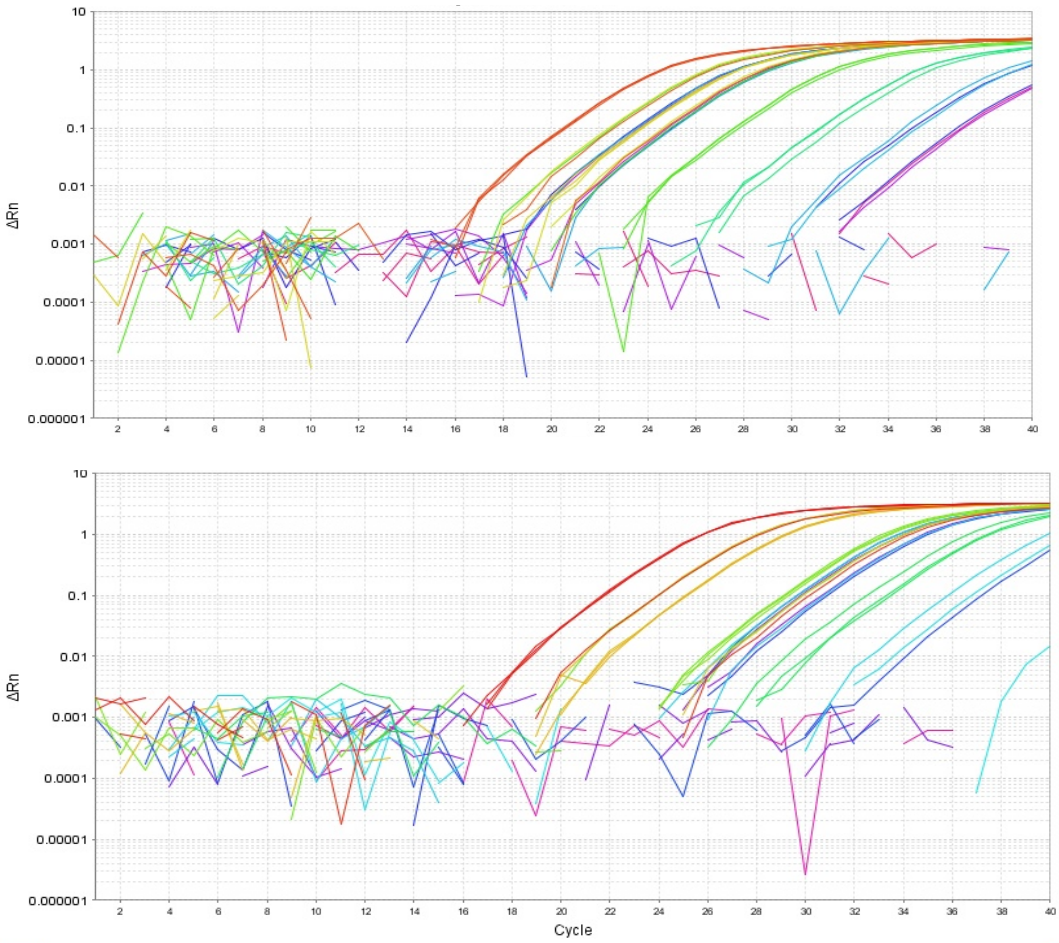


Figure 5.3.2.1 Amplification of *Synechococcus* sp. WH7803 cDNA with phosphoenol pyruvate carboxylase (top) and T3 PKS (bottom) primer sets. Different colours represent different DNA concentrations – red the highest, purple – lowest.

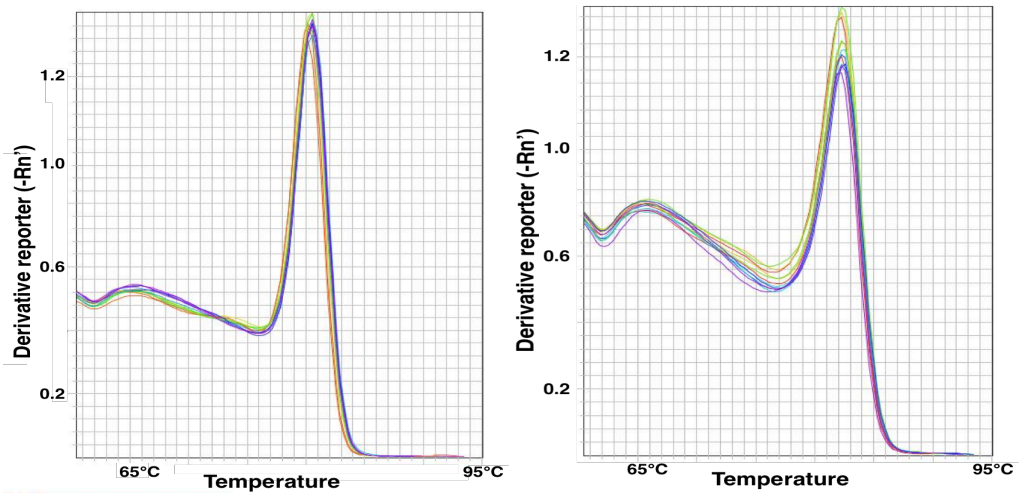


Figure 5.3.2.2 Melting curves for phosphoenol pyruvate carboxylase (left) and T3 PKS (right) primer set.

Table 5.3.2.1 C_T, ΔC_T, $\Delta\Delta C_T$ values and fold change in expression of T3 PKS and phosphoenol pyruvate carboxylase genes in exponential and stationary phase of growth of *Synechococcus* sp. WH7803.

Sample	**Average C_T**		**ΔC_T**	**$\Delta\Delta C_T$**	**Fold change ($2^{-\Delta\Delta C_T}$)**
	T3 PKS	**phosphoenol pyruvate carboxylase**			
exponential phase	29.53	23.69	5.85	0.34	1.27
stationary phase	30.59	25.08	5.51		

5.3.3 Construction and growth of a *Synechococcus* sp. WH7803 T3 PKS mutant

The T3 PKS gene was chosen for inactivation by insertional mutagenesis due to its predicted function in secondary metabolite biosynthetic pathways which may link it to the m/z 392 metabolite observed in chapter 4, the presence of this putative T3 PKS gene cluster in other *Synechococcus* strains suggesting a more general role across this genus, and indeed its prevalence in a wide range of other bacteria. A ~500 bp internal fragment of the gene was chosen based on its location, ability to disrupt binding sites and availability of sequences that can be used for designing primers of good quality (Figure 5.3.3.1). The disruption of the T3 PKS gene and segregation of the mutant were confirmed by PCR with appropriate primers (Figure

5.3.3.2; see also section 2.8 and Figure 5.3.3.3). No products were expected and seen for confirmation of plasmid presence (primers pgp3533_F and PKS7803_835_R; product approximately 600 bp) in the wild type culture and for segregation check (primers PKS7803_118_F and PKS7803_835_R; product approximately 700 bp) in the mutant cultures. All cultures were expected to give and gave products for confirmation of gene presence (primers PKS7803_252_Xba and PKS7803_835_R; product approximately 600 bp).

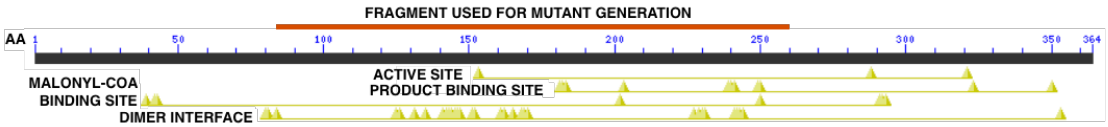


Figure 5.3.3.1 Putative active and binding sites in the T3 PKS gene of *Synechococcus* sp. WH7803.

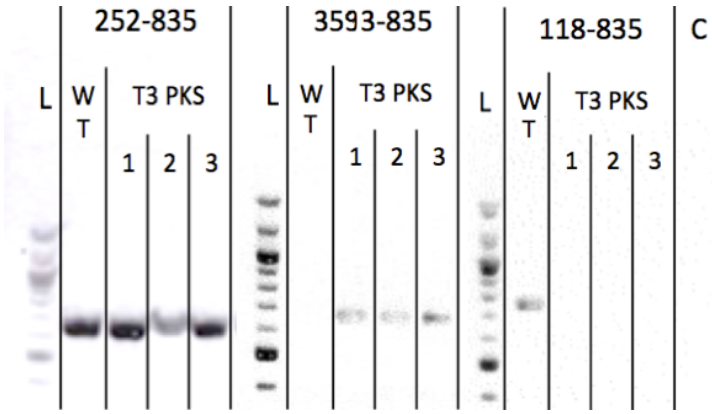


Figure 5.3.3.2 Confirmation of gene disruption and segregation in *Synechococcus* sp. WH7803 T3 PKS mutants. Confirmation of T3 PKS gene presence (left), plasmid presence (middle) and segregation (right) in wild type (WT) and three T3 PKS mutant cultures (T3 PKS 1-3). L – ladder, C – no template negative control. Numbers represent primer locations (see Figure 5.3.3.3). Ladder bands from the top: 1517 bp, 1200 bp, 1000 bp, then every 100 bp until 100 bp (thicker bands: 1000 bp and 500 bp).

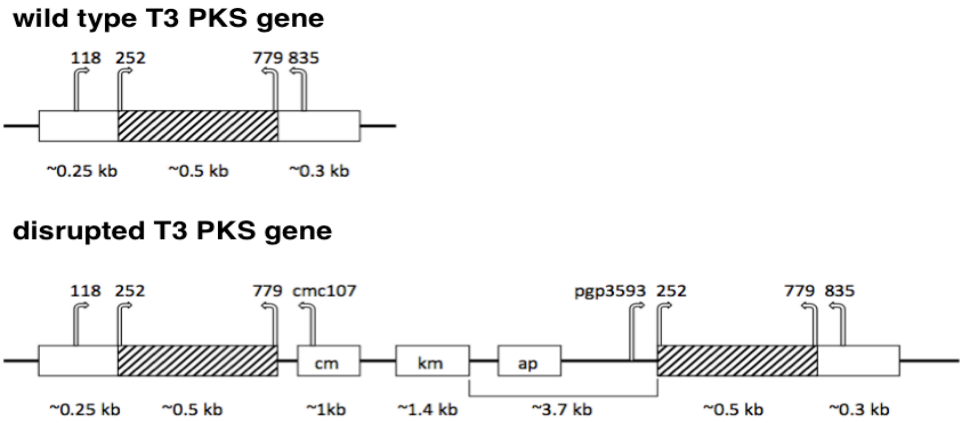


Figure 5.3.3.3 T3 PKS gene (*synWH7803_1003*) in the wild type and mutant *Synechococcus* sp. WH7803. Numbers above the illustration mark locations where PCR primers start, numbers below – approximate size of the expected products.

Approximately half of the *Synechococcus* sp. WH7803 T3 PKS mutant cultures survive under standard growth conditions (see section 2.2) until late exponential phase (7 out of 15 cultures inoculated at the same time, with the same inoculum), compared to all wild type cultures (6 out of 6; Figure 5.3.3.4). The T3 PKS mutant cultures that survive do not show any significant difference in cell counts per milliliter nor $OD_{750\ nm}$ compared to wild type cultures, irrespective of the presence of kanamycin or heterotrophic bacteria (Figure 5.3.3.5).

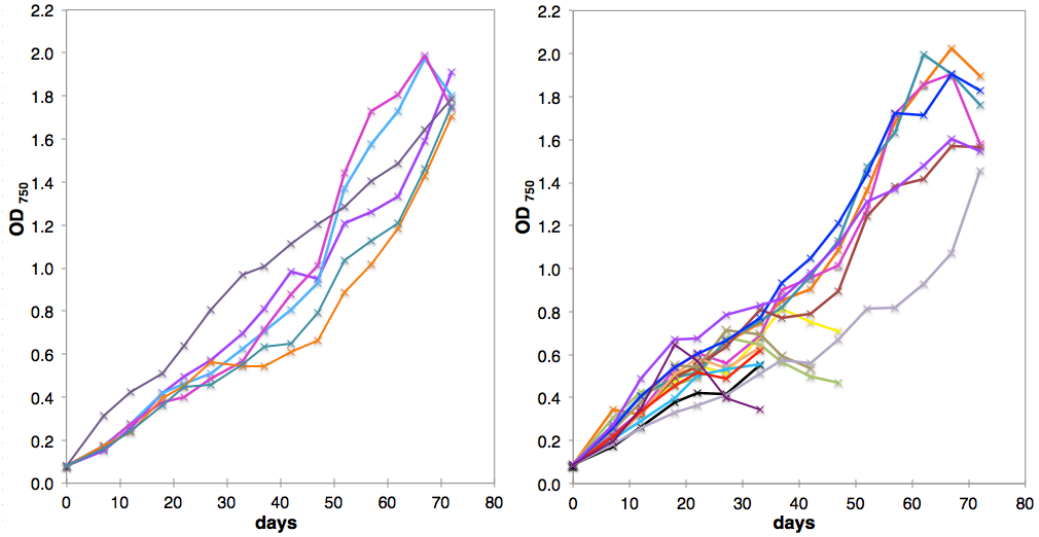


Figure 5.3.3.4 Growth of six *Synechococcus* sp. WH7803 wild type (left) and fifteen T3 PKS mutant axenic cultures (right) monitored by spectrophotometry (optical density at 750 nm).

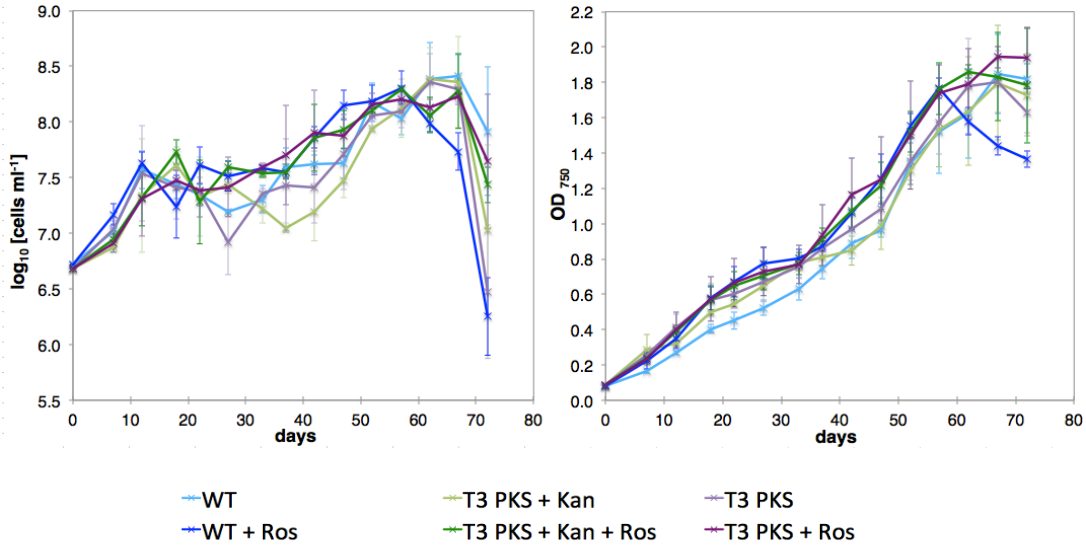


Figure 5.3.3.5 Growth of *Synechococcus* sp. WH7803 wild type (WT) and T3 PKS mutant cultures (T3 PKS with and without kanamycin) in axenic cultures and co-cultures with *R. pomeroyi* (Ros) monitored by flow cytometry (cell count per ml of culture; left) and spectrophotometry (optical density at 750 nm; right). Error bars represent standard deviation.

Whilst wild type and T3 PKS mutant *Synechococcus* sp. WH7803 have the same absorption at 750 nm, the T3 PKS mutant cultures have a slightly

higher absorbance in the UV range (Figure 5.3.3.6) and lower cell counts than wild type cultures (i.e. 1.2 x 10^7 cells corresponds to $OD_{750\ nm}$=0.54 for T3 PKS mutant and $OD_{750\ nm}$=0.44 for wild type cells). Using flow cytometry although the Forward Scatter (FSC) of T3 PKS mutant cultures is very similar to that of the wild type cultures, the Side Scatter (SSC) histogram shows a skewed distribution of counts in T3 PKS mutant cultures (see Figure 5.3.3.7).

