Dynamics of the bacterial community associated with *Phaeodactylum tricornutum* cultures:

a novel approach to scaling up microalgal cultures

A PhD thesis by:

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Dynamics of the bacterial community associated with *Phaeodactylum tricornutum* cultures: a novel approach to scaling up microalgal cultures

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presented by

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Date of the oral examination:

For my family: past, present and future

Voor mijn familie: verleden, heden en toekomst

Kwa familia yangu : kutoka zamani, sasa na katika siku zijazo

Kũrĩ andũ aitũ: aria matarĩ ho, aria me ho, na aria magakorũo ho

"Everything you see exists together in a delicate balance."

– *The Lion King*

Walt Disney Pictures, 1994

Foreword

The research carried out in this PhD thesis was one of fourteen PhD Early Stage Research projects that form AccliPhot, a Marie Curie Initial Network project funded by the European Commission (grant agreement number PITN-GA-2012-316427). The aim of **AccliPhot** is to investigate and understand short-term **Accli**mation mechanisms to changes in light conditions in **Phot**osynthetic organisms. The acclimation processes are studied on various scales using both experimental and theoretical methods. The investigated scales encompass the molecular signalling mechanisms inducing the responses, the implications for metabolism, and whole-organism behaviour, with emphasis on particular growth and biomass yield. Our aim is to employ this understanding to optimise and upscale biotechnological exploitation of photosynthetic microalgae for the production of biofuels and high-value commodities.

The contribution of this PhD research project to the overall aims of AccliPhot was the feasibility of large-scale cultures at a pilot plant and help towards the systematic understanding of microalgal culture dynamics at different scales providing us with important data for model development by other partners. The project aims to evaluate the efficiency of *Phaeodactylum tricornutum* for biofuel production in large-scale industrial outdoor bioreactors and assess the extent to which the models developed for controlled laboratory conditions are applicable to outdoor, industry-scale bioreactors.

Majority of the research was carried out at Daithi O'Murchu Marine Research Station (DOMMRS) in Bantry in the west of County Cork, Ireland.

DOMMRS has been in operation since 1991 when it was part of the Aquaculture Development Centre (ADC), University College Cork. However in 2005 it was established as an independent research station with a hatchery. It has sister companies with commercial scale shellfish and finfish farms (60ha). Research at the station has focused on aquaculture husbandry, macro-algal culture and integrated multitrophic aquaculture (IMTA), disease and fouling control, biofuel production, minimising waste in the aquaculture and fisheries production process, developing new products from waste, environmental monitoring and harmful algal blooms research and prediction. Aside from AccliPhot, some current projects include; IDREEM "Increasing Industrial Resource Efficiency in European Mariculture" (FP7 Environment theme), SAFI "Services for the Aquaculture and Fishing Industry" (FP7-Space), and ODIN "Food based solutions for optimal vitamin D nutrition and health through the life cycle" (FP7 – Agriculture). In November 2013 DOMMRS was awarded the Copernicus Masters Award from the European Space Agency for "Best service for European citizens" (using satellite data) for work carried out on algal blooms

Chapter descriptions

Each Chapter is written in manuscript form, allowing the reader to understand the Chapters independent of each other.

Chapter I provides an extended literature review exploring marine diatoms and their relationship with marine bacteria. It also introduces *P. tricornutum* and its industrial relevance.

Chapter II describes the initial experimental approaches designed to study the population responses of up-scaled *P. tricornutum* cultures and

introduces the main experimental design flaw: the up-scaled cultures had a variety of biological contaminants.

Chapter III is a manuscript in preparation for submission to a scientific journal. It delves into the identification of the bacterial community present in *P. tricornutum* cultures and aims to understand the role of each member of the bacterial community. Co-authors include Dr Ovidiu Popa, Dr Antonella Succurro, Dr Julie Maguire and Jun. Prof. Oliver Ebenhöh.

Chapter IV contains preliminary results from systematic co-culture experiments of *P. tricornutum* and species of the identified bacterial factions identified in Chapter III.

The future of algal-bacterial co-cultivation with respect to a bio-based economy is discussed in Chapter V, as well as a brief introduction to a spin-off mini-project investigating the role of chitin synthase in *P. tricornutum*.

Chapter VI is a special chapter based on my personal idea that algal biotechnology can have an impact on international and sustainable development efforts in developing countries, with special focus on Kenya. This Chapter was co-authored by Karin Moejes and is a manuscript in preparation for submission to the African Journal of Agriculture.

Summary (English)

The pennate diatom *Phaeodactylum tricornutum* is a model organism able to synthesise a number of industrially relevant molecules. Realising the industrial potential of microalgal-derived products relies on keeping large-scale monocultures which are prone to contamination by other organisms. However, little is known about the identity and characteristics of the invading organisms. In nature, diatoms are not found as isolated entities but rather are active members of a complex ecosystem, which is poorly understood. Bacteria, which have co-existed with diatoms for more than 200 million years, form a crucial part of this ecosystem and have been shown to enhance the growth of diatoms. Increased understanding of the interactions could allow for the exploration of 'synthetic ecology' as a novel scaling up technique. To gain insight into the dynamics of the bacterial communities associated with diatoms, the complexity of a natural system was translated into a reproducible, systematic experimental approach where the microbiome of batch grown non-axenic cultures of *P. tricornutum* was investigated using barcoded 16S-V6-Next-Generation-Sequencing. The results identified four main families, Alteromondaceae, Pseudoalteromonadaceae, Flavobacteriaceae and Pseudomonadaceae, were major players within the microbiome and changed in abundance over time. A network of putative interactions between *P. tricornutum* and each of the bacterial factions was proposed, thus providing a framework to understanding the dynamics of diatom-associated microbial communities. Species-specific co-culture experiments were carried out, which further corroborating the positive impact of bacterial co-cultivation on *P. tricornutum* growth efficiency.

Zusammenfassung (Deutsch)

Die pennate Kieselalge *Phaeodactylum tricornutum* ist ein Modellorganismus, welcher in der Lage ist, eine Reihe von industriell relevanten Molekülen zu synthetisieren. Das industrielle Potenzial von Mikroalgen zur Gewinnung von relevanten Produkten beruht auf der Kultivierung von großflächigen Monokulturen. Diese sind jedoch anfällig für Verschmutzungen durch Fremdorganismen. Aktuell ist nur sehr wenig erforscht welche Arten von solchen Organismen die Monokulturen befallen und welchen Einfluss sie auf diese haben. In der Natur findet man Diatomeen nicht als isolierte Einheiten sondern sie sind aktiver Bestandteil eines komplexes Ökosystems, welches zur Zeit nur grob beschreiben ist. Bakterien leben seit mehr als 200 Millionen Jahren eng mit Diatomeen zusammen und bilden einen wichtigen Teil dieses Ökosystems. Untersuchungen auf diesem Gebiet haben gezeigt, dass Bakterien einen positiven Einfluss auf das Wachstum von Diatomeen haben können. Ein besseres Verständnis der Wechselwirkungen zwischen Bakterien und Diatomeen unterstützt die Erforschung der „synthetischen Ökologie" als neuartige Scaling-up-Methode. Um einen Einblick in die Dynamik der Bakteriengemeinschaft in Verbindung mit Diatomeen zu gewinnen, wurde die Komplexität eines natürlichen Systems in einem reproduzierbaren, systematischen, experimentellen Ansatz umgesetzt. Hierfür wurde das Mikrobiom von nicht axenische *P. tricornutum*–Kulturen über die verschiedenen Wachstumsphasen der Kieselalge mit Hilfe der 16S-V6 Next-Generation-Sequenzierung charakterisiert. Als Ergebnis konnten vier Hauptfamilien von Bakterien identifiziert werden, Alteromondaceae, Pseudoalteromonadaceae, Flavobacteriaceae und Pseudomonadaceae, die

ein dynamisches Wachstum in Abhängigkeit von den Wachstumsphasen der Kieselalge aufwiesen. Weiterhin wurde ein Netzwerk möglicher Wechselwirkungen zwischen *P. tricornutum* und den einzelnen Bakterienfraktionen entwickelt, welches die Grundlage für das Verständnis der die Interaktion der Diatomeen und ihrer assoziierten Mikrobiome bildet. Um die positive Einflussnahme der assoziierten Bakterien zu bestätigen, wurden weitere Versuche zu artspezifischen Co-Kulturen durchgeführt. Hierdurch konnte die positive Wirkung von spezifischen Bakterien auf die Wachstumseffizienz von *P. tricornutum* Kulturen gezeigt werden.

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1 Introduction

"Diatoms are so exquisite it is hard to believe that they are also enormously important. They are, one could argue, the most vital plants on Earth. They bob, drift, and sometimes glide through most of the waters of the world in incredible numbers. Just one liter of sea water may contain as many as 1 million of these one-celled specks of algae- the primary foodstuff of the sea. Even land-dwelling creatures, including man, are in their debt, for diatoms that team in the upper few meters of the ocean produce, through photosynthesis, much of the oxygen we breathe."

– **Richard B. Hoover** in National Geographic 155(6):870, June 1997

1 Diatoms: key players in our marine biome

With our oceans constituting approximately 70.9% of the Earth's surface, it is the largest biome on our planet, yet it is probably the least studied. Derived from the Greek word *planktos* (made to wander or drift), plankton is the generic term for the microbiome which inhabits this vast ecosystem. Plankton are immensely diverse, spanning all three Domains of life, and are the microbial engines that drive global cycling of the six major elements (hydrogen, carbon, nitrogen, oxygen, sulphur and phosphorus) (Falkowski *et al.*, 2008; Strom, 2008).

Phytoplankton form the photosynthetic functional subgroup of plankton. Derived from the Greek words *phyton* (plant) and *planktos* (made to wander or drift), phytoplankton are photosynthetic unicellular organisms spanning a number of taxonomic Kingdoms and Phyla, including Bacteria such as cyanobacteria and prochlorophytes, polyphyletic Chromalveolata such as heterokonta (including diatoms), haptophytes (including coccolithophores) and dinoflagellates, and/as well as Plantae such as chlorophyta and charophyta (both green alga) (Cavalier-Smith, 1998; Adl *et al.*, 2005, 2012). However, with advances in genome-scale phylogenetic analyses the taxonomic classifications are always changing.

These ubiquitous photosynthetic microbes fix more than a hundred million tons of carbon in the form of carbon dioxide into organic material, which amounts to approximately half of global primary production (Field, 1998; Behrenfeld *et al.*, 2006). Of the total marine primary production, diatoms

alone account for roughly 40%; one-fifth of global primary production (Nelson *et al.*, 1995; Aumont *et al.*, 2003).

Diatoms are microalgae found in all aquatic and moist environments (Bradbury, 2004); their marine-based counterparts can be both planktonic and benthic. Diatoms belong to the Phylum *Heterokontophyta* and the Class *Bacillariophyceae* (Dangeard, 1933). They are the result of a secondary endosymbiotic event that took place around one billion years ago between a red alga (Rhodophyta) and a heterotrophic eukaryote (Bhattacharya *et al.*, 2007), and according to Mann and Vanormelingen (2013), 'the number of extant species of diatoms is estimated to be at least 30,000 and probably circa 100,000, by extrapolation from an eclectic sample of genera and species complexes'.

Diatoms come in a wide variety of shapes and sizes, ranging from 5µm to 2mm (Falciatore and Bowler, 2002). When nutrient levels are high, the more widely studied smaller diatoms (5 to 50µm) are able to 'float' and stay in the euphotic zone. When nutrient levels become suboptimal, these diatoms can aggregate and rapidly sink out of the euphotic zone where they become food for deep-water organisms. A small fraction that settles on the sea floor is sequestered over geological timescales in sediments and rocks (Sarthou *et al.*, 2005; Armbrust, 2009). Although ubiquitous in our oceans, less is known about giant diatoms (2-5mm). Whereas planktonic diatoms are found in all open water bodies, benthic diatoms can be found on the surfaces of macroalgae, rocks, and on the ocean floor (Falciatore and Bowler, 2002).

Perhaps its most unique feature, diatoms have a transparent, amorphous silica cell wall composed of hydrated silicon dioxide, SiO_2, in a 3-

dimensional network of -Si-O-Si-O- units, called a frustule (Kröger and Poulsen, 2008). Depending on the symmetry of the frustule, diatoms are categorized as either centric (radially symmetrical) or pennate (bilaterally symmetrical).

The combination of specific environmental factors (nutrient bioavailability, temperature, light availability, turbidity and water chemistry) can cause the proliferation of microalgal growth in both saline and freshwater bodies. When cell counts (per mL) reach certain concentrations, a phenomena known as an 'algal bloom' is said to have occurred. Harmful Algal Blooms (HABs) are when the blooms have an adverse effect on other organisms within the ecosystem by various methods including toxin production. HABs are colloquially known as 'red tides' due to the pigmentation of the species dominating most blooms. Red tides have been mentioned throughout recorded human history. The Bible contains verses such as "And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river …" (Exodus 7:21 King James Version) and "The second angel poured out his vial upon the sea; and it became as the blood of a dead man: and every living soul died in the sea." (Revelation 16:3 King James Version), which all point towards descriptions of algal blooms. Of the estimated known phytoplankton species (~4 000; including diatoms and dinoflagellates), 7% (~300) have been reported to cause HABs. Approximately 2% of the ~300 species are in fact harmful or toxic, with dinoflagellates estimated to compose up to 90% of the ~70 harmful HAB-causing species (Smayda, 1997).

Despite the devastating socio-economic as well as ecological effects of HABs, the specific etiology of HAB formation is still poorly understood.

2 Fundamentals of diatom-bacterial interactions

But diatoms are only one of the members of the relatively unexplored marine microbiome. In 2009, the *Tara* Oceans project was launched aiming to greatly improve our understanding of this mysterious world. The three-year study abroad the schooner *Tara* implemented optical and genomic methods to describe members of the plankton ecosystem (viruses, bacteria, archaea, protists, and metazoans) in their natural habitats (Karsenti *et al.*, 2011). In May 2015, Sunagawa *et al.* (2015) published the metagenomics data from 243 samples collected from 68 unique locations.

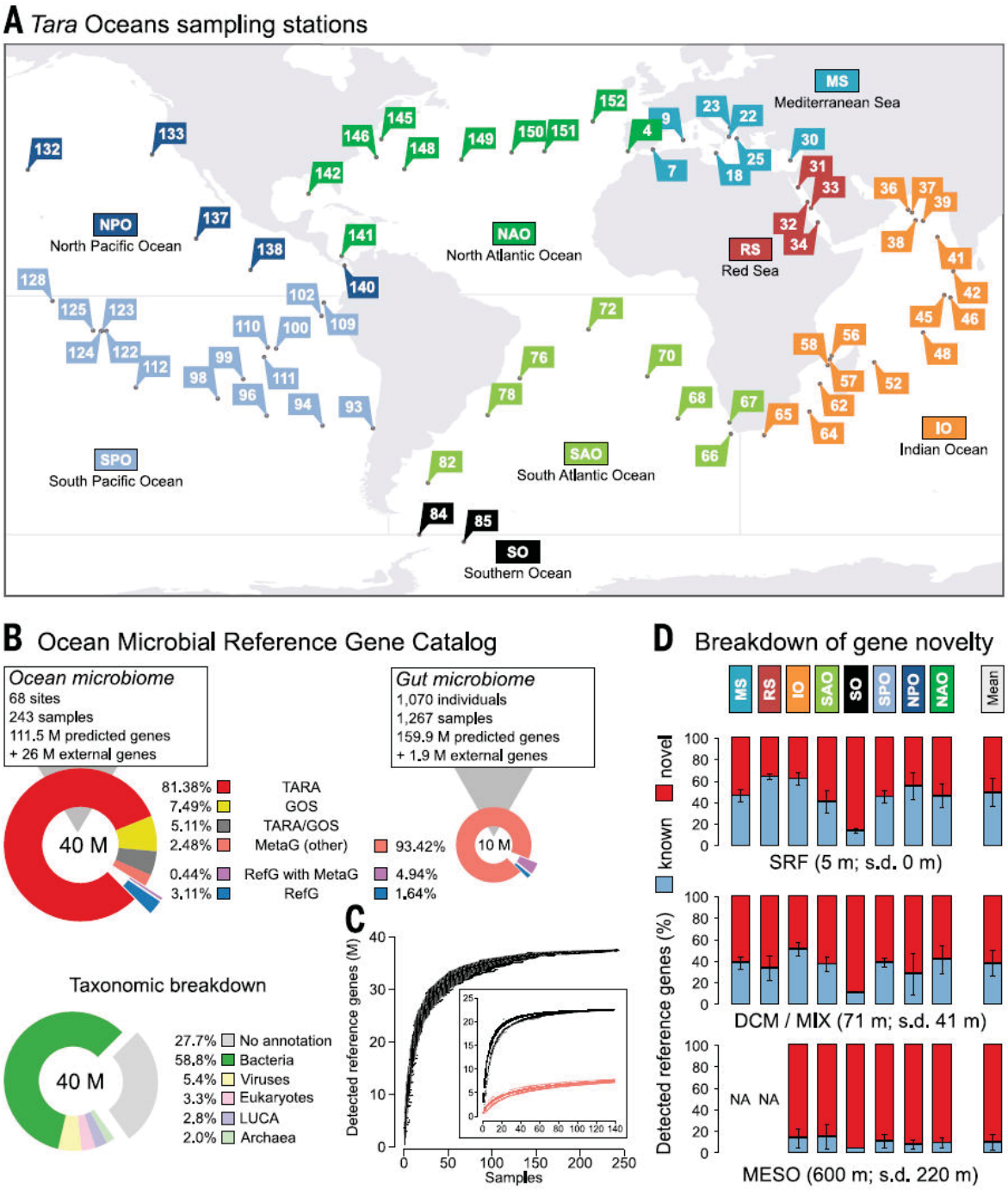

Figure 1: Genetic diversity in the global ocean microbiome identified by *Tara* Oceans project (figure taken from Sunagawa *et al.*, 2015). **(A)** Geographic distribution of 68 (out of over 200 in total) representative *Tara* Oceans sampling stations at which seawater samples and environmental data were collected from multiple depth layers. **(B)** Deep Illumina shotgun sequencing of 243 samples, followed by metagenomic assembly and gene prediction, resulted in the identification of more than 111.5 x 10^6 gene-coding sequences. Genes identified in this study were clustered together with over 26 x 10^6 sequences from publicly available data to yield a set of more than 40 x 10^6 reference genes (top left), which equals more than four times the number of genes

in the human gut microbial reference gene catalogue (top right; Li *et al.*, 2014). The combined clustering of genes identified in *Tara* Oceans samples with those obtained from public resources allowed for the annotation of genes according to the composition of each cluster. More than 81% of the genes were found only in samples collected by *Tara* Oceans when compared to previous studies. A breakdown of taxonomic annotations (bottom left) shows that the reference gene catalogue is mainly composed of bacterial genes (LUCA denotes genes that could not unambiguously be assigned to a domain of life). **(C)** Rarefaction curve of detected genes for 100-fold permuted sampling orders shows only a small increase in newly detected genes toward the end of sampling. The subplot compares sequencing depth-normalised rarefaction curves for 139 prokaryotic ocean samples (black) mapped to the prokaryotic subset of the Ocean Microbial Reference Gene catalogue (24.4×10^6 genes) and the same number of random (100-fold permuted) human gut samples (pink) mapped to a human gut gene catalogue (Li *et al.*, 2014), suggesting that the ocean harbours a greater genetic diversity than the human gut. **(D)** For the subset of 139 prokaryotic samples analysed, the fraction of detected genes that had previously been available in public databases (blue) are compared to those that were newly identified in samples collected by *Tara* Oceans (red).The breakdown by ocean region and depths shows that the Southern Ocean and the mesopelagic zone had been vastly under sampled prior to *Tara* Oceans.

Abbreviations: MS, Mediterranean Sea; RS, Red Sea; IO, Indian Ocean; SAO, South Atlantic Ocean; SO, Southern Ocean; SPO, South Pacific Ocean; NPO, North Pacific Ocean; NAO, North Atlantic Ocean; GOS, Sorcerer II Global Ocean Sampling expedition; MetaG, genes of metagenomic origin; RefG, genes from reference genome sequences; LUCA, last universal common ancestor; SRF, surface water layer; DCM, deep chlorophyll maximum layer; MIX, subsurface epipelagic mixed layer; MESO, mesopelagic zone.

The data showed that 58.8% of the sequences belonged to Bacteria, even though bacterial densities (10^5 to 10^6 per gram of seawater) in our oceans are orders of magnitudes less than those found in sediments (10^8 cells per gram), humans (10^{14} cells per gram), or soil (10^9 cells per gram) (Whitman *et al.*, 1998; Amin *et al.*, 2012). All members of the plankton ecosystem co-exist and therefore have established a symbiotic relationship within their respective phyla – but also with other phyla and even domains. With the data from *Tara* confirming that the sheer amplitude of genetic material

belongs to bacteria within our oceans, and the fact that bacteria have co-existed with diatoms in biomes throughout our oceans for more than 200 million year, we can begin to ask ourselves how exactly are the bacteria communicating and interacting between themselves as well as with the diatoms.

This is not a revolutionary idea, however. In 1933, Waksman *et al.* investigated the saprophytic characteristic of marine bacteria, and in 1937 correlated dead and decaying diatom cultures to the growth of bacteria (1933, 1937). A decade later, looking more broadly at all algae, Lucas postulated that in waters there are 'non-predatory relationship based on the release and biological influence of metabolites, ranging from toxins to vitamins and hormones' (Lucas, 1947). Provasoli went a step further to suggest that bacteria can enhance the growth of algae in his 1958 review (Provasoli, 1958). In the decade following Provasoli's review numerous papers provided ample evidence of algal-bacterial interactions, but little light was shone on species-specific interactions until Delucca and McCracken carried out a co-culture study on the effect of naturally-collected bacteria on a number of algal species (Delucca and Mccracken, 1977).

In plant biology, the term 'rhizosphere' was introduced by Dr Lorenz Hiltner in 1904 to describe the region of soil which is subjected to the influence of plant roots (Hiltner, 1904; Hartmann *et al.*, 2008). Bell and Mitchell felt that an aquatic equivalent would be fitting; and thus coining the term 'phycosphere' in 1972, denoting the region extending outwards from the algal cell in which bacterial growth is stimulated by extracellular products of the alga (Bell and Mitchell, 1972).

The ‘paradox of the plankton’ asks how it is possible for many species to coexist in nutrient limiting ecosystems, such as the marine pelagic zone, given the tendency for competition to exclude species (Hutchinson, 1961). This goes against the competitive exclusion principle – which states that one species alone would outcompete all the others so that in a final equilibrium situation the assemblage would reduce to a population of a single species (Hardin, 1960; Hutchinson, 1961).

In nature, no single organism is an isolated sovereign entity that is not influenced by its environment including other organisms. Symbiosis attempts to explain the interaction between two organisms which are fundamentally dissimilar in phenotype and genotype, yet are living in close physical association for an extended period of time. Whether symbiosis is a purely mutualist relationship or if it can be defined by a number of mechanisms of interaction (e.g. mutualism, commensalism, parasitism, amensalism), is highly debated amongst scientists. By applying the broader definition of symbiosis we are able to get an extended understanding of the interaction between bacteria and diatoms.

Just as we have several types of interpersonal relationships that are created, dissolved and maintained throughout our lives, ecological communities also display this fluidity of relationships (Moon *et al.*, 2010). We will attempt to define and describe different direct relationship types between bacteria and diatom, as described by previous studies.

The first relationship type is **competition**. This relationship occurs when two organisms compete for the same resource; whether it be food, space, sexual partners etc. Phosphorus is essential for the survival and growth of both diatoms and bacteria – phosphorus is quite literally the ‘staff of life’; the

scaffolding on which all biomass is built (Karl, 2000). Both organisms primarily utilise orthophosphate as a source of phosphorus. However, because orthophosphates are locked in dissolved organic phosphate (DOP) compounds present in the predominantly nutrient-limited marine biome, both diatoms and bacteria developed mechanisms such as the excretion of enzymes, including phosphatases, to liberate orthophosphate (PO_4^{3-}) from DOP. The mechanism is not species-specific, which consequently means the 'free' orthophosphates can be acquired by any phosphate-requiring organism (Persson *et al.*, 1988). Some oceanic habitats have orthophosphate concentrations of less than 50pM, essentially limiting uptake via diffusion. Therefore, one would assume selection for very small organisms with large surface-to-volume ratios. However, on a mass-for-mass basis, small bacteria require more phosphorus than larger phytoplankton, including diatoms, suggesting that the bacteria act as a 'phosphorus storage service' instead of remineralising agents of phosphorus (Karl, 2000). When orthophosphate is limiting, the competitive relationship between diatoms and bacteria could potentially influence not only the species composition of the phytoplankton and bacterial communities, but also the fundamental functioning of the microbial ecosystem by shifting the balance between phytoplankton and bacteria and the bacterial degradation of organic material (Thingstad *et al.*, 1993).

The second relationship type is **parasitism**. The parasite benefits from this relationship at the expense of the host. In diatom-bacterial interactions either party could play either role. Several studies have shown diatoms to produce antibacterial compounds. Desbois *et al.* showed that the diatom *Phaeodactylum tricornutum* excreted fatty acids (such as eicosapentaenoic acid or EPA), nucleotides, peptides, and pigment derivatives not only to 'kill-

off' the competition (i.e. the bacteria) but also to protect themselves against opportunistic attack or pathogenic damage (see Desbois *et al.*, 2009 for further references).

Reversing these roles makes the bacteria algicidal. The bacteria inhibit diatom growth either through attachment of the bacteria to the diatom or mediated by the excretion of extracellular molecules. Furusawa *et al.* showed the filamentous marine bacteria *Saprospira sp.* attach and partially degrade the cell wall of the diatom *Chaetoceros ceratosporum* in order to penetrate the cell causing them to completely lyse (Furusawa *et al.*, 2003). Another study conducted by Jung *et al.* showed that the freshwater bacteria *Pseudomonas fluorescens* (species SK09) directly injected an algicidal compound into the cytoplasm of the freshwater diatom *Stephanodiscus hantzschii*, causing the degradation of over 90% of the diatom culture within five days. This study also proved that the bacterium was in fact species-specific as it did not supress the growth of a number of other diatom cultures (Jung *et al.*, 2008). Algicidal activity of extracellular molecules was shown by Lee *et al.* where the concentrated supernanent of a marine bacterium *Pseudoalteromonas sp.* strain A28 contained various enzymes including proteases, DNases, cellulases, and amylases, capable of causing the lysis of the diatom *Skeletonema costatum* (Lee *et al.*, 2000). *Kordia algicida*, as its nomenclature suggests, is an algicidal bacterium isolated from a red tide in Masan Bay, Korea, excrete diffusible enzymes larger than 30kDa to interfere with diatom growth (Sohn, 2004; Paul and Pohnert, 2011). The algicidal substances excretes by *K. algicida* were not species-specific and significantly inhibited the growth of *Skeletonema costatum*, *Phaeodactylum tricornutum*, and *Thalassiosira weissflogii* (Paul and Pohnert, 2011). The studies mentioned were associated with the study

of harmful algal bloom mechanics and potential biocontrol agents that could hinder the development of the destructive blooms.

The next relationship types are interconnected; **commensalism**, **mutualism** and **neutralism**. Commensalism describes a relationship where one organism is positively affected by the relationship and the other is not affected. Mutualism is when both partners benefit from the relationship. And neutralism is when the relationship neither hinders nor benefits either partner (Moon *et al.*, 2010). These relationship types are based on a biological barter trade system between diatoms and bacteria – where substances such as trace metals, vitamins, and nutrients (nitrate, phosphate, silicate, dissolved organic carbon).