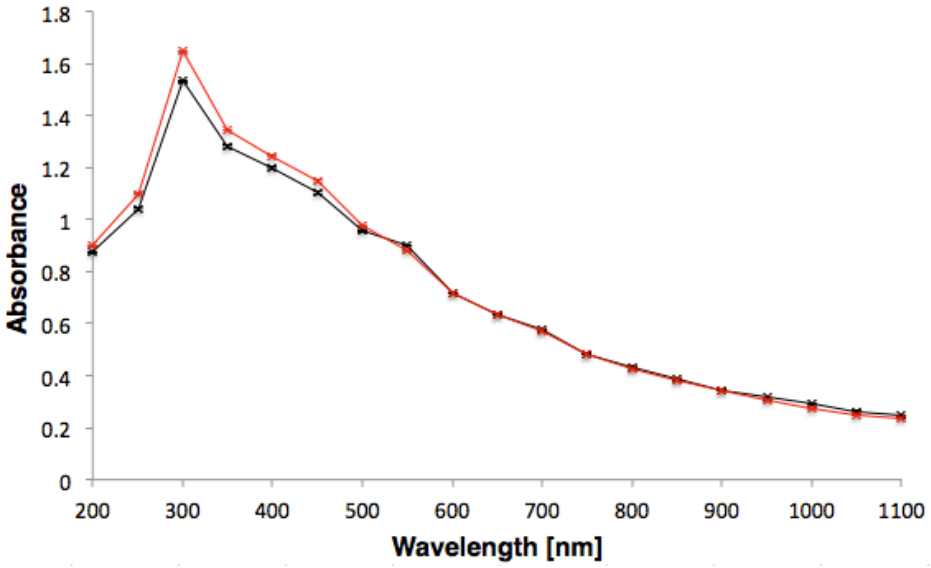


Figure 5.3.3.6 Absorbance spectra of axenic *Synechococcus* sp. WH7803 wild type (black) and T3 PKS mutant cultures (red). Error bars represent standard deviation.

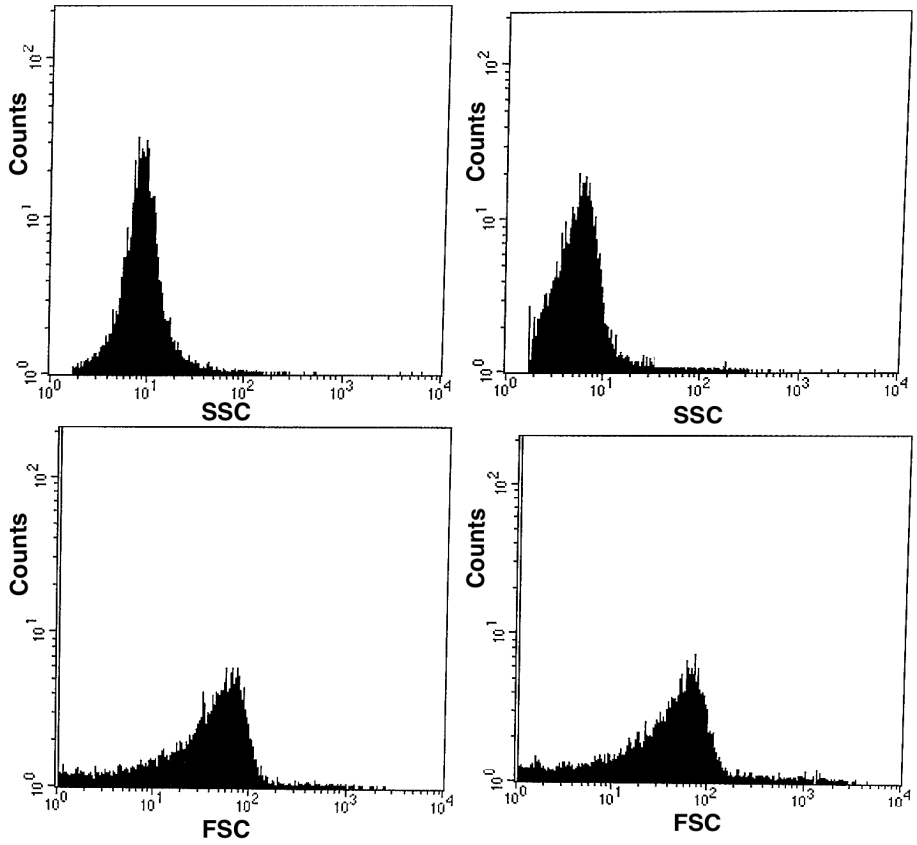


Figure 5.3.3.7 Side (top) and Forward Scatter (bottom) of axenic *Synechococcus* sp. WH7803 wild type (left) and T3 PKS mutant cells (right).

Levels of the six molecules identified previously (see section 4.3.1) in mutant cultures were approximately an order of magnitude lower than in wild type cultures (Figure 5.3.3.8). Apart from the six metabolites described previously, two more molecules with m/z=380 and m/z=437 were identified to be affected by heterotroph addition and the disruption of T3 PKS gene in *Synechococcus* sp. WH7803 (Figure 5.3.3.9). Unfortunately due to low signal intensity, it was not possible to generate high resolution MS m/z value. However, based on mass differences with other identified compounds, some predictions can be made – the m/z=380.18 molecule could be $[C_{17}H_{34}NO_6S]^+$ (the difference between m/z=380.18 and m/z=364.2156 molecules is 16 – mass of extra oxygen), while the m/z=437.23 molecule could be $[C_{19}H_{37}N_2O_7S]^+$ (the difference between m/z=437.23 and m/z=421.23 molecules is 16 – mass of extra oxygen).

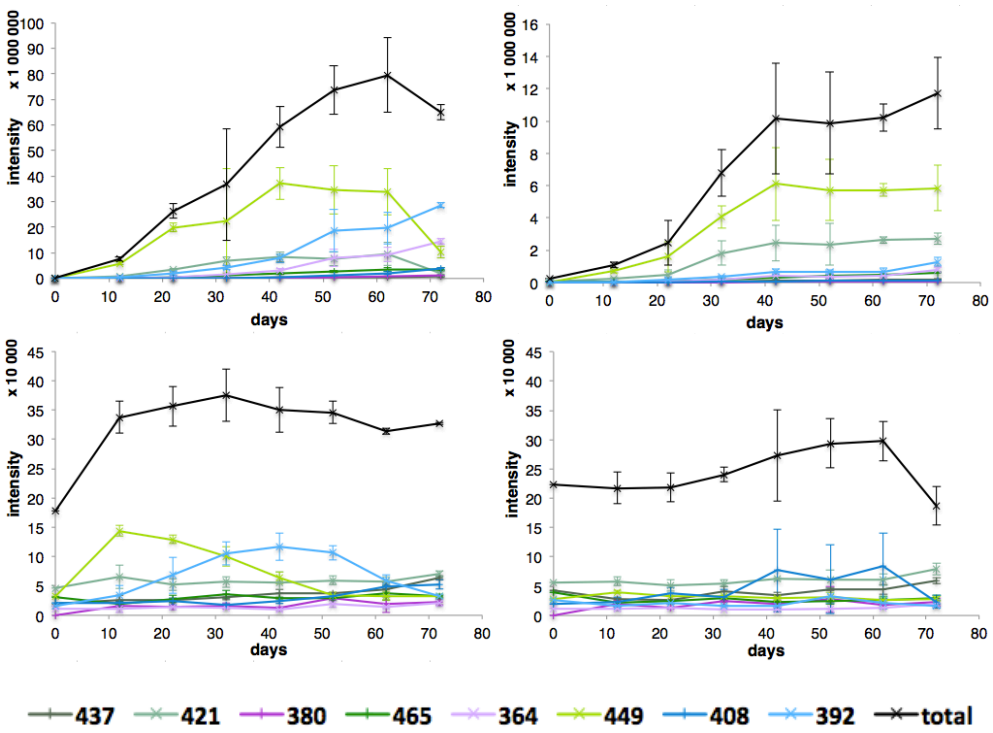


Figure 5.3.3.8 Levels of metabolites present in filtered supernatant of *Synechococcus* sp. WH7803 wild type (left) and T3 PKS mutant (right) cultures grown with (bottom) and without (top) *Ruegeria pomeroyi*. Error bars represent standard deviation.

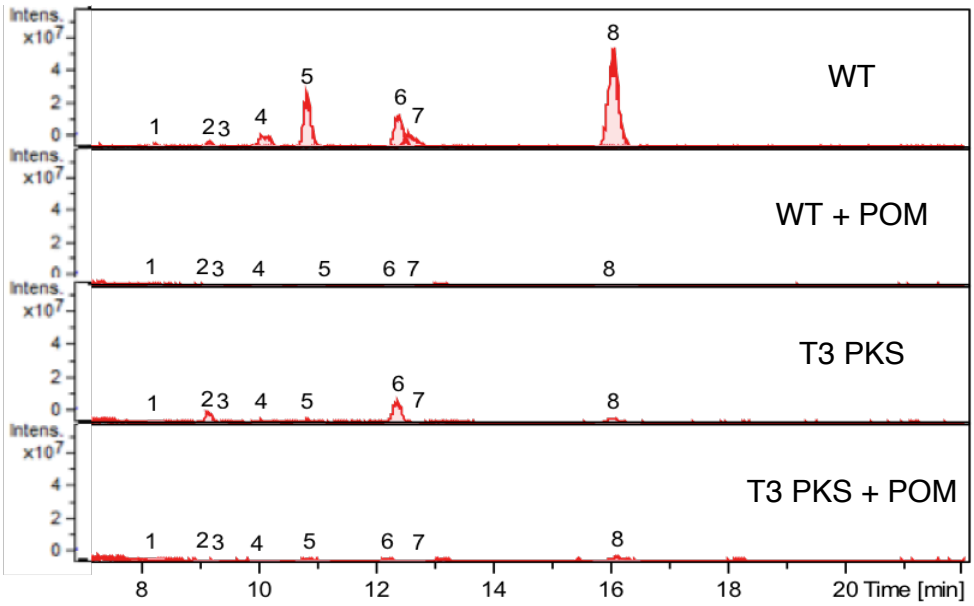


Figure 5.3.3.9 Levels of the six metabolites described in chapter 4 and m/z=380 and 437 molecules observed during the mutant characterisation experiments in *Synechococcus* sp. WH7803 wild type (WT) and T3 PKS (T3 PKS) mutant cultures – axenic and with *Ruegeria pomeroyi* (+POM). Molecules: m/z=437 (1), m/z=421 (2), m/z=380 (3), m/z=465 (4), m/z=364 (5), m/z=449 (6), m/z=408 (7) and m/z=392 (8).

All eight molecules were present at much lower concentrations, at the intensity of background noise (10^4), in co-cultures of both wild type and the T3 PKS mutant of *Synechococcus* sp. WH7803 with *Ruegeria pomeroyi*. When normalised to cell counts, there was no clear trend of a changed production of the eight molecules with stage of growth, but rather an increase in intensity per 1000 cells caused by a temporary drop in cell numbers in the mid exponential phase (axenic cultures) and due to cell death (both wild type cultures and axenic T3 PKS mutant culture) during late stationary phase (Figure 5.3.3.10; cell counts in Figure 5.3.3.5 and metabolite levels in Figure 5.3.3.9).

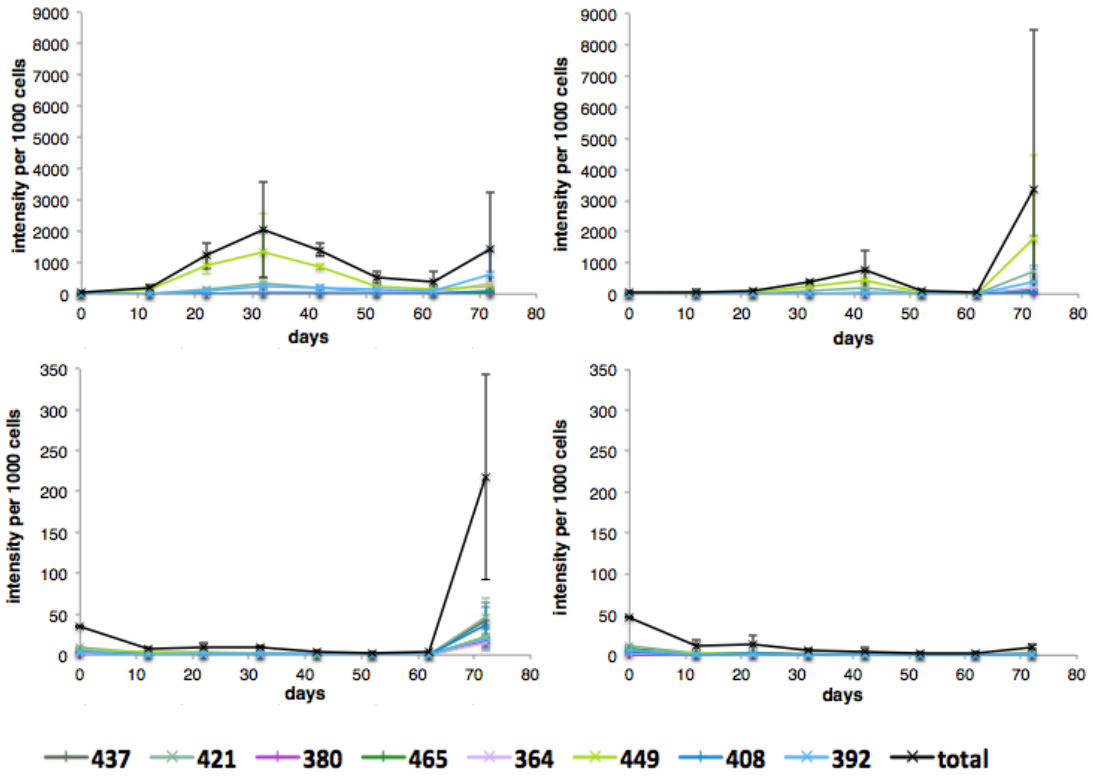


Figure 5.3.3.10 Levels of metabolites present in filtered supernatant of *Synechococcus* sp. WH7803 wild type (left) and T3 PKS mutant (right) cultures grown with (bottom) and without (top) *Ruegeria pomeroyi* normalised to cell numbers (per 1000 cells). Error bars represent standard deviation.

5.3.4 Assessing the effect of nutrient limitation on growth and exometabolite production in *Synechococcus* sp. WH7803 T3 PKS mutant

Both the wild type and T3 PKS mutant cultures of *Synechococcus* sp. WH7803 were affected by nutrient limitation in terms of growth (Figure 5.3.4.1). Iron limited T3 PKS mutant cultures started to show a decline in cell numbers 10 days earlier than the wild type culture, which suggests that the mutant may be more sensitive to iron depletion. Metabolite levels in wild type cultures of *Synechococcus* sp. WH7803 grown in nutrient depleted media showed the same pattern of metabolite level increase with growth and the same peak of the m/z=449 molecule before a peak in the m/z=392 metabolite as cultures grown in nutrient replete media (Figure 5.3.4.2;

compared with Figure 5.3.3.8 – a drop in m/z=449 started when m/z=392 molecule increased). T3 PKS mutant cultures again show an approximately one order of magnitude lower concentration of all eight metabolites compared to the wild type. There is also a steep increase in the level of m/z=449 and m/z=421 molecules at the time of a sharp decrease in cell numbers (days 30-40) in the T3 PKS mutant grown under standard conditions and m/z=392, m/z=449 and m/z=364 molecules in T3 PKS mutant in P-deplete growth conditions (Figure 5.3.4.2 bottom left two panels).