2.1 Iron

Iron acquisition by diatoms is required for essential biological processes such as photosynthesis, respiration and nitrogen fixation. High-nutrient, low-chlorophyll (HNLC) areas cover over 20% of the world's open ocean surface waters, where there is abundant sunlight and the excess presence of major nutrients (nitrate, phosphate an silicate), yet the phytoplankton community remains small (Martin *et al.*, 1994). One explanation for this phenomena is the 'iron limitation hypothesis' (Pitchford and Brindley, 1999). Several iron-fertilisation experiments in HNLC areas led to the creation of algal blooms often dominated by diatoms, supporting the theory (see Boyd *et al.*, 2004 for further references). The fact that diatoms dominated the blooms suggests that diatoms have developed methods of surviving in iron-limiting oceans, but are able to allow for the subsequent rapid growth

when iron becomes available (Morrissey and Bowler, 2012). Most bioactive trace metals, including iron, exist at nanomolar (10^{-9} M) to picomolar (10^{-12} M) concentrations in our oceans, approximately one-millionth of the intracellular concentration in diatoms (Bruland *et al.*, 1991; Morel and Price, 2003). The iron present is found as iron (III), more than 99% of which is bound to organic ligands forming ferric compounds (Vraspir and Butler, 2009).

But how do diatoms meet the intracellular demand for iron? There are a number of mechanisms employed by diatoms including a reductive iron uptake (via e.g. high-affinity ferric reductase, multi-copper oxidase, and iron (III) permease); iron storage (ferritin); and a non-reductive mechanism via siderophores (Sutak *et al.*, 2012; Groussman *et al.*, 2015).

Greek for 'iron carrier', siderophores area group of iron scavengers that act by chelating iron (III). Siderophores are produced and excreted by bacteria, and some cyanobacteria, which then reuptake the siderophores with bound iron (III) via outer-membrane transporters that are siderophore-specific (Vraspir and Butler, 2009). Diatoms are not known to produce siderophores (Soria-Dengg and Horstmann, 1995; Amin *et al.*, 2009). However, based on genome sequence analyses, the presence of a gene orthologue of a bacterial ferrichrome binding protein; suggests the possibility of iron (III)-siderophore utilisation by *P. tricornutum*. Furthermore, experiments carried out by Soria-Dengg and Horstmann showed that *Phaeodactylum tricornutum* was able to uptake siderophores ferrioxamines B and E (1995). Bacterial siderophores could potentially supply diatoms with their daily dose of iron.

2.2 Domoic acid and Harmful Algal Blooms

In the summer of 1961, a Californian newspaper reported an invasion of 'crazed seabirds' that 'pelted the shores of North Monterey Bay, California' with regurgitated anchovies, which provided inspiration for Alfred Hitchcock's 1963 thriller *The Birds*. Since we have yet to discover time travel, a group from Louisiana State University led by Dr Sibel Bargu carried out post-mortems on the seagulls killed during the summer of 1961, and found that they had been poisoned by domoic acid (Bargu *et al.*, 2011). Domoic acid is a neurotoxin produced by diatoms that accumulates in bivalves, echinoderms and small fish, and when these are consumed by humans can cause amnesic shellfish poisoning (ASP). The role of domoic acid and its biosynthetic pathway in diatoms is relatively unknown, although a number of hypotheses exist based on experimental evidence. Rue and Bruland

demonstrated the role of domoic acid acts as a chelator of copper and iron (Rue and Bruland, 2001), but is debated whether domoic acid production was affected by increased or decreased copper and iron bioavailability (Bates *et al.*, 2000; Maldonado *et al.*, 2002; Wells *et al.*, 2005; Trick *et al.*, 2010). Also, domoic acid could be synthesised to acquire copper, an important component in iron acquisition, although is also debatable (Wells *et al.*, 2005; Lelong *et al.*, 2013). Other parameters influencing the production of domoic acid by diatoms are organic sources of nitrogen (ureas and glutamine), changes in pH, CO_2, and salinity (see Lelong *et al.*, 2012 for further references). Furthermore, the diatom *Pseudo-nitzschia multiseries* was shown to lose most of its ability to produce domoic acid when cultured axenically, but recovered this ability by the reintroduction into a non-axenic culture (Bates *et al.*, 1995). Furthermore, the study by

Kobayashi *et al.* showed that direct contact between the diatom and bacterial consortia is necessary for *P. multiseries* to produce high levels of domoic acid (2009). A study by Prince *et al.* in 2013 demonstrated a decrease in *Skeletonema marinoi* cells and an increase in *Pseudo-nitzschia delicatissima* cells when grown in co-culture in the presence of domoic acid under iron replete conditions, concluding that domoic acid can improve the competitive edge of *P. delicatissima* and that iron is likely involved (Prince *et al.*, 2013).

2.3 Vitamins

In 1912, Polish biochemist Kazimierz (later anglicised to Casimir) Funk conceptualised 'vitamines', or 'vital amines' (the 'e' at the end of 'vitamine' was later dropped when it was realised that not all vitamins are amines) (Funk, 1912). Vitamins, especially B vitamins, are small vital/essential water soluble molecules that act as growth factors that function as coenzymes required in majority of important metabolic pathways (Madigan *et al.*, 2015).

Research on B vitamins has largely focused on vitamins B_1 (thiamine), B_7 (biotin) and B_{12} (cobalamin) as they are required, alone or in combination, by most marine diatoms and bacteria (Provasoli, 1958; Provasoli and Carlucci, 1974; Sañudo-Wilhelmy *et al.*, 2014). Earlier research on diatoms and their vitamin requirements focused mainly on elucidating the mechanisms that trigger phytoplankton blooms (see Discussion section in Ohwada and Taga, 1972 for further references of earlier studies). A study of the distributions and seasonal variations thiamine, biotin and cobalamin in the North Pacific Ocean and the Pacific coastline of Japan in 1972 showed

that the geographical and vertical distributions of thiamine and biotin in the sea generally showed similar patterns to that of chlorophyll *a* whereas cobalamin did not always follow the trend (Ohwada and Taga, 1972).

Diatoms are generally regarded as autotrophs – only requiring light and a mixture of inorganic nutrients to grow. However, experimental data generated in the 50s, 60s and 70s illustrated that without an adequate supply of vitamins, many species of phytoplankton do not grow (i.e. many species are vitamin auxotrophs) (Provasoli and Carlucci, 1974; Panzeca *et al.*, 2006). Supporting literature-based evidence was provided by Croft *et al.* in 2006 who found that of >300 species of algae, more than half required cobalamin, 22% required thiamine and 5% required biotin (Croft *et al.*, 2006). Looking at just the *Bacillariophyceae* reported in Croft's paper, of the 54 diatoms investigated, 61% require cobalamin, 13% require thiamine, and none were biotin auxotrophs.

The fact that auxotrophy of all three vitamins does not discriminate based on the phyla the algae comes from, suggests that multiple appearances and disappearances of this trait must have occurred throughout evolution, driven by environmental factors (Croft *et al.*, 2006; Cooper and Smith, 2015).

Prokaryotes are believed to be the main producers of B vitamins (Provasoli, 1963; Provasoli and Carlucci, 1974). However, Sañudo-Wilhelmy *et al.* evaluated vitamin auxotrophy in whole genome sequenced representative bacteria belonging to those taxa most commonly found in marine environment including; Cyanobacteria, Alpha- and Gammaproteobacteria, and Bacteriodetes (note: these are all culturable bacteria and therefore do not represent their physiologies in *in situ* uncultured communities). In total, 37%, 76% and 78% of the marine bacteria had *de novo* pathways for

synthesising cobalamin, thiamine and biotin, respectively. This suggests that bacteria also require vitamins and therefore must compete with the diatoms. Although we mentioned the percentage of diatoms unable to synthesise cobalamin, thiamine or biotin, some diatoms synthesise and in fact excrete excess vitamins (Sañudo-Wilhelmy *et al.*, 2014), including *P. tricornutum* which synthesises its own cobalamin, thiamine and biotin (Croft *et al.*, 2006). In summary, the bacteria could supply diatoms with vitamins, or *vice versa* – diatoms could in fact be the ones excreting excess vitamins for other diatoms and bacteria to use. A vitamin-based relationship can, therefore, also be a competitive one.

2.4 Dissolved Organic Carbon (DOC)

The carbon concentrating mechanism (CCM), a mechanism to facilitate an ample flux of CO_2 to photosynthesis under CO_2 limitation, is known to occur in the few marine diatoms studied so far (Colman and Rotatore, 1995; Johnston and Raven, 1996; Tortell et al., 1997; Badger et al., 1998; Matsuda et al., 2001a). However, it is estimated that up to 50% of carbon fixed via phytoplankton-mediated photosynthesis is utilised by marine bacteria (Azam *et al.*, 1983), mainly as dissolved organic carbon (DOC) compounds, defined as the organic material <0.7µm in size (Stocker, 2012). DOC from diatoms originates either from live cells or recently lysed or grazed cells, which determines the type of DOCs available, and therefore likely determining the bacterial consortia associated with the diatom (Amin *et al.*, 2012). Glycolate is a small two-carbon molecule synthesised during photorespiration, which constitutes a large majority of the total carbon excreted by certain diatom species in culture (see Edenborn and Litchfield,

1987 for further references). The functional gene *glcD*, which encodes the D-subunit of glycolate oxidase, was found to be present in only a small subset of marine bacteria, suggesting that glycolate-utilising bacterial consortia would benefit from associating with diatoms excreting excess glycolate (Lau and Armbrust, 2006). Primers developed for *glcD* in this study were implemented primers in follow-up experiment to identified and follow the progression of glycolate-utilising bacteria during a phytoplankton bloom. A clear pattern emerged with three groups of glycolate users identified; early-bloom, late-bloom, and those who increased in relative abundance and the bloom progressed. However, the overall *glcD* sequence diversity decreased as the phytoplankton progressed (Lau *et al.*, 2007). This proposes a dynamic complexity within the bacterial consortia based solely on the type of DOC provided by the diatom, in this case the competition for glycolate. A list of known marine N_2-fixing organisms is listed in Karl *et al.* (2002).

2.5 Nitrogen

Nitrogen is an important component of proteins and nucleic acid and essential for the survival and growth of diatoms. Nitrogen is found in the ocean as well as in the atmosphere as gaseous dinitrogen (N_2). However, diatoms can only assimilate inorganic (nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) or ammonia (NH_3)) or organic (urea ($CO(NH_2)_2$), amino acids) sources of nitrogen (Bowler *et al.*, 2010). N_2-fixing microorganisms, which are exclusively prokaryotic (including Bacteria and Archaea), are central to meeting this demand for nitrogen by diatoms by converting N_2 into the useable bio-available forms of nitrogen mentioned above (further

references to known N_2-fixing bacteria are mentioned in Karl *et al.*, 2002). Nitrogen-fixing bacteria utilise the enzyme nitrogenase to reduce N_2 into NH_3 in strictly anaerobic conditions. Subsequently, two separate groups of aerobic bacteria firstly oxidise NH_3 (or NH_4^+?) into NO_2^- and finally oxidise NO_2^- into NO_3^-. Both processes require molecular oxygen, supplied by photoautotrophic organisms (Falkowski, 1997).

2.6 Phytohormones

Phytohormones (including auxin, abscisic acid, cytokinin, ethylene, and gibberellins) are a class of small molecules that act as chemical messengers to coordinate cellular processes in higher plants. It is hypothesised that higher plant hormone systems have evolved from similar systems in microalgae (Kenrick and Crane, 1997), and therefore, fragments of this system could potentially still exist in modern diatoms and microalga. Bioactive forms of all main phytohormones have been detected in a wide range of algal lineages (see Lu and Xu, 2015 for further references). However, controversy surrounds scientific data available partaking to the functional roles of most phytohormones in microalgae. This is because nearly all experimental evidence has been derived from the effect of exogenous higher plant phytohormones on the microalgae (Lu and Xu, 2015). Although the trend is changing – a couple of functional studies have been carried out exploring the effect of endogeneous microalgal phytohormones (e.g. Lu *et al.*, 2014), none of these studies focused on diatoms.

The phytohormone, auxin is probably one of the most well-known phytohormones, the most abundant of which is indole-3-acetic acid (IAA).

IAA has been detected in natural seawater samples since the 1960s (Maruyama *et al.*, 1989; Mazur and Homme, 1993; Bentley, 2009). More specifically, a study by Maruyama *et al.* showed IAA being produced by bacteria, especially in the presence of tryptophan which correlates with findings in terrestrial bacteria (see Maruyama *et al.*, 1989 for further references). A recent study conducted by Amin *et al.* corroborate these earlier experiments by showing a species-specific interaction between a coastal diatom, *Pseudo-nitzschia multiseries*, and a bacterial *Sulfitobacter* species (SA11), where the bacteria was shown to promote diatom cell division via secretion IAA, synthesised by the bacterium using diatom secreted and endogenous tryptophan. The IAA and tryptophan act as signalling molecules in this intricate diatom-bacteria relationship (Amin *et al.*, 2015).

2.7 Sulphur

Sulphur is an essential element required in the building of proteins, including amino acids and coenzymes such as thiamine, biotin and coenzyme A. Most of the Earth's sulphur stock is in our oceans, mainly in the stable form of dissolved sulphate and in sedimentary (Sievert *et al.*, 2007). Diatoms have two central metabolic roles for assimilated sulphate is utilised by diatoms in two main ways; 1) as polysaccharides, 6-deoxy-6-sulfoglucose or sulfoquinovose (SQ), which can be assembled into phosphorus-free lipids such as sulfoquinovosyl diacylglycerol (SQDG), an intermediate of which is sulfopropanediol or 2,3-dihydroxy-1-propanesulfonate (DHPS) which has been described as an 'excretion product of all diatoms' (Benson and Lee, 1972; Mayer *et al.*, 2010). A study

conducted by Durham *et al.* (2015) identified DHPS, which has no previously known role in the microbial food web, as an abundant metabolite in a natural diatom bloom. Subsequent co-culture studies between the bacteria *Ruegeria pomeroyi* DSS-3 and the diatom *Thalassiosira pseudonana* CCMP1335, showed that ten of the most highly up-regulated genes encoded genes for the transport and catabolism of DHPS by *R. pomeroyi*. In contrast, *T. pseudonana* downregulated any sulphur-related genes including those involved in the biosynthesis of SQDG. These results suggest that certain bacteria are able to utilise DHPS as a carbon source and diatoms can use it as a 'currency' in exchange for e.g. vitamin B_{12}, driving both carbon and sulphur cycles in the ocean (Durham *et al.*, 2015). 2) in the production of methionine and cysteine and following a number of intracellular reactionary steps, diatoms can convert methionine into dimethylsulphoniopropionate (DMSP) (Gage *et al.*, 1997). Diatoms accumulate excess DMSP with increased salinity, increased light intensity and nitrogen starvation, where it acts as an osmolyte, antioxidant, and possibly as an overflow metabolite (Kettles *et al.*, 2014). DMSP can subsequently be cleaved by the enzyme DMSP lyase in the diatoms into a number of products, including the volatile dimethylsulphide (DMS). DMS acts as a sulphur transporter supplying the relatively sulphur-limiting terrestrial biome with sulphur via the atmosphere where the DMS oxidises to form aerosol particles which provide the majority of the Cloud Condensation Nuclei (CCN) over the oceans as well as directly reflecting solar radiation, contributing to maintaining global mean temperatures (Andreae, 1990).

2.8 Communication

We all know communication is an important component of a successful relationship, and diatom-bacterial relationships are not exempt from this rule. Unfortunately, interkingdom communication in the ocean biome is a relatively unexplored field. Bacteria are able to communicate with each other using the comparatively well-known language known as quorum sensing. Quorum sensing in bacteria was initially proposed to explain luminescence in dense populations of two marine bacteria in response to the accumulation of a secreted autoinducer signalling molecules (Nealson and Hastings, 1979). The concentration of autoinducers in the extracellular environment increases in correlation to the increase in population density. When the concentration reaches a critical threshold, it synchronically alters gene expression, and therefore the phenotypic behaviour of the bacterial population (see Fuqua *et al.*, 1994 and Waters and Bassler, 2005 for further references). A group autoinducers synthesised and utilised by bacteria in the marine biome known to interact with eukaryotic organisms, are acyl homoserine lactones (AHLs). AHLs are comprised of a slightly hydrophilic homoserine lactone ring and a hydrophobic side chain, creating an amphipathic molecule capable of passively diffusing through the phospholipid cell membrane as well as the aqueous extracellular and intracellular environments (Fuqua *et al.*, 2001).

Although much less is known about quorum sensing-like activity of diatoms, they do synthesise AHL-like pheromones which have similar molecular characteristics allowing for transmembrane-free crossing of the cell membrane (Amin *et al.*, 2012). A number of pheromones have been

identified in cultures of freshwater diatoms (Jüttner and Müller, 1979; Pohnert and Boland, 1996; Wendel and Jüttner, 1996; Hombeck and Boland, 1998), but only one study has identified such molecules in marine diatoms. Based on previous findings, Derenbach and Pesando postulated that two marine diatoms, *Skeletonema costatum* and *Lithodesmium undulatum*, were possibly be secreting pheromones to initiate female gamete formation. Experimental results revealed that *S. costatum* cultures conclusively contained the known brown macroalgal pheromone ectocarpene. *L. undulatum* also contained traces of the pheromone (Derenbach and Pesando, 1986). Although examples of this cross-species communication exists, none have been characterised between diatoms and bacteria. Although, in theory, diatoms and bacteria could use autoinducers and pheromones as a language, further research is required to characterise the 'language' spoken.

3 A peculiar diatom called *Phaeodactylum tricornutum*

Phaeodactylum tricornutum is a diatom first described by Bohlin in 1897 when he found it in samples collected off the coast of Plymouth, United Kingdom. Unlike most diatoms, which have the distinct ability to precipitate soluble silicic acid to form a silica cell wall, *P. tricornutum* has a poorly silicified cell wall and therefore does not have an obligate requirement for silicic acid (Montsant *et al.*, 2005; Martino *et al.*, 2007). As indicated by its species name '*tricornutum*', Bohlin described a 'three-pointed star shaped cell' or triradiate diatom. Although never formally described, Allen and Nelson isolated a novel marine diatom in 1910 which they referred to as *Nitzschia closterium.* It was only in 1946 that Wilson correlated Bohlin's initial find with the organism found by Allen and Nelson, and deduced that the *Nitzschia closterium* strain was in fact *Phaeodactylum tricornutum* (Wilson, 1946).

The spindle-like shaped cells were maintained in the culture collection of the Plymouth Marine Laboratory and distributed around the world. In the decades following Allen and Nelson's discovery, several laboratories began to report the presence of oval, spindle-like (fusiform), and cells shaped like three-pointed stars (triradiate) in their cultures (Lewin *et al.*, 1958).

Although *P. tricornutum* is not ubiquitous in our oceans, it is found in coastal regions such as rock pools and estuaries where aquatic environmental parameters (salinity, temperature) vary greatly as a consequence of tidal changes and solar irradiation (Martino *et al.*, 2011). Its habitual characteristics and peculiar ability to form oval, fusiform, and triradiate cells, as well as its poorly silicified cell wall, has led to the exponential increase in scientific research on *P. tricornutum*.

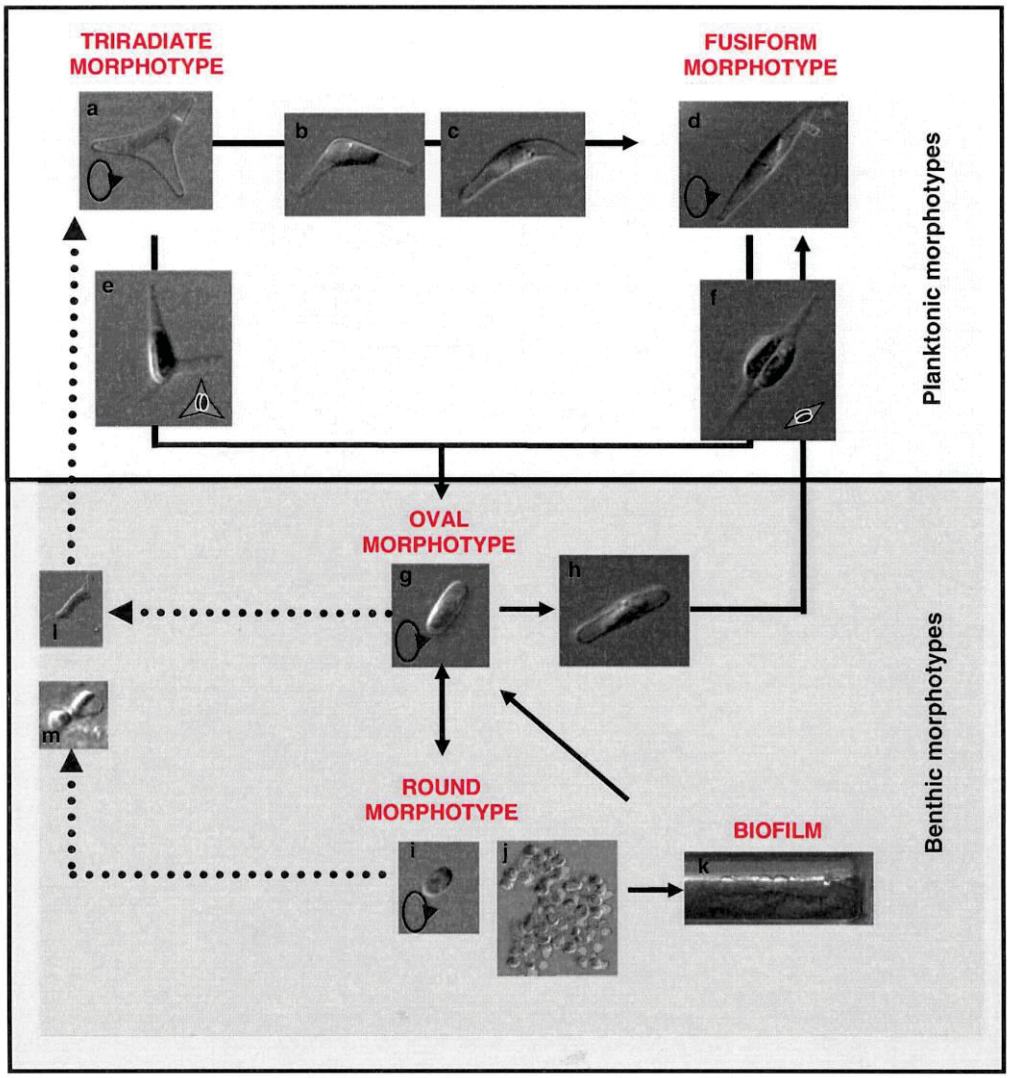

Figure 2: Schematic representation of *P. tricornutum* life cycle and morphotype changes in response to environmental cues (figure taken from Martino *et al.*, 2011). The triradiate **(a)** and fusiform **(d)** morphotypes are planktonic and convert to the adhesive, motile and benthic oval morphotype through intermediate shapes;

boomerang shapes **(b,c)** or directly **(e,f)** in response to hyposalinity and cold temperatures (15°C) (Martino *et al.*, 2011; Willis *et al.*, 2013). A fourth 'round' morphotype **(i)** was also proposed by (Martino *et al.*, 2007) as a possible resting cell which form in response to prolonged conditions of stress and can form aggregates **(j)** that can generate biofilms **(k)**. All conversions are reversible except for fusiform to triradiate. Conversion from triradiate or fusiform to oval always occurs by division whereas the reversible process occurs by elongation **(h)**. Small triradiates have been occasionally observed **(l)** and they could represent an intermediate state between round and triradiate cells **(l,m)** (Martino *et al.*, 2011).

Both the fusiform and triradiate morphotypes can be observed in laboratory cultures as well as environmental water samples, however, the fusiform type is found to be the most stable (Martino *et al.*, 2007; Tesson *et al.*, 2009). The oval morphotype has the classical diatom characteristic of a silica valve that is embedded in the cell wall (Lewin *et al.*, 1958). Oval morphotypes are typically found on either solid media or in unagitated liquid cultures (Lewin *et al.*, 1958; Bartual *et al.*, 2008).

Furthermore, interest in *P. tricornutum* gained momentum in 1989 where Siron *et al.* showed that *P. tricornutum* has the ability to produce the poly unsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) in high proportion to the total fatty acid content; EPA constitutes approximately 20 – 40% of the total fatty acids (Siron *et al.*, 1989; Rebolloso-Fuentes *et al.*, 2001; Fajardo *et al.*, 2007). Regarding advances in biofuel technology, Chisti (Chisti, 2007) showed that *P. tricornutum* produces between 20 – 30% oil (% dry weight) which can be converted to biofuels (e.g. biodiesel by transesterification) that can be directly used in existing engines and transport infrastructure (Scott *et al.*, 2010).

3.1 Genetic characteristics of *Phaeodactylum tricornutum*

Following the genome sequencing of *P. tricornutum* in 2008, and the subsequent generation of expressed sequence tag (ESTs) databases of

P. tricornutum, light was shed on its unique characteristics relating to its acclimation capabilities to the marine environment as well as the ever-changing regions it naturally habituates (Montsant *et al.*, 2005; Martino *et al.*, 2007; Bowler *et al.*, 2008). Therefore, advances in the genetics of *P. tricornutum* make it an excellent model organism. Latest version of the genome annotation database, Phatr3, is available on http://protists.ensembl.org/Phaeodactylum_tricornutum/.

The availability of the whole-genome sequence *of P. tricornutum* allows for its use as a model organism in the laboratory. The genetic information also allows for the potential use in pharmaceuticals and synthetic biology as a 'chassis' to carry genes since we are able to genetically modify certain strains of *P. tricornutum*. Since it is a eukaryote, in theory, it has the cellular machinery capable of synthesising or modify eukaryotic genes (unlike prokaryotes such as *Escherichia coli* currently used in research and industry.

4 Algaenomics: from bench to bank

Driven by photosynthesis, diatoms store solar energy by converting carbon dioxide (CO_2) into carbohydrates, lipids, proteins, and other cellular components. They are sunlight-driven cell factories that convert carbon dioxide into potential biofuels, foods, feeds and high-value bio-actives (Chisti, 2007). *Phaeodactylum tricornutum* is able to synthesise a number of industrially relevant molecules using photosynthesis as the energy provider, whose applications include; in aquaculture as feed in e.g. bivalve, echinoderm, crustacean and fish hatcheries, as biomass for biofuels, pharmaceuticals, nutraceuticals, nanotechnology, and bioremediation industries. The bioactive molecules utilised in these industries are described below.

4.1 Fatty acids

P. tricornutum biomass contains, on average, 18% lipids (Kates and Volcani, 1966; Rebolloso-Fuentes *et al.*, 2001). The lipid component consists of the following lipid classes; monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulphoquinovosyldiacylglycerol (SQDG), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylinositol (PI), triacylglycerol (TAG), and a small number of unidentified lipids (Arao *et al.*, 1987). Attached to lipids within each class are any one of the many fatty acids, which include palmitic acid (16:0), palmitoleic acid (16:1n-7) eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA;

22:6n-3) (Siron *et al.*, 1989). Plant-derived alpha-linolenic acid (ALA; 18:3n-3), and marine-derived EPA and DHA, colloquially known as omega-3 polyunsaturated fatty acids (PUFAs), are important in human nutrition with health benefits such as reduced cardiovascular morbidity and mortality, reduced risk of premature births and improved cognitive and behavioural development of the foetus, and benefiting patients with atherosclerosis, hypertension and neurological/neuropsychiatric diseases (Yashodhara *et al.*, 2009). The main source of omega-3 PUFAs in human diet is through the consumption of oily fish species such as salmon, mackerel and herring (Strobel *et al.*, 2012), which accumulate these essential fatty acids through their diet. Fisheries are producing the maximum fish stocks per year required to meet demand for human consumption, as well as supplying feed for industrial fish farms and fish oil supplements, exerting great pressure on the ocean ecosystem (Dulvy *et al.*, 2003). Furthermore, South American anchovy fisheries are the main suppliers of crude EPA and DHA and it is predicted that they will no longer be able to meet market demand (Ismail, 2010). In 2013, the wholesale price of algal-derived omega-3 PUFAs was between $80 and $160, which was higher than the wholesale price of fish-derived omega-3 PUFAs (Borowitzka, 2013). Phytoplankton, including diatoms, are the primary synthesisers of omega-3 PUFAs, and other organisms further up the marine food web only accumulate them through their diet. *P. tricornutum* has the ability to synthesise EPA at a high proportion of the total fatty acid, constituting between 20% and 40% of the total fatty acid content (Siron *et al.*, 1989; Rebolloso-Fuentes *et al.*, 2001; Fajardo *et al.*, 2007), and its high maximum specific growth rate, make it a promising sustainable alternative source of omega-3 PUFAs (Lenihan-Geels *et al.*, 2013). The issue of traceability will also not be an issue with diatom-

derived omega-3. Desbois *et al.* identified EPA as the bioactive molecule with antibacterial characteristics excreted by *P. tricornutum* that is active against a range of Gram-negative and Gram-positive bacteria including multidrug-resistant Staphylococcus aureus (MRSA) which is of increased concern in healthcare institutions worldwide (Desbois *et al.*, 2009).

4.2 Sterols

Sterols are a class of lipids that are an essential building block of eukaryotic cell membranes, controlling membrane fluidity and permeability. They are also precursors for a number of hormones and bioactive secondary metabolites (Piironen *et al.*, 2000). Unlike animals, which have one main type of sterol, cholesterol, plants can produce a diverse group of complex sterols collectively known as phytosterols. Phytosterols are products of the isoprenoid pathway – a dedicated pathway to sterol synthesis in photosynthetic plants occurs at the squalene stage through the activity of squalene synthetase (Piironen *et al.*, 2000).

The main types of phytosterols are campesterol (a 24-methyl sterol), stigmasterol and sitosterol (two 24-ethyl sterols), and brassicasterol, and their less abundant saturated derivatives called stanols (John *et al.*, 2007; Dufourc, 2008).

Cholesterol is an amphipathic molecule obtained by cells either from endogenous (biosynthetic) or exogenous (dietary) sources. Due to its amphipathic characteristic, cholesterol is optimally transported in the blood plasma within lipoproteins; mainly low-density (LDL) and high-density lipoproteins (HDL) (Johnson *et al.*, 1991). LDLs are the primary carriers of cholesterol and when continuously elevated in the blood plasma has been

implicated in the initiation and progression of atherosclerosis (thickening of artery walls), leading to myocardial infarctions, stroke, and heart failure (Brunzell *et al.*, 2008). HDLs, on the other hand, is involved in the process of 'reverse cholesterol transport' whereby cholesterol is transported to the liver for catabolism and removal from the body (Johnson *et al.*, 1991). However, raising HDL levels alone does not lower the risks associated with high blood plasma cholesterol because the HDL levels affect the concentration of all other lipoproteins (Singh *et al.*, 2007).

Strategies for lowering cholesterol in blood plasma include dietary restrictions limiting exogenous sources of cholesterol, and the intake of drugs known as 'statins' which inhibit cholesterol biosynthesis in humans. Phytosterols are structurally related to cholesterol, differing in their side-chain configuration, which have experimentally been shown to be effective cholesterol-lowering agents (Ling and Jones, 1995). Although the exact mechanism of how phytosterols decrease blood plasma cholesterol levels is unknown, several theories exist: (1) cholesterol is the intestine is precipitated into a non-absorbable state; (2) displacement of cholesterol by phytosterols in 'micelles' required for the cholesterol to pass through the intestines and into the blood plasma (Moreau *et al.*, 2002).

The utilisation of phytosterol-based treatments in the fight against cholesterol-associated diseases has been present since the 1950s when initial studies showed that although plant-derived sitosterol was poorly absorbed by rabbits, it did prevent cholesterol absorption if present in excess (Pollak, 1953). In 1957, a sitosterol-derived drug called Cytellin was introduced to the market by Eli Lilly but was quickly removed from the market when its poor solubility and bioavailability deemed it ineffective

unless taken at very high doses (up to 50g a day) (Moreau *et al.*, 2002). With the discovery of 'statins', and the issues encountered in the 1950s, it wasn't until the 1990s when a revival of phytosterol-based treatments began to receive attention again. This can mainly be attributed by the work of The Raisio Group based in Finland who solved the issue of poor solubility and bioavailability by esterifying a specific type of phytosterol, stanols, with fatty acids to form a crystalline powder (Miettinen *et al.*, 1996). The resulting powder can be added to a wide range of foods. The Raisio Group's first phytosterol-containing product was Benecol margarine (Raisio Group, Raisio, Finland) that was demonstrated in subsequent clinical studies to conclusively lower LDL cholesterol even at 2-3g per day doses (Moreau *et al.*, 2002; Luo *et al.*, 2015). However, various studies both proved and disproved a theory that phytosterol or stanol margarines reduced beta-carotene and alpha-tocopherol levels in the blood plasma, as well as studies reporting association between increased plasma levels of phytosterols and increased risk of coronary heart disease (further references in Kritchevsky and Chen, 2005).

Phytosterols are also applicable to other nutraceutical and pharmaceutical markets as bioactive molecules demonstrating anti-inflammatory, anti-oxidant, anti-cancer, and anti-diabetic responses (further references available in Moreau *et al.*, 2002 and Luo *et al.*, 2015). Recent studies confirmed that phytosterols are able to cross the blood-brain-barrier and can accumulate in the brain (Vanmierlo *et al.*, 2012). Mice fed with diets enriched with the phytosterol stigmasterol showed protection mechanisms in the brain, proposing that the addition of phytosterols containing mainly stigmasterol might be beneficial in preventing Alzheimer's disease (Burg *et al.*, 2013).

Despite the increase in research and commercial interest of phytosterol compounds very little is known about the biochemistry of diatom-derived sterols. Rampen *et al.* characterised the sterol composition of over 100 diatom cultures, and found 44 unique sterols with 11 forming the majority (Rampen *et al.*, 2010). In fact, *P. tricornutum* was shown to accumulate C-28 sterols, mainly brassicasterol and campesterol (Rampen *et al.*, 2010; Fabris *et al.*, 2014), which are both bioactive molecules displaying a number of health benefits as highlighted above. Brassicasterol has been shown to have cholesterol-lowering properties (Demonty *et al.*, 2007), and campesterol demonstrates cholesterol-lowering, anti-cancer (Kazłowska *et al.*, 2013), anti-oxidant (Lv *et al.*, 2015) and anti-angiogenic (Choi *et al.*, 2007) properties.

As a summary, with the global phytosterol market in 2013 standing at approximately $300 million and growing about 7 to 9% per annum (Borowitzka, 2013), diatom-derived sterols have the potential to form part of that market in both nutraceutical and pharmaceutical applications. However, this potential remains relatively unexplored.

4.3 Biofuels

Biomass is the biological material derived from living, or recently living organisms. The alluring prospect of utilising plant and algal biomass as a source of biofuels is that, in comparison with other renewable energy sources such as wind, tidal, and solar, they allow solar energy to be stored and exploited to produce biofuels. Diatoms can give rise to several different types of renewable biofuels, including biodiesel and biohydrogen (Kapdan and Kargi, 2006) and biogas from the biomass (Spolaore *et al.*, 2006). The

advantages of using diatoms instead of terrestrial crops, such as those used for first and second-generation biofuels, are that they represent much higher potential biofuel yields per (Chisti, 2007), can be harvested throughout most of the year, thus giving a regular supply of biomass, and do not require freshwater (Chisti, 2008). More importantly, it will not compete with the land needed for food crops, therefore overcoming the food-versus-fuel predicament presented by most first and second-generation biofuels. Chisti showed that *P. tricornutum* produces between 20 – 30% oil (% dry weight) which can be converted to biofuels (e.g. biodiesel by transesterification) that can be directly used in existing engines and transport infrastructure (Chisti, 2007; Scott *et al.*, 2010).

On the 23rd of February 2012, the current President of the United States of America, Barack Obama remarked on the energy crisis by stating that:

"We're making new investments in the development of gasoline and diesel and jet fuel that's actually made from a plant-like substance – algae. … If we can figure out how to make energy out of that, we'll be doing all right. Believe it or not, we could replace up to 17 percent of the oil we import for transportation with this fuel that we can grow right here in the United States."

- (excerpt from The White House Office of the Press Secretary)

Despite these positive remarks, and all the aforementioned advantages, algal- or diatom-derived biofuels are not a commercial reality simply because they are not economically feasible. The cost of production and thus the market price of algal biofuels simply cannot compete with the comparably low cost of fossil fuel prices (Norsker *et al.*, 2011).

4.4 High-value compounds

Algal-derived high-value compounds, however, have shown commercial promise, based on the high market price of these compounds. High-value compounds include carotenoids contained in the chloroplasts of diatoms such as beta-carotene, fucoxanthin, diatoxanthin and diadinoxanthins (Pennington *et al.*, 1988). The market price of beta-carotene is between $300 and $700 per kilogram (Markou and Nerantzis, 2013). Fucoxanthin the main carotenoid found in the thylakoids of *P. tricornutum* where it forms light harvesting complexes with Chlorophyll a (Owens and Wold, 1986).

In nature, fucoxanthin contributes to more than 10% of the total carotenoids (Matsuno, 2001). In human health, fucoxanthin can act as an antioxidant, chemo-preventive agent against obesity, cancer, inflammation, angiogenesis, and diabetes, and has protective properties over numerous organs including the skin, liver, brain blood vessels, bones and eyes (Peng *et al.*, 2011). In 2012, Kim *et al.* confirmed that *P. tricornutum* is indeed a rich source of fucoxanthin (15.71 mg per gram of freeze-dried *P. tricornutum*, atleast 10 times more abundant than in macroalgae) that can be easily extracted with ethanol (Kim *et al.*, 2012). The wholesale market price of fucoxanthin was discussed and announced in February 2015, with a copy of the report going from approximately £1000 up to £1800 (see the following website for the price and purchase links http://www.researchandmarkets.com/research/t3hksq/global).

4.5 Silaffins

Silaffins are matrix peptides and proteins which, along with intracellular silicic acid, form the amorphous hydrated silicon dioxide cell wall of diatoms (Kröger *et al.*, 1999). Silaffins are comprised of Long Chain Polyamines (LCPAs) that precipitate silica forming nano- and micro-particles shaped into spheres and plates containing many pores. Silaffin biotechnology can be applied in a range of fields, including molecular separation, chemical probing, implantology, nanoelectronics and microfluidics (Pamirsky and Golokhvast, 2013). Unlike most diatoms, which have the distinct ability to precipitate soluble silicic acid to form a silica cell wall, *P. tricornutum* has a poorly silicified cell wall and therefore does not have an obligate requirement for silicic acid (Montsant *et al.*, 2005; Martino *et al.*, 2007), making *P. tricornutum* not the first choice for this application. However, due to the in-depth knowledge of its genome, we could use *P. tricornutum* as a model organism during the initial research stages.

4.6 Bioremediation

Heavy metals have been used in a wide range of applications for thousands of years, from lead in water pipes, mercury in early medicine, and cadmium in Claude Monet paintings and more recently in rechargeable batteries. Heavy metal pollution in coastal and estuarine waters due to human activities poses a significant threat to human health with modern medicine attributing a number of human ailments, such as carcinogenesis, neurological and neuropsychiatric disease, and cardiovascular disease to an excess exposure to such heavy metals (Jarup, 2003). When exposed to

heavy metals, diatoms synthesise phytochelatins (PC) which have a general structure (gamma-glutamic acid-cysteine)n-glycine, with n values generally ranging between 2 and 6 (Grill *et al.*, 1985). *P. tricornutum* showed an increased accumulation of PC within a few hours from cadmium, lead or copper exposure (Morelli and Scarano, 2001, 2004). Morelli extended this finding and successfully used *P. tricornutum* to assess metal pollution in aquatic environments based on PC production (Morelli and Fantozzi, 2008). Not only can diatoms be used as potential biomarkers to assess the extent of the heavy metal pollution, they have the potential to be effectively used as bioremediation agents, but the specificity and speed of bio-absorption should be evaluated in further studies (Lebeau, 2003). Another bioremediation application for diatoms is in the treatment of wastewater rich in phosphorus and nitrogen. Craggs *et al.* showed the successful removal of over 80% of ammonium and 100% of orthophosphates from diluted sewage effluent by *P. tricornutum* and a cyanobacterium, *Oscillatoria* sp. (Craggs *et al.*, 1995, 1997). The characteristic of 'sweeping up' excess phosphorus and nitrogen in wastewater could form a fundamental element in Integrated Multitrophic Aquaculture (IMTA) where the waste from aquaculture ponds could provide the necessary phosphorus and nitrogen for diatom cultures which in turn can be used as feed in the system as demonstrated by Lefebvre *et al.* (1996).

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2 Large-scale cultivation

exploring the upscaling of two strains of ***Phaeodactylum tricornutum***

"Men moet de huid niet verkopen voordat de beer geschoten is."

(translated from Dutch: *"Don't sell the fur until the bear has been shot"*)

– Dutch proverb

Summary

The research project was conducted as part of the Marie Curie Initial Training Networks project, AccliPhot, funded by European Commission. AccliPhot aims to investigate and understand short-term acclimation mechanisms to changes in light conditions in photosynthetic organisms. The investigated scales encompass the molecular signalling mechanisms inducing the responses, the implications for metabolism, and whole-organism behaviour, in particular growth and biomass yield. The aim is to employ this understanding to optimise and upscale biotechnological exploitation of photosynthetic microalgae for the production of biofuels and high-value bio-actives. The contribution of this PhD research project to the overall aims of AccliPhot is the feasibility of large-scale cultures and working towards the systematic understanding of the population-level response in microalgal cultures at different scales providing important data for model development by other project partners.

A systematic preliminary approach is described in this Chapter where different accessions of *P. tricornutum* were cultivated in culture vessels of different sizes and dimensions under both controlled as well as natural outdoor conditions, and their growth characteristics monitored.

1 Introduction

Driven by photosynthesis, *Phaeodactylum tricornutum* strains are capable for synthesising a number of industrially relevant molecules, applicable to various industries. In aquaculture, *P. tricornutum* is used as feed in e.g. bivalve, echinoderm, crustacean and fish. *P. tricornutum* biomass contains, on average, 18% lipids making it a potential candidate for biofuel production (Rebolloso-Fuentes *et al.* 2001; Kates & Volcani 1966). Furthermore, *P. tricornutum* has the ability to produce the poly-unsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in high proportions of the total fatty acid content (Fajardo *et al.* 2007; Siron *et al.* 1989; Rebolloso-Fuentes *et al.* 2001). Marine-derived EPA and DHA, colloquially known as omega-3 PUFAs, are important in human nutrition with a number of health benefits (Yashodhara *et al.*, 2009). *P. tricornutum* is therefore an ideal source of omega-3 PUFAs for the pharma- and nutraceutical industries.

Fucoxanthin is the main carotenoid found in the thylakoids of *P. tricornutum* where it forms light harvesting complexes with Chlorophyll a. Up to 15.71 mg fucoxanthin per gram of freeze-dried *P. tricornutum* can be produced, at least 10 times more abundant than in macroalgae, that can be easily extracted with ethanol (Owens and Wold, 1986; Kim *et al.*, 2012). In human health, fucoxanthin can act as an antioxidant, a chemo-preventive agent against obesity, cancer, inflammation, angiogenesis, and diabetes, and has protective action on numerous organs including the skin, liver, brain blood vessels, bones and eyes (Peng *et al.*, 2011).

The production of microalgal biomass requires relatively simple photosynthetic growth conditions: light, carbon dioxide, water and inorganic salts. The system must also include a means of agitation or mixing of the culture to prevent the settling of the microalgal cells, the elimination of thermal stratification (temperature layering effect that occurs in water), distribution of carbon dioxide and inorganic salts, removal of the photosynthetically produced oxygen, and the enhancement of light utilisation efficiency (Terry and Raymond, 1985). There are two main land-based cultivation methods implemented for the large-scale cultivation of microalgae; raceway ponds and photobioreactors. A comparison summary can be found in Table 1.

Table 1: Comparison of raceway ponds and photobioreactors (derived from Pulz, 2001)

CULTURE SYSTEM	RACEWAY PONDS	PHOTOBIOREACTORS
Required space	High	For photobioreactor itself, low
Water loss	Very high, may also cause salt precipitation	Low
CO_2 loss	High, depending on pond depth	Low
Oxygen concentration	Usually low enough because of continuous spontaneous outgassing	Build-up in closed system requires gas exchange devices (O_2 must be removed to prevent inhibition of photosynthesis and photo oxidative damage)
Temperature	Highly variable, some control possible by pond depth	Cooling often required (by spraying water on photobioreactor or immersing tubes in cooling baths)
Shear	Usually low (gentle mixing)	Usually high (fast and turbulent flows required for good mixing, pumping through gas exchange devices)
Cleaning	No issue	Required (wall-growth and dirt reduce light intensity), but causes abrasion, limiting photobioreactor life-time
Contamination risk	High (limiting the number of species that can be grown)	Medium to low
Biomass quality	Variable	Reproducible
Biomass concentration	Low, between 0.1 and 0.5g/l	High, generally between 0.5 and 8g/l
Production flexibility	Only a few species possible, difficult to switch	High, switching possible
Process control and reproducibility	Limited (flow speed, mixing, temperature only by pond depth)	Possible within certain tolerances
Weather dependence	High (light intensity, temperature, rainfall)	Medium (light intensity, cooling required)
Start-up	6 – 8 weeks	2 – 4 weeks
Capital costs	High ~ US$100000 per hectare	Very high ~US$250000 to US$1000000 per hectare (photobioreactor plus supporting systems)
Operating costs	Low (paddlewheel, CO_2 addition)	Higher (CO_2 addition, oxygen removal, cooling, cleaning, maintenance)
Harvesting costs	High, species dependent	Lower due to high biomass concentration and better control over species and conditions

1.1 Raceway ponds

Raceway ponds are simple open-air microalgal biomass cultivation systems which have been used for the large-scale cultivation of microalgae since the 1950s. They are typically made of a closed loop recirculation channel that is typically 0.3m deep. The mixing (and circulation) is achieved by the implementation of a paddlewheel (see Figures 1 and 2) which must operate continuously to prevent sedimentation. Broth is harvested behind the paddlewheel. The building materials utilized for raceway ponds are concrete, compact earth, and may be lined with white plastic (Chisti, 2007).

As carbon dioxide is the carbon source for algae, its sequestration from the air into the culture limits the growth rate. Other major bottlenecks of raceway ponds are the lack of temperature control (any cooling is only achieved by evaporation, and any heating will add to the production cost), and the susceptibility to contamination by other organisms including other algal strains that might out-compete the desired species (Borowitzka, 1999).

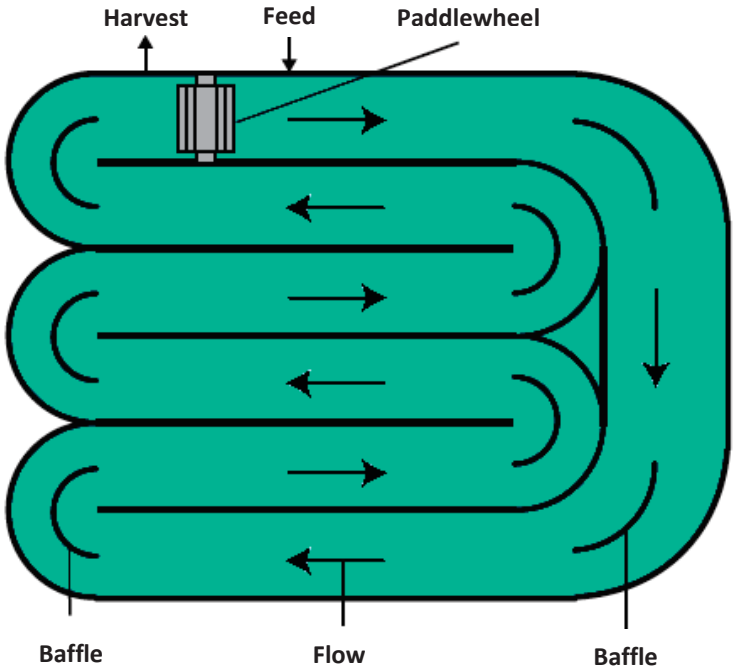


Figure 1: A schematic aerial view of a raceway pond (modified from Chisti, 2007)

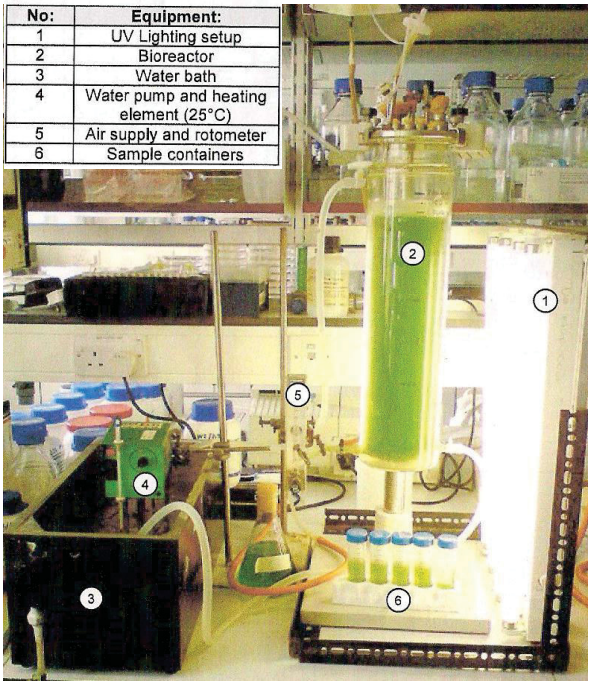
Figure 2: Photograph of a 250m^2 open raceway ponds (taken November 2013; Application Centre for Renewable Resources (ACRRES; Wageningen University initiative) in Lelystad)

1.2 Photobioreactors

Photobioreactors are closed microalgal biomass cultivation devices that allow for the production of a monoseptic culture which is fully isolated from a potentially contaminating environment (Grima and Fernández, 1999). Although there are a number of photobioreactor configurations available, they all have the same basic principles and parameters. The main parameter that affects photobioreactor design is provision for light penetration; i.e. high surface-to-volume ratio, crucial if one wants to improve the photosynthetic efficiency. In order to achieve a high surface-to-volume ratio, several designs have been developed which can be grouped into three basic types; tubular, flat-plate and fermenter-type (Carvalho *et al.*, 2006). Tubular and flat-plate photobioreactors are specifically designed for efficient sunlight harvesting and discussed in Figures 3 – 6.

A

No:	Equipment:
1	UV Lighting setup
2	Bioreactor
3	Water bath
4	Water pump and heating element (25°C)
5	Air supply and rotometer
6	Sample containers



B

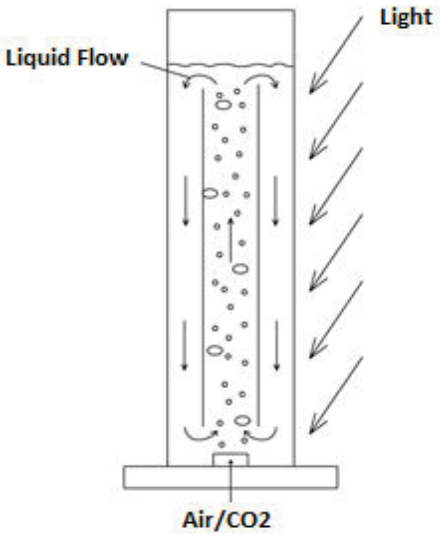


Figure 3: A modified chemostat setup as an airlift bioreactor (A) and a schematic airlift photobioreactor (B). Vertical Tubular Reactors (VTRs) are simple airlift and bubble columns which are usually constructed from transparent glass or plastic (polyethylene) vertical tubing, thus allowing for sufficient light penetration. CO_2 is supplied via bubbling, which also provides mixing and the efficient removal of 0_2 (Chisti, 2007; Carvalho *et al.*, 2006). Photograph and illustration modified from Xu and Weathers, 2009; Bangert, 2010; Moejes, 2011 Masters thesis (unpublished)).

Figure 4: Photograph of a Horizontal Tubular Reactor (HTR) (taken in November 2013; AlgaePARC, Wageningen University). HTRs are composed of horizontal transparent glass or plastic (polyethylene) tubing. Gas transfer takes place in the tube connection or via a dedicated gas-exchange unit, and the angle toward sunlight is particularly adequate for efficient light harvesting (Carvalho *et al.*, 2006).

Figure 5: Helical tubular PBR (HeTRs) in Murdoch University, Australia (Chisti, 2007). HeTRs are an alternative to straight tubular reactors which are composed of a set of polyethylene tubes coiled in an open circular framework, coupled with a gas exchange tower and a heat exchange system; a centrifugal pump drives the culture broth through the long tube to the gas exchange tower (Carvalho *et al.*, 2006; Chisti, 2007).