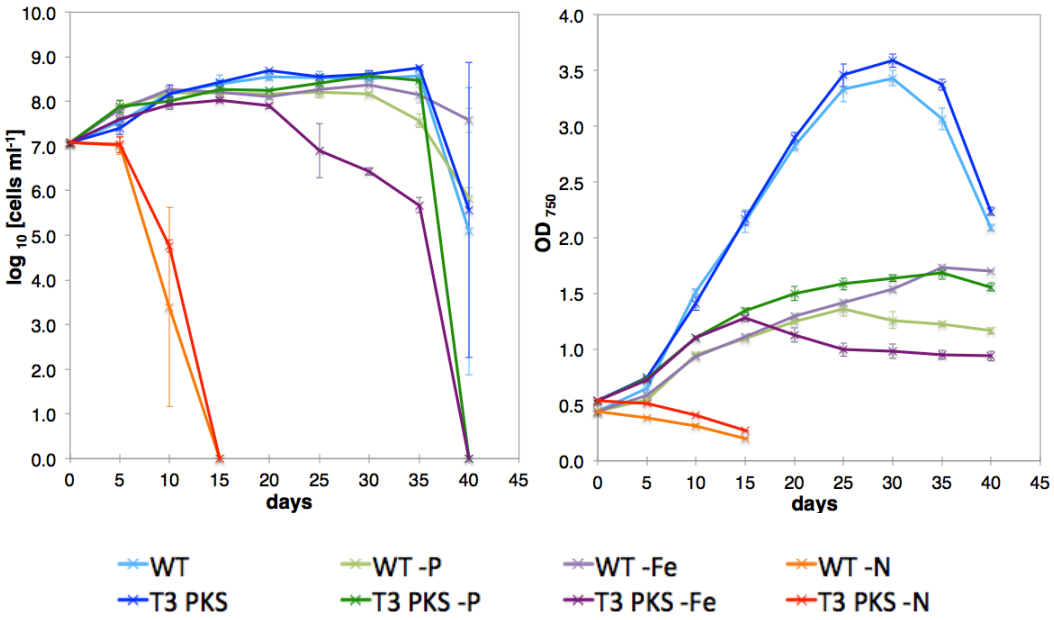


Figure 5.3.4.1 Growth of axenic *Synechococcus* sp. WH7803 wild type (WT) and T3 PKS mutant cultures (T3 PKS) in standard ASW and ASW depleted of phosphorus (-P), iron (-Fe) and nitrogen (-N) monitored by flow cytometry (cell counts per ml of culture; left) and spectrophotometry (optical density at 750 nm; right). Error bars represent standard deviation.

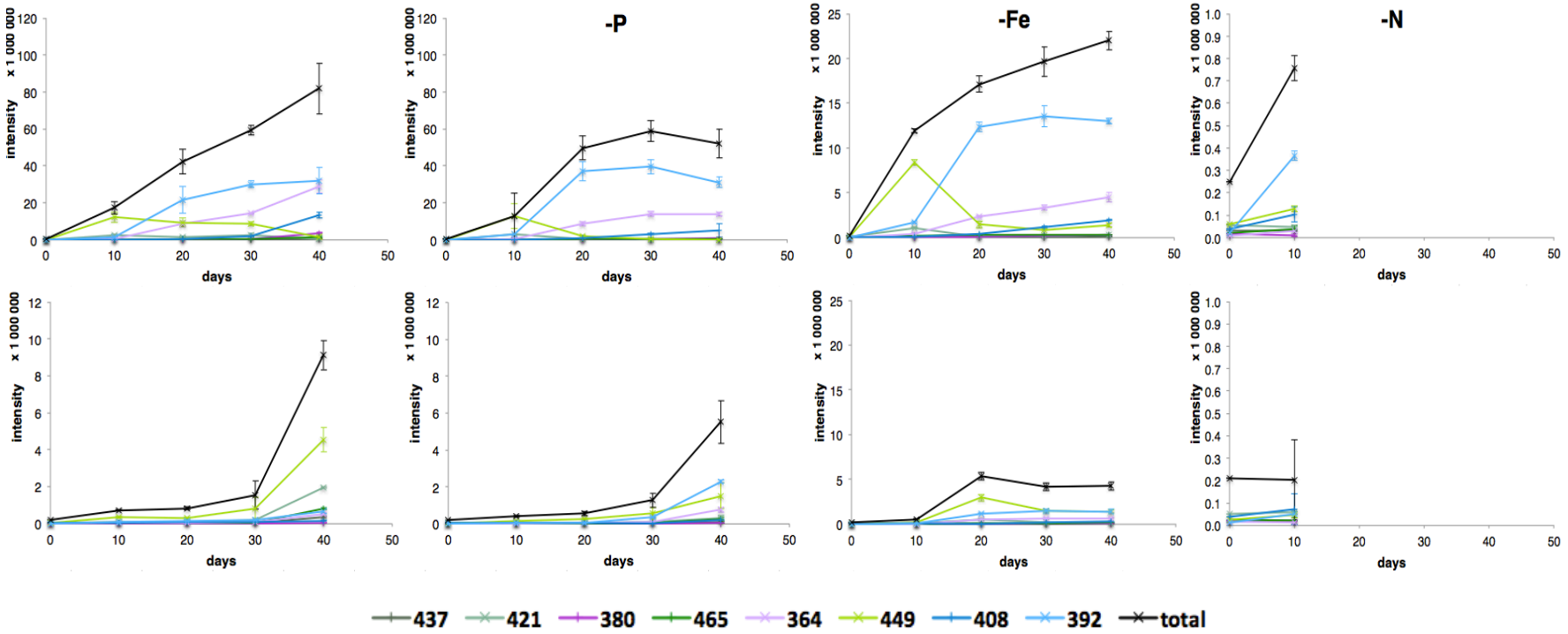
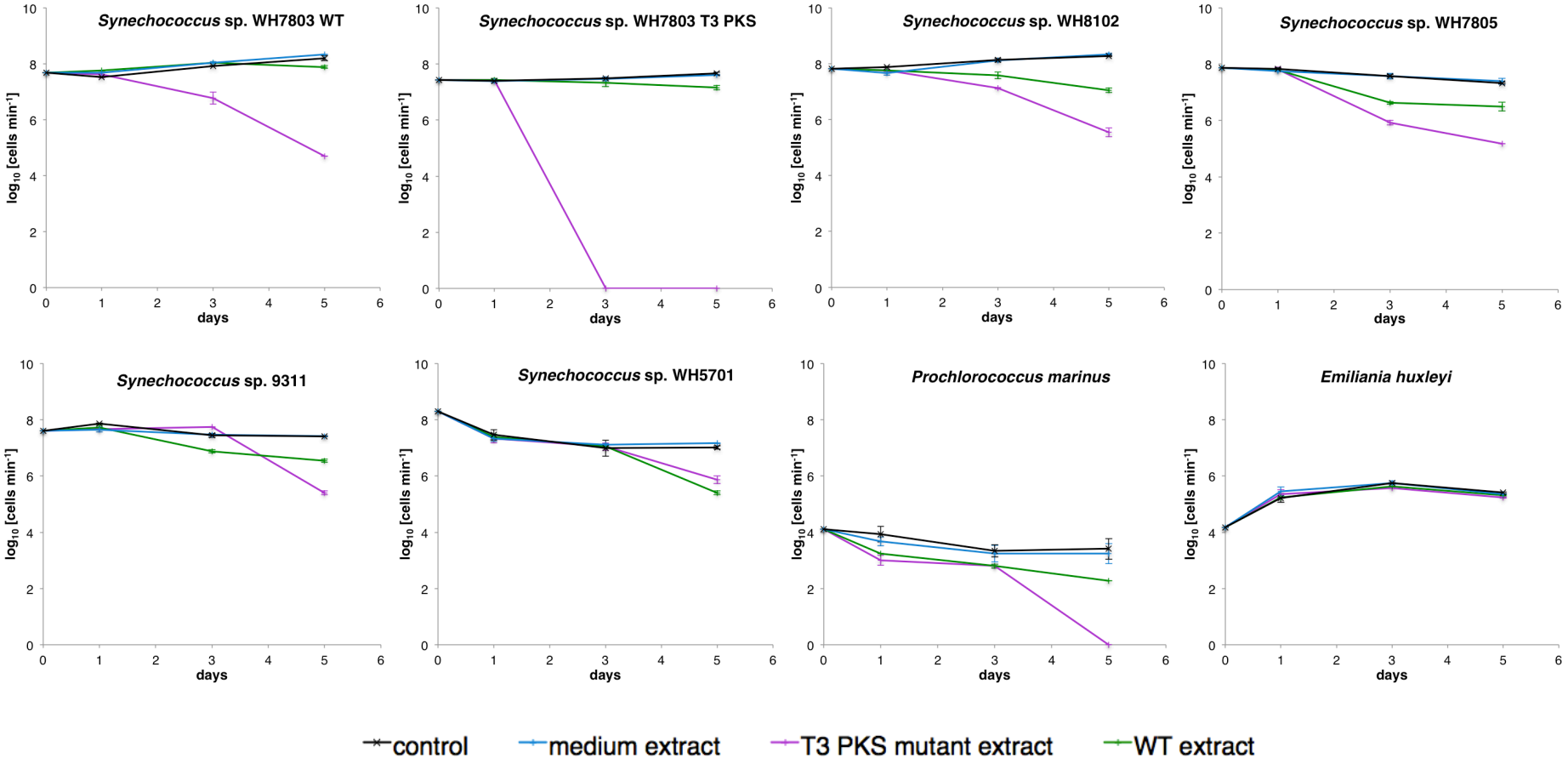


Figure 5.3.4.2 Levels of metabolites present in filtered supernatant of *Synechococcus* sp. WH7803 wild type (top) and T3 PKS mutant (bottom) cultures grown in standard ASW and ASW depleted of phosphorus (-P), iron (-Fe) and nitrogen (-N). Error bars represent standard deviation.

5.3.5 Assessing the effect of extract addition on the growth of a range of phototrophs

Growth of the various phototroph cultures (see Table 5.2.3.1) under standard conditions was similar in both the presence or absence of ASW extract (Figure 5.3.5.1), suggesting little or no growth effect of ASW extract addition on the phototrophs. *Emiliania huxleyi* and the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, were not affected by the addition of either the *Synechococcus* sp. WH7803 wild type extract or the T3 PKS mutant extract. In contrast, *Ostreococcus tauri* and the *Synechococcus* sp. WH7803 T3 PKS mutant itself were most affected by the T3 PKS mutant extract, with no cells detected after 3 days. In most cultures, the T3 PKS mutant extract seemed to have a stronger negative effect on the growth of phototrophs than the wild type extract.


Synechococcus sp. WH7803 WT
Synechococcus sp. WH7803 T3 PKS
Synechococcus sp. WH8102
Synechococcus sp. WH7805
Synechococcus sp. 9311
Synechococcus sp. WH5701
Prochlorococcus marinus
Emiliania huxleyi
log10 [cells min-1]
days
control
medium extract
T3 PKS mutant extract
WT extract

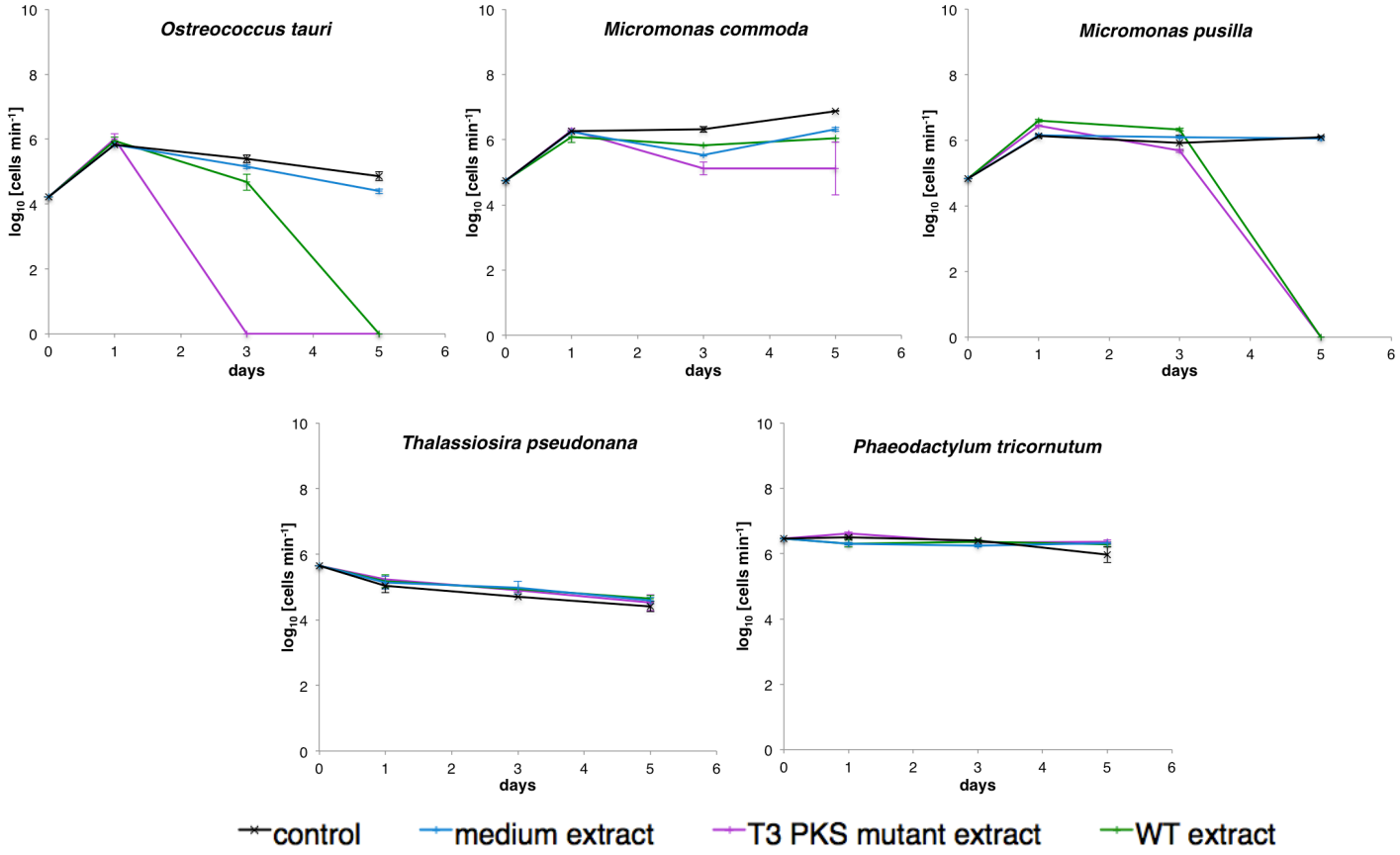


Figure 5.3.5.1 Growth of phototroph cultures after the addition of ASW medium extract (blue), *Synechococcus* sp. WH7803 wild type (green) and T3 PKS mutant (pink) supernatant extracts re-dissolved in seawater. Error bars represent standard deviation.

5.4 Discussion and conclusions

The presence of twenty three putative biosynthetic gene clusters in the genome of *Synechococcus* sp. WH7803 suggests that the cyanobacterium is able to produce a range of different secondary metabolites. As discussed earlier, free-living, single celled marine cyanobacteria are known to produce secondary metabolites, such as prochlorosins (Li et al., 2010), despite having relatively small genomes, often living in oligotrophic areas and at low cell densities.

Cyanobacteria were shown to have an average of five NRPS/PKS gene clusters per genome, forming about 5% of their genomes (Shih et al., 2015). Thus, whilst it is perhaps not surprising to find a T3 PKS gene cluster in a number of *Synechococcus* spp. and *Prochlorococcus* spp. the number of PKS/NRPS in *Synechococcus* sp. WH7803 appears well below this average of five. With 80% of these gene clusters associated with unknown products (Calteau et al., 2014), it is also difficult to predict the biosynthetic pathways. The problem of encountering proteins of no known function, with no conserved domains that could help in predicting their function, was noted previously, during proteomics studies of various marine *Synechococcus* strains (Christie-Oleza et al., 2015).

Genes of known function in the T3 PKS gene cluster of *Synechococcus* sp. WH7803 (see Table 5.3.1.2) are related mainly to membrane associated proteins and transport systems. Of the seven genes present in *Synechococcus* sp. WH7803 that have their counterparts in all other *Synechococcus* spp. and *Prochlorococcus* spp. strains tested, three are annotated as hypothetical proteins (*synWH7803_1001*, *synWH7803_1018*, *synWH7803_1019*). However, based on a conserved domains search, it looks like the first of the three genes may be a S-adenosylmethionine-dependent methyltransferase dehydrogenase (*synWH7803_1001*) suggesting a role of cysteine or methionine in formation of the metabolite. Describing the role of all of the seven genes would be most helpful for

studying the biosynthetic pathway involved in the production of the secondary metabolite.

The presence of genes with high amino acid identity to the *Synechococcus* sp. WH7803 T3 PKS gene in genomes of a range of marine bacteria, including marine heterotrophic bacteria, suggests the possibility of horizontal gene transfer. Moreover, studies showed that NRPS/T3 PKSs are more common in more recent branches of the phylogenetic cyanobacterial tree and that NRPS/PKS diversification and species clustering based on NRPS/T3 PKS pathways do not match species phylogeny (Calteau et al., 2014; Ehrenreich et al., 2005). However, Calteau et al. also note that the diversity of pathways observed required complex evolution, including gene duplication and shuffling, indels, inversions and domain diversification.