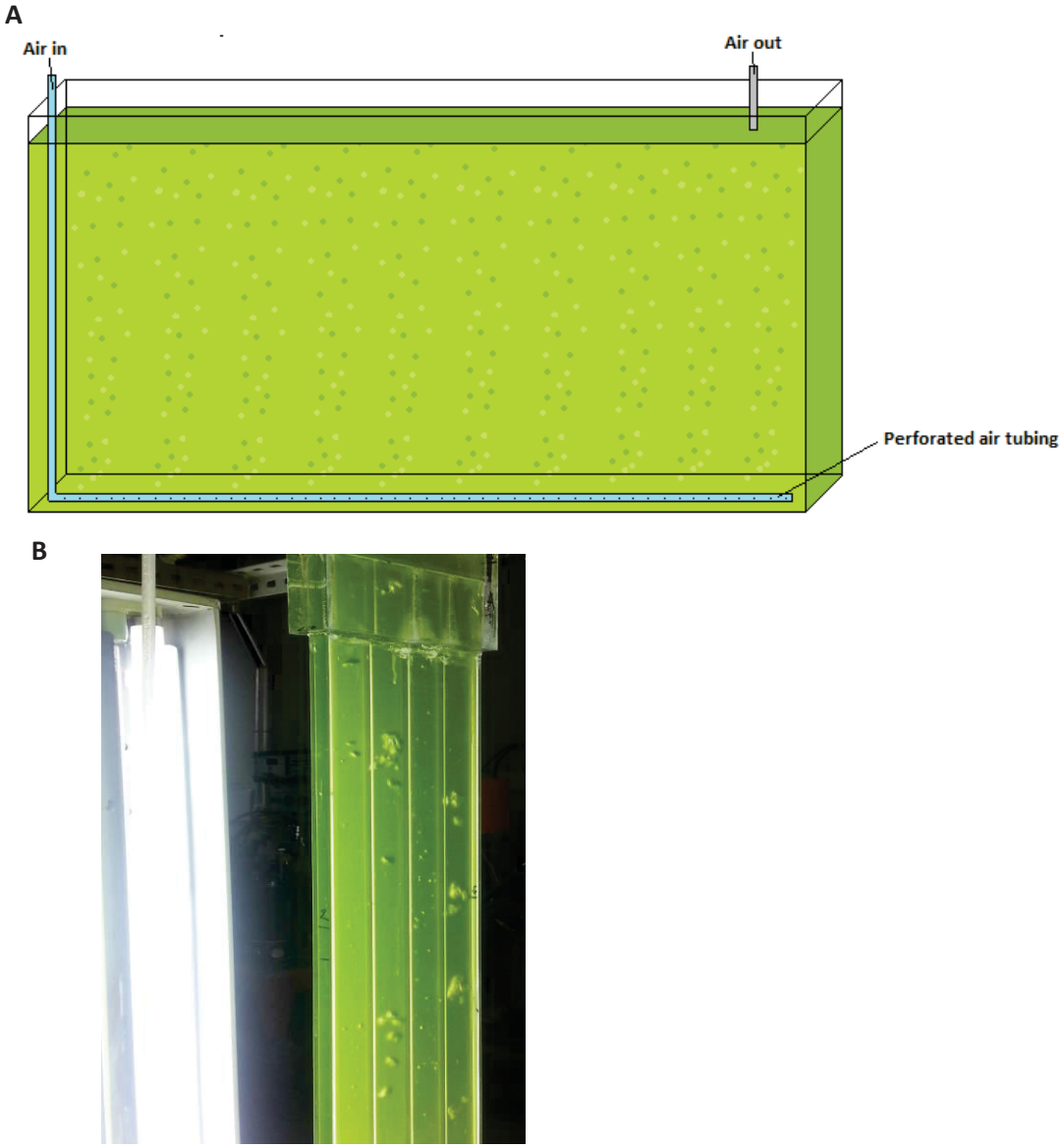


Figure 6: Schematic diagram of a flat-plate photobioreactor. Flat-Plate Reactors (FPRs) are designed to make efficient use of sunlight by constructing narrow panels so as to attain high area-to volume ratios **(A)**. Panels can be constructed from polyethylene bags supported by a metal framework, or from acrylic plastic walls. CO_2 is supplied via bubbling, which also provides mixing and the efficient removal of 0_2. They can either be placed outside and utilize natural sunlight or be placed indoors and utilize an artificial light source **(B)** (picture taken in November 2013, Laboratório Nacional de Energia e Geologia (LNEG), Lisbon, Portugal; schematic diagram based on designs seen at A4F AlgaeFarm).

Irrespective of the cultivation method, they both rely on keeping monocultures of the desired species, especially if the final product is a bioactive molecule for human consumption (Mata *et al.*, 2010). Photobioreactors (PBRs) are closed systems that allow for the production of monoseptic cultures, fully isolated from potential contamination if cultivation protocols are followed correctly (Grima and Fernández, 1999). However, high operational costs of PBRs would increase production costs. The other option, open raceway ponds, are highly susceptible to contamination, and unless the desired species is a halophile or thermophile (Parmar *et al.*, 2011), it is hard to maintain monocultures. Irrespective of the cultivation method, the establishment of unwanted organisms such as amoeba, ciliates, rotifers, bacteria, viruses, and other photosynthetic organisms in microalgal cultures, is a serious obstacle in large-scale microalgae cultivation (Day *et al.*, 2012; Wang *et al.*, 2013). Although much research is carried out in the field of microalgal culture upscaling, very little is known about the identity and characteristics of these organisms, even though they are the root of microalgal culture 'crashes' which lead to loss of biomass, and therefore, loss of revenue.

1.3 Strains of *Phaeodactylum tricornutum*

Phaeodactylum tricornutum strain CCAP 1055/1 (Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, CCMP 632; isolated off the coast of Blackpool, United Kingdom; circa 1956) was the strain selected for sequencing by Bowler *et al.* in 2008 (Bowler *et al.*, 2008). The genome has since then been re-annotated and made available on http://protists.ensembl.org/Phaeodactylum_tricornutum/Info/Index/. Most original research articles in the last decade carried out their work on

P. tricornutum strain CCAP 1055/1; presumably due to the availability of genomic data. However, previous research experiments carried out at Daithi O'Murchu Marine Research Station (DOMMRS) showed favourable growth with another strain, CCAP 1052/1B (personal communication; unpublished work; MABFUEL Emma Seale). Work focused on biomass and fatty acid content. *P. tricornutum* strain CCAP 1052/1B was isolated from English Channel, off the coast of Plymouth, United Kingdom. The CCAP 1052/B accession is a derivative of CCAP 1052/A, the first *P. tricornutum* strain to be isolated that was initially wrongly characterised as *Nitzschia closterium* W. Sm. forma *minutissima* by E. J. Allen circa 1910 (Allen and Nelson, 1910). Subsequent research cited this strain as *Nitzschia closterium* until the 1950s when J. C. Lewin noted a resemblance of this strain to other characterised strains of *P. tricornutum* isolated in the decades prior (Lewin *et al.*, 1958). 1958 saw the reclassification of *Nitzschia closterium* W. Sm. forma *minutissima* as *P. tricornutum*. Clonal isolates of the early CCAP 1052/1A accession (when it was still known as *Nitzschia closterium*) were taken in the 1930s which were able to grow in freshwater (Martino *et al.*, 2007). This isolate was given the strain reference CCAP 1052/1B.

Although the clonal isolate CCAP 1052/1B was able to grow in freshwater, the Culture Collection for Algae and Protozoa at the Scottish Association for Marine Science (Oban, Scotland) grow this accession in F/2 + Si marine media (http://www.ccap.ac.uk/strain_info.php?Strain_No=1052/1B).

2 Experimental procedure

2.1 Strains and culture conditions

All *Phaeodactylum tricornutum* strains were obtained from the Culture Collection of Algae and Protozoa (CCAP) based in Oban, Scotland (http://www.ccap.ac.uk/our-cultures.htm). All strains are obtained non-axenic. Investigated the following strains; **CCAP 1052/1B** (isolated from English Channel, off the coast of Plymouth, United Kingdom; 1930s); and **CCAP 1055/1** (clonal isolate from CCAP 1055/3, 2003; isolated off the coast of Blackpool, United Kingdom; circa 1956; genome fully sequenced in 2008) (Martino *et al.*, 2007; Bowler *et al.*, 2008).

All strains were cultured in Guillard's medium for diatoms (F/2 + Si, (Ryther and Guillard, 1962; Guillard, 1975)) in natural seawater passed through a UV-light filter and a 1 µM filter bag. The filtered natural seawater is then either chemically sterilised with sodium hypochlorite and sodium thiosulphate pentahydrate, or autoclaved at 121°C and 100 kPa (15psi) for 15 minutes. F/2 + Si media was either made de novo as per the recipe (see http://www.ccap.ac.uk/pdfrecipes.htm), or with the use of Cell-Hi F2P powder (http://www.variconaqua.com/). Cell-Hi F2P is Guillard's F/2 medium in powdered form that contains the same concentration of nitrates, phosphates, trace elements and vitamins. 1kg of Cell-Hi F2P powder makes 10,000ltrs of F/2 medium. For diatoms, 30g of sodium metasilicate (silicate source) is added for every 1000ltrs of F/2 medium. This is implemented for

cultures over 10L. Also investigated growth in minimal media with only a source of nitrogen ($NaNO_3$) and phosphorus ($NaH_2PO_4.2H_2O$) at the same concentration as in the F/2 medium recipe provided.

Cultures were kept at 18-20°C in a temperature-controlled room (nicknamed 'The Shed'; see Figure 7) and 24hr light at an average of 132.3 µmol m^{-2} s^{-1} using Phillips TL-D 58W 33-640 M9 fluorescent tube lights. All cultures had a modified aeration system provided by either 2mL or 10mL pipettes, or silicon tubing weighed down by weights (for larger cultures), attached to the main pressurised air supply via 0.2µm air filters (Filter Membrane LifeASSURE PFS020 PTFE 3M).

Figure 7: Photographs of 'The Shed'. Cultivation room built for a previous project, MABFUEL. Room contains a lighting panel, shelving and culturing frame (to hang bags), laminar flow unit, refrigerator for samples, a bag sealer, sink, UV water treatment system, and an air-conditioning system to control the room temperature.

Figure 8: Photograph of the outdoor culturing frame. An outdoor replica of the culturing frame used to hang the bags was built for outdoor experimentation. The culturing frame was placed in the poly tunnel and an air supply system identical to the indoor system implemented.

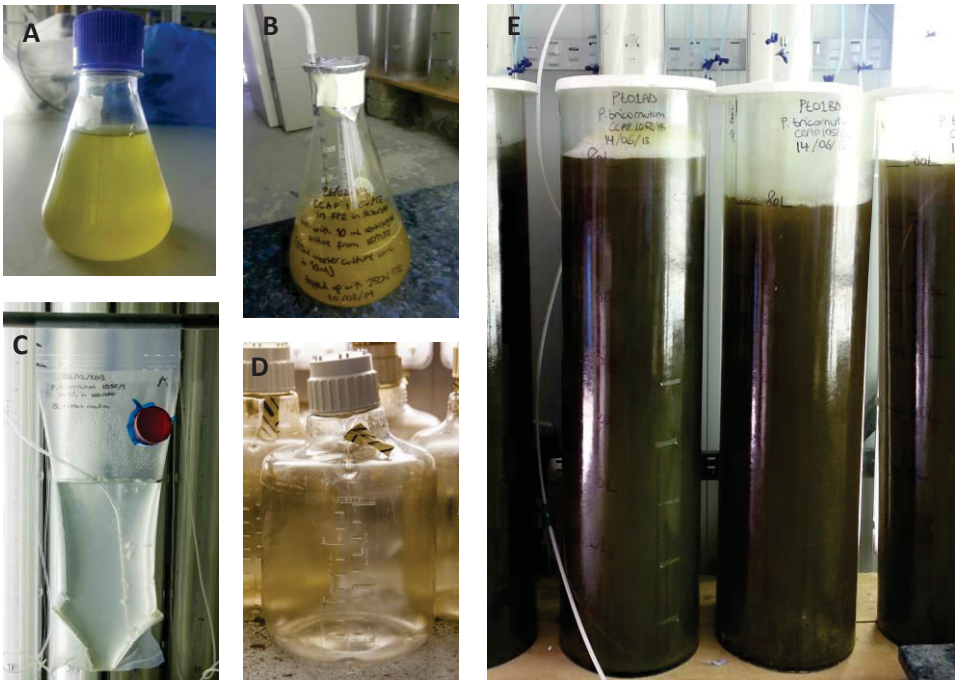


Figure 9: Various cultivation vessels. (A) 250 mL and **(B)** 1 L Erlenmeyer flasks, **(C)** 5 L polyethylene 'V' shaped bags, **(D)** 10 L polyethylene carboys and **(E)** 80 L vertical polyethylene columns.

2.2 Growth measurements

Growth was monitored every 24 to 48 h using a light microscope and carrying out cell counts of each culture in quadruplicates for each culture. During the cell counts the ratios of the four different morphotypes (oval, fusiform, triradiate and cruciform) were recorded, and descriptions of each culture noted. Samples of each culture were subsequently taken using a sterile 10ml syringe and placed in 50ml Falcon centrifuge tubes and placed in -20°C freezer.

2.3 Environmental parameter readings

Light intensity, temperature, salinity, conductivity, dissolved oxygen saturation, luminescent dissolved oxygen, and the pH are all recorded. The

light intensity is recorded prior to the cultures being set up using an Apogee Quantum Meter (http://www.apogeeinstruments.co.uk/mq-100-quantum-meter/) that records the Lux of the artificial light sources. The Lux readings were recorded by inserting the quantum meter into an empty culture vessels to mimic the light penetration inside the flasks and placing it in the exact position of where the cultures will be placed; in the back (closest to light source), middle and front, and an average taken. For outdoor cultures, the Apogee Quantum Meter had a setting that records the light intensity every 30 minutes. The sensor was left in strategic locations that would provide a fair recording of the light intensity for all cultures being cultivated outdoors at any given time. Hach HQ40d Probe Series Field Kit (http://www.hach.com/hq40d-portable-ph-conductivity-dissolved-oxygen-orp-and-ise-multi-parameter-meter/) was used to record the temperature, salinity, conductivity, saturation, luminescent dissolved oxygen, and the pH of all the cultures. There are three separate probes that can be connected to the Hach HQ40d Portable Multi-Parameter Meter. These include an IntelliCAL™ CDC401 Rugged Conductivity Probe for recording the salinity, temperature and conductivity; an IntelliCAL™ LDO101 Rugged Luminescent/Optical Dissolved Oxygen Probe for recording temperature, the dissolved oxygen saturation, and the luminescent dissolved oxygen; and finally an IntelliCAL™ PHC101 Standard Gel Filled pH Electrode. The probes are used by inserting them into the cultures and recording the readings displayed on the Hach HQd Portable Multi-Parameter Meter display. The pH electrode was calibrated in pH4.01, pH7.00 and pH10.01 buffering solutions. Because the probes are intended for field work they are quite large and robust, therefore, care was taken to rinse the probes with ethanol and distilled water before testing each culture. Smaller cultures were moved into the fume cupboard prior to testing.

3 Results

3.1 Effect of culture scale on *Phaeodactylum tricornutum* growth

The temporal evolution of *P. tricornutum* growth of strains CCAP 1055/1 and CCAP 1052/1B was recorded in 0.25 L and 1 L Erlenmeyer flasks, 10 L plastic carboys, and 80 L vertical columns (Figure 9). All cultures were inoculated with master cultures achieving an average culture density of 0.77 x 10^6 cells/mL for CCAP 1052/1B cultures and 0.45 x 10^6 cells/mL for CCAP 1055/1 cultures on Day 1. Maximal cell densities reached by each strain in all four culture scales are represented in Table 2 and the specific growth rates (μ d^{-1}) of *P. tricornutum* at different cultivation scales in Table 3.

Table 2: Maximal cell densities of two strains of *P. tricornutum* at varying vessel scales

Strain	Cultivation scale (cells/mL)			
	0.25 L	**1 L**	**10 L**	**80 L**
CCAP 1052/1B	20.61 x 10^6 ± 8.33	10.33 x 10^6 ± 1.1	7.26 x 10^6 ± 0.34	9.61 x 10^6 ± 1.72
CCAP 1055/1	15.54 x 10^6 ± 1.04	7.54 x 10^6 ± 1.96	5.99 x 10^6 ± 0.19	6.24 x 10^6 ± 0.41

Table 3: Specific growth rates (μ d^{-1}) of two strains of *P. tricornutum* at varying vessel scales

Strain	Specific growth rate (μ d^{-1})			
	$\mu_{0.25L}$	μ_{1L}	μ_{10L}	μ_{80L}
CCAP 1052/1B	1.65 ± 0.26 d^{-1}	0.38 ± 0.11 d^{-1}	0.16 ± 0.02 d^{-1}	0.15 ± 0.01 d^{-1}
CCAP 1055/1	0.57 ± 0.02 d^{-1}	0.35 ± 0.01 d^{-1}	0.21 ± 0.01 d^{-1}	0.13 ± 0.01 d^{-1}

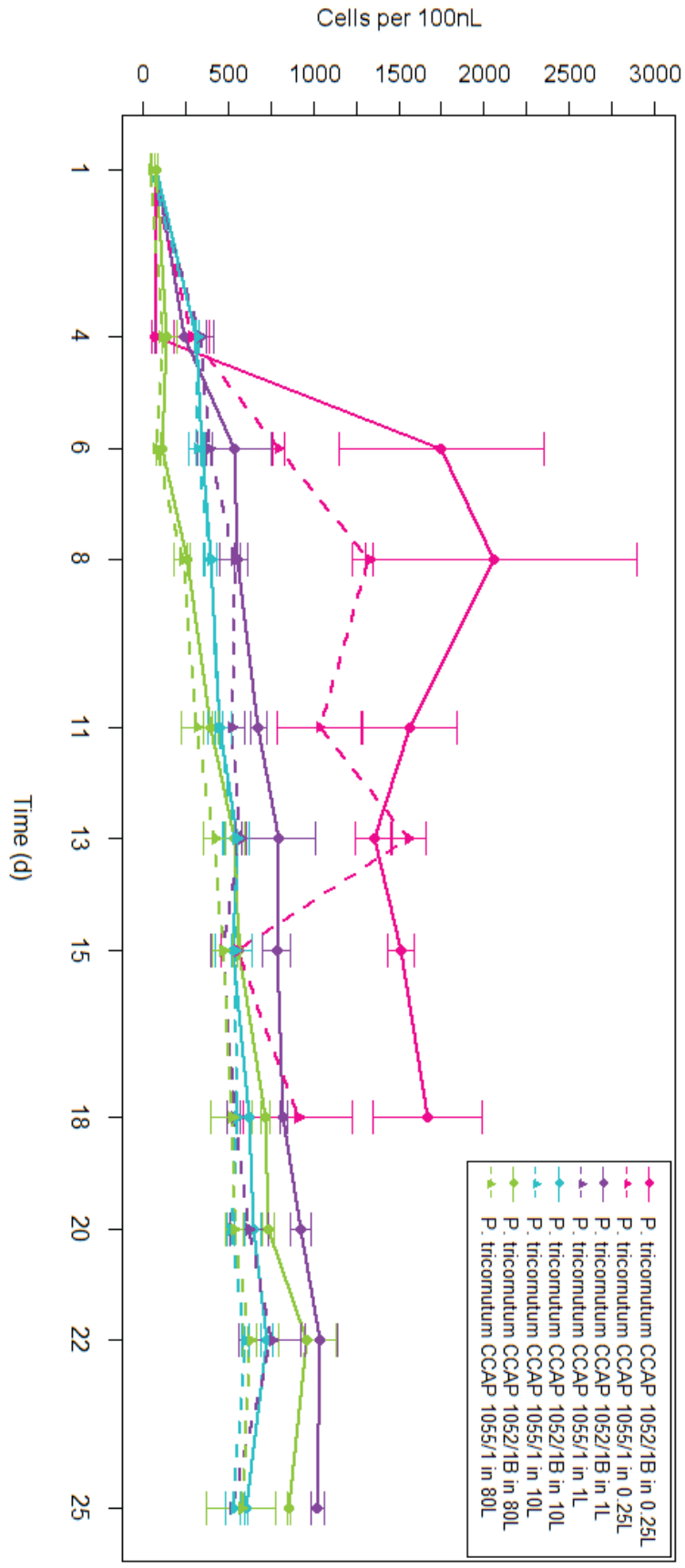


Figure 10: Growth of two strains of P. tricornutum at different cultivation scales

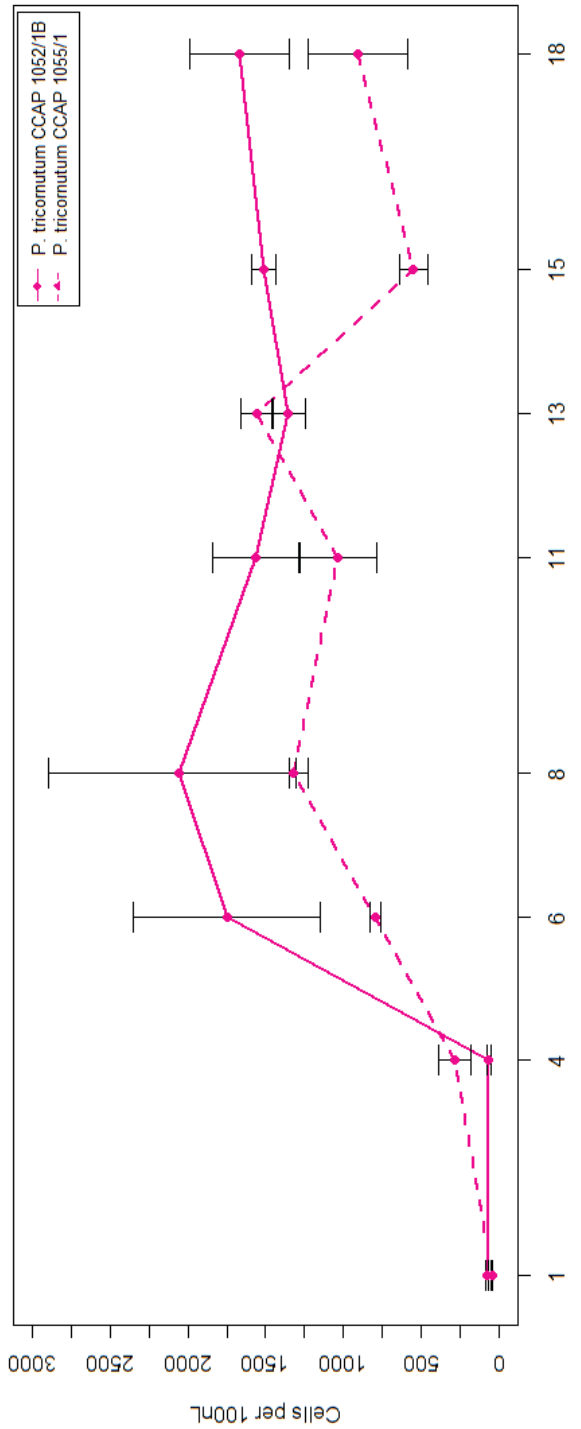


Figure 11: Growth of two strains of P. tricornutum in 0.25L flasks

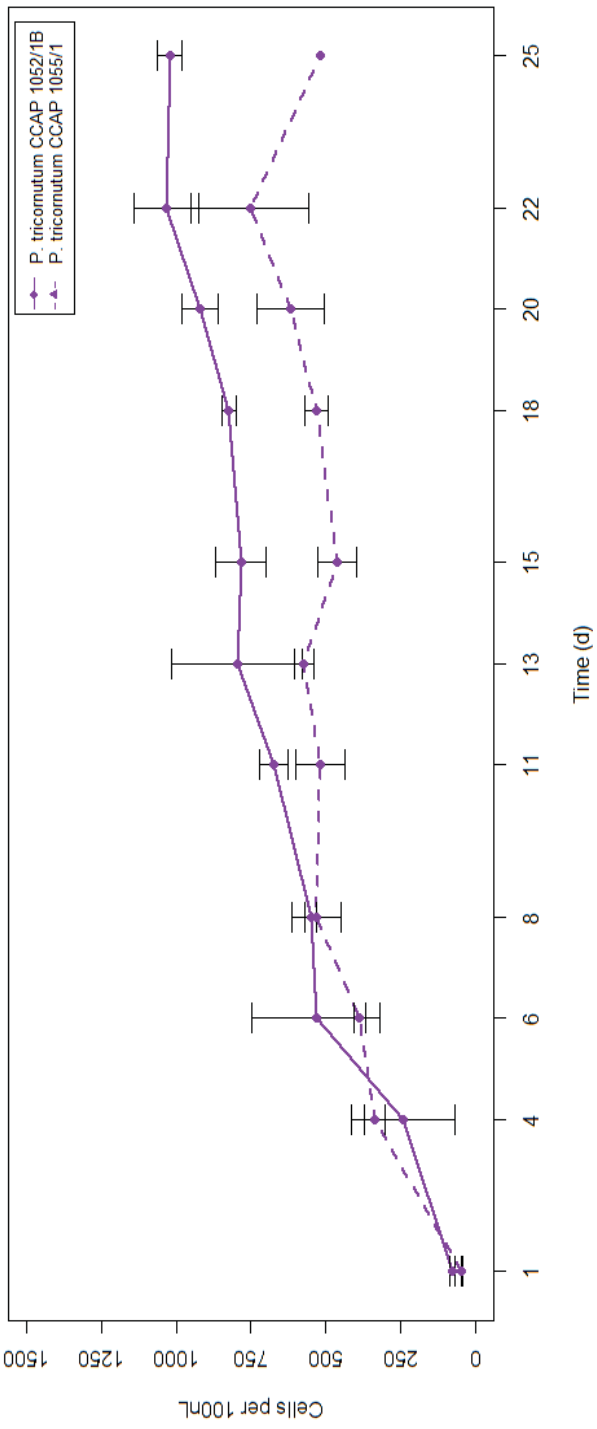


Figure 12: Growth of two strains of P. tricornutum in 1L flasks

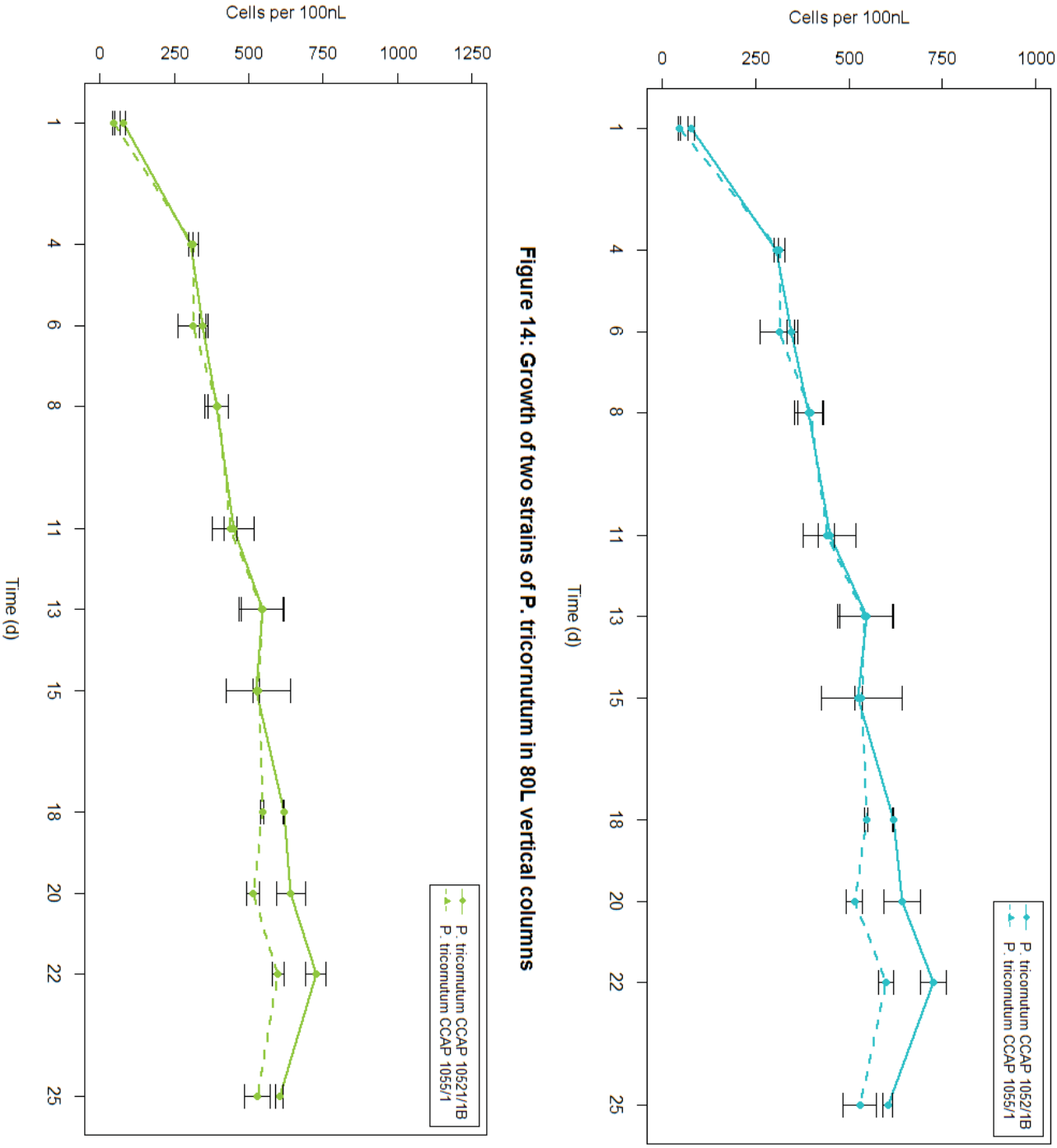


Figure 13: Growth of two strains of P. tricornutum in 10L carboys

Figure 14: Growth of two strains of P. tricornutum in 80L vertical columns

3.2 Growth efficiency of two strains of *Phaeodactylum tricornutum* under differing conditions

Whilst investigating the effect of vessel scale, the efficiency of two strains of *P. tricornutum* were analysed, as illustrated in Figures 10-14. Below are the Student's t-test *p*-values showing the effect of strain on the maximal cell densities and specific growth rate at different scales. These will be discusses further in the Discussions section.

Table 4: Student's t-test *p*-values comparing maximal cell densities of two strains of *P. tricornutum*

Vessel	0.25 L	1 L	10 L	80 L	
***p*-value**	0.3546	0.098	0.0048	0.0299	***p = < 0.05***

Table 5: Student's t-test *p*-values comparing specific growth rates (μ d^{-1}) of two strains of *P. tricornutum*

Vessel	0.25 L	1 L	10 L	80 L	
***p*-value**	0.002	0.6625	0.0179	0.0705	***p = < 0.05***

Given the initial indication of a difference in the growth of the two strains (CCAP 1052/1B and CCAP 1055/1), a more thorough study was carried out. The two strains were cultivated in 5L polyethylene 'V' bags, in complete F/2 + silicate (F/2 + Si) and minimal nitrate and phosphate (N + P) media, and under controlled indoor and unpredictable outdoor conditions. The minimal media contains the same concentrations of nitrate and phosphate present in the F/2 + Si media.

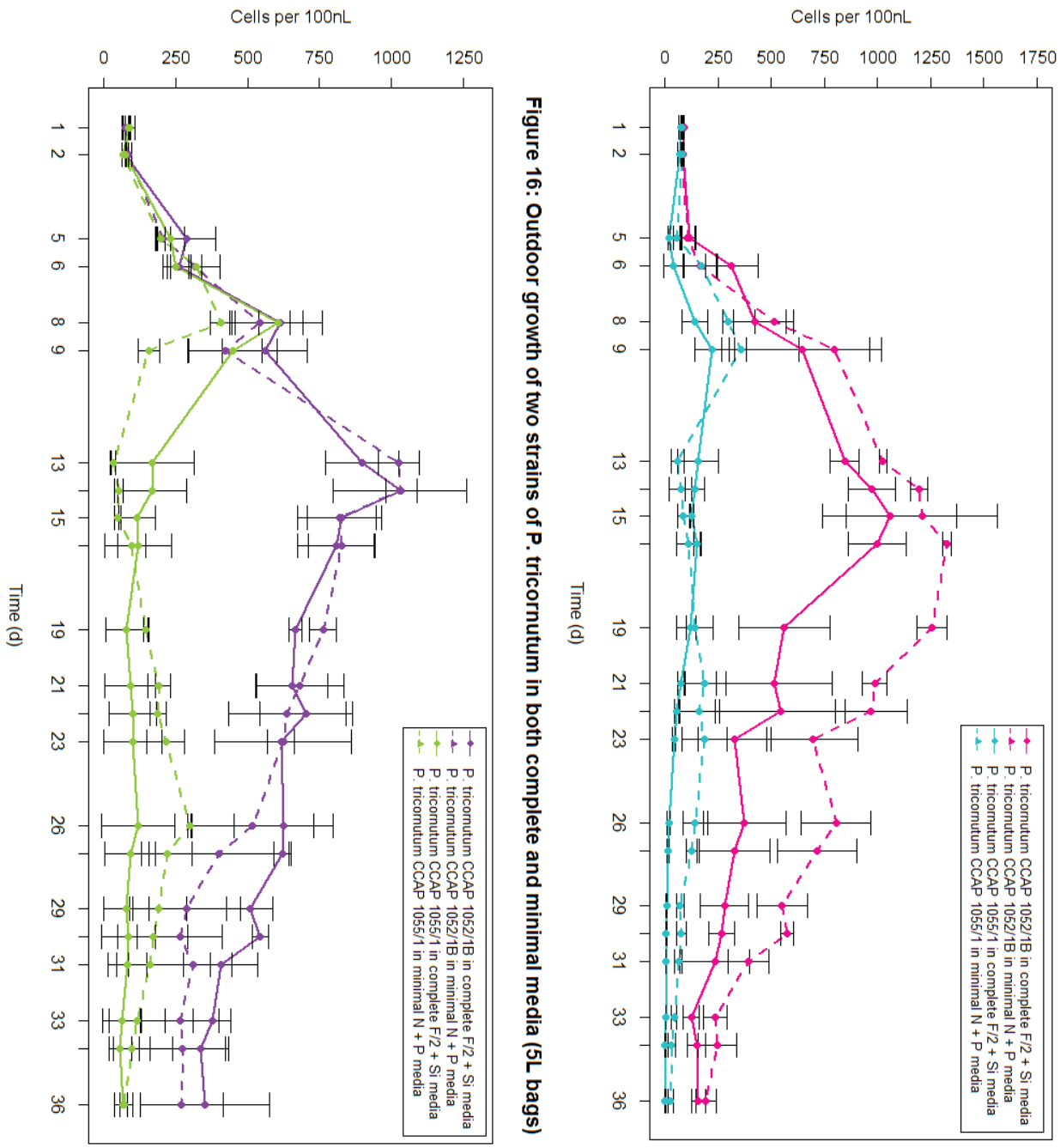


Figure 15: Indoor growth of two strains of P. tricornutum in both complete and minimal media (5L bags)

Figure 16: Outdoor growth of two strains of P. tricornutum in both complete and minimal media (5L bags)

Maximal cell densities reached by each strain in all cultivation conditions are represented in Table 6 and the specific growth rates (μ d^{-1}) of *P. tricornutum* in all cultivation conditions in Table 7. *'Max_indoor'* represents the cultures in complete F/2 + Si media cultivated indoors. *'Min_indoor'* represents the cultures in minimal N + P media cultivated indoors. *'Max_outdoor'* represents the cultures in complete F/2 + Si media cultivated outdoors. '*Min_indoor'* represents the cultures in minimal N + P media cultivated outdoors.

Table 6: Maximal cell densities of two strains of *P. tricornutum* in different conditions

Strain	Cell densities (cells/mL)			
	Max_indoor	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
CCAP 1052/1B	10.57 x 10^6 ± 3.14	13.28 x 10^6 ± 1.93	10.35 x 10^6 ± 0.55	10.30 x 10^6 ± 2.33
CCAP 1055/1	2.19 x 10^6 ± 0.81	3.55 x 10^6 ± 0.28	6.07 x 10^6 ± 1.52	4.07 x 10^6 ± 0.37

Table 7: Specific growth rates (μ d^{-1}) of two strains of *P. tricornutum* in different conditions

Strain	Specific growth rate (μ d^{-1})			
	Max_indoor	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
CCAP 1052/1B	0.43 ± 0.07 d^{-1}	0.41 ± 0.08 d^{-1}	0.33 ± 0.02 d^{-1}	0.22 ± 0.07 d^{-1}
CCAP 1055/1	0.63 ± 0.37 d^{-1}	0.47 ± 0.07 d^{-1}	0.22 ± 0.05 d^{-1}	0.15 ± 0.05 d^{-1}

3.3 Characterisation of two strains of *Phaeodactylum tricornutum* morphology under differing cultivation conditions

Two strains of *P. tricornutum* (CCAP 1052/1B and CCAP 1055/1) were cultivated in minimal and complete media and under either controlled indoor conditions or outdoor conditions, and the ratio of fusiform, triradiate and oval cells elucidated over time.

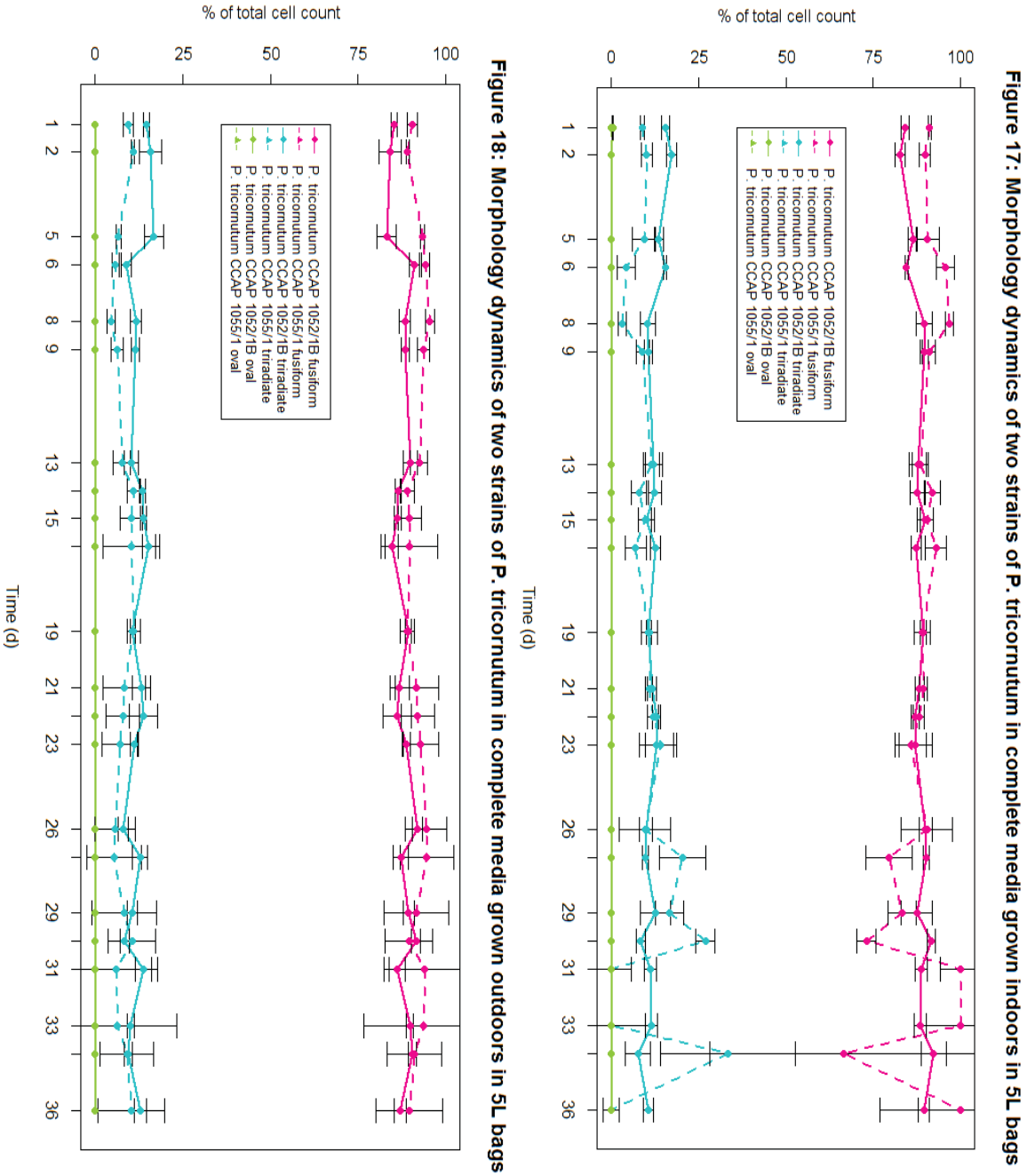


Figure 17: Morphology dynamics of two strains of P. tricornutum in complete media grown indoors in 5L bags

Figure 18: Morphology dynamics of two strains of P. tricornutum in complete media grown outdoors in 5L bags

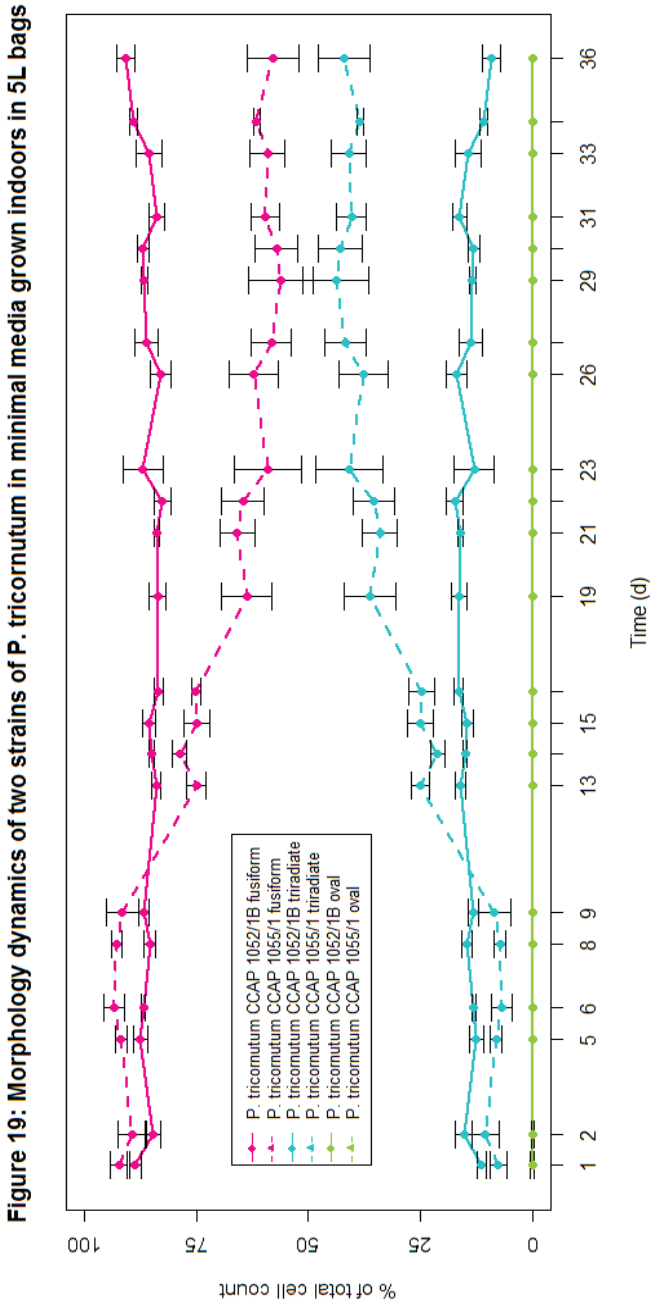


Figure 19: Morphology dynamics of two strains of P. tricornutum in minimal media grown indoors in 5L bags

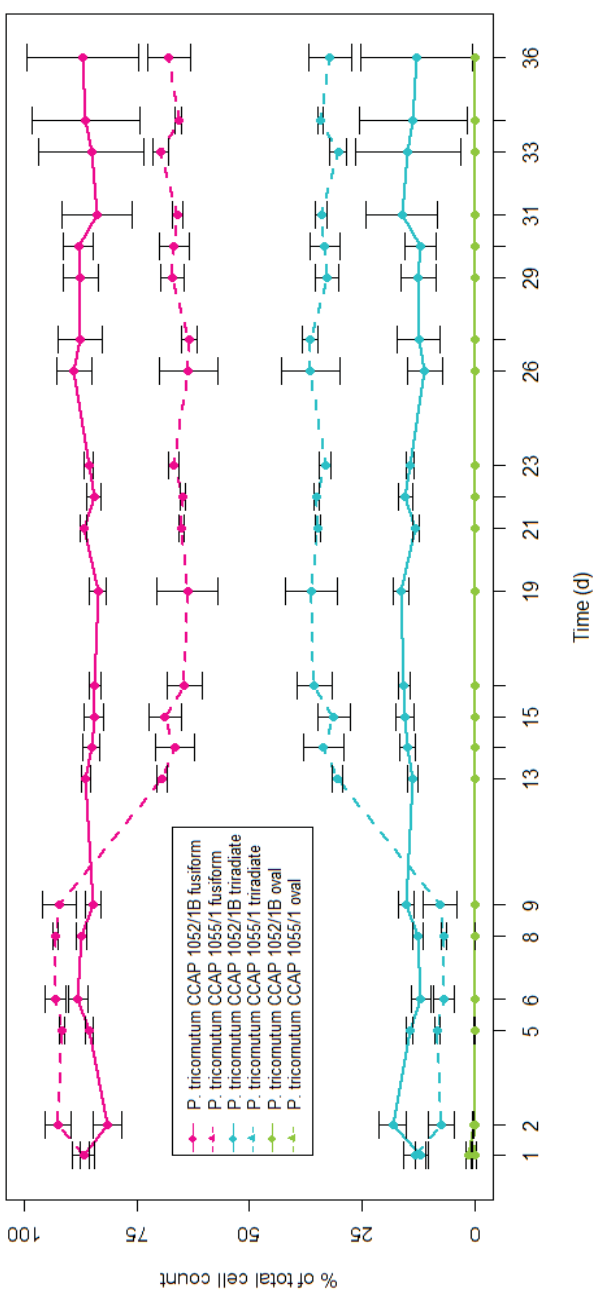


Figure 20: Morphology dynamics of two strains of P. tricornutum in minimal media grown outdoors in 5L bags

4 Discussion

4.1 Cultivation scale and dimension

The null hypotheses adopted are that vessel scale does not affect the maximal cell densities as well as the specific growth rates (μ d^{-1}) of either strain of *P. tricornutum*. A secondary null hypothesis is that the strain also has no effect on the maximal cell density nor the specific growth rates (μ d^{-1}), expanded on later in the Discussion section.

Table 8: Student's t-test for *P. tricornutum* CCAP 1052/1B maximal cell densities in varying vessel scales

	***p*-values**			
	0.25 L	**1 L**	**10 L**	**80 L**
0.25 L		0.1014	0.0501	0.0886
1 L	*0.1014*		0.0099	0.5743
10 L	*0.0501*	*0.0099*		0.081
80 L	*0.0886*	*0.5743*	*0.081*	

***p* = < 0.05**

Table 9: Student's t-test for *P. tricornutum* CCAP 1055/1 maximal cell densities in varying vessel scales

	***p*-values**			
	0.25 L	**1 L**	**10 L**	**80 L**
0.25 L		0.0034	0.0001	0.0001
1 L	*0.0034*		0.2445	0.3237
10 L	*0.0001*	*0.2445*		0.3922
80 L	*0.0001*	*0.3237*	*0.3922*	

***p* = < 0.05**

Table 10: Student's t-test for *P. tricornutum* CCAP 1052/1B specific growth rates (μ d^{-1}) in varying vessel scales

	***p*-values**			
	0.25 L	**1 L**	**10 L**	**80 L**
0.25 L		0.0015	0.0006	0.0006
1 L	*0.0015*		0.0271	0.0226
10 L	*0.0006*	*0.0271*		0.4818
80 L	*0.0006*	*0.0226*	*0.4818*	

p = < 0.05

Table 11: Student's t-test for *P. tricornutum* CCAP 1055/1 specific growth rates (μ d^{-1}) in varying vessel scales

	***p*-values**			
	0.25 L	**1 L**	**10 L**	**80 L**
0.25 L		0.0001	0.0001	0.0001
1 L	*0.0001*		0.0001	0.0001
10 L	*0.0001*	*0.0001*		0.0006
80 L	*0.0001*	*0.0001*	*0.0006*	

p = < 0.05

Tables 8-11 above contain the *p*-values from the Student's t-test comparing the maximal cell densities as well as specific growth rates (μ d^{-1}) of the two strains at different vessel scales. Adopting a 95% confidence interval, we can reject the null hypothesis in the context of specific growth rates (μ d^{-1}); vessel scale does, indeed, have a direct influence on the specific growth rate of two strains of *P. tricornutum*. However, the maximal cell densities reveal mixed results; we can neither reject nor accept the null hypothesis with regards to the cell densities, suggesting other factors may limit the maximal densities that *P. tricornutum*, strains can reach. These factors are addressed below.

Our scale-up approach utilised cultivation vessels based on the fundamentals of photobioreactor design: (1) provision of a high surface-to-volume ratio for light penetration and (2) the provision of carbon dioxide which is coupled to the mixing of the culture to avoid biomass

accumulation on the surface of the culture vessel. We must consider that whilst extended research efforts are in place, we have yet to assimilate reliable scale-up methods for photobioreactors (Grima and Fernández, 1999)**,** but several factors must be taken in consideration when scaling up microalgal cultures which were addressed in the scale-up experiment. These factors could explain why the maximal cell densities were not conclusively affected by the vessel scale.

4.1.1 Effect of light availability on growth

Photosynthesis converts light energy, in this case from the artificial light sources provided, into chemical energy. When light is the only limiting factor, microalgal productivity becomes proportional to the light conversion efficiency (Richmond *et al.*, 2003; Kumar *et al.*, 2010). Provision of a high surface-to-volume ratio is essential for light penetration. The Lambert-Beer Law relates to the light absorption potential of a specific material that has been extended to include light scattering (Klok *et al.*, 2013), where the most dominant effect of light scattering is the increase distance the light must travel through the microalgal culture thus increasing the probability of light absorption (Blanken *et al.*, 2016). Put simply: the further the light must travel, the more it is absorbed or lost.

Furthermore, a model developed by Huisman et al (Huisman *et al.*, 2002) explores the principles of light-limitation in a modified chemostat that operates comparably as a continuous mode photobioreactor. The Huisman model derives the microalgal density $A(t) \geq 0$ using the following Ordinary Differential Equation:

$$\frac{d}{dt}A = H(A) := \underbrace{\frac{\mu_{max}}{z_{max}} \ln\left(\frac{H_p + I_{in}}{H_p + I_{out}}\right) \frac{A}{kA + \mu_{max}}}_{gain} \underbrace{- h_r A - D_r A}_{loss}$$

(Thornton *et al.*, 2010)

Where:

I_{in}	incoming light
I_{out}	outgoing light
K_{bg}	background turbidity
z_{max}	mixing depth
h_r	dilution/outflow
μ_{max}	maximum specific growth rate
H_p	half saturation of photosynthesis
k	specific light attenuation
D_r	specific maintenance (death rate, δ d^{-1})

One characteristic accounted for in the Huisman model is that light intensity decreases with depth due to the 'shading effect' (Ugwu *et al.*, 2008) from other microalgae in the culture. This is comparable to the (Klok *et al.*, 2013) model addressing the probability of light absorption the further the light must travel. In the Huisman model the inverse proportionality with respect to z_{max} implies that increasing the shallowness of the cultivation vessel, increases the microalgal growth. Thornton et al. (Thornton *et al.*, 2010) show that halving the z_{max} has a much greater effect of microalgal growth than doubling the I_{in}.

As the vessel scale increases, the depth of the microalgal culture increase, and thus, the distance light must travel. This is clearly shown in Table 2 and 3 where both the maximal cell densities and specific growth rates decreased as the vessel scale increased. Furthermore, the lowest maximal cell density

was recorded in the 10 L cultures, which can be explained by the lower surface-to-volume ratio presented by the dimensions of the 10 L polyethylene carboys. The vertical 80 L polyethylene columns recorded higher maximal cell densities than the 10 L cultures as well as the *P. tricornutum* CCAP 1055/1 1 L cultures. The dimensions of the 80 L columns allowed for higher surface-to-volume ratios and are capable of reaching maximal cell densities and specific growth rates that are comparable to those recorded in more technologically advanced and expensive narrow tubular photobioreactors (Sánchez Mirón *et al.*, 2002; Ugwu *et al.*, 2008).

4.1.2 Gas-liquid mass transfer: availability of carbon dioxide (CO_2)

As with all photosynthetic organisms, microalgae predominantly use CO_2 as a carbon source. No growth can occur in the absence of CO_2, and an insufficient supply of CO_2 is often the limiting factor in productivity. Natural dissolution of atmospheric CO_2 into the water is not enough; atmospheric CO_2 levels are at approximately 0.0387%, which are not sufficient to support the high microalgal growth rates and productivities needed for large-scale biofuel production. Usual sources of CO_2 for microalgae are atmospheric CO_2, CO_2 from industrial exhaust gases (e.g. flue gas and flaring gas, which typically contain about 4% to 15% of CO_2), and CO_2 chemically fixed in the form of soluble carbonates (e.g. $NaHCO_3$ and Na_2CO_3) (Kumar *et al.*, 2010). Uptake of these inorganic forms of carbon also has the potential to increase the pH within the cultures (Hansen, 2002). Oxygen (O_2) is a product of photosynthesis. Oxygen levels above atmospheric O_2 levels (0.2247 mol O_2 m^{-3}) can inhibit photosynthesis in a number of microalgal species, even when the CO_2 levels remain elevated (Aiba, 1982). Furthermore, elevated levels of O2 coupled with high irradiance can lead to photo-oxidation (Richmond, 1990; Camacho Rubio *et al.*, 1999).

To avoid the accumulation of inhibitory levels of O_2, the cultures must be sufficiently aerated. Aeration was provided to all cultures by bubbling pressurised atmospheric air through all cultures. Based on the maximal cell densities reached and the specific growth rates recorded, light limitation contributed more to the differences between the vessel scales than either CO_2 limitation or photo-oxidation.

Figures 10-14 and Tables 4 and 5 in the Results section highlighted the initial differences in strain efficiency of *P. tricornutum* CCAP 1052/1B and CCAP 1055/1. A significant difference in maximal cell densities reached by both strains is observed as the vessel scale increased. With respect to the specific growth rates, this is not observed. This suggests that there is no clear correlation between vessel scale and strain efficiencies. This is further discussed below.

Ideally, vessels of different volumes but identical dimensions would have provided more insight into the population dynamics of *P. tricornutum* cultures when scaled up.

4.2 Growth efficiency of two strains of *Phaeodactylum tricornutum*

Figures 15 and 16 show a clear difference between the growth efficiency of the two *P. tricornutum* strains (CCAP 1052/1B and CCAP 1055/1), both in minimal (N + P) and complete (F/2 + Si) media, as well as controlled indoor and outdoor conditions. Overall, a greater growth efficiency of the Plymouth isolate (CCAP 1052/1B) when compared to the Blackpool isolate (CCAP 1055/1) is observed.

The null hypothesis adopted is that the media composition or whether the cultures are indoors or outdoors does not affect the maximal cell densities and the specific growth rates (μ d^{-1}) of either strain of *P. tricornutum*. The second null hypothesis is that the strain also has no effect on the maximal cell density nor the specific growth rates (μ d^{-1}).