Constitutive expression, across growth phase, of the T3 PKS gene from *Synechococcus* sp. WH7803 under standard growth conditions (see section 5.3.2) confirms that the gene cluster is not silent, but does not explain the changing metabolite levels per cell throughout growth of the culture. However, the one order of magnitude decrease in concentration of the eight molecules in the *Synechococcus* sp. WH7803 T3 PKS mutant does suggest a clear link between the gene and the metabolites observed in chapters 4 and 5. A question remains how the molecules are produced at all, with a disrupted T3 PKS gene. There is some evidence of successful PKS gene disruption, with production of the compound of interest completely stopped, but unfortunately not in cyanobacteria (Yu et al, 2007). There could be a compensation mechanism, less efficient, but capable of performing a similar function to that of the product of the T3 PKS gene. Although no other PKS genes were found in the genome of *Synechococcus* sp. WH7803, there could be a hypothetical protein of a similar function somewhere in the genome.

Another explanation is that the gene was not fully inactivated by insertional mutagenesis. Although the malonyl-CoA binding site was separated from the active site and product binding site, the active site itself was not

disrupted and the enzyme could have remained partially active. Generating a clean mutant, with T3 PKS gene completely removed instead of inactivated by plasmid insertion, would solve this problem.

There seems to be a pattern of molecule m/z=449 peaking before the m/z=392 compound, which could suggest that the m/z=449 is an intermediate step in the production of the m/z=392 molecule. A chemical structure and biosynthetic pathway elucidation would be crucial to determine the relationship between the eight molecules. The two compounds observed for the first time during this study, m/z=380 and m/z=437, based on their mass to charge ratio and elution times, could be $[C_{17}H_{34}NO_6S]^+$ and $[C_{19}H_{37}N_2O_7S]^+$ respectively. This suggests a set of eight compounds, four of which have one extra oxygen, which could suggest sulphur oxidation, and four – an additional C_2H_4 group, which suggests a longer carbon chain (Table 5.4.1).

Table 5.4.1 Predicted chemical formulae for eight compounds produced by *Synechococcus* sp. WH7803.

Extra C_2H_4	**Extra oxygen**	
	no	**yes**
no	$[C_{17}H_{34}NO_5S]^+$ $[C_{19}H_{37}N_2O_6S]^+$	$[C_{17}H_{34}NO_6S]^+$ $[C_{19}H_{37}N_2O_7S]^+$
yes	$[C_{19}H_{38}NO_5S]^+$ $[C_{21}H_{41}N_2O_6S]^+$	$[C_{19}H_{38}NO_6S]^+$ $[C_{21}H_{41}N_2O_7S]^+$

The difference in cell numbers at the same $OD_{750\ nm}$ value between *Synechococcus* sp. WH7803 wild type and T3 PKS mutant cultures, as well as a difference in side scatter, suggest a change in cell granularity. Slightly higher absorbance in the UV-B region by the T3 PKS mutant also points towards a change in internal structures or a change in UV protection mechanisms.

In plants and some bacteria, expression of chalcone synthase, a T3 PKS, is regulated by a photoreceptor in response to red, blue or UV light (plants) (Kreuzaler et al., 1983; Martin, 1993) and infrared light (bacteria) (Jiang et al., 1999). Flavonoids, for production of which chalcone synthase is the first committed enzyme, can absorb UV-B to protect plant cells (Jenkins et al.,

2001). The authors suggest that the pathway is not mediated by oxidative stress signalling, as hydrogen peroxide added to the cell culture did not stimulate chalcone synthase expression. Whether the *Synechococcus* T3 PKS is affected by exposure to light or plays a role in UV protection is unresolved. Some genes, although annotated as chalcone synthase, are predicted to have different roles, using different starter molecules and producing different compounds (Dixon, 2001; Moore & Hopke, 2001; Schroder, 1997).

As only approximately half of the *Synechococcus* sp. WH7803 T3 PKS mutant cultures survive until typical late exponential phase compared to all wild type cultures, there is a clear growth disadvantage due to disruption of the T3 PKS gene. Although all cultures were inoculated with the same medium and kept in the same conditions, sudden culture collapse did not affect all of them. The T3 PKS mutant cultures were checked at the end of the experiment for presence of the plasmid and to confirm segregation, but there was no difference between the cultures that did survive and those that died early.

A possible explanation could be a toxic intermediate that cannot be removed from the culture due to T3 PKS gene disruption, which is not produced constitutively, but rather in bursts. If the burst coincides with a temporary drop in cell numbers or stress (e.g. a change in light intensity and temperature when cultures are removed from the incubator for sampling), the culture may not recover. Another explanation could be that the m/z=392 molecule plays a protective role in cultures – when in wild type cultures concentration of the compound peaks between mid-exponential and stationary phases, i.e. after the m/z=449 molecule peaks, there is no such peak in axenic *Synechococcus* sp. WH7803 T3 PKS mutant cultures and some of the cultures die (see Figure 5.3.2.7). On the other hand, it could also be argued that rather than the absence of the m/z=392, it is the rather higher relative concentrations of the m/z=449 and m/z=421 molecules that is the problem. In wild type cultures, the molecules with the highest

concentration are those with m/z=392, m/z= 449 and m/z=364, compared to m/z=449 and m/z=421 in *Synechococcus* sp. WH7803 T3 PKS mutant cultures.

To test whether molecules in the *Synechococcus* sp. WH7803 T3 PKS mutant culture have any effect on the growth of phototrophs, including *Synechococcus* sp. WH7803 wild type and T3 PKS mutant cultures, large volume supernatant extracts of wild type and T3 PKS mutant cultures redissolved in seawater were added to 13 different phototrophs. The growth of all *Synechococcus* spp., *Prochlorococcus* and some green algae were affected more by the addition of the T3 PKS extract and, with the exception of *Synechococcus* sp. WH5701, all showed culture decline or death after 5 days of culture. This suggests that the molecules produced by *Synechococcus* sp. WH7803 T3 PKS mutant cultures are more toxic than those produced by the *Synechococcus* sp. WH7803 wild type culture. To determine which molecules are responsible for the effect of higher toxicity or which molecule has a protective influence on the culture, fractionation of the extract could be performed. A fraction of the extract found to be active could then be analysed further to purify the compound(s) responsible for the effect. A study of antimicrobial and cytotoxic activity of cyanobacterial extracts suggested that marine *Synechococcus* sp. extracts can induce apoptosis in eukaryotic cells and cause inhibition of Gram-positive bacteria, but do not affect Gram-negative bacteria (Martins et al., 2008). A study focusing on purified compounds instead of crude extracts could improve our understanding of the mechanisms behind these effects. Also, a study of toxicity of marine *Synechocystis* and *Synechococcus* extracts on marine invertebrates suggested that a crude extract may have a stronger negative effect on survival of invertebrates than a partially purified extract due to a possible synergistic effect of different toxic compounds (Martins et al., 2007).

There is no evidence of cyanobactin gene clusters present in *Synechococcus* spp. genomes (Leikoski et al., 2013) and no bacteriocidal

activity was observed for prochlorosins (Li et al., 2010). Given the high rate of discovery of novel cyanobacterial compounds, it is possible that the molecules are a novel class of antibacterial compounds. However, it is important to remember that, taking into account cyanobacteria cell densities in the open ocean, the likelihood of close phototroph – phototroph interactions is not high and the compounds may in fact have a different role, perhaps related to metabolism of the cell, environmental sensing, protection from stress or interaction with heterotrophic bacteria. To improve our understanding of the function of these compounds, a broad –omics approach would be advisable. A proteomics study would provide some insights into what happens inside the T3 PKS mutant cell compared to a wild type cell and suggest what to focus on for further physiological experiments.

To conclude, the T3 PKS gene cluster in *Synechococcus* sp. WH7803 is not silent and plays a role in the production of at least eight different compounds, produced in all nutrient conditions, in both axenic cultures and co-cultures. Insertional inactivation of the T3 PKS gene may cause early death of a culture and the changes in the molecules released from the cells into the surrounding environment can have a negative effect on the growth of other phototrophs and the *Synechococcus* sp. WH7803 T3 PKS mutant itself.

6. Conclusions and future work

The overall aim of this work was to improve our understanding of marine phototroph – heterotroph interactions. Despite the importance of marine microorganisms for our planet, surprisingly little is known about the interactions between two major groups of microbes – phototrophs that produce every second molecule of oxygen we breathe and heterotrophs that drive the biogeochemical processes in the ocean.

By trying to determine which heterotrophic bacteria are the most frequent partners in *Synechococcus* spp. non-axenic cultures, a better appreciation of its community interactions was achieved (Chapter 3). Over half of all the cultures tested were hosting the same 4 heterotrophic bacteria belonging to the following genera: *Nitratireductor* spp., *Rhodobacteraceae, Muricauda* spp., *Phyllobacteriacae.* All of these genera are known to be associated with seawater and have been observed in cultures of other phototrophs or in environmental enrichments previously.

To determine the nature of the relationships, a broad –omics approach study would be advisable, together with co-culturing physiological experiments to confirm the predicted interactions. A look into the proteomics of co-cultures, similar to that of *Synechococcus* sp. – *R. pomeroyi* (Christie-Oleza et al., 2015, 2017), would provide some overview of what the relationship is giving to and requiring from its members, for example whether it is provision of a specific resource, remineralisation of organic matter to provide nutrients or removal of specific toxic compounds that keeps the partners together. Once an overview of metabolic or other interactions between a model *Synechococcus* sp. and its most frequently occurring heterotrophic partners is available from proteomics data, physiological studies, accessing growth of partners and relationship dynamics can determine if the relationship is beneficial to both, one or none of the partners.

Ultimately, to advance the field of interactions between marine bacteria, a model community should be developed. Focusing the scientific community's efforts on understanding a simple, yet representative system, would speed up research and enable us to push the field much more than by studying various systems separately – just like focusing efforts on studying *E. coli* as a model bacterium advanced the field of microbiology. With known metabolic relationships and including genome-sequenced representatives of most dominant phyla, a simple, yet robust oceanic community could be formed – a concept similar to the development of a maize community model system, which includes 7 strains from three of the four most dominant bacterial phyla found in maize roots (Niu et al., 2017). Studies like this one provide invaluable data on preliminary community member selection for development of such a system, especially those including one of the most abundant phototrophs in oceanic systems.

Although the metadata collected for the non-axenic *Synechococcus* sp, cultures did not explain the observed differences in community composition, it is important to remember that the sample size, especially for specific geographic locations, was very small and thus could have been inadequate to observe any differences, if they in fact exist. For future studies, it would be important to include parameters such as nutrient concentration, water temperature, light level and cell densities at the place of sample collection, as these types of information could give more insights on what drives specific relationships and would characterise the natural environment of the microorganisms much better than values such as latitude, longitude or depth of collection. Unfortunately, very rarely full datasets containing all these types of information are available, especially for cultures isolated twenty and more years ago, so it might mean designing a completely new study from the point of sample collection to sequencing a clonal isolate maintained in culture for a few years.

The study of how partner community changes during clonal purification of *Synechococcus* sp. was based on a very low number of samples, and as

such should be treated with caution, and as a preliminary experiment highlighting the challenges and opportunities that would be associated with a similar large-scale study. Especially, the questions to what extent is the process of heterotroph survival stochastic and to what extent is it driven by metabolic interactions rather than phylogeny, seems worth pursuing in the future to better understand the formation of phototroph-heterotroph relationships and their specificity.

Taking into account the presence of *Rhodobacteraceae* in more than half of all non-axenic *Synechococcus* sp. WH7803 cultures and multiple reports of the presence of *Roseobacter* in the open ocean (Gonzalez et al., 2000), a model system of *Synechococcus* sp. WH7803 – *Ruegeria pomeroyi* was chosen for further work (Chapter 4). An exometabolomics approach targeting differences between the model phototroph – heterotroph co-culture compared to the axenic phototroph culture revealed a group of potentially novel compounds produced by *Synechococcus* sp. WH7803 in high concentrations only in axenic cultures. The same effect was observed in 14 out of 16 *Synechococcus* sp. WH7803 – heterotrophic bacteria co-cultures, suggesting it is probably a response of the phototroph to the presence of heterotrophic bacteria, rather than a species-specific interaction.

Ruegeria pomeroyi addition to an axenic *Synechococcus* sp. WH7803 culture caused a sharp decrease in molecule m/z=449 concentration (no longer detected in the media within 10-20 days) suggesting that the molecule can be degraded – directly or indirectly – by the heterotroph. The m/z=392 molecule was not affected by the presence of the heterotroph, suggesting that it cannot be broken down and used as a source of nutrients. Molecule structure determination by NMR would help to highlight the differences between the two molecules and provide some insight into what sorts of biochemical machinery is required to break down the compounds, which in turn could be a source of speculation about the function and the pathway or organism targeted by the molecule.

Another advantage of elucidating a full chemical structure of the molecule(s) would be the ability to predict steps of their biosynthesis which would help in determining which genes are involved in the production of the compounds. Once the specific genes are known, their expression could be tested in axenic cultures and co-cultures, as well as in different growth conditions which would provide more insight into the function of the molecules and regulation of their production in co-cultures.

One of the major challenges for structure determination is the low yield of the molecules of interest. The issue has been reported previously for prochlorosins produced by *Prochlorococcus* at the level of 10 µg from 20 l of culture (Li et al., 2010). Feeding a culture of *Synechococcus* sp. WH7803 with ^{13}C sodium bicarbonate could strengthen the NMR signal and help with full chemical structure elucidation. Once biosynthetic genes are known, use of a heterologous expression host, for example *Synechocystis* sp. or *E. coli*, becomes a possibility for specific molecules needed in high quantities. The time invested in developing such a system could be compensated for by higher growth speeds and yields of heterologous hosts compared to the native host.

Further experiments to help determine the function of the molecules could include generation of a knockout mutant(s) in the genes involved in the production of the compounds and looking for any differences between the mutants and the wild type. Addition of purified molecules to the axenic culture of the phototroph, or a range of photo- and heterotrophs, could also be interesting.

The fragmentation pattern of the m/z=392 compound suggested that it may belong to the polyketide synthases group of secondary metabolites due to possible presence of a characteristic fatty-acid-like chain of carbons. *Synechococcus* sp. are known to have T3 PKS gene clusters in their genomes (Shih et al., 2013) and *Synechococcus* sp. WH7803 was shown to contain only one of these clusters (Chapter 5).

Unfortunately the T3 PKS gene cluster in *Synechococcus* sp. WH7803 contains some hypothetical proteins, for which not even a search for conserved domains is of any help in determining their function. As in the case of earlier studies of the same and a similar model system (Aharonovich & Sher, 2016; Christie-Oleza et al., 2015), this is a major obstacle in interpreting genomic data. Future development and verification of gene, protein, metabolite and other database entries is of great importance and would help not only in natural products discovery, but also in physiological studies of the metabolism of co-cultures.

Seven genes were found to be conserved in all T3 PKS *Synechococcus* spp. and *Prochlorococcus* spp. gene clusters, out of which three are annotated as hypothetical. Trying to determine the function of these three genes in the first instance would be of major help in describing the biosynthetic pathway they form. Also, a PCR check of cDNA generated from RNA of *Synechococcus* sp. WH7803 could determine which genes are transcribed together, which could also provide some insight about the biosynthetic pathway.

A mutant generated for the T3 PKS gene in *Synechococcus* sp. WH7803 (Chapter 5) showed that growth of the axenic culture is negatively affected by the disruption of the gene – 'every other' mutant culture dies suddenly during the mid-exponential phase of growth. This unpredictability of growth of mutant cultures, as well as the results of antimicrobial assays of wild type and T3 PKS mutant extracts against a range of phototrophs, suggests that there is a toxic effect of the disrupted gene on the health of cultures. To determine whether the cause of this is any specific metabolite, the extracts could be fractionated and added to growing cultures – if a fraction causes a decrease in cell numbers, it should be further analysed, including purification and structure elucidation of any compounds present. A proteomics comparison of wild type and T3 PKS mutant cultures could also provide some insight into differences in cell metabolism and help determine the pathways affected by disruption of the gene.