Table 12: Student's t-test for *P. tricornutum* CCAP 1052/1B maximal cell densities in different conditions

				***p* = < 0.05**
	***p*-values**			
	Max_indoor	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
Max_indoor		0.2718	0.9106	0.9106
Min_indoor	0.2718		0.0647	0.1632
Max_outdoor	0.9106	0.0647		0.9729
Min_outdoor	0.9106	0.1632	0.9729	

Table 13: Student's t-test for *P. tricornutum* CCAP 1055/1 maximal cell densities in different conditions

				***p* = < 0.05**
	***p*-values**			
	Max_indoor	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
Max_indoor		0.0515	0.0175	0.0216
Min_indoor	0.0515		0.0476	0.1242
Max_outdoor	0.0175	0.0476		0.0912
Min_outdoor	0.0216	0.1242	0.0912	

Table 14: Student's t-test for *P. tricornutum* CCAP 1052/1B specific growth rates (μ d^{-1}) in different conditions

				***p* = < 0.05**
	***p*-values**			
	Max_indoor	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
Max_indoor		0.8066	0.0626	0.0202
Min_indoor	0.8066		0.1269	0.0322
Max_outdoor	0.0626	0.1269		0.0639
Min_outdoor	0.0202	0.0322	0.0639	

Table 15: Student's t-test for *P. tricornutum* CCAP 1055/1 specific growth rates (μ d^{-1}) in different conditions

				***p* = < 0.05**
	***p*-values**			
	Max_indoor	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
Max_indoor		0.4847	0.1272	0.0883
Min_indoor	0.4847		0.0079	0.0035
Max_outdoor	0.1272	0.0079		0.1463
Min_outdoor	0.0883	0.0035	0.1463	

Tables 12-15 above contain the *p*-values from the Student's t-test comparing the maximal cell densities as well as specific growth rates (μ d^{-1}) of the two strains under different cultivation conditions. Adopting a 95% confidence interval, we can reject the null hypothesis in the context of specific growth rates (μ d^{-1}); the media as well as whether the culture is indoors or outdoors does, indeed, have a direct influence on the specific growth rate of two strains of *P. tricornutum*. However, the maximal cell densities reveal mixed results. For *P. tricornutum* CCAP 1055/1, the different cultivation conditions does have an impact on the maximal cell density. However, for *P. tricornutum* CCAP 1052/1B the different cultivation conditions does not have an impact on the maximal cell density.

Table 16: Student's t-test *p*-values comparing maximal cell densities of two strains of *P. tricornutum*

				***p* = < 0.05**
Condition	**Max_indoor**	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
***p*-value**	0.011	0.001	0.0101	0.0102

Table 17: Student's t-test *p*-values comparing specific growth rates (μ d-1) of two strains of *P. tricornutum*

				***p* = < 0.05**
Condition	**Max_indoor**	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
***p*-value**	0.39	0.4044	0.0217	0.2106

Tables 16 and 17 conclusively show that the Plymouth isolate (CCAP 1052/1B) reaches greater maximal cell densities regardless of the cultivation conditions when compared to the Blackpool isolate (CCAP 1055/1). However, when comparing the specific growth rates, the Plymouth isolate (CCAP 1052/1B) only has a greater specific growth rate when in complete media and outdoors when compared to the Blackpool isolate (CCAP 1055/1). This suggests that the Plymouth strain is a more robust strain.

4.3 Morphology

P. tricornutum is a highly pleiormorphic pennate diatom capable of changing morphology between fusiform, triradiate and oval forms. This unique characteristic can be attributed to its distinct cell wall that unlike other diatoms is only poorly silicified (Lewin *et al.*, 1958; Borowitzka and Volcani, 1978). However, the physiological significance of the ability to change morphology is unknown (Martino *et al.*, 2007). Previous studies by De Martino *et al.* (2007) showed that the Plymouth strains are either 95% to 100% fusiform (CCAP 1052/1A) or 60% to 75% oval (CCAP 1052/1B). The Blackpool strain is usually also 95% to 100% fusiform. The systematic cultivation of both strains in minimal and complete media and under controlled indoor and varying outdoor conditions in this study, contradict these findings. Tables 18 and 19 contain the average percentage of fusiform, triradiate and oval morphologies elucidated over the 36-day growth period. The null hypothesis is that the percentage of each morphology of either strain of *P. tricornutum* is not affected by the media composition nor whether it is cultivated indoors or outdoors.

Table 18: Average percentage of fusiform, triradiate and oval morphologies of indoor cultures of two strains of *P. tricornutum* in minimal and complete media

Strain	Minimal media			Complete media		
	Fusiform	Triradiate	Oval	Fusiform	Triradiate	Oval
CCAP 1052/1B	85.78 ± 2.09	14.22 ± 2.09	0	88.20 ± 2.33	11.78 ± 2.33	0.02 ± 0.09
CCAP 1055/1	71.84 ± 14.08	25.16 ± 14.08	0	89.30 ± 8.14	10.70 ± 8.14	0

Table 19: Average percentage of fusiform, triradiate and oval morphologies of outdoor cultures of two strains of *P. tricornutum* in minimal and complete media

Strain	Minimal media			Complete media		
	Fusiform	Triradiate	Oval	Fusiform	Triradiate	Oval
CCAP 1052/1B	85.89 ± 1.78	14.03 ± 1.79	0.08 ± 0.29	87.87 ± 2.47	12.17 ± 2.47	0
CCAP 1055/1	73.16 ± 11.74	26.84 ± 11.74	0	91.90 ± 2.10	8.10 ± 2.10	0

Tables 18 and 19, as well as Figures 17-20 show that in both complete and minimal media, and in both indoor and outdoor conditions, the Plymouth strain, CCAP 1052/1B, maintained an average of 87% fusiform, 13% triradiate and less than 0.08% oval in complete and minimal media, and in indoor and outdoor conditions. Over time, the ratios remained unchanged. The Blackpool strain shows a similar characteristic when cultivated in complete media in both indoor and outdoor conditions (Figures 17 and 18) with an average of 82% fusiform, 18% triradiate and 0% oval in complete and minimal media, and in indoor and outdoor conditions. However, when the Blackpool strain was cultivated in minimal conditions (Figures 19 and 20), irrespective of whether the cultures were indoors or outdoors, we see a clear shift from Day 13 (both indoor and outdoor) in the ratio of fusiform to triradiate to approximately 65% fusiform and 35% triradiate.

The growth curves of Blackpool strain has distinct lag, exponential growth, stationary and decline phases. However, after the decline phase, it enters what is known as the long-term stationary phase where the remaining

P. tricornutum cells maintain a constant cell density. The long-term stationary phase is unusual to algal growth curves but relatively common with bacterial growth curves. It is during this fifth growth phase, that the morphology of the Blackpool strain in minimal media begins to change. Please note that the average cell density is about 1.19 x 10^6 ± 0.83 cells/mL (indoor) and 1.61 x 10^6 ± 0.95 cells/mL (outdoor).

Table 20: Average percentage of fusiform and triradiate morphologies of *P. tricornutum* CCAP 1055/1 during long-term stationary phase (Day 13 - 36)

	Indoor		Outdoor	
	Minimal media	**Complete media**	**Minimal media**	**Complete media**
Fusiform	64.32 ± 7.49	88.08 ± 9.17	66.25 ± 2.01	91.59 ± 1.97
Triradiate	35.68 ± 7.49	11.92 ± 9.17	33.75 ± 2.01	8.41 ± 1.97

Table 21: Student's t-test for average percentage of trirdiate morphology of *P. tricornutum* CCAP 1055/1 in different conditions

				***p* = < 0.05**
	***p*-values**			
	Max_indoor	**Min_indoor**	**Max_outdoor**	**Min_outdoor**
Max_indoor		0.0254	0.5522	0.0158
Min_indoor	0.0254		0.0037	0.6886
Max_outdoor	0.5522	0.0037		0.0001
Min_outdoor	0.0158	0.6886	0.0001	

Table 21 contains the *p*-values for the average percentage of triradiate morphology of the Blackpool strain in different conditions. *'Max_indoor'* represents the cultures in complete F/2 + Si media cultivated indoors. *'Min_indoor'* represents the cultures in minimal N + P media cultivated indoors. *'Max_outdoor'* represents the cultures in complete F/2 + Si media cultivated outdoors. '*Min_indoor'* represents the cultures in minimal N + P media cultivated outdoors. The *p*-values indicate that we can reject the null hypothesis when it comes to the effect of media composition on the percentage of each morphology of the Blackpool strain. The minimal media composition leads to a statistically significant increase in the percentage of

triradiate-shaped and a decrease in fusiform-shaped *P. tricornutum* cells of the Blackpool strain in long-term stationary phase, regardless of whether it is cultivated indoors or outdoors.

The triradiate morphotype is known to be difficult to maintain under laboratory conditions (Lewin *et al.*, 1958). An Expressed Sequence Tag (EST) analysis of triradiate cells carried out by De Martino *et al.* (2011) showed that transcriptional regulators and certain classes of transporters are activated which could be involved in osmoregulation and buoyancy adjustments, allowing triradiate cells to float.

Several hypotheses could be postulated to explain why the Blackpool strain begins to increase its composition of triradiate cells in minimal media in the long-term stationary phase. In nature, increased buoyancy could allow the cells to float closer to the surface and increase its photosynthetic rate. The question is why this characteristic of increased triradiate cells occurs only when the cells are in minimal media? Could the cells have adapted to minimal media conditions, upregulating genes needed for the expression of vitamins such as vitamin B_{12}, or for the expression of siderophore utilisation?

The physiological differences that each morphotype expresses could explain differential ecological advantages of one morphotype over the other in nature remains an unexplored field, and the results presented in this Chapter have led to further questions regarding the physiology of the different morphotypes. Further experiments would need to be carried out in order to concretely provide evidence of this elicited morphological change in the Blackpool strain.

4.3.1 Cruciform morphology

On various occasions whilst carrying out cell counts, a fifth morphotype was identified; the cruciform morphotype. The first few encounters were approached with scepticism and assumed that this was purely an illusion caused by the refraction of the light from the microscope or simply two fusiforms crossing over one another. However, in April 2014, a study carried out by He *et al.* confirmed that what was seen was not a fragment of the imagination (He *et al.*, 2014). The study isolated and stabilised a long-term culture of a strain *of P. tricornutum* (CCMM 2004) that maintained a proportion of more than 31.3% cruciform morphology. Further insight into the polymorphic nature of the strain showed that the cruciform cells were capable of transforming into oval cells.

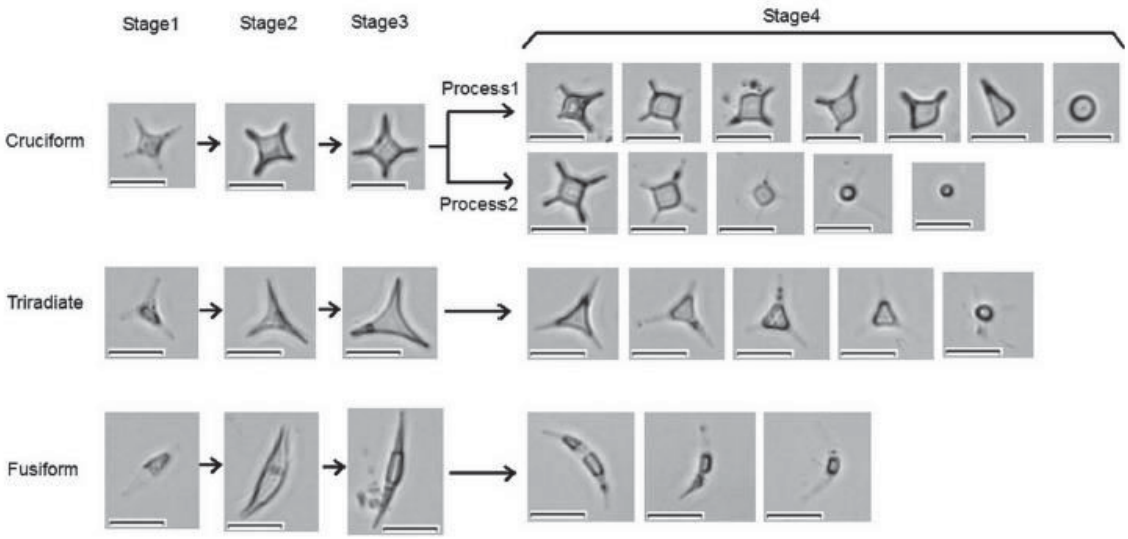


Figure 21: Transformation from cruciform, triradiate and fusiform to oval morphotype (figure from (He *et al.*, 2014)). This transformation involves four stages. In stage 1; the three morphotypes show the characteristics of a thin form indicating that the content of intracellular substances is poor. In stage 2, the cells are plump indication of many intracellular substances. In stage 3, the overall outline of the cells begins to change. In stage 4, the arms of the three morphotypes gradually disappear and the oval form is observed. Scale bar = 10 μm.

Increasing our understanding of the physiological characteristics of each morphotype, including the cruciform morphotype, would allow us to deduce differences in growth rates and metabolism, essential for biotechnological applications.

4.4 Contamination of large-scale cultures

Types of contaminants, their known effects on microalgal cultures. Short intro into synthetic biology as a scale up approach.

Microalgal cultivation methods rely on keeping monocultures of the desired species, especially if the final product is a bioactive molecule for human consumption (Mata *et al.*, 2010). Photobioreactors are closed systems that allow for the production of monoseptic cultures, fully isolated from potential contamination if cultivation protocols are followed correctly (Grima and Fernández, 1999). The other option is open raceway ponds that are highly susceptible to contamination, and unless the desired species is a halophile or thermophile (Parmar *et al.*, 2011), it is hard to maintain monocultures. Irrespective of the cultivation method, the establishment of unwanted organisms such as amoeba, ciliates, rotifers, bacteria, viruses, and other photosynthetic organisms in microalgal cultures, is a serious obstacle in large-scale microalgae cultivation (Day *et al.*, 2012; Wang *et al.*, 2013).

Predatory zooplankton such as ciliates and rotifers are commonly found in large-scale cultures and are capable of reducing microalgal cell densities in a matter of just a few days (Rosetta and McManus, 2003; Lürling and Beekman, 2006; Benemann, 2008). A study by Moreno-Garrido and Cañavate showed the grazing capacity of a ciliate to reduce the cell density of large-scale (400 L to 2000 L) outdoor *Dunaliella salina* cultures from

0.75 x 10^6 cells/mL to 0.15 x 10^6 cells/mL within 48h to 72h (Moreno-Garrido and Cañavate, 2000).

Viruses are important members of natural ecosystems. Virus-infected cells are lysed and release Dissolved Organic Matter (DOM) utilised by other members of the ecosystem (Suttle, 2005). Nagasaki et al. (Nagasaki *et al.*, 1999) investigated the algicidal efficiency of the *Heterosigma akashiwo* virus clone 01 (HaV01) on the Harmful Algal Bloom (HAB) causing microalga *Heterosigma akashi*wo. Their results showed that almost all cells had lost motility within 24 h and a culture 'crash' from 1.27 x 10^5 cells/mL to 10^1 cells/mL.

The presence of unwanted microalgal species which are capable of out-growing the microalgae of choice is a common phenomenon. The interaction between the two microalgal species could be based on, but not limited to, competition for space and resources, and allelopathy (Wang *et al.*, 2013).

Bacteria are present in all of the Earths' biomes (Dykhuizen, 1998), including oceans where they have co-existed with microalgae, including diatoms, for more than 200 million years (Amin *et al.*, 2012). The presence of bacteria in microalgal cultures will be discussed further in Chapter III. Although much research is carried out in the field of microalgal culture upscaling, very little is known about the identity and characteristics of these organisms, even though they are the root of microalgal culture 'crashes' which lead to loss of biomass, and therefore, loss of revenue.

4.5 Acknowledgements

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Community dynamics

dynamics of the ***bacterial community associated with*** ***Phaeodactylum tricornutum*** *cultures*

"When we try to pick out anything by itself, we find it bound fast by a thousand invisible cords that cannot be broken, to everything else in the Universe"

– **John Muir** in the John Muir Papers, University of the Pacific: Journal in Fox 1981:291

27 July 1869

Summary

The pennate diatom *Phaeodactylum tricornutum* is a model organism able to synthesise a number of industrially relevant molecules. Realising the industrial potential of microalgal-derived products relies on keeping large-scale monocultures which are prone to contamination by other organisms. However, little is known about the identity and characteristics of the invading organisms. In nature, diatoms are not found as isolated entities but rather are active members of a complex ecosystem, which is poorly understood. Bacteria, which have co-existed with diatoms for more than 200 million years, form a crucial part of this ecosystem and have been shown to enhance the growth of diatoms (Bruckner *et al.*, 2011; Amin *et al.*, 2015). Increased understanding of the interactions could allow for the exploration of 'synthetic ecology' as a novel scaling up technique (Kazamia *et al.*, 2012). To gain insight into the dynamics of the bacterial communities associated with diatoms, we translated the complexity of a natural system into a reproducible, systematic experimental approach where we investigated the microbiome of batch grown non-axenic cultures of *P. tricornutum* (CCAP 1052/1B) using barcoded 16S-V6-Next-Generation-Sequencing. Our results reveal that the bacterial community associated with *P. tricornutum* cultures changes over time. We identified four main families, Alteromondaceae, Pseudoalteromonadaceae, Flavobacteriaceae and Pseudomonadaceae, as major players within the microbiome. From our results, we propose a network of putative interactions between

P. tricornutum and each of the bacterial factions, thus providing a framework to understanding the dynamics of diatom-associated microbial communities. Further species-specific co-culture experiments coupled with a metabolic profiling approach are on-going. Preliminary results show increased growth rates and maximal cell densities when *P. tricornutum* is co-cultured with representative members of the four identified families.

1 Introduction

With our oceans constituting approximately 70.9% of the Earth's surface, it is the largest biome on our planet, yet it is probably the least understood. Derived from the Greek words *phyton* (plant) and *planktos* (made to wander or drift), phytoplankton are immensely diverse photosynthetic unicellular organisms spanning a number of taxonomic kingdoms and phyla. They are the microbial engines that drive global cycling of the six major elements necessary for life (hydrogen, carbon, nitrogen, oxygen, sulphur and phosphorus) (Falkowski *et al.*, 2008; Strom, 2008).

These ubiquitous photosynthetic microbes fix more than a hundred million tonnes of carbon in the form of carbon dioxide into organic material, which amounts to approximately half of global primary production (Field, 1998; Behrenfeld *et al.*, 2006). Of the total marine primary production, diatoms alone account for roughly 40%; one-fifth of global primary production (Nelson *et al.*, 1995; Aumont *et al.*, 2003).

Diatoms belong to the phylum Heterokontophyta and the class Bacillariophyceae (Dangeard, 1933). They are the result of a secondary endosymbiotic event that took place around one billion years ago between a red alga (Rhodophyta) and a heterotrophic eukaryote (Bhattacharya *et al.*, 2007). And according to Mann and Vanormelingen (2013), 'the number of extant species of diatoms is estimated to be at least 30 000 and probably circa 100 000'.

Phaeodactylum tricornutum is a diatom first described by Bohlin in 1897 when he found it in samples collected off the coast of Plymouth, United Kingdom. Unlike most diatoms, which have the distinct ability to precipitate soluble silicic acid to form a silica cell wall, *P. tricornutum* has a poorly silicified cell wall and therefore does not have an obligate requirement for silicic acid (Montsant *et al.*, 2005; Martino *et al.*, 2007). *P. tricornutum* is found in coastal regions such as rock pools and estuaries where aquatic environmental parameters (salinity, temperature) vary greatly as a consequence of tidal changes and solar irradiation (Martino *et al.*, 2011). Its habitual characteristics, peculiar ability to form oval, fusiform, and triradiate cells, as well as its poorly silicified cell wall, have triggered a tremendous increase in scientific research on *P. tricornutum*. The genome sequencing of *P. tricornutum* was completed in 2008, and the subsequent generation of expressed sequence tag (ESTs) databases of *P. tricornutum*, shed light on its unique characteristics relating to its acclimation capabilities to the marine environment as well as the versatile regions it naturally inhabits (Montsant *et al.*, 2005; Martino *et al.*, 2007; Bowler *et al.*, 2008). This makes *P. tricornutum* an excellent model organism.

Driven by photosynthesis, *P. tricornutum* is able to synthesise a number of industrially relevant molecules, applicable to various industries. In aquaculture, *P. tricornutum* is used as feed in e.g. bivalve, echinoderm, crustacean and fish (Ryther and Goldman, 1975; Tredici *et al.*, 2009). *P. tricornutum* biomass contains, on average, 18% lipids making it a potential candidate for biofuel production (Rebolloso-Fuentes *et al.* 2001; Kates & Volcani 1966). Furthermore, *P. tricornutum* has the ability to produce the poly-unsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in high proportions of the total

fatty acid content (Fajardo *et al.* 2007; Siron *et al.* 1989; Rebolloso-Fuentes *et al.* 2001). Marine-derived EPA and DHA, colloquially known as omega-3 PUFAs, are important in human nutrition with health benefits such as reduced cardiovascular morbidity and mortality, reduced risk of premature births and improved cognitive and behavioural development of the foetus, as well as benefiting patients with atherosclerosis, hypertension, and neurological and neuropsychiatric diseases (Yashodhara *et al.*, 2009). *P. tricornutum* is therefore an ideal source of omega-3 PUFAs for the pharma- and nutraceutical industries.

Fucoxanthin is the main carotenoid found in the thylakoids of *P. tricornutum* where it forms light harvesting complexes with Chlorophyll a. Up to 15.71 mg fucoxanthin per gram of freeze-dried *P. tricornutum* can be produced, at least 10 times more abundant than in macroalgae, that can be easily extracted with ethanol (Owens and Wold, 1986; Kim *et al.*, 2012). In human health, fucoxanthin can act as an antioxidant, a chemo-preventive agent against obesity, cancer, inflammation, angiogenesis, and diabetes, and has protective action on numerous organs including the skin, liver, brain blood vessels, bones and eyes (Peng *et al.*, 2011).

To realise the industrial potential of *P. tricornutum* derived products, substantial amounts of microalgal biomass produced at low cost is required. This is achieved by implementation of large-scale cultivation methods such as open raceway ponds and various types of photobioreactors. Microalgal cultivation methods rely on keeping monocultures of the desired species, especially if the final product is a bioactive molecule for human consumption (Mata *et al.*, 2010). Photobioreactors (PBRs) are closed systems that allow for the production of monoseptic cultures, fully isolated from potential contamination if cultivation protocols are followed correctly

(Grima and Fernández, 1999). However, high operational costs of PBRs would increase production costs. The other option is open raceway ponds, which are simple open-air cultivation systems that have been in use since the 1950s (Chisti, 2007). They are highly susceptible to contamination, and unless the desired species is a halophile or thermophile (Parmar *et al.*, 2011), it is hard to maintain monocultures. Irrespective of the cultivation method, the establishment of unwanted organisms such as amoeba, ciliates, rotifers, bacteria, viruses, and other photosynthetic organisms in microalgal cultures, is a serious obstacle in large-scale microalgae cultivation (Day *et al.*, 2012; Wang *et al.*, 2013). Although much research is carried out in the field of microalgal culture upscaling, very little is known about the identity and characteristics of these organisms, even though they are the root of microalgal culture 'crashes' which lead to loss of biomass, and therefore, loss of revenue.

The establishment of non-target organisms in microalgal cultures should not come as a surprise. Microalgae are not found in monoculture in nature and imposing such an environment is counterintuitive leaving unstable cultures. Rather than looking at these organisms as contaminants, understanding them could allow for the exploration of 'synthetic ecology' as a novel scaling up technique, a concept proposed by Kazamia *et al.* in 2012 (Kazamia *et al.*, 2012). The cornerstone of synthetic ecology is the Competitive Exclusion Principle, or Gause's Law, which states 'as a result of competition two species scarcely ever occupy similar niches, but displace each other in such a manner that each takes possession of certain peculiar kinds of food and modes of life in which it has an advantage over its competitor' (Gause, 1934; Hardin, 1960). By 'synthesising' a community of organisms that fills every niche in the ecosystem (i.e. the microalgal culture)

supporting the growth of the desired microalgae, we prevent the establishment of other, potentially harmful organisms in the culture, and optimise the utilisation of nutrients (Kazamia *et al.*, 2012).

In order for synthetic ecology to be a legitimate contender as a novel scaling up technique, greater understanding of species-specific interactions is required. Bacteria are present in all of the Earths' biomes (Dykhuizen, 1998), including oceans where they have co-existed with diatoms for more than 200 million years (Amin *et al.*, 2012). In 1958, Provasoli suggested that bacteria can enhance the growth of algae (Provasoli 1958). In the subsequent decades, species-specific studies have further corroborated his initial idea (Delucca and McCracken, 1977; Suminto and Hirayama, 1997). Furthermore, Bruckner *et al.* (2011) showed an increase in growth of *P. tricornutum* when co-cultured with an Alphaproteobacterium strain as well as when cultured in the spent media of the bacteria. A recent study conducted by Amin *et al.* shows a species-specific interaction between a coastal diatom, *Pseudo-nitzschia multiseries*, and a bacterial *Sulfitobacter* species (SA11), where the bacteria was shown to promote diatom cell division via secretion indole-3-acetic acid IAA, synthesised by the bacterium using diatom secreted and endogenous tryptophan. The IAA and tryptophan act as signalling molecules in this intricate diatom-bacteria relationship (Amin *et al.*, 2015).

With respect to the application to industry, the bacteria act as probiotics for the microalgae culture, just as bacterial probiotics have been successfully implemented in human diet by the pharma- and nutraceutical industries (Parvez and Malik, 2006), poultry industries (Kabir, 2009), and aquaculture industries (Qi *et al.*, 2009), to name a few. By identifying the bacterial community in non-axenic *P. tricornutum* cultures we can start to identify

and characterise those that may have a beneficial role in the cultures. Subsequently, a suitable candidate to fill a certain niche in the hypothetical synthetic ecosystem could be chosen.

2 Experimental procedure

2.1 Strains and culture conditions

All *Phaeodactylum tricornutum* cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP) based in Oban, Scotland (http://www.ccap.ac.uk/our-cultures.htm). All cultures are obtained non-axenic. Based on previous experimental evidence (unpublished data), the *P. tricornutum* strain CCAP1052/1B displayed optimal growth in 5L cultures. *P. tricornutum* was cultured in Guillard's medium for diatoms (F/2 + Si) in filtered natural seawater chemically sterilised using sodium hypochlorite and sodium thiosulphate pentahydrate. *P. tricornutum* was grown in two media conditions; (1) complete F/2 medium with the addition of sodium metasilicate as the source of silicon, as per (Ryther and Guillard, 1962; Guillard, 1975), and (2) minimal media with a source of nitrogen ($NaNO_3$) and phosphorus ($NaH_2PO_4.2H_2O$) at the same concentration as in the F/2 medium recipe. Recipe was obtained from the Culture Collection of Algae and Protozoa website (see http://www.ccap.ac.uk/pdfrecipes.htm). All cultures were grown in hanging 5L polyethylene bags with a 'V' shaped bottom prepared using a heat sealer (Figure 1).

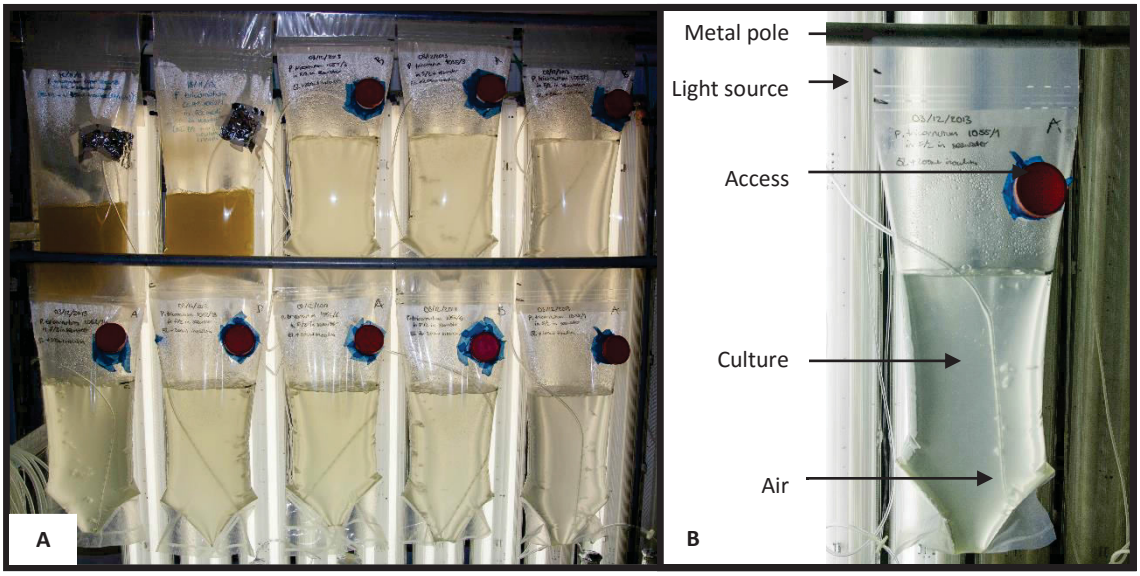


Figure 1: Non-axenic *Phaeodactylum tricornutum* culture set up. 5L polyethylene bags with a 'V' shaped bottom were created using the heat sealer machine. The bags were then rinsed and filled with 5L of filtered seawater. Afterwards each bag was sealed and hung approximately 30 cm from the light source. A small incision was made to insert the aeration tubing. This consists of a 10ml pipette attached to silicon tubing which is attached to a sterile air filter connecting it to the main air supply. A modified access port was created to take samples and measure the environmental parameters *(Photographs courtesy of Maria Rubio Bernal)*

All cultures had a modified aeration system provided by a 10ml pipette attached to the main pressurised air supply via 0.2 µm sterile air filters (Filter Membrane LifeASSURE PFS020 PTFE 3M). A modified access port was created to allow for sampling and measurement of environmental parameters. Cultures were kept at 18-20°C and 24hr light at an average of 132.3 µmol m^{-2} s^{-1} using Phillips TL-D 58W 33-640 M9 fluorescent tube lights. All cultures, irrespective of media condition, were inoculated with 250ml from the same 5L stock culture of actively growing non-axenic *P. tricornutum*.

2.2 Growth measurements

Growth was monitored every 24 to 48 h using a light microscope and carrying out cell counts of each culture in quadruplicates for each culture. During the cell counts the ratios of the four different morphotypes (oval, fusiform, triradiate and cruciform) were recorded, and descriptions of each culture noted. Samples of each culture were subsequently taken using a sterile 10ml syringe and placed in 50ml Falcon centrifuge tubes and placed in -20°C freezer.

2.3 Genomic DNA extraction

All samples from Day 1, 8, 15, and 22 were thawed in a water bath set at 25°C. As per (de Gouvion Saint Cyr *et al.*, 2014), samples were centrifuged for 5mins at 2 000g to gather the *P. tricornutum* in the pellet while particles such as debris, other organisms, bacteria, and soluble substances remain in the supernatant. Because the bacteria might be attached to the *P. tricornutum* cells in the pellet, the pellet was washed with deionised water and then centrifuged for 5mins at 2 000g. This was repeated twice. Genomic DNA extraction was carried out in the Aquaculture and Fisheries Development Centre and University College Cork. The Mo Bio's PowerWater® DNA Isolation Kit (catalogue no. 14900-100-NF) was utilised to carry out the genomic DNA extraction. The protocol provided with the kit was followed. Presence of gDNA was detected by running a 1% agarose-ethidium bromide gel with 72 wells. The samples were sent on dry ice to Heinrich-Heine University, Düsseldorf, for the V6 16S sequencing.

2.4 Barcoded 16S-V6-Next Generation Sequencing

Ion Torrent™ barcoded Next Generation Sequencing protocol was used to sequence the bacterial gDNA (Quail *et al.*, 2012; Grada and Weinbrecht, 2013). Amplification of the V6 hyper variable region of 16S rRNA with forward and reverse primers (Table 1) was carried out. Ion Reporter™ software assembles all the raw sequencing data and sorts all the reads using the unique sample-specific barcode sequences and removes them from the reads. The outcome is raw FASTQ files which are ready for analysis using bioinformatics tools.

Table 1: 16S V6 rRNA primer sequences. 'Max' is the complete media. 'Min' is the minimal media. 'A', 'B', and 'C' are the three replicates

Description	Barcode Name	Adaptor A Sequence	Barcode Sequence	Forward V6 Primer Sequence	Reverse V6 Primer Sequence
Day 1_Max_A	IonXpress_071	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TGAGGCTCCGAC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 1_Max_B	IonXpress_072	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGAAGGCCACAC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 1_Max_C	IonXpress_073	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCTGCCTGTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 1_Min_A	IonXpress_074	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGATCGGTTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 1_Min_B	IonXpress_075	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCAGGAATAC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 1_Min_C	IonXpress_076	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGGAAGAACCTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 8_Max_A	IonXpress_077	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGAAGCGATTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 8_Max_B	IonXpress_078	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGCCAATTCTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 8_Max_C	IonXpress_079	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CCTGGTTGTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 8_Min_A	IonXpress_080	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCGAAGGCAGGC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 8_Min_B	IonXpress_081	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CCTGCCATTCGC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 8_Min_C	IonXpress_082	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGGCATCTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 15_Max_A	IonXpress_083	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAGGACATTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 15_Max_B	IonXpress_084	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTTCCATAAC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 15_Max_C	IonXpress_085	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CCAGCCTCAAC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 15_Min_A	IonXpress_086	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTTGGTTATTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 15_Min_B	IonXpress_087	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TTGGCTGGAC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 15_Min_C	IonXpress_088	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CCGAACACTTC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 22_Max_A	IonXpress_090	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAACCACGGC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 22_Max_B	IonXpress_091	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGGAAGGATGC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 22_Max_C	IonXpress_092	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAGGAACCGC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 22_Min_A	IonXpress_093	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTTGTCCAATC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 22_Min_B	IonXpress_094	CCATCTCATCCCTGCGTGTCTCCGACTCAG	TCCGACAAGC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT
Day 22_Min_C	IonXpress_095	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CGGACAGATC	MWACGCGARGAACCTTACC	CGACARCCATGCANCACCT

2.5 Bioinformatics

A total of 87,077,374 reads were identified. The smallest sample was just over 1 million reads; the largest sample was just under 10 million reads. The sequencing data was subjected to a pipeline adapted and modified from Pylro *et al.* (2014). Primers were trimmed with fastq-mcf (version 1.04.807) (Aronesty, 2011), the resulting sequences were quality filterted and clustered into OTUs (operational taxonomic units) with usearch (version 8.0.1517; 32Bit – opensource) (Edgar 2010; 2013). Taxonomy assignment was done by QIIME (version 1.9.0) (Caporaso *et al.*, 2010) with the implemented uclust classifier based on 97% sequence identity to the reference 16S sequences from SILVA 111 database (Quast C. *et al.* 2013). Statistical analyses were performed in R.

The complete protocol containing all processing steps is available upon request.

3 Results

3.1 Characteristics of *Phaeodactylum tricornutum* culture growth

There is a significant difference (p=0.042, unpaired Wilcoxon signed rank) in the maximal cell density when *P. tricornutum* is cultivated in complete (9.3 x 10^6 cells/mL) or minimal media (11.2 x 10^6 cells/mL). The growth rates during the exponential phase in both cultures were $\mu_{complete}$ = 0.43 ± 0.07 d^{-1} and $\mu_{minimal}$ = 0.51 ± 0.04 d^{-1} respectively. In contrast, the death rates when the cultures 'crash' are $\delta_{complete}$ = 0.09 ± 0.02 d^{-1} and $\delta_{minimal}$ = 0.08 ± 0.04 d^{-1} respectively.

3.2 Bacterial community profile of *Phaeodactylum tricornutum* cultures

The 16S rRNA gene sequences were clustered to defined Operational Taxonomic Units (OTUs) at ≥97% sequence identity. Most OTUs could be assigned to the genera level (Figure 2). Of the 9 727 OTUs identified, 8 109 corresponded to known sequences in the SILVA database (v.118) (Quast *et al.*, 2013). The OTU abundance at the phylum level showed that 99.97% of all OTUs belonged to Proteobacteria, Bacteriodetes, Actinobacteria and Firmicutes (Figure 3). A comparison of the number of individual reads to the number of unique OTUs showed that the high number of reads per phyla is not the result of a single OTU (Figure 4). OTUs with hits to known 16S *P. tricornutum* chloroplast sequences were omitted.

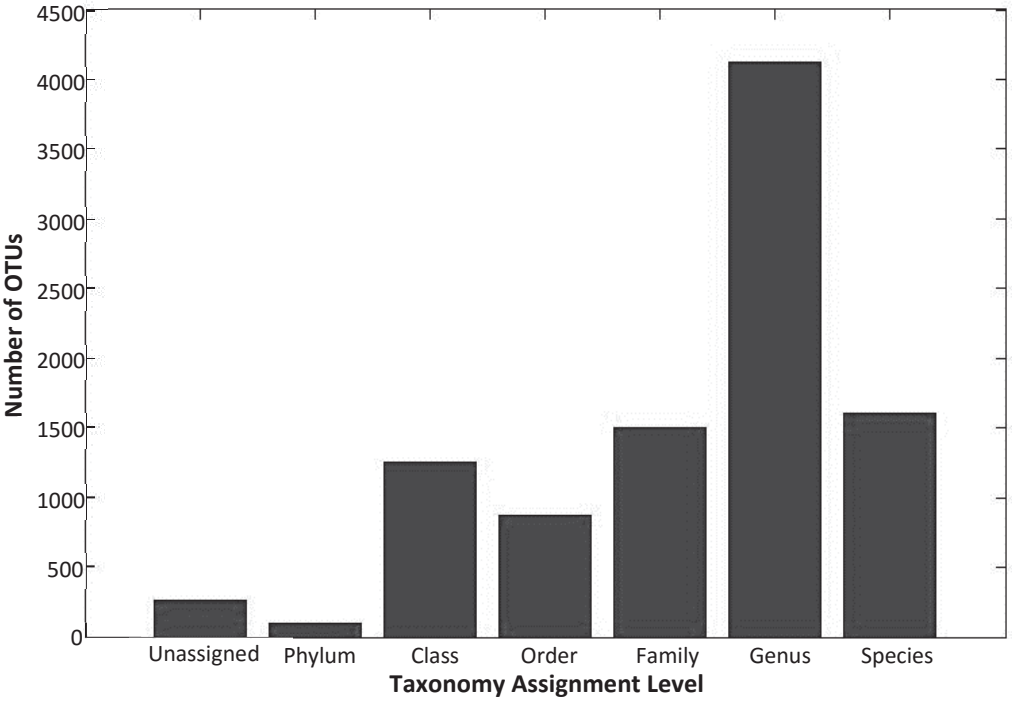


Figure 2: Operational Taxonomic Unit (OTU) Taxonomy Assignment Level. The 16S rRNA gene sequences were clustered to defined Operational Taxonomic Units (OTUs) at ≥97% sequence identity. Most OTUs could be assigned to the genera level, using the SILVA database (v.118) (Quast *et al.*, 2013).

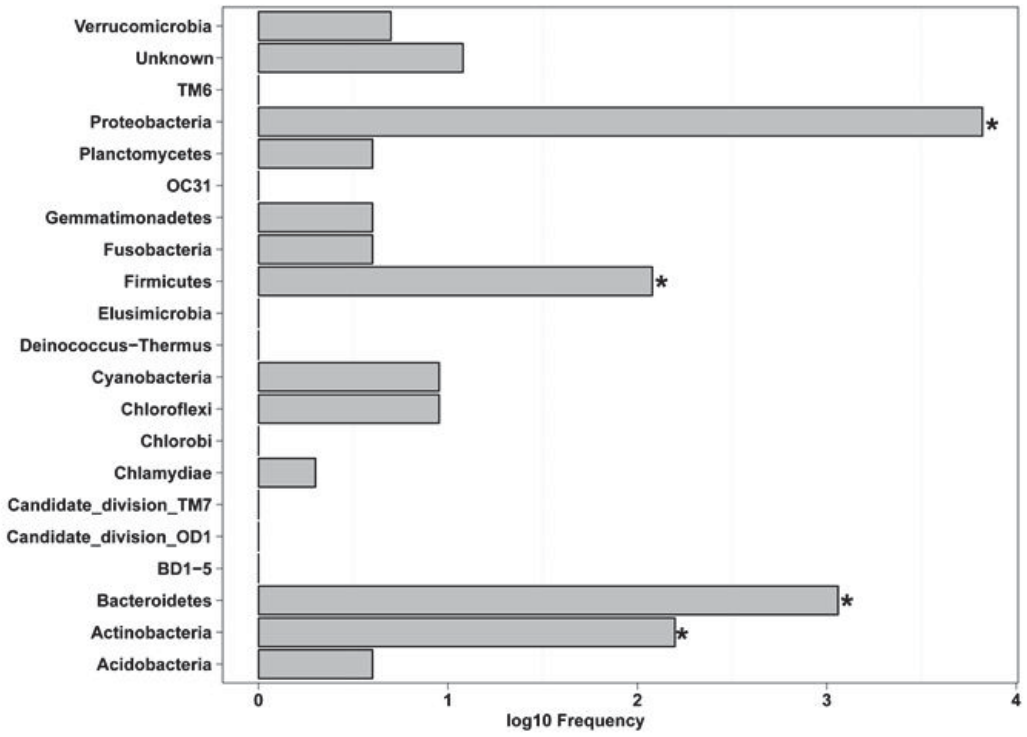


Figure 3 (previous page): Distribution of Operational Taxonomic Unit (OTU) abundance (LOG scaled) within phyla from complete data set. The bins marked with asterisks correspond to 99.97% of all which belong to Proteobacteria, Bacteriodetes, Actinobacteria and Firmicutes.

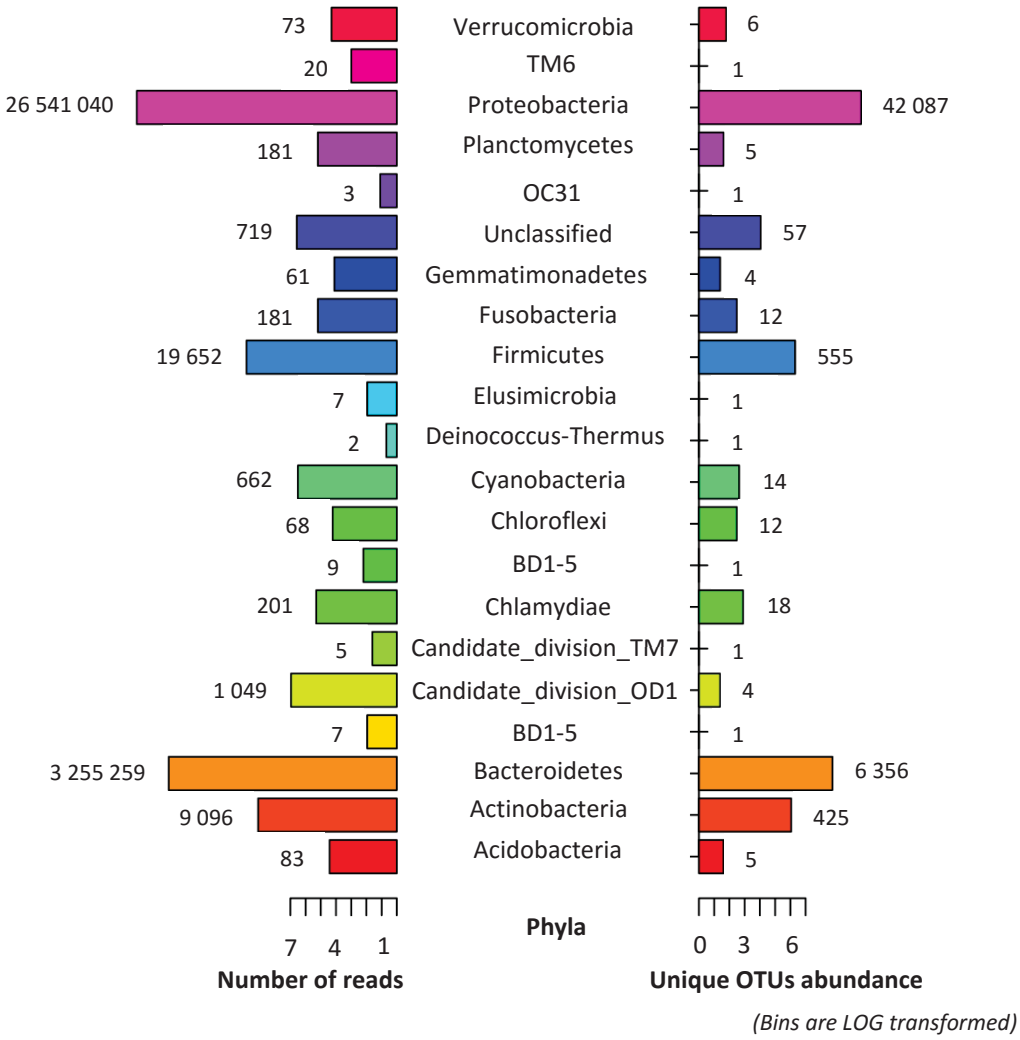


Figure 4: Number of reads per unique OTU abundance (at the phylum level). A comparison of the number of individual reads to the number of unique OTUs shows that phyla with high number of reads do not result in single OTUs.

Rarefaction curves were used to evaluate the Alpha diversity in the different media conditions as well as at the different time points (Figure 5). Species richness in both minimal and complete media was ~3 000. Species richness over time remained between ~2 400 and 2 600, with reduced species

richness (~1 300) on Day 8 (both minimal and complete media) possibly due to elevated levels of 16S *P. tricornutum* chloroplast reads which had to be omitted. Greatest species richness (~ 3 000) was shown on Day 22. Overall, all datasets showed less increase in the number of unique species the sample size increased, confirming adequate species richness in all culture conditions.

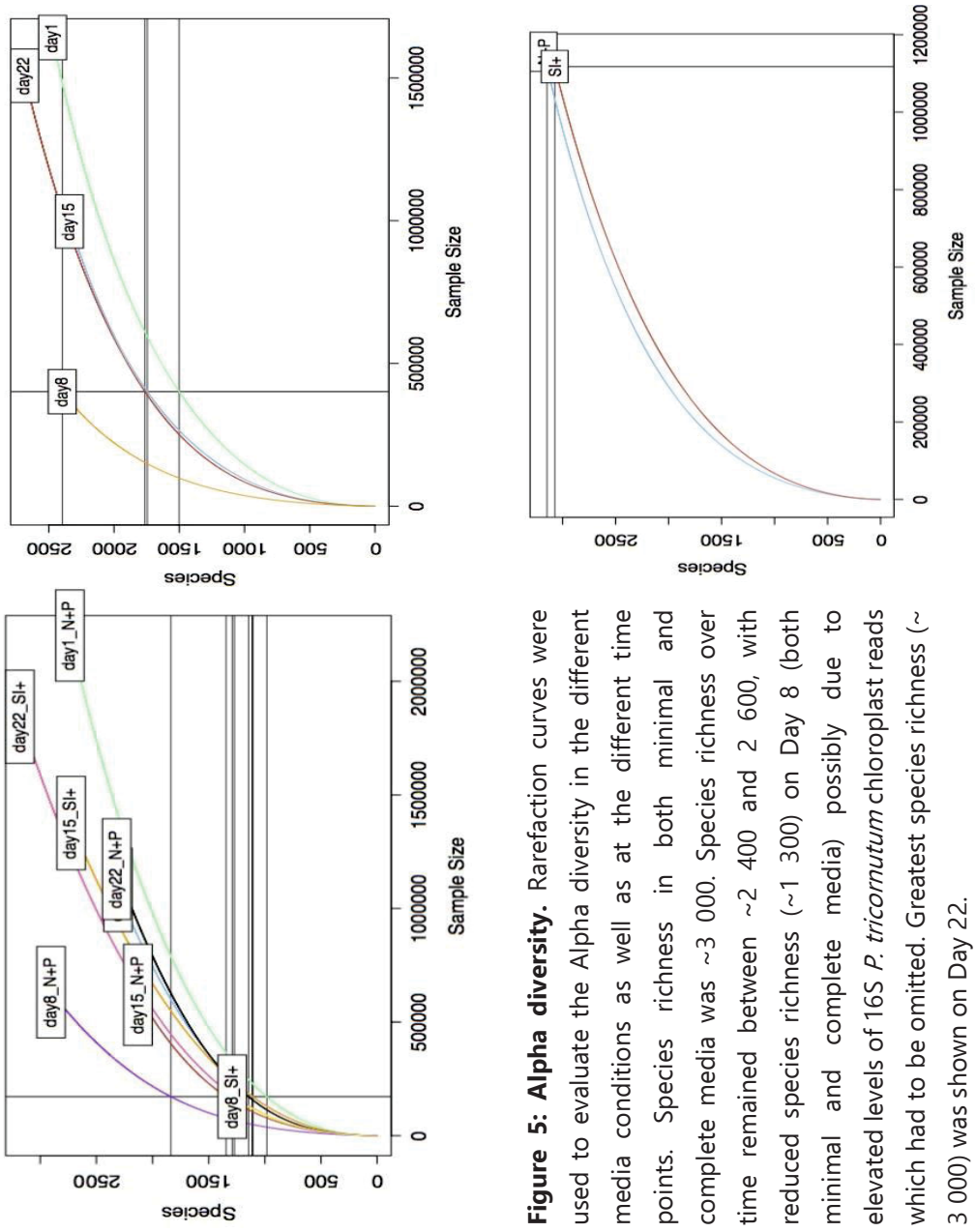


Figure 5: Alpha diversity. Rarefaction curves were used to evaluate the Alpha diversity in the different media conditions as well as at the different time points. Species richness in both minimal and complete media was ~3 000. Species richness over time remained between ~2 400 and 2 600, with reduced species richness (~1 300) on Day 8 (both minimal and complete media) possibly due to elevated levels of 16S *P. tricornutum* chloroplast reads which had to be omitted. Greatest species richness (~ 3 000) was shown on Day 22.

To compare the species composition between the different samples (days / media) we used a non-metric multidimensional scaling (NMDS) function based on generalised UniFrac distances (Chen *et al.*, 2012). We observed a clear divergence in the bacterial community in the two media conditions. Ordination based on the sampling day indicated that the bacterial community was dynamic with a clear divergence visible between Day 1 and the other three sampling days. Day 15 and 22 showed a slight overlap (Figure 6). An adapted version of PermanovaG was used to carry out permutational multivariate analysis of variance using multiple distance matrices which were previously calculated based on the generalised UniFrac distance (Chen *et al.*, 2012). The significance for the test was assessed by 5000 permutations. The results of the PermanovaG tests support the NMDS ordination, confirming a statistically significant effect in the bacterial community profile at the different sampling points and in the two media conditions whereas no significant effect was found in the experimental replicates (Figure 7).

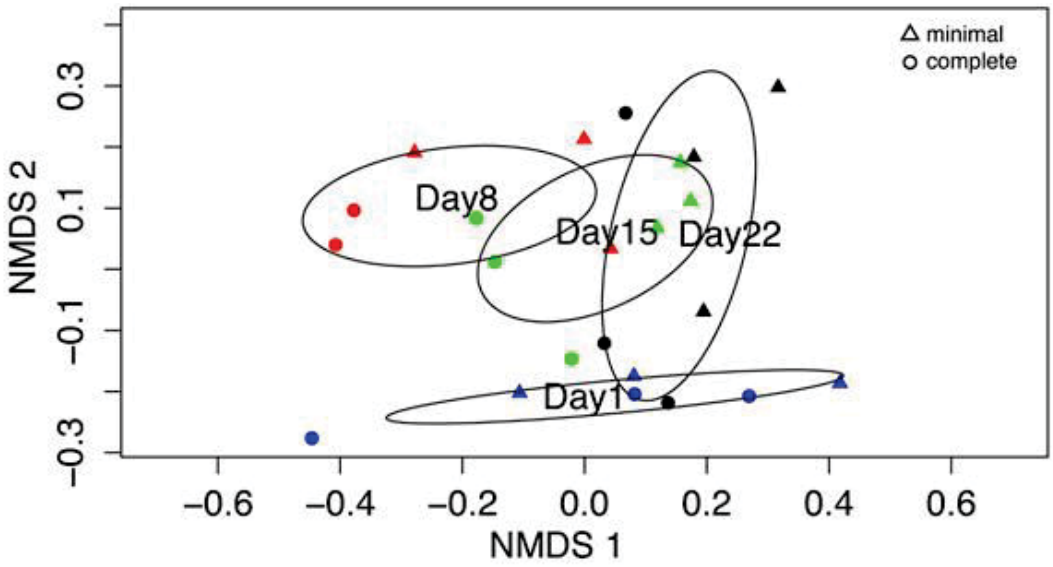


Figure 6. Ordination plot of bacterial community in the two media conditions for all sampling points. To compare the species composition between the different samples (days / media) we used a non-metric multidimensional scaling (NMDS) function based on generalised UniFrac distances (Chen *et al.*, 2012). Triangles and circles correspond to minimal media and complete media conditions, respectively. Blue represents Day 1. Red represents Day 8. Green represents Day 15. Black represents Day 22. The ellipses correspond to the 99% confidence interval to each group centroid.