All the metabolites discovered to be at lower levels in co-cultures with *R. pomeroyi* than in axenic *Synechococcus* sp. WH7803 were affected by the disruption of the T3 PKS gene, which suggests that the T3 PKS gene plays a role in their biosynthesis. An approximately one order of magnitude change in the concentration of these molecules was observed instead of a complete disappearance of the molecules – a clean knockout mutant of the T3 PKS gene could help determine whether the cause of this is the enzyme retaining some of its function during insertional mutagenesis, an alternative, less efficient backup pathway for production of the compounds or another compensation mechanism that affected the pathway indirectly. Generating T3 PKS mutants in other picocyanobacteria and looking for changes in growth and metabolite profiles should also be considered in future work to characterize the products of these gene clusters in other, also environmentally important, genera.

There is also the question of non-biological roles of the molecules. As many cyanobacterial secondary metabolites are of interest to industry (Burja et al., 2001), it would be interesting to screen this new group of compounds for potential antimicrobial, anticancer, anti-inflammatory activity and other activity of potential clinical interest. By learning about small molecules produced by microscopic organisms in the vast global ocean, we may in fact not only improve our understanding of the planet we live on, but also expand our knowledge of molecules useful to ourselves.

List of References

Adachi, M., Matsubara, T., Okamoto, R., Nishijima, T., Itakura, S., Yamaguchi, M. (2002) “Inhibition of cyst formation in the toxic dinoflagellate *Alexandrium* (*Dinophyceae*) by bacteria from Hiroshima Bay, Japan”, *Aquatic Microbial Ecology*, 26, 223-233.

Aharonovich, D., Sher, D. (2016) “Transcriptional response of *Prochlorococcus* to co-culture with a marine *Alteromonas*: differences between strains and the involvement of putative infochemicals”, *International Society for Microbial Ecology Journal*, 10 (12), 2892-2906.

Alavi, M., Miller, T., Erlandson, K., Schneider, R., Belas, R. (2001) “Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures”, *Environmental Microbiology*, 3 (6), 380-396.

Althoff, K., Schutt, C., Steffen, R., Batel, R., Muller, W.E.G. (1998) “Evidence for a symbiosis between bacteria of the genus *Rhodobacter* and the marine sponge *Halichondria panicea*: harbor also for putatively toxic bacteria?”, *Marine Biology*, 130 (3), 529-536.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., (1990) “Basic local alignment search tool”, *Journal of Molecular Biology,* 215, 403–410.

Amin, S.A., Parker, M.S., Armbrust, E.V. (2012) “Interactions between diatoms and bacteria”, *Microbiology and Molecular Biology Reviews*, 76 (3), 667-684.

Amin, S.A., Kupper, F.C., Green, D.H., Harris, W.R., Carrano, C.J. (2007) “Boron binding by a siderophore isolated from marine bacteria associated with the toxic dinoflagellate *Gymnodinium catenatum*”, *Journal of Americal Chemistry Society*, 129 (3), 478-479.

Amin, S.A., Green, D.H., Hart, M.C., Kupper, F.C., Sunda, W.G., Carrano, C.J. (2009) “Photolysis of iron-siderophore chelates promotes bacterial-algal mutualism”, *Proceedings of the National Academy of Sciences of the United States of America*, 106 (40), 17071-17076.

Amin, S.A., Hmelo, L.R., van Tol, H.M., Durham, B.P., Carlson, L.T., Heal, K.R., Morales, R.L., Berthiaume, C.T., Parker, M.S., Djunaedi, B., Ingalls, A.E., Parsek, M.R., Moran, M.A., Armbrust, E.V. (2015) “Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria”, *Nature*, 522 (7554), 98-101.

Apple, J.K., Strom, S.L., Palenik, B., Brahamsha, B. (2011) “Variability in protist grazing and growth on different marine *Synechococcus* isolates”, *Applied and Environmental Microbiology*, 77 (9), 3074-3084.

Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S., Allen, A.E., Apt, K.E., Bechner, M., Brzezinski, M.A., Chaal, B.K., Chiovitti, A., Davis, A.K., Demarest, M.S., Detter, J.C., Glavina, T., Goodstein, D., Hadi, M.Z., Hellsten, U., Hildebrand, M., Jenkins, B.D., Jurka, J., Kapitonov, V.V., Kroger, N., Lau, W.W., Lane, T.W., Larimer, F.W., Lippmeier, J.C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Parker, M.S., Palenik, B., Pazour, G.J., Richardson, P.M., Rynearson, T.A., Saito, M.A., Schwartz, D.C., Thamatrakoln, K., Valentin, K., Vardi, A., Wilkerson, F.P., Rokhsar, D.S. (2004) “The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism”, *Science*, 306 (5693), 79-86.

Arrigo, K.R. (2005) “Marine microorganisms and global nutrient cycles”, *Nature*, 437 (7057), 349-355.

Arun, A. B., Chen, W. M., Lai, W. A., Chao, J. H., Rekha, P. D., Shen, F. T., (2009) “*Muricauda lutaonensis* sp. nov., a moderate thermophile isolated from a coastal hot spring”, *International Journal of Systematic and Evolutionary Microbiology*, 59, 2738–2742.

Azam, F., Malfatti, F., (2007) “Microbial structuring of marine ecosystems”, *Nature Reviews Microbiology*, 5, 782–791.

Bahr, M., Stams, A.J., De la Rosa, F., Garcia-Encina, P.A., Munoz, R. (2011) “Assessing the influence of the carbon oxidation-reduction state on

organic pollutant biodegradation in algal-bacterial photobioreactors", *Applied Microbiology and Biotechnology*, 90 (4), 1527-1536.

Bambauer, A., Rainey, F.A., Stackebrandt, E., Winter, J. (1998) "Characterization of *Aquamicrobium defluvii* gen. nov. sp. nov., a thiophene-2-carboxylate-metabolizing bacterium from activated sludge", *Archives of Microbiology*, 169, 293–302.

Baquero, F., Moreno, F. (1984) "The microcins", *FEMS Microbiology Letters*, 23, 117-124.

Barbara, G.M., Mitchell., J.G. (2003) "Bacterial tracking of motile algae", *FEMS Microbiology Ecology*, 44, 79-87.

Barboza, G.F.O., Gorlach-Lira, K., Sassi, C.F.C., Sassi, R. (2017) "Microcystins production and antibacterial activity of cyanobacterial strains of *Synechocystis*, *Synechococcus* and *Romeria* from water and coral reef organisms (Brazil)", *Revista de Biologia Tropical*, 65 (3), 890-899.

Bates, S.S., Douglas, D.J., Doucette, G.J. and Leger, C. (1995) Enhancement of domoic acid production by reintroducing bacteria to axenic cultures of the diatom *Pseudonitzschia multiseries*, Natural Toxins, 3, 428–435.

Bates, S.S., Gaudet, J., Kaczmarska, I., Ehrman, J.M. (2004) "Interaction between bacteria and the domoic-acid-producing diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle; can bacteria produce domoic acid autonomously?", *Harmful Algae*, 3 (1), 11-20.

Beliaev, A.S., Romine, M.F., Serres, M., Bernstein, H.C., Linggi, B.E., Markillie, L.M., Isern, N.G., Chrisler, W.B., Kucek, L.A., Hill, E.A., Pinchuk, G.E., Bryant, D.A., Wiley, H.S., Fredrickson, J.K., Konopka, A. (2014) "Inference of interactions in cyanobacterial-heterotrophic co-cultures via transcriptome sequencing", *International Journal of Systematic and Evolutionary Microbiology*, 8 (11), 2243-2255.

Bertani, G., (1951) “Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*”, *Journal of Bacteriology*, 62 (3), 293–300.

Biller, S.J., Coe, A., Chisholm, S.W. (2016) “Torn apart and reunited: impact of a heterotroph on the transcriptome of *Prochlorococcus*”, *International Society for Microbial Ecology Journal*, 10 (12), 2831-2843.

Biller, S.J., Schubotz, F., Roggensack, S.E., Thompson, A.W., Summons, R.E., Chisholm, S.W. (2014) “Bacterial Vesicles in Marine Ecosystems”, *Science*, 343, 6167, 183-186.

Bolch, C.J., Subramanian, T.A., Green, D.H. (2011) “The Toxic Dinoflagellate *Gymnodinium catenatum* (*Dinophyceae*) Requires Marine Bacteria for Growth”, *Journal of Phycology*, 47 (5), 1009-1022.

Bolch. C.J., Bejoy. T.A., Green. D.H., (2017) “Bacterial associates modify growth dynamics of the dinoflagellate *Gymnodinium catenatum*”, *Frontiers in Microbiology*, 8, 670.

Brito, A., Gaifem, J., Ramos, V., Glukhov, E., Dorrestein, P.C., Gerwick, W.H., Vasconcelos, V.M., Mendes, M.V., Tamagnini, P. (2015) “Bioprospecting Portuguese Atlantic coast cyanobacteria for bioactive secondary metabolites reveals untapped chemodiversity”, *Algal Research*, 9, 218-226.

Bruins, A. P., (1998) “Mechanistic aspects of electrospray ionization”, *Journal of Chromatography*, 794, 1-2, 345-357.

Bruns, A., Philipp, H., Cypionka, H., Brinkhoff, T. (2003) “*Aeromicrobium marinum* sp. nov., an abundant pelagic bacterium isolated from the German Wadden Sea”, *International Journal of Systematic and Evolutionary Microbiology*, 53 (Pt 6), 1917-1923.

Bruns, A., Rohde, M., Berthe-Corti, L. (2001) “*Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment”, *International Journal of Systematic and Evolutionary Microbiology*, 51, 1997-2006.

Buchan, A., Gonzalez, J.M., Moran, M.A. (2005) “Overview of the marine *Roseobacter* lineage”, *Applied and Environmental Microbiology*, 71 (10), 5665-5677.

Buchan, A., LeCleir, G.R., Gulvik, C.A., González, J.M. (2014) “Master recyclers: features and functions of bacteria associated with phytoplankton blooms”, *Nature Reviews Microbiology*, 12, 686–698.

Bunkley-Williams, L., Williams, E.H. (1998) “Ability of Pederson Cleaner Shrimp to remove juveniles of the parasitic *Cymothoid* Isopod, *Anilocra haemuli*, from the host”, *Crustaceana*, 71 (8), 862-869.

Burja, A.M., Banaigs, B., Abou-Mansour, E., Burgess, J.G., Wright, P.C. (2001) “Marine cyanobacteria – a prolific source of natural products”, *Tetrahedron*, 57, 9347-9377.

Calteau, A., Fewer, D.P., Latifi, A., Coursin, T., Laurent, T., Jokela, J., Kerfeld, C.A., Sivonen, K., Piel, J., Gugger, M. (2014) “Phylum-wide comparative genomics unravel the diversity of secondary metabolism in *Cyanobacteria*”, *BioMed Central Genomics*, 15, 977.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., (2010) “QIIME allows analysis of high-throughput community sequencing data” *Nature Methods*, 7, 335–336.

Carmichael, W.W., Li, R. (2006) “Cyanobacteria toxins in the Salton Sea”, *Saline Systems*, 2, 5.

Carvalho, L.R., Costa-Neves, A., Conserva, G.A.A., Brunetti, R.L., Hentschke, G.S., Malone, C.F.S., Torres, L.M.B., Sant’Anna, C.L., Rangel, M. (2013) “Biologically active compounds from cyanobacteria extracts: in vivo and in vitro aspects”, *Revista Brasileira de Farmacognosia*, 23 (3), 471-480.

Chang, Z., Sitachitta, N., Rossi, J.V., Roberts, M.A., Flatt, P.M., Jia, J., Sherman, D.H., Gerwick, W.H. (2004) “Biosynthetic pathway and gene

cluster analysis of curacin A, an antitubulin natural product from the tropical marine cyanobacterium *Lyngbya majuscula*", *Journal of Natural Products*, 67 (8), 1356-1367.

Chen, F., Wang, K., Kan, J., Suzuki, M.T., Wommack, K.E. (2006) "Diverse and unique picocyanobacteria in Chesapeake Bay, revealed by 16S-23S rRNA internal transcribed spacer sequences", *Applied and Environmental Microbiology*, 72 (3), 2239-2243.

Cho, B.C., Azam, F. (1988) "Major role of bacteria in biogeochemical fluxes in the ocean's interior", *Nature*, 332, 441-443.

Cho, D.H., Ramanan, R., Heo, J., Lee, J., Kim, B.H., Oh, H.M., Kim, H.S. (2015) "Enhancing microalgal biomass productivity by engineering a microalgal-bacterial community", *Bioresource Technology*, 175, 578-585.

Christie-Oleza, J.A., Scanlan, D.J., Armengaud, J. (2015) ""You produce while I clean up", a strategy revealed by exoproteomics during *Synechococcus-Roseobacter interactions*", *Proteomics*, 15 (20), 3454-3462.

Christie-Oleza, J.A., Sousoni, D., Lloyd, M., Armengaud, J., Scanlan, D.J. (2017) "Nutrient recycling facilitates long-term stability of marine microbial phototroph–heterotroph interactions", *Nature Microbiology*, 2, 17100.

Cirés, S., Casero, M.C., Quesada, A. (2017) "Toxicity at the Edge of Life: A Review on Cyanobacterial Toxins from Extreme Environments.", *Marine Drugs*, 15 (7), E233.

Cole, J.J. (1982) "Interactions between bacteria and algae in aquatic ecosystems", *Annual Review of Ecology and Systematics*, 13, 291-314.

Cortes, J., Haydock, S.F., Roberts, G.A., Bevitt, D.J., Leadlay, P.F. (1990) "An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*", *Nature*, 348 (6297), 176-178.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G. (2005) “Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria”, *Nature*, 438 (7064), 90-93.

Crosa, J.H., Walsh, C.T. (2002) “Genetics and Assembly Line Enzymology of Siderophore Biosynthesis in Bacteria”, *Microbiology and Molecular Biology Reviews*, 66 (2), 223-249.

D'Onofrio, A., Crawford, J.M., Stewart, E.J., Witt, K., Gavrish, E., Epstein, S., Clardy, J., Lewis, K. (2010) “Siderophores from neighboring organisms promote the growth of uncultured bacteria”, *Chemistry & Biology*, 17 (3), 254-264.

De-Bashan, L.E., Hernandez, J.P., Morey, T., Bashan, Y. (2004) “Microalgae growth-promoting bacteria as “helpers” for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater”, *Water Research*, 38 (2), 466-474.

Desbois, A.P., Lebl, T., Yan, L., Smith, V.J. (2008) “Isolation and structural characterisation of two antibacterial free fatty acids from the marine diatom, *Phaeodactylum tricornutum*”, *Applied Microbiology and Biotechnology*, 81 (4), 755-764.

Dittmann, E., Gugger, M., Sivonen, K., Fewer, D.P. (2015) “Natural Product Biosynthetic Diversity and Comparative Genomics of the Cyanobacteria”, *Trends in Microbiology*, 23 (10), 642-652.

Dixon, R.A. (2001) “Natural products and plant disease resistance”, *Nature*, 411 (6839), 843-847.

Droop, M.R. (2007) “Vitamins, phytoplankton and bacteria: symbiosis or scavenging?”, *Journal of Plankton Research*, 29 (2), 107-113.

Durham, B.P., Sharma, S., Luo, H., Smith, C.B., Amin, S.A., Bender, S.J., Dearth, S.P., Van Mooy, B.A.S., Campagna, S.R., Kujawinski, E.B., Armbrust, E.V., Moran, M.A. (2015) “Cryptic carbon and sulfur cycling

between surface ocean plankton", *Proceedings of the National Academy of Sciences of the United States of America*, 112 (2), 453-457.

Edgar, R.C. (2013) "UPARSE: highly accurate OTU sequences from microbial amplicon reads", *Nature Methods*, 10, 996–998.

Edgar, R.C. (2016) "SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences", *bioRxiv,* doi: https://doi.org/10.1101/074161.

Edgar, R.C. (2017) "UNBIAS: An attempt to correct abundance bias in 16S sequencing, with limited success (preprint)", *bioRxiv*, doi:10.1101/124149.

Edgar, R.C., Flyvbjerg, H. (2015) "Error filtering, pair assembly and error correction for next-generation sequencing reads", *Bioinformatics,* 31 (21), 3476–3482.

Ehrenreich, I.M., Waterbury, J.B., Webb, E.A. (2005) "Distribution and diversity of natural product genes in marine and freshwater cyanobacterial cultures and genomes", *Applied and Environmental Microbiology*, 71 (11), 7401-7413.

Eilers, H., Pernthaler, J., Glockner, F.O., Amann, R. (2000) "Culturability and In Situ Abundance of Pelagic Bacteria from the North Sea", *Applied and Environmental Microbiology*, 66 (7), 3044-3051.