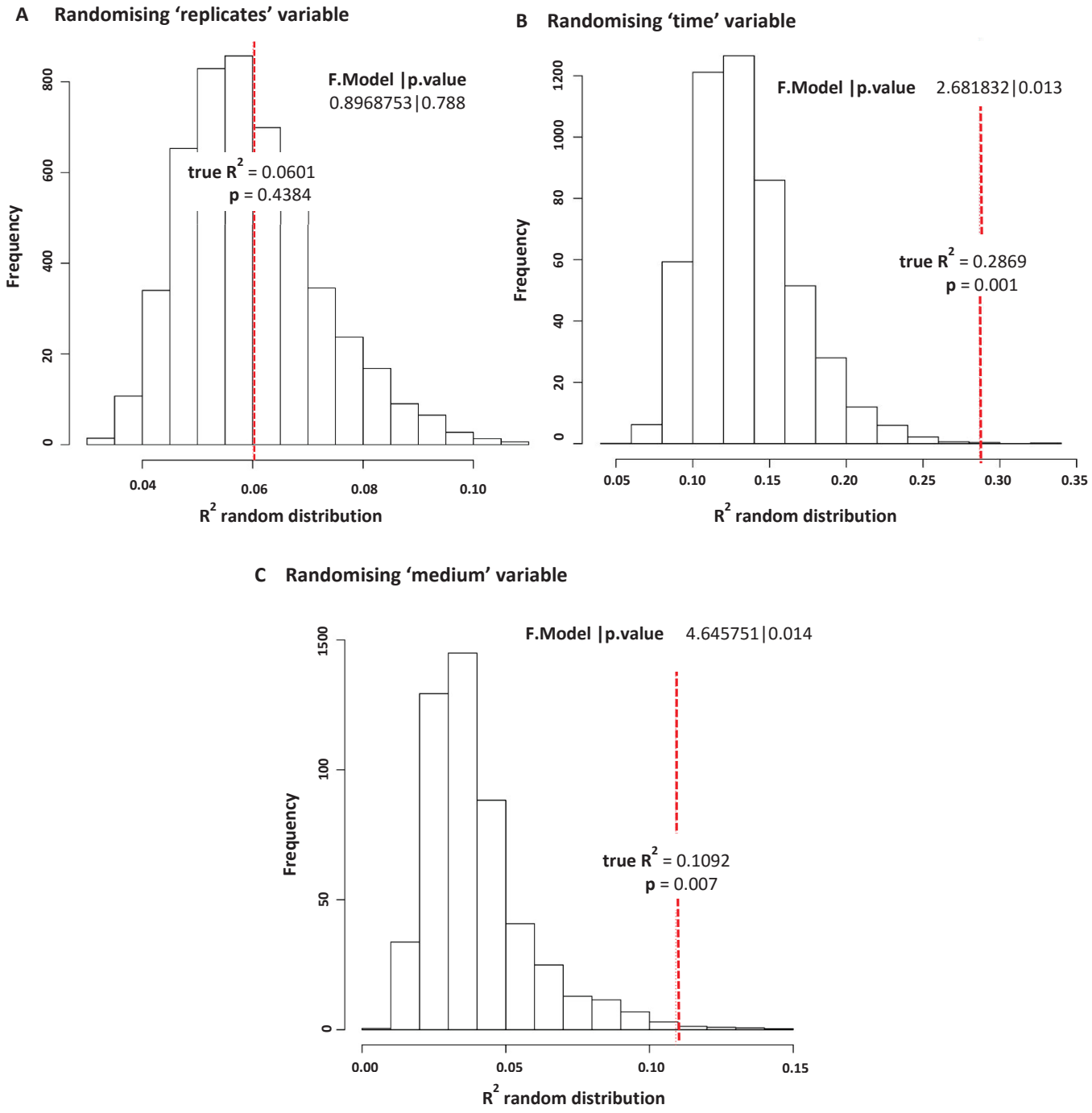
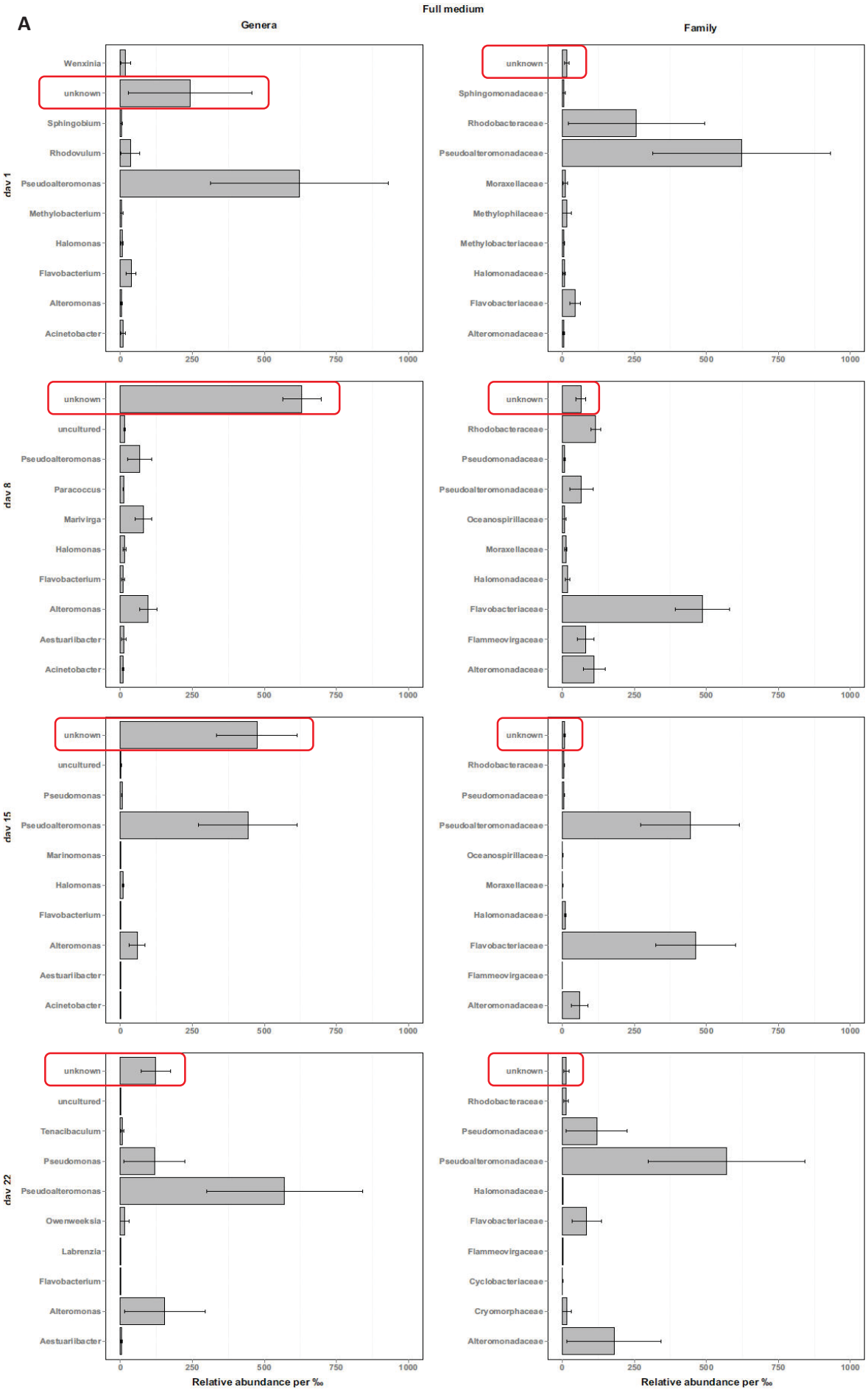


Figure 7: Beta diversity. A modified version of PermanovaG was used to carry out permutational multivariate analysis of variance using multiple distance matrices. The distance matrices [24x24] were previously calculated based on the generalised UniFrac distance (Chen *et al.*, 2012), weighted UniFrac and unweighted UniFrac (Lozupone and Knight, 2005) distance. The significance for the test was assessed by 5000 permutations. **(A)** shows no significant effect between the replicates (p-value of 0.4384). **(B)** shows a significant effect for the time variable (p-value of 0.001). **(C)** shows also shows a significant effect for the medium variable (p-value of 0.007)

3.3 Effect of temporal evolution and media composition on the bacterial community profile

We compared the bacterial community profiles over time and in the different media conditions at the family level so as to avoid diluting the signal of the less abundant genera. Figure 8 shows no dynamical difference within the genera that cannot be observed at the family level. By investigating the bacterial community dynamics at the family level, we also include taxonomical information that is unavailable at the genus level.


A
Full medium
Genera
Family
day 1
day 8
day 15
day 22
Wenxinia
unknown
Sphingobium
Rhodovulum
Pseudoalteromonas
Methylobacterium
Halomonas
Flavobacterium
Alteromonas
Acinetobacter
uncultured
Paracoccus
Marivirga
Aestuariibacter
Pseudomonas
Marinomonas
Tenacibaculum
Owenweeksia
Labrenzia
Sphingomonadaceae
Rhodobacteraceae
Pseudoalteromonadaceae
Moraxellaceae
Methylophilaceae
Methylobacteriaceae
Halomonadaceae
Flavobacteriaceae
Alteromonadaceae
Pseudomonadaceae
Oceanospirillaceae
Flammeovirgaceae
Cyclobacteriaceae
Cryomorphaceae
0
250
500
750
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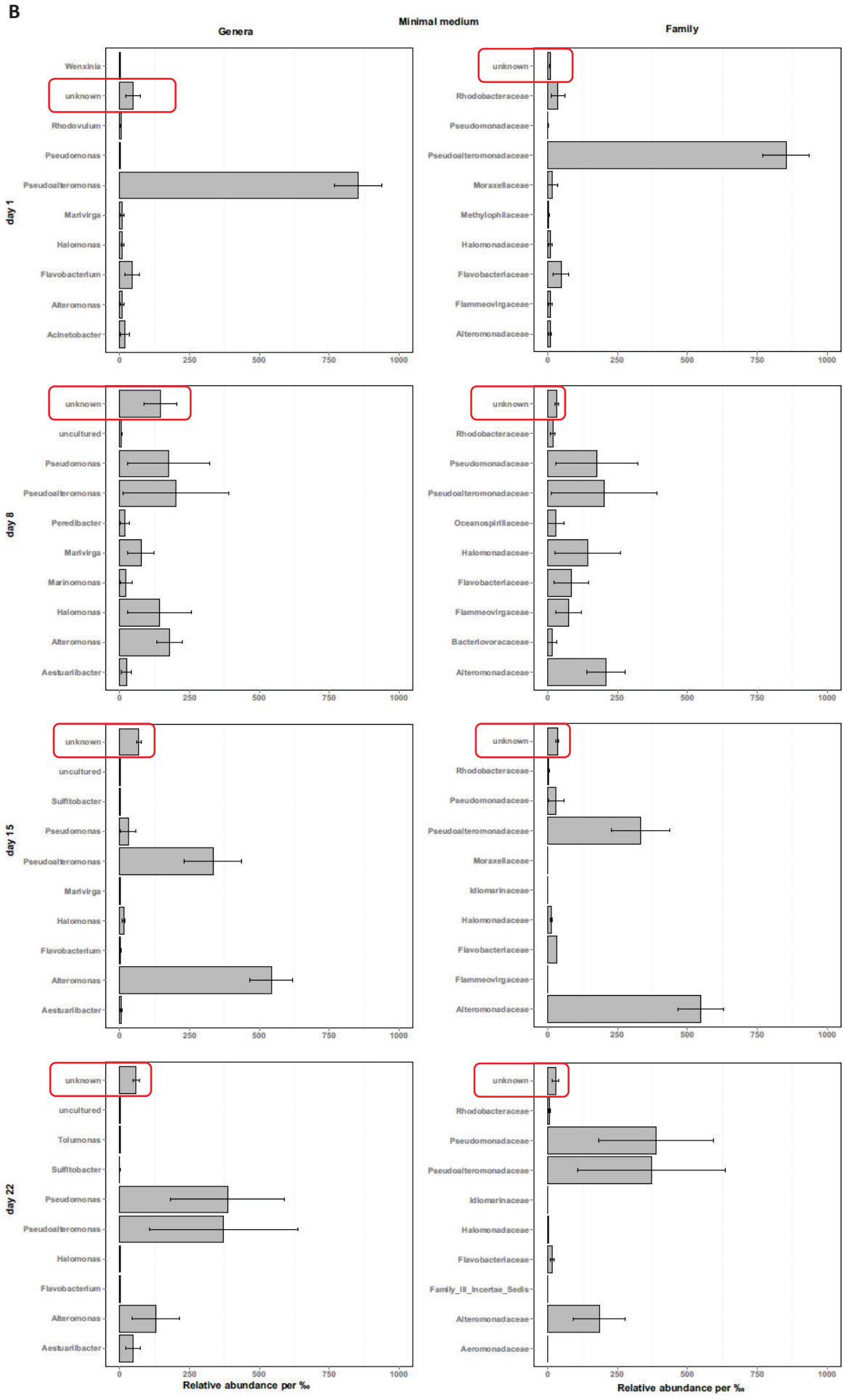
Relative abundance per ‰


B
Minimal medium
Genera
Family
day 1
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day 22
Wenxinia
unknown
Rhodovulum
Pseudomonas
Pseudoalteromonas
Marivirga
Halomonas
Flavobacterium
Alteromonas
Acinetobacter
uncultured
Peredibacter
Marinomonas
Aestuariibacter
Sulfitobacter
Tolumonas
Rhodobacteraceae
Pseudomonadaceae
Pseudoalteromonadaceae
Moraxellaceae
Methylophilaceae
Halomonadaceae
Flavobacteriaceae
Flammeovirgaceae
Alteromonadaceae
Oceanospirillaceae
Bacteriovoracaceae
Idiomarinaceae
Family_III_Incertae_Sedis
Aeromonadaceae
0
250
500
750
1000
Relative abundance per ‰

Figure 8 (previous pages): Comparison between bacterial community at genera level and family level. (A) in complete media. **(B)** in minimal media. We show no dynamical difference within the genera that cannot be observed at the family level. Encircled in red, there are a greater number of OTUs that could not be assigned a taxonomy ('unknowns') at the genera level than at the family level. By investigating the bacterial community dynamics at the family level, we also include taxonomical information that is unavailable at the genus level.

Overall, the families over-represented in all samples are Pseudoalteromonadaceae, Alteromonadaceae, Flavobacteriaceae and Pseudomonadaceae. Figure 9 illustrates temporal evolution of the bacterial community in both minimal and complete media. When looking at the temporal evolution of the bacterial community a clear difference in the community profile is seen. A remarkable feature is that at all investigated time points there exist one or two dominant families.

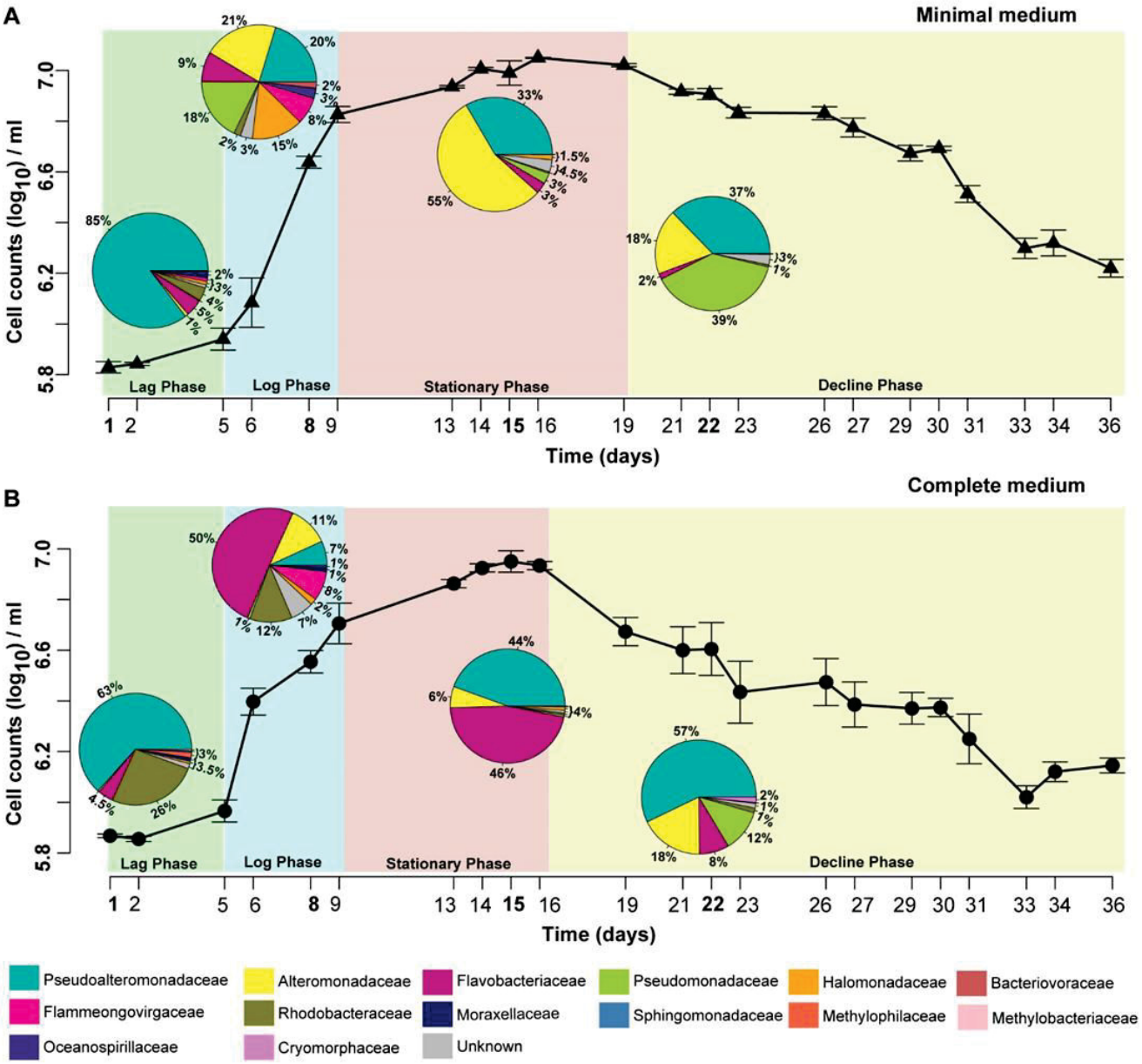

Figure 9: Bacterial community profile of *Phaeodactylum tricornutum* cultures over time and in differing media conditions. Both panels illustrate the growth of *P. tricornutum* (CCAP 1052/1B) over a 36 day period. The growth curves have been partitioned into lag (green), log (blue), stationary (red), and decline (yellow) phases. The abundance (%) of the 'Top Ten' bacterial families (corresponding colours described in the key) is depicted in pie charts on Days 1, 8, 15 and 22 in both media conditions. The existence of one dominant family at each investigated time point is a peculiar characteristic. In **minimal media (A)**, the lag phase of *P. tricornutum* growth is dominated by Pseudoalteromonadaceae (85%). However, during the log phase, a wide diversity of bacterial families is observed, with members of the Alteromonadaceae family (21%) beginning to dominate. During the stationary phase, a clear dominance of Alteromonadaceae species (55%) in the community can be

observed. The decline phase, however, shows the Pseudomonadaceae (39%) as a dominant family, with Pseudoalteromonadaceae species (37%) increasing in abundance again. In **complete media (B)**, the lag phase is also dominated by Pseudoalteromonadaceae (63%). During the log phase, 50% of the community is composed of members of the Flavobacteriaceae family, with the other 50% distributed amongst a number of different families. Flavobacteriaceae (46%) remain high in abundance during the stationary phase, with Pseudoalteromonadaceae species (44%) beginning to increase in abundance again. As for minimal media (A), Pseudoalteromonadaceae (57%) show clear dominance of the community during the decline phase.

3.4 Bacterial community in complete media

The community in complete media over time showed the dominant family when *P. tricornutum* cell densities are low to be Pseudoalteromonadaceae (63% and 57% on Day 1 and Day 22, respectively). Flavobacteriaceae species dominated (50%) when the *P. tricornutum* culture is growing exponentially (Day 8). Day 15, when *P. tricornutum* cell densities are at their highest, shows co-dominance of both Flavobacteriaceae (46%) and Pseudoalteromonadaceae (44%).

3.5 Bacterial community in minimal media

The community in minimal media also shows the dominant Family as Pseudoalteromonadaceae on Day 1 when *P. tricornutum* cell densities are low. However, on Day 22 Pseudomonadaceae (39%) and Pseudoalteromonadaceae (37%) are both overrepresented. When the *P. tricornutum* culture is growing exponentially (Day 8) a cluster of Families dominate; namely Alteromonadaceae (21%), Pseudoalteromonadaceae (20%), Pseudomonadaceae (18%), Halomonadaceae (15%) and Flavobacteriaceae (9%). When the cell density of *P. tricornutum* peaks (Day 15), the Alteromonadaceae species take over (55%).

The bacterial communities within the two media conditions on Day 1 are more closely related than the communities on days 8 and 15 (see Table 2 for generalised UniFrac distances). As the cultures begin to 'crash' (Day 22), the bacterial communities in the two media conditions increase in similarity again.

In general, the main families identified show a distinct pattern of disappearance and regeneration within the bacterial community. In the complete media, Pseudoalteromonadaceae species start at 63% (Day 1), drops in abundance to 7% (Day 8) then recovers to 57% (Day 22). Flavobacteriaceae species, in complete media, start at 4.5% (Day 1), increases in abundance to 50% (Day 8), and then falls back to 8% (Day 22). In the minimal media, Alteromonadaceae species have an abundance of only 1% (Day 1), peaks at 55% (Day 15), but drops down to 18% (Day 22).

Table 2: Generalised UniFrac distances of bacterial communities in complete and minimal media over time. Generalised UniFrac distance contains an extra parameter α controlling the weight on abundant lineages so the distance is not dominated by highly abundant lineages. $\alpha = 0.5$ has overall the best power.

		DAY 1		DAY 8		DAY 15		DAY 22	
		Minimal	Complete	Minimal	Complete	Minimal	Complete	Minimal	Complete
DAY 1	Minimal	0	0.5158104	0.7195151	0.8637909	0.6668939	0.7504169	0.6655156	0.6608732
	Complete	0.5158104	0	0.7020723	0.7203046	0.7176411	0.7125498	0.7019796	0.6733559
DAY 8	Minimal	0.7195151	0.7020723	0	0.6641379	0.5844515	0.6637369	0.5905962	0.497263
	Complete	0.8637909	0.7203046	0.6641379	0	0.8558383	0.5896856	0.8340209	0.7723285
DAY 15	Minimal	0.6668939	0.7176411	0.5844515	0.8558383	0	0.6605545	0.4569922	0.5042935
	Complete	0.7504169	0.7125498	0.6637369	0.5896856	0.6605545	0	0.630498	0.5720828
DAY 22	Minimal	0.6655156	0.7019796	0.5905962	0.8340209	0.4569922	0.630498	0	0.4597439
	Complete	0.6608732	0.6733559	0.497263	0.7723285	0.5042935	0.5720828	0.4597439	0

4 Discussion

In nature, *Phaeodactylum tricornutum* is not an isolated sovereign entity impassive to its environment including other organisms. In fact, it is part of a complex ecosystem, which is poorly understood. To reduce the complexity of a natural system, but nevertheless to gain valuable insights into the dynamics of the bacterial communities associated with diatoms, we investigated here non-axenic cultures of laboratory strains of *P. tricornutum*. The Results section showed the trends of the bacterial community dynamics during the batch growth of the *P. tricornutum*.

To progress towards the goal of creating a synthetic community, an in-depth understanding of the naturally occurring diatom-bacterial interactions, which are predominantly based on a 'biological barter trade system' between diatoms and bacteria – where substances such as trace metals, vitamins, and nutrients (nitrate, phosphate, silicate, carbon) are traded – is necessary. Based on previous studies on diatom-bacterial interactions and on existing characterisation of known species from each family, we will in the following derive hypotheses regarding the role of the dominant bacterial families in the *P. tricornutum* cultures.

The growth dynamics of *P. tricornutum* in the two media conditions showed an accelerated 'culture crash' in the complete media compared to the minimal media, which could suggest a more stable culture in the minimal media (Figure 9). Simultaneously, the dynamics of the bacterial community reveals that the community in the minimal media increases in complexity

over time. The link between ecosystem complexity and stability based on theoretical and experimental data has been debated by ecologists for over half a century (MacArthur, 1955; Elton, 1958; Gardner and Ashby, 1970; Pimm, 1984). Our observations are in agreement with more recent hypotheses indicating that diversity generally increases the stability of an ecosystem (McCann, 2000).

4.1 Potential role of dominant bacterial families.

The putative roles of each of the dominant families is illustrated in Figure 10.

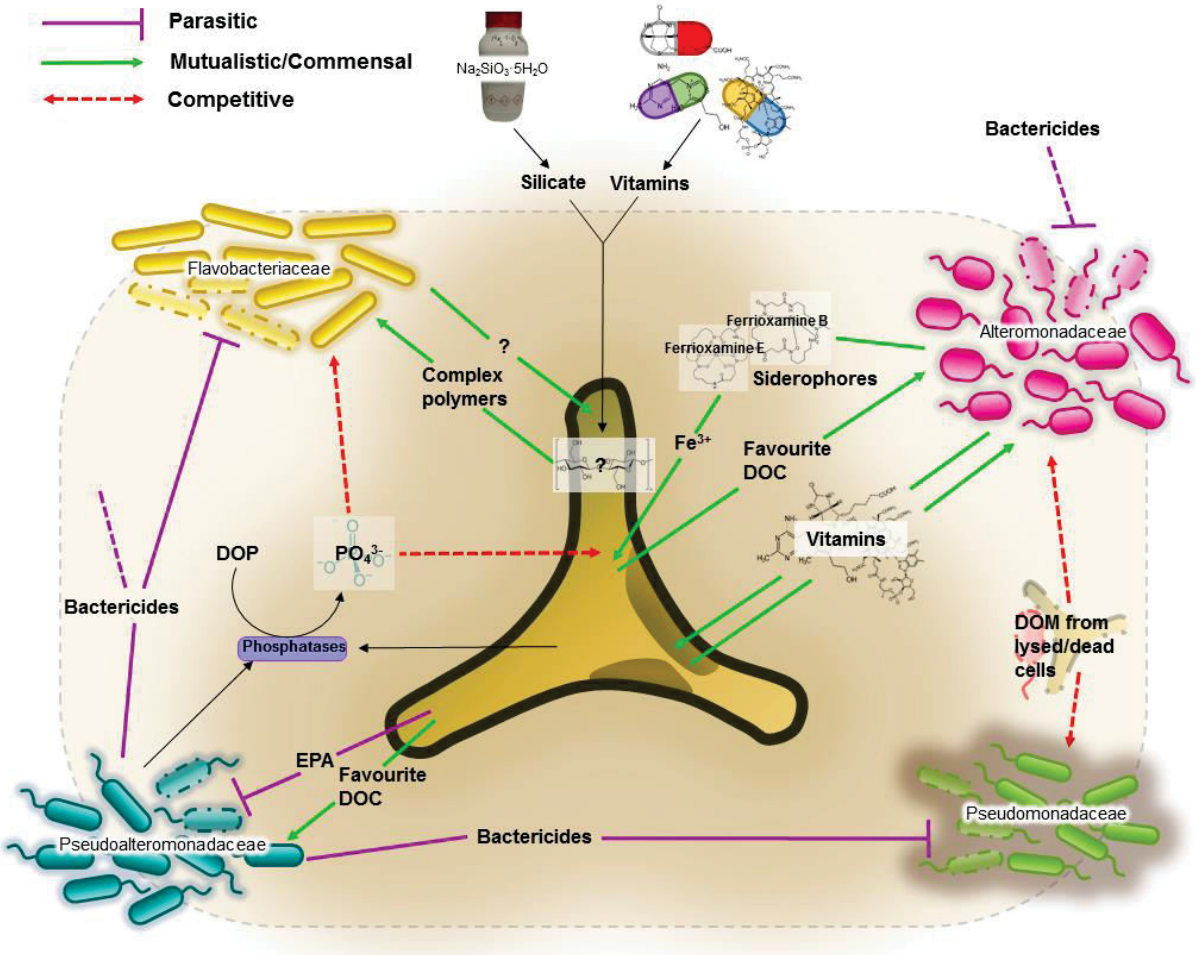

Figure 10 (previous page): Network of putative interactions between *Phaeodactylum tricornutum* and identified bacterial families. The dotted grey line depicts the **'phycosphere'**; a term coined by Bell and Mitchell in 1972 as an aquatic equivalent of the 'rhizosphere', denoting the region extending outwards from the algal cell in which bacterial growth is stimulated by extracellular products of the alga (Bell and Mitchell, 1972). **Bactericidal Effects.** Several species of the Pseudoalteromonadaceae family have been reported to possess bactericidal effects (Bowman, 2007). *P. tricornutum*, however, can excrete fatty acids (such as eicosapentaenoic acid or EPA), nucleotides, peptides, and pigment derivatives to protect themselves against opportunistic attack or pathogenic damage (Desbois *et al.*, 2009). **Iron.** Siderophores are a group of iron scavengers that act by chelating iron (III). Siderophores are produced and excreted by bacteria, and some cyanobacteria, which then reuptake the siderophores with bound iron (III) via outer-membrane transporters that are siderophore-specific (Vraspir and Butler, 2009). Diatoms are not known to produce siderophores (Soria-Dengg and Horstmann, 1995; Amin *et al.*, 2009). However, based on genome sequence analyses, the presence of a gene orthologue of a bacterial ferrichrome binding protein