Fandino, L.B., Riemann, L., Steward, G.F., Long, R.A., Azam, F. (2001) "Variations in bacterial community structure during a dinoflagellate bloom analyzed by DGGE and 16S rDNA sequencing", *Aquatic Microbial Ecology*, 23, 119-130.

Fautin, D.G. (1991) "The Anemonefish Symbiosis: What is Known and What is Not", *Symbiosis*, 10, 23-46.

Ferrão-Filho, A.S., Kozlowski-Suzuki, B. (2011) "Cyanotoxins: Bioaccumulation and Effects on Aquatic Animals", *Marine Drugs*, 9 (12), 2729–2772.

Ferrero, E.M., de Godos, I., Rodríguez, E.M., García-Encina, P.A., Muñoz, R., Bécares, E. (2012) "Molecular characterization of bacterial communities

in algal–bacterial photobioreactors treating piggery wastewaters", *Ecological Engineering*, 40, 121-130.

Ferrier, M., Martin, J.L., Rooney-Varga, J.N. (2002) "Stimulation of *Alexandrium fundyense* growth by bacterial assemblages from the Bay of Fundy", *Journal of Applied Microbiology*, 92 (4), 706-716.

Field, C.B. (1998) "Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components", *Science*, 281 (5374), 237-240.

Findlay, J.A., Patil, A.D. (1984) "Antibacterial constituents of the diatom *Navicula delognei*", *Journal of Natural Products*, 47 (5), 815-818.

Fiore, C.L., Longnecker, K., Kido Soule, M.C., Kujawinski, E.B. (2015) "Release of ecologically relevant metabolites by the cyanobacterium *Synechococcus elongates* CCMP 1631", *Environmental Microbiology*, 17 (10), 3949-3963.

Flombaum, P., Gallegos, J.L., Gordillo, R.A., Rincon, J., Zabala, L.L., Jiao, N. (2013) "Present and future global distributions of the marine cyanobacteria *Prochlorococcus* and *Synechococcus*", *Proceedings of the National Academy of Sciences,* 110, 9824–9829.

Fuentes, J.L., Garbayo, I., Cuaresma, M., Montero, Z., Gonzalez-Del-Valle, M., Vilchez, C. (2016) "Impact of Microalgae-Bacteria Interactions on the Production of Algal Biomass and Associated Compounds", *Marine Drugs*, 14 (5).

Fuller, N.J., Tarran, G.A., Yallop, M., Orcutt, K.M., Scanlan, D.J. (2006) "Molecular analysis of picocyanobacterial community structure along an Arabian Sea transect reveals distinct spatial separation of lineages", *Limnology and Oceanogrpahy*, 51 (6), 2515-2526.

Fuller, N.J., Marie, D., Partensky, F., Vaulot, D., Post, A.F., Scanlan, D.J. (2003) "Clade-Specific 16S Ribosomal DNA Oligonucleotides Reveal the Predominance of a Single Marine Synechococcus Clade throughout a

Stratified Water Column in the Red Sea", *Applied and Environmental Microbiology*, 69 (5), 2430-2443.

Funa, N., Ohnishi, Y., Fujii, I., Shibuya, M., Ebizuka, Y., Horinouchi, S. (1999) "A new pathway for polyketide synthesis in microorganisms", *Nature*, 400 (6747), 897-899.

Furusawa, G., Yoshikawa, T., Yasuda, A., Sakata, T. (2003) "Algicidal activity and gliding motility of *Saprospira* sp. SS98-5", *Canadian Journal of Microbiology*, 49 (2), 92-100.

Gardes, A., Iversen, M.H., Grossart, H.P., Passow, U., Ullrich, M.S. (2011) "Diatom-associated bacteria are required for aggregation of *Thalassiosira weissflogii*", *International Society for Microbial Ecology Journal*, 5 (3), 436-445.

Geng, H., Belas, R. (2010) "Molecular mechanisms underlying *Roseobacter*-phytoplankton symbioses", *Current Opinion in Biotechnology*, 21 (3), 332-338.

Giovannoni, S.J., Stingl, U. (2005) "Molecular diversity and ecology of microbial plankton", *Nature*, 437 (7057), 343-348.

Gleason, F.K., Paulson, J.L. (1984) "Site of action of the natural algicide, cyanobacterin, in the blue-green alga, *Synechococcus* sp.", *Archives of Microbiology*, 138, 273-277.

Gonzalez, J.M., Simo, R., Massana, R., Covert, J.S., Casamayor, E.O., Pedros-Alio, C., Moran, M.A. (2000) "Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom", *Applied and Environmental Microbiology*, 66 (10), 4237-4246.

Gonzalez, J.M., Fernandez-Gomez, B., Fernandez-Guerra, A., Gomez-Consarnau, L., Sanchez, O., Coll-Llado, M., Del Campo, J., Escudero, L., Rodriguez-Martinez, R., Alonso-Saez, L., Latasa, M., Paulsen, I., Nedashkovskaya, O., Lekunberri, I., Pinhassi, J., Pedros-Alio, C. (2008) "Genome analysis of the proteorhodopsin-containing marine bacterium

Polaribacter sp. MED152 (Flavobacteria)", *Proceedings of the National Academy of Sciences of the United States of America*, 105 (25), 8724-8729.

Green, D.H., Echavarri-Bravo, V., Brennan, D., Hart, M.C. (2015) "Bacterial Diversity Associated with the Coccolithophorid Algae *Emiliania huxleyi* and *Coccolithus pelagicus f. braarudii*", *BioMed Research International*, 2015, 194540.

Green, D.H., Llewellyn, L.E., Negri, A.P., Blackburn, S.I., Bolch, C.J. (2004) "Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum"*, FEMS Microbiology Ecology, 47, 345–357.

Grossart, H.P. (1999) "Interactions between marine bacteria and axenic diatoms (*Cylindrothecafusiformis*, *Nitzschia laevis* and *Thalassiosira weissflogii*) incubated under various conditions in the lab", *Aquatic Microbial Ecology*, 19, 1-11.

Grossart, H.P., Czub, G., Simon, M. (2006) "Algae-bacteria interactions and their effects on aggregation and organic matter flux in the sea", *Environmental Microbiology*, 8 (6), 1074-1084.

Grossart, H.P., Levold, F., Allgaier, M., Simon, M., Brinkhoff, T. (2005) "Marine diatom species harbour distinct bacterial communities", *Environmental Microbiology*, 7 (6), 860-873.

Grossart, H.P., Simon, M. (2007) "Interactions of planktonic algae and bacteria: effects on algal growth and organic matter dynamics", *Aquatic Microbial Ecology*, 47, 163-176.

Guan, T.W., Xiao, J., Zhao, K., Luo, X.X., Zhang, X.P., Zhang, L.L. (2010) "*Halomonas xinjiangensis* sp. nov., a halotolerant bacterium isolated from a salt lake", *International Journal of Systematic and Evolutionary Microbiology*, 60 (2), 349-352.

Guannel, M.L., Horner-Devine, M.C., Rocap, G. (2011) “Bacterial community composition differs with species and toxigenicity of the diatom *Pseudo-nitzschia*”, *Aquatic Microbial Ecology*, 64 (2), 117-133.

Guerrini, F., Mazzotti, A., Boni, Pistocchi, R. (1998) “Bacterial-algal interactions in polysaccharide production”, *Aquatic Microbial Ecology*, 15, 247-253.

Haines, K.C., Guillard, R.R.L. (1974) “Growth of vitamin B_{12} requiring marine diatoms in mixed laboratory cultures with vitamin B_{12} producing bacteria”, Journal of Phycology, 10, 245–252.

Haynes, K., Hofmann, T.A., Smith, C.J., Ball, A.S., Underwood, G.J., Osborn, A.M. (2007) “Diatom-derived carbohydrates as factors affecting bacterial community composition in estuarine sediments”, *Applied and Environmental Microbiology*, 73 (19), 6112-6124.

Henley, W.J., Yin, Y., (1998) “Growth and photosynthesis of marine *Synechococcus* (*Cyanophyceae*) under iron stress”, *Journal of Phycology*, 34, 94–103.

Helliwell, K.E., Wheeler, G.L., Leptos, K.C., Goldstein, R.E., Smith, A.G. (2011) “Insights into the evolution of vitamin B_{12} auxotrophy from sequenced algal genomes”, *Molecular Biology and Evolution*, 28 (10), 2921-2933.

Hertweck, C. (2009) “The biosynthetic logic of polyketide diversity”, *Angewandte Chemie International Edition,* 48 (26), 4688-4716.

Hernandez, D., Riano, B., Coca, M., Garcia-Gonzalez, M.C. (2013) “Treatment of agro-industrial wastewater using microalgae-bacteria consortium combined with anaerobic digestion of the produced biomass”, *Bioresource Technology*, 135, 598-603.

Ho, C.S., Lam, C.W., Chan, M.H., Cheung, R.C., Law, L.K., Lit, L.C., Ng, K.F., Suen, M.W., Tai, H.L. (2003) “Electrospray ionisation mass spectrometry: principles and clinical applications”, *Clinical Biochemistry Reviews*, 24 (1), 3-12.

Hold, G.L., Smith, E.A., Birkbeck, T.H., Gallacher, S. (2001) “Comparison of paralytic shellfish toxin (PST) production by the dinoflagellates *Alexandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 in the presence and absence of bacteria”, *FEMS Microbiology Ecology*, 36 (2-3), 223-234.

Hosoya, S., Yokota, A. (2007) “*Loktanella atrilutea* sp. nov., isolated from seawater in Japan”, *International Journal of Systematic and Evolutionary Microbiology*, 57 (9), 1966-1969.

Huber, R., Langworthy, T.A., Konig, H., Thomm, M., Woese, C.R., Sleytr, U.B., Stetter, K.O. (1986) “*Thermotoga maritima*, sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C”, *Archives of Microbiology,* 144, 324–333

Hunken, M., Harder, J., Kirst, G.O. (2008) “Epiphytic bacteria on the Antarctic ice diatom *Amphiprora kufferathii* Manguin cleave hydrogen peroxide produced during algal photosynthesis”, *Plant Biology*, 10 (4), 519-526.

Huntemann, M., Teshima, H., Lapidus, A., Nolan, M., Lucas, S., Hammon, N., Deshpande, S., Cheng, J.F., Tapia, R., Goodwin, L.A. (2012) “Complete genome sequence of the facultatively anaerobic, appendaged bacterium *Muricauda ruestringensis* type strain (B1T)”, *Standards in Genomic Sciences,* 6, 185-193.

Hwang, C.Y., Kim, M.H., Bae, G.D., Zhang, G.I., Kim, Y.H., Cho, B.C. (2009) “*Muricauda olearia* sp. nov., isolated from crude-oil-contaminated seawater, and emended description of the genus *Muricauda*”, *International Journal of Systematic and Evolutionary Microbiology*, 59, 1856–1861.

Ito, Y., Butler, A. (2005) “Structure of synechobactins, new siderophores of the marine cyanobacterium *Synechococcus* sp. PCC 7002”, *Limnology and Oceanography*, 50 (6), 1918-1923.

Jang, G.I., Hwang, C.Y., Cho, B.C. (2011) “*Nitratireductor aquimarinus* sp. nov., isolated from a culture of the diatom *Skeletonema costatum*, and

emended description of the genus *Nitratireductor*", *International Journal of Systematic and Evolutionary Microbiology*, 61 (11), 2676-2681.

Jardillier, L., Zubkov, M.V., Pearman, J., Scanlan, D.J. (2010) "Significant CO_2 fixation by small prymnesiophytes in the subtropical and tropical northeast Atlantic Ocean", *The International Society for Microbial Ecology Journal*, 4 (9), 1180-1192.

Jasti, S., Sieracki, M.E., Poulton, N.J., Giewat, M.W., Rooney-Varga, J.N. (2005) "Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium* spp. and other phytoplankton", *Applied and Environmental Microbiology*, 71 (7), 3483-3494.

Jauzein, C., Evans, A., Erdner, D., (2015) "The impact of associated bacteria on morphology and physiology of the dinoflagellate *Alexandrium tamarense*", *Harmful Algae*, 50, 65-75.

Jenkins, G.I., Long, J.C., Wade, H.K., Shenton, M.R., Bibikova, T.N. (2001) "UV and blue light signalling: pathways regulating chalcone synthase gene expression in *Arabidopsis*", *New Phytologist*, 151, 121-131.

Jiang, Z., Swem, L.R., Rushing, B.G., Devanathan, S., Tollin, G., Bauer, C.E. (1999) "Bacterial photoreceptor with similarity to photoactive yellow protein and plant phytochromes", *Science* 285 (5426), 406-409.

Johnson, Z.I., Zinser, E.R., Coe, A., McNulty, N.P., Woodward, E.M.S., Chisholm, S.W. (2006) "Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients", *Science*, 311, 1737–1740.

Kaczmarska, I., Ehrman, J.M., Bates, S.S., Green, D.H., Léger, C., Harris, J. (2005) "Diversity and distribution of epibiotic bacteria on *Pseudo-nitzschia multiseries* (*Bacillariophyceae*) in culture, and comparison with those on diatoms in native seawater", *Harmful Algae*, 4 (4), 725-741.

Kang, H.S., Yang, H.L., Lee, S.D., (2009) "*Nitratireductor kimnyeongensis* sp nov., isolated from seaweed", *International Journal of Systematic and Evolutionary Microbiology* , 59, 1036–1039.

Kaniga, K., Delor, I., Cornelis, G.R. (1991) “A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the blaA gene of *Yersinia enterocolitica*”, *Gene*, 109, 137-141.

Kim, B.-H., Ramanan, R., Cho, D.-H., Oh, H.-M., Kim, H.-S. (2014) “Role of *Rhizobium*, a plant growth promoting bacterium, in enhancing algal biomass through mutualistic interaction”, *Biomass and Bioenergy*, 69, 95-105.

Kim, J.M., Jeong, S.-Y., Lee, S.-J. (2008) “Isolation, identification, and algicidal activity of marine bacteria against *Cochlodinium polykrikoides*”, *Journal of Applied Phycology*, 20 (6), 1069-1078.

Kim, J.M., Jin, H.M., Jeon, C.O. (2013) “*Muricauda taeanensis* sp. nov., isolated from a marine tidal flat”, *International Journal of Systematic and Evolutionary Microbiology,* 63 (7), 2672-2677.

Kim, K.H., Roh, S.W., Chang, H.W., Nam, Y.D., Yoon, J.H., Jeon, C.O., Oh, H.M., Bae, J.W., (2009) “*Nitratireductor basaltis* sp. nov., isolated from black beach sand”, *International Journal of Systematic and Evolutionary Microbiology* , 59, 135–138.

King, R., Bonfiglio, R., Fernandez, Metzler, C., Miller, Stein, C., Olah, T., (2000) “Mechanistic investigation of ionization suppression in electrospray ionization”, *Journal of the American Society for Mass Spectrometry*, 11, 942–950.

Klindworth, A., Pruesse, E., Schweer, T., Peplles, J., Quast, C., Horn, M., Glockner, F.O. (2013) “Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies”, *Nucleic Acids Research*, 41 (1), 1-11.

Kobayashi, K., Takata, Y., Kodama, M. (2009) “Direct contact between *Pseudo-nitzschia multiseries* and bacteria is necessary for the diatom to produce a high level of domoic acid”, *Fisheries Science*, 75 (3), 771-776.

Kobayashi, K., Shimogawa, H., Sakakura, A., Teruya, T., Suenaga, K., Kigoshi, H. (2004) “Spongiacysteine, a Novel Cysteine Derivative from Marine Sponge Spongia sp”, *Chemistry Letters*, 33 (10), 1262-1263.

Kovach, M.E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., Peterson, K. M. (1995) “Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes”, *Gene*, 166 (1), 175-176.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., Peterson, K.M. (1994) “pBBR1MCS: a broad-host-range cloning vector”, *Biotechniques*, 16 (5), 800-802

Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D.N., Hahlbrock, K. (1983) “UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*”, *Proceedings of the National Academy of Sciences of the United States of America*, 80 (9), 2591-2593.

Labbé, N., Juteau, P., Parent, S., Villemur, R., (2003) “Bacterial Diversity in a Marine Methanol-Fed Denitrification Reactor at the Montreal Biodome, Canada”, *Microbial Ecology,* 46 (1), 12-21.

Lage, O.M., Bondoso, J. (2012) “Bringing *Planctomycetes* into pure culture”, *Frontiers in Microbiology*, 3, 405.

Lai Q., Wang L., Liu Y., Yuan J., Sun F., Shao Z. (2011a). “*Parvibaculum indicum* sp. nov., isolated from deep-sea water”, *International Journal of Systematic and Evolutionary Microbiology*, 61 (6), 271–274.

Lai Q., Yu Z., Yuan J., Sun F., Shao Z. (2011b). “*Nitratireductor indicus* sp. nov., isolated from deep-sea water”, *International Journal of Systematic and Evolutionary Microbiology*, 61 (6), 295–298.

Lau, N.S., Matsui, M., Abdullah, A.A. (2015) “Cyanobacteria: Photoautotrophic Microbial Factories for the Sustainable Synthesis of Industrial Products”, *BioMed Research International*, 2015, 754934.

Lavin, P., Gomez, P., Gonzalez, B., Ulloa, O. (2008) "*Synechococcus* assessed by terminal restriction fragment length polymorphisms of 16S-23S rRNA internal transcribed spacer sequences", *Revista Chilena de Historia Natural*, 81, 515-531.

Leao, P.N., Engene, N., Antunes, A., Gerwick, W.H., Vasconcelos, V. (2012) "The chemical ecology of cyanobacteria", *Natural Product Reports*, 29 (3), 372-391.

Leao, P.N., Costa, M., Ramos, V., Pereira, A.R., Fernandes, V.C., Domingues, V.F., Gerwick, W.H., Vasconcelos, V.M., Martins, R. (2013) "Antitumor activity of hierridin B, a cyanobacterial secondary metabolite found in both filamentous and unicellular marine strains", *PLoS One*, 8 (7), e69562.

Lee, J.J. (2006) "Algal symbiosis in larger foraminifera", *Symbiosis*, 42, 63-75.

Lee, S.O., Kato, J., Takiguchi, N., Kuroda, A., Ikeda, T., Mitsutani, A., Ohtake, H. (2000) "Involvement of an extracellular protease in algicidal activity of the marine bacterium *Pseudoalteromonas* sp. strain A28", *Applied and Environmental Microbiology*, 66 (10), 4334-4339.

Lee, S.Y., Park, S., Oh, T.K., Yoon, J.H., (2012) "*Muricauda beolgyonensis* sp. nov., isolated from a tidal flat*", International Journal of Systematic and Evolutionary Microbiology*, 62 (5), 1134-1139.

Leikoski, N., Liu, L., Jokela, J., Wahlsten, M., Gugger, M., Calteau, A., Permi, P., Kerfeld, C.A., Sivonen, K., Fewer, D.P. (2013) "Genome mining expands the chemical diversity of the cyanobactin family to include highly modified linear peptides", *Chemistry & Biology*, 20 (8), 1033-1043.

Letscher, R.T., Knapp, A.N., James, A.K., Carlson, C.A., Santoro, A.E., Hansell, D.A. (2015) "Microbial community composition and nitrogen availability influence DOC remineralization in the South Pacific Gyre", *Marine Chemistry*, 177, 325-334.

Li, B., Sher, D., Kelly, L., Shi, Y., Huang, K., Knerr, P.J., Joewono, I., Rusch, D., Chisholm, S.W., van der Donk, W.A. (2010) “Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria”, *Proceedings of the National Academy of Sciences of the United States of America*, 107 (23), 10430-10435.

Li, Y., Muller, R., (2009) “Non-modular polyketide synthases in myxobacteria”, *Phytochemistry,* 13 (15–16), 1850–1857.

Lim, G.E., Haygood, M.G. (2004) ““*Candidatus* Endobugula glebosa”, a specific bacterial symbiont of the marine bryozoan *Bugula simplex*”, *Applied and Environmental Microbiology*, 70 (8), 4921-4929.

Lipski, A., Kampfer, P. (2012) “*Aquamicrobium ahrensii* sp. nov. and *Aquamicrobium segne* sp. nov., isolated from experimental biofilters”, *International Journal of Systematic and Evolutionary Microbiology*, 62 (10), 2511-2516.

Livak, K.J., Schmittgen, T.D. (2001) “Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method”, *Methods*, 25, 402-408.

Llewellyn, C.A., White, D.A., Martinez-Vincente, V., Tarran, G., Smyth, T.J. (2012) “Distribution of mycosporine-like amino acids along a surface water meridional transect of the Atlantic”, *Micobiology of Aquatic Systems*, 64, 320-333.

Long, R.A., Azam, F. (2001) “Antagonistic interactions among marine pelagic bacteria”, *Applied and Environmental Microbiology*, 67 (11), 4975-4983.

Lubarsky, H.V., Hubas, C., Chocholek, M., Larson, F., Manz, W., Paterson, D.M., Gerbersdorf, S.U. (2010) “The stabilisation potential of individual and mixed assemblages of natural bacteria and microalgae”, *PLoS One*, 5 (11), e13794.

Lupette, J., Lami, R., Krasovec, M., Grimsley, N., Moreau, H., Piganeau, G., Sanchez-Ferandin, S. (2016) “Marinobacter Dominates the Bacterial Community of the *Ostreococcus tauri* Phycosphere in Culture”, *Frontiers in Microbiology*, 7, 1414.

Maldonado, M.T., Hughes, M.P., Rue, E.L., Wells, M.L. (2002) “The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*”, *Limnology and Oceanography*, 47 (2), 515-526.

Malfatti, F., Azam, F. (2009) “Atomic force microscopy reveals microscale networks and possible symbioses among pelagic marine bacteria”, *Aquatic Microbial Ecology*, 58, 1-14.

Malfatti, F., Samo, T.J., Azam, F. (2010) “High-resolution imaging of pelagic bacteria by Atomic Force Microscopy and implications for carbon cycling”, *The International Society for Microbial Ecology Journal*, 4 (3), 427-439.

Martin, A.P., Zubkov, M.V., Burkill, P.H., Holland, R.J. (2005) “Extreme spatial variability in marine picoplankton and its consequences for interpreting Eulerian time-series”, *Biology Letters*, 1, 366–369.

Martin, C.R. (1993) “Structure, function and regulation of the chalcone synthase”, *International Review of Cytology*, 147, 233-284.

Martins, R., Fernandez, N., Beiras, R., Vasconcelos, V. (2007) “Toxicity assessment of crude and partially purified extracts of marine *Synechocystis* and *Synechococcus* cyanobacterial strains in marine invertebrates”, *Toxicon*, 50 (6), 791-799.

Martins, R.F., Ramos, M.F., Herfindal, L., Sousa, J.A., Skaerven, K., Vasconcelos, V.M. (2008) “Antimicrobial and cytotoxic assessment of marine cyanobacteria – *Synechocystis* and *Synechococcus*”, *Marine Drugs*, 6 (1), 1-11.

Mas, A., Jamshidi, S., Lagadeuc, Y., Eveillard, D., Vandenkoornhuyse, P. (2016) “Beyond the Black Queen Hypothesis”, *The International Society for Microbial Ecology Journal*, 10, 2085-2091.

Mayali, X., Franks, P.J.S., Azam, F. (2007) “Bacterial induction of temporary cyst formation by the dinoflagellate *Lingulodinium polyedrum*”, *Aquatic Microbial Ecology*, 50, 51-62.

Mayali, X., Franks, P.J., Tanaka, Y., Azam, F. (2008) “Bacteria-Induced Motility Reduction in *Lingulodinium polyedrum* (*Dinophyceae*)”, *Journal of Phycology*, 44 (4), 923-928.

Mayali, X., Azam, F. (2004) “Algicidal Bacteria in the Sea and their Impact on Algal Blooms”, *Journal of Eukaryotic Microbiology*, 51 (2), 139-144.

Mazard, S., Ostrowski, M., Partensky, F., Scanlan, D.J. (2012) “Multi-locus sequence analysis, taxonomic resolution and biogeography of marine *Synechococcus*”, *Environmental Microbiology*, 14 (2), 372-386.

Mazur-Marzec, H., Błaszczyk, A., Felczykowska, A., Hohlfeld, N., Kobos, J., Toruńska-Sitarz, A., Devi, P., Montalvão, S., D’souza, L., Tammela, P., Mikosik, A., Bloch, S., Nejman-Faleńczyk, B., Węgrzyn, G. (2015) “Baltic cyanobacteria – a source of biologically active compounds”, *European Journal of Phycology*, 50 (3), 343-360.

McCarren, J., Becker, J.W., Repeta, D.J., Shi, Y., Young, C.R., Malmstrom, R.R., Chisholm, S.W., DeLong, E.F. (2010) “Microbial community transcriptomes reveal microbes and metabolic pathways associated with dissolved organic matter turnover in the sea”, *Proceedings of the National Academy of Sciences of the United States of America*, 107 (38), 16420-16427.

McLean, B.M., Baskaran, K., Connor, M.A. (2000) The use of algal-bacterial biofilms to enhance nitrification rates in lagoons: experience under laboratory and pilot-scale conditions, *Water Science & Technology*, 42 (10-11), 187-194.

Mendez-Perez, D., Begemann, M.B., Pfleger, B.F. (2011) “Modular synthase-encoding gene involved in alpha-olefin biosynthesis in *Synechococcus* sp. strain PCC 7002”, *Applied and Environmental Microbiology*, 77 (12), 4264-4267.

Micallef, M.L., D'Agostino, P.M., Al-Sinawi, B., Neilan, B.A., Moffitt, M.C. (2015) “Exploring cyanobacterial genomes for natural product biosynthesis pathways”, *Marine Genomics*, 21, 1-12.

Miller, V.L., Mekalanos, J.J. (1988) “A Novel Suicide Vector and Its Use in Construction of Insertion Mutations: Osmoregulation of Outer Membrane Proteins and Virulence Determinants in *Vibrio cholerae* Requires *toxR*”, *Journal of Bacteriology*, 170 (6), 2575-2583.

Moore, L.R., Chisholm, S.W. (1999) “Photophysiology of the marine cyanobacterium *Prochlorococcus*: ecotypic differences among cultured isolates”, *Limnology & Oceanography*, 44, 628–638.

Moore, L.R., Post, A.F., Rocap, G., Chisholm, S.W., (2002) “Utilization of different nitrogen sources by the marine cyanobacteria, *Prochlorococcus* and *Synechococcus*”, *Limnology and Oceanography,* 47 (4), 989-966.

Moore, B.S., Hopke, J.N. (2001) “Discovery of a new bacterial polyketide biosynthetic pathway”, *ChemBioChem*, 2 (1), 35-38.

Moran, M.A., Belas, R., Schell, M.A., Gonzalez, J.M., Sun, F., Sun, S., Binder, B.J., Edmonds, J., Ye, W., Orcutt, B., Howard, E.C., Meile, C., Palefsky, W., Goesmann, A., Ren, Q., Paulsen, I., Ulrich, L.E., Thompson, L.S., Saunders, E., Buchan, A. (2007) “Ecological genomics of marine *Roseobacters*”, *Applied and Environmental Microbiology*, 73 (14), 4559-4569.

Moran, M.A., Buchan, A., Gonzalez, J.M., Heidelberg, J.F., Whitman, W.B., Kiene, R.P., Henriksen, J.R., King, G.M., Belas, R., Fuqua, C., Brinkac, L., Lewis, M., Johri, S., Weaver, B., Pai, G., Eisen, J.A., Rahe, E., Sheldon, W.M., Ye, W., Miller, T.R., Carlton, J., Rasko, D.A., Paulsen, I.T., Ren, Q., Daugherty, S.C., Deboy, R.T., Dodson, R.J., Durkin, A.S., Madupu, R.,

Nelson, W.C., Sullivan, S.A., Rosovitz, M.J., Haft, D.H., Selengut, J., Ward, N. (2004) “Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment”, *Nature*, 432 (7019), 910-913.

Morris, J.J., Lenski, R.E., Zinser, E.R. (2012) “The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss”, *mBio*, 3 (2), 1-7.

Morris, J.J., Kirkegaard, R., Szul, M.J., Johnson, Z.I., Zinser, E.R. (2008) “Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by “helper” heterotrophic bacteria”, *Applied and Environmental Microbiology*, 74 (14), 4530-4534.

Morris, J.J., Johnson, Z.I., Szul, M.J., Keller, M., Zinser, E.R. (2011) “Dependence of the cyanobacterium *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface”, *PLoS One*, 6 (2), e16805.

Moustafa, A., Evans, A.N., Kulis, D.M., Hackett, J.D., Erdner, D.L., Anderson, D.M., Bhattacharya, D. (2010) “Transcriptome profiling of a toxic dinoflagellate reveals a gene-rich protist and a potential impact on gene expression due to bacterial presence”, *PLoS One*, 5 (3), e9688.

Nakagawa, S., Takai, K., (2008) “Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance”, *FEMS Microbiology Ecology*, 65, 1–14.

Natrah, F.M.I., Bossier, P., Sorgeloos, P., Yusoff, F.M., Defoirdt, T. (2014) “Significance of microalgal-bacterial interactions for aquaculture”, *Reviews in Aquaculture*, 6 (1), 48-61.

Natrah, F.M.I., Kenmegne, M.M., Wiyoto, W., Sorgeloos, P., Bossier, P., Defoirdt, T. (2011) “Effects of micro-algae commonly used in aquaculture on acyl-homoserine lactone quorum sensing”, *Aquaculture*, 317 (1-4), 53-57.

Ni, J., Tao, F., Wang, Y., Yao, F., Xu, P. (2016) “A photoautotrophic platform for the sustainable production of valuable plant natural products from CO_2”, *Green Chemistry*, 18 (12), 3537-3548.

Osada, M., Stewart, J.E. (1997) “Gluconic acid/gluconolactone: physiological influences on domoic acid production by bacteria associated with *Pseudo-nitzschia multiseries*”, *Aquatic Microbial Ecology*, 12, 203-209.

Paerl, H.W., Otten, T.G., Joyner, A.R., (2016) “Moving towards adaptive management of cyanotoxin-impaired water bodies”, *Microbial Biotechnology*, 9 (5), 641-651.

Palenik, B., Ren, Q., Dupont, C.L., Myers, G.S., Heidelberg, J.F., Badger, J.H., Madupu, R., Nelson, W.C., Brinkac, L.M., Dodson, R.J., Durkin, A.S., Daugherty, S.C., Sullivan, S.A., Khouri, H., Mohamoud, Y., Halpin, R. and Paulsen, I.T. (2006) “Genome sequence of *Synechococcus* CC9311: Insights into adaptation to a coastal environment”, *Proceedings of the National Academy of Sciences of the United States of America*, 103, 13555-13559.

Park, S., Park, J.M., Jung, Y.T., Won, S.M., Yoon, J.H. (2015) “*Primorskyibacter insulae* sp. nov., isolated from the junction between the ocean and a freshwater spring”, *International Journal of Systematic and Evolutionary Microbiology*, 65 (11), 3971-3976.

Partensky, F., Hess, W.R., Vaulot, D. (1999) “*Prochlorococcus*, a marine photosynthetic prokaryote of global significance”, *Microbiology and Molecular Biology Reviews*, 63 (1), 106-127.

Paul, C., Pohnert, G. (2011) “Interactions of the algicidal bacterium *Kordia algicida* with diatoms: regulated protease excretion for specific algal lysis”, *PLoS One*, 6 (6), e21032.

Paulsen, M.L., Dore, H., Garczarek, L., Seuthe, L., Muller, O., Sandaa, R-A., Bratbak, G., Larsen, A. (2016) “*Synechococcus* in the Atlantic Gateway to the Arctic Ocean”, Frontiers in Marine Science, 3, 1-14.

Paz-Yepes, J., Brahamsha, B., Palenik, B. (2013) “Role of a microcin-C-like biosynthetic gene cluster in allelopathic interactions in marine *Synechococcus*”, *Proceedings of the National Academy of Sciences of the United States of America*, 110 (29), 12030-12035.

Peng, J., Shen, X., El Sayed, K.A., Dunbar, D.C., Perry, T.L., Wilkins, S.P., Hamann, M.T., Bobzin, S., Huesing, J., Camp, R., Prinsen, M., Krupa, D., Wideman, M.A. (2003) “Marine Natural Products as Prototype Agrochemical Agents”, *Journal of Agricultural and Food Chemistry*, 51, 2246-2252.

Prokic, I., Brümmer, F., Brigge, T., Görtz, H.D., Gerdts, G., Schütt, C., Elbrächter, M., Müller, W.E.G., (1998) “Bacteria of the genus *Roseobacter* associated with the toxic dinoflagellate *Prorocentrum lima*”, *Protist,* 149, 347–357.

Ramanan, R., Kim, B.H., Cho, D.H., Oh, H.M., Kim, H.S. (2016) “Algae-bacteria interactions: Evolution, ecology and emerging applications”, *Biotechnology Advances*, 34 (1), 14-29.

Ribalet, F., Intertaglia, L., Lebaron, P., Casotti, R. (2008) “Differential effect of three polyunsaturated aldehydes on marine bacterial isolates”, *Aquatic Toxicology*, 86 (2), 249-255.

Risgaard-Petersen, N., Nicolaisen, M.H., Revsbech, N.P., Lomstein, B.A. (2004) “Competition between ammonia-oxidizing bacteria and benthic microalgae”, *Applied and Environmental Microbiology*, 70 (9), 5528-5537.

Roeselers, G., van Loosdrecht, M.C., Muyzer, G. (2007) “Heterotrophic pioneers facilitate phototrophic biofilm development”, *Microbial Ecology*, 54 (3), 578-585.

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., (2016) “VSEARCH: a versatile open source tool for metagenomics”, *PeerJ Preprints*, 4:e2409v1.

Romanenko, L.A., Tanaka, N., Svetashev, V.I., Kalinovskaya, N.I., (2012) “*Poseidonocella pacifica* gen. nov., sp. nov. and *Poseidonocella sedimentorum* sp. nov., novel alphaproteobacteria from the shallow sandy sediments of the Sea of Japan.”, *Archives of Microbiology*,194 (2), 113-121.

Roth, P.B., Twiner, M.J., Mikulski, C.M., Barnhorst, A.B., Doucette, G.J. (2008) “Comparative analysis of two algicidal bacteria active against the red tide dinoflagellate *Karenia brevis*”, *Harmful Algae*, 7 (5), 682-691.

Rowan, R. (1998) “Diversity and Ecology of *Zooxanthellae* on Coral Reefs”, *Journal of Phycology*, 34 (3), 407-417.

Rue, E., Bruland, K. (2001) “Domoic acid binds iron and copper: a possible role for the toxin produced by the marine diatom *Pseudo-nitzschia*”, *Marine Chemistry*, 76, 127-134.

Saito, M. A., Rocap, G., Moffett, J. W. (2005) “Production of cobalt binding ligands in a *Synechococcus* feature at the Costa Rica upwelling dome”, *Limnology and Oceanography,* 50, 279–290.

Santos, C.A., Reis, A. (2014) “Microalgal symbiosis in biotechnology”, *Applied Microbiology and Biotechnology*, 98 (13), 5839-5846.

Sapp, M., Schwaderer, A.S., Wiltshire, K.H., Hoppe, H.G., Gerdts, G., Wichels, A. (2007) “Species-specific bacterial communities in the phycosphere of microalgae?”, *Microbial Ecology*, 53 (4), 683-699.

Scanlan, D.J., Ostrowski, M., Mazard, S., Dufresne, A., Garczarek, L., Hess, W.R., Post, A.F., Hagemann, M., Paulsen, I., Partensky, F. (2009) “Ecological genomics of marine picocyanobacteria”, *Microbiology and Molecular Biology Reviews*, 73 (2), 249-299.

Schafer, H., Abbas, B., Witte, H., Muyzer, G., (2002) “Genetic diversity of ‘satellite’ bacteria present in cultures of marine diatoms”, FEMS Microbiology Ecology, 42, 25–35.

Schlesner, H., (1994) “The development of media suitable for the microorganisms morphologically resembling *Planctomyces* spp., *Pirellula* spp., and other *Planctomycetales* from various aquatic habitats using dilute media”, *Systematic and Applied Microbiology,* 17, 135-145.

Schmidt, J.R., Wilhelm, S.W., Boyer, G.L. (2014) “The fate of microcystins in the environment and challenges for monitoring”, *Toxins,* 6, 3354–3387.

Schroder, J. (1997) “A family of plant-specific polyketide synthases: facts and predictions”, *Trends in Plant Science*, 2 (10), 373-378.

Segev, E., Wyche, T.P., Kim, K.H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N., Paulson, J.N., Chai, L., Clardy, J., Kolter, R. (2016) "Dynamic metabolic exchange governs a marine algal-bacterial interaction", *eLife*, 5.

Seyedsayamdost, M.R., Carr, G., Kolter, R., Clardy, J. (2011a) "Roseobacticides: small molecule modulators of an algal-bacterial symbiosis", *Journal of American Chemical Society*, 133 (45), 18343-18349.

Seyedsayamdost, M.R., Case, R.J., Kolter, R., Clardy, J. (2011b) "The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*", *Nature Chemistry*, 3 (4), 331-335.

Sher, D., Thompson, J.W., Kashtan, N., Croal, L., Chisholm, S.W. (2011) "Response of *Prochlorococcus* ecotypes to co-culture with diverse marine bacteria", *The International Society for Microbial Ecology Journal*, 5 (7), 1125-1132.

Shih, P.M., Wu, D., Latifi, A., Axen, S.D., Fewer, D.P., Talla, E., Calteau, A., Cai, F., Tandeau de Marsac, N., Rippka, R., Herdman, M., Sivonen, K., Coursin, T., Laurent, T., Goodwin, L., Nolan, M., Davenport, K.W., Han, C.S., Rubin, E.M., Eisen, J.A., Woyke, T., Gugger, M., Kerfeld, C.A. (2013) "Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing", *Proceedings of the National Academy of Sciences of the United States of America*, 110 (3), 1053-1058.

Simon, R., Priefer, U., Puhler, A. (1983) "A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria", *Nature Biotechnology*, 1, 784-791.

Sivonen, K., Leikoski, N., Fewer, D.P., Jokela, J. (2010) "Cyanobactins-ribosomal cyclic peptides produced by cyanobacteria", *Applied Microbiology and Biotechnology*, 86 (5), 1213-1225.

Skerratt, J., Bowman, J., Hallegraeff, G., James, S., Nichols, P. (2002) "Algicidal bacteria associated with blooms of a toxic dinoflagellate in a temperate Australian estuary", *Marine Ecology Progress Series*, 244, 1-15.

Smith, D.C., Steward, G.F., Long, R.A., Azam, F. (1995) “Bacterial mediation of carbon fluxes during a diatom bloom in a mesocosm”, *Deep Sea Research II*, 42 (1), 75-97.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M., Herndl, G.J., (2006) “Microbial diversity in the deep sea and the underexplored ‘rare biosphere’”, *Proceedings of the National Academy of Sciences of the United States of America,* 103, 115–120.

Sohm, J.A., Ahlgren, N.A., Thomson, Z.J., Williams, C., Moffett, J.W., Saito, M.A., Webb, E.A., Rocap, G. (2016) “Co-occuring *Synechococcus* ecotypes occupy four major oceanic regimes defined by temperature, macronutrients and iron”, *The International Society for Microbial Ecology Journal*, 10, 333-345.

Strom, S.L. (2008) “Microbial ecology of ocean biogeochemistry: a community perspective”, *Science*, 320 (5879), 1043-1045.

Su, J.Q., Yang, X.R., Zheng, T.L., Tian, Y., Jiao, N.Z., Cai, L.Z., Hong, H.S. (2007) “Isolation and characterization of a marine algicidal bacterium against the toxic dinoflagellate *Alexandrium tamarense*”, *Harmful Algae*, 6 (6), 799-810.

Subashchandrabose, S.R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., Naidu, R. (2011) “Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential”, *Biotechnology Advances*, 29 (6), 896-907.

Swain, S.S., Paidesetty, S.K., Padhy, R.N. (2017) “Antibacterial, antifungal and antimycobacterial compounds from cyanobacteria”, *Biomedicine & Pharmacotherapy*, 90, 760-776.

Tai, V., Paulsen, I.T., Phillippy, K., Johnson, D.A., Palenik, B. (2009) “Whole-genome microarray analyses of *Synechococcus-Vibrio* interactions”, *Environmental Microbiology*, 11 (10), 2698-2709.

Tan, L.T. (2007) “Bioactive natural products from marine cyanobacteria for drug discovery”, *Phytochemistry*, 68 (7), 954-979.

Tan, L.T. (2013) “Pharmaceutical agents from filamentous marine cyanobacteria”, *Drug Discovery Today*, 18 (17-18), 863-871.

Tang, W., van der Donk, W.A. (2012) “Structural characterization of four prochlorosins: a novel class of lantipeptides produced by planktonic marine cyanobacteria”, *Biochemistry*, 51 (21), 4271-4279.

Tang, Y.Z., Koch, F., Gobler, C.J. (2010) “Most harmful algal bloom species are vitamin B1 and B12 auxotrophs”, *Proceedings of the National Academy of Sciences of the United States of America*, 107 (48), 20756-20761.

Thingstad, T., Skjoldal, E., & Bohne, R. (1993) Phosphorus cycling and algal-bacterial competition in Sandsfjord, western Norway, *Marine Ecology Progress Series*, 99 (3), 239-259.

Tillett, D., Dittmann, E., Erhard, M., von Dohren, H., Borner, T., Neilan, B.A. (2000) “Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system”, *Chemistry & Biology*, 7 (10), 753-764.

Tripp, H.J., Kitner, J.B., Schwalbach, M.S., Dacey, J.W., Wilhelm, L.J., Giovannoni, S.J. (2008) “SAR11 marine bacteria require exogenous reduced sulphur for growth”, *Nature*, 452 (7188), 741-744.

Unson, M.D., Holland, N.D., Faulkner, D.J. (1994) “A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the spongetissue”, *Marine Biology*, 119, 1-11.

Uribe, P., Espejo, R.T. (2003) “Effect of Associated Bacteria on the Growth and Toxicity of *Alexandrium catenella*”, *Applied and Environmental Microbiology*, 69 (1), 659-662.

van der Ha, D., Nachtergaele, L., Kerckhof, F.M., Rameiyanti, D., Bossier, P., Verstraete, W., Boon, N. (2012) “Conversion of biogas to bioproducts by

algae and methane oxidizing bacteria", *Environmental Science and Technology*, 46 (24), 13425-13431.

Vijayakumar, S., Menakha, M. (2015) "Pharmaceutical applications of cyanobacteria—A review", *Journal of Acute Medicine*, 5 (1), 15-23.

Wagner-Dobler, I., Biebl, H. (2006) "Environmental biology of the marine *Roseobacter* lineage", *Annual Review of Microbiology*, 60, 255-280.

Wang, H., Fewer, D.P., Sivonen, K. (2011) "Genome mining demonstrates the widespread occurrence of gene clusters encoding bacteriocins in cyanobacteria", *PLoS One*, 6 (7), e22384.

Wang, H., Hill, R.T., Zheng, T., Hu, X., Wang, B. (2016) "Effects of bacterial communities on biofuel-producing microalgae: stimulation, inhibition and harvesting", *Critical Reviews in Biotechnology*, 36 (2), 341-352.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., (2007) "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy", *Applied and Environmental Microbiology*, 73, 5261–5267.

Wang, R., Gallant, E., Seyedsayamdost, M.R. (2016b) "Investigation of the Genetics and Biochemistry of Roseobacticide Production in the *Roseobacter* Clade Bacterium *Phaeobacter inhibens*", *MBio*, 7 (2), e02118.

Wang, X., Li, Z., Su, J., Tian, Y., Ning, X., Hong, H., Zheng, T. (2010) "Lysis of a red-tide causing alga, *Alexandrium tamarense*, caused by bacteria from its phycosphere", *Biological Control*, 52 (2), 123-130.

Wang, Y., Tang, S.K., Lou, K., Mao, P.H., Jin, X., Jiang, C.L., Xu, L.H., Li, W.J. (2008) "*Halomonas lutea* sp. nov., a moderately halophilic bacterium isolated from a salt lake", *International Journal of Systematic and Evolutionary Microbiology*, 58 (9), 2065-2069.

Wells., M.L., Trick, C.G., Cochlan, W.P., Hughes, M.P., Trainer, V.L. (2005) "Domoic acid: The synergy of iron, copper, and the toxicity of diatoms", *Limnology and Oceanography*, 50 (6), 1908-1917.

Whitman, W.B., Coleman, D.C., Wiebe, W.J., (1998) “Prokaryotes: The unseen majority”, *Proceedings of the National Academy of Sciences*, 95, 6578–6583.

Wichard, T., Gerecht, A., Boersma, M., Poulet, S.A., Wiltshire, K., Pohnert, G. (2007) “Lipid and fatty acid composition of diatoms revisited: rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success”, *ChemBioChem*, 8 (10), 1146-1153.

Willey, J.M., van der Donk, W.A. (2007) “Lantibiotics: peptides of diverse structure and function”, *Annual Review of Microbiology*, 61, 477-501.

Windler, M., Bova, D., Kryvenda, A., Straile, D., Gruber, A., Kroth, P.G. (2014) “Influence of bacteria on cell size development and morphology of cultivated diatoms”, *Phycological Research*, 62 (4), 269-281.

Wu, Y., Yu, P., Zhou, Y., Xu, L., Wang, C., Wu, M., (2013) “*Muricauda antarctica* sp. nov., a marine member of the *Flavobacteriaceae* isolated from Antarctic seawater”, *International Journal of Systematic and Evolutionary Microbiology*, 63, 3451–3456.

Wu, Z., Wang, F., Gu, C., Zhang, Y., Yang, Z., Wu, X., Jiang, X., (2014) “*Aquamicrobium terrae* sp. nov., isolated from the polluted soil near a chemical factory”, *Antonie van Leeuwenhoek*, 105 (6), 1131–1137.

Wyman, M., Gregory, R.P.F., Carr, N.G., (1985) “Novel role for phycoerythrin in a marine cyanobacterium, *Synechococcus* strain DC2”, *Science*, 230, 818–820.

Yang, C., Li, Y., Guo, Q., Lai, Q., Wei, J., Zheng, T., Tian, Y., (2013) “*Muricauda zhangzhouensis* sp. nov., isolated from mangrove sediment.”, *International Journal of Systematic and Evolutionary Microbiology*, 63 (6), 2320-2325.

Yi, H., Lim, Y.W., Chun, J. (2007) “Taxonomic evaluation of the genera *Ruegeria* and *Silicibacter*: a proposal to transfer the genus *Silicibacter* Petursdottir and Kristjansson 1999 to the genus *Ruegeria* Uchino et al.

1999", *International Journal of Systematic and Evolutionary Microbiology*, 57 (4), 815-819.

Yoon, J.H., Kang, S.J., Jung,Y.T., Oh, T.K., (2008) "*Muricauda lutimaris* sp. nov., isolated from a tidal flat of the Yellow Sea", *International Journal of Systematic and Evolutionary Microbiology*, 58 (7), 1603-1607.

Yu, D., Xu, F., Zeng, J., Zhan, J. (2012) "Type III polyketide synthases in natural product biosynthesis", *IUBMB Life*, 64 (4), 285-295.

Yu, F., Zaleta-Rivera, K., Zhu, X., Huffman, J., Millet, J.C., Harris, S.D., Yuen, G., Li, X.C., Du, L. (2007) "Structure and biosynthesis of heat-stable antifungal factor (HSAF), a broad-spectrum antimycotic with a novel mode of action", *Antimicrobial Agents and Chemotherapy*, 51 (1), 64-72.

Zacharof, M.P., Lovitt, R.W. (2012) "Bacteriocins produced by lactid acid bacteria", *APCBEE Procedia*, 2, 50-56.

Zhang, Z., Gao, X., Qiao, Y., Wang, Y., Zhang, X.H., (2015) "*Muricauda pacifica* sp. nov., isolated from seawater of the South Pacific Gyre", *International Journal of Systematic and Evolutionary Microbiology*, 65 (11), 4087-4092.

Zhu, L., Zancarini, A., Louati, I., De Cesare, S., Duval, C., Tambosco, K., Bernard, C., Debroas, D., Song, L., Leloup, J., Humbert, J.F., (2016) "Bacterial communities associated with four cyanobacterial genera display structural and functional differences: evidence from an experimental approach" Frontiers in Microbiology, 7, 1–11.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., Burkill, P.H. (2001) "Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea", *Environmental Microbiology*, 3 (5), 304-311.

Zwirglmaier, K., Jardillier, L., Ostrowski, M., Mazard, S., Garczarek, L., Vaulot, D., Not, F., Massana, R., Ulloa, O. and Scanlan, D.J. (2008) "Global phylogeography of marine *Synechococcus* and *Prochlorococcus* reveals a

distinct partitioning of lineages among oceanic biomes", *Environmental Microbiology*, 10, 147–161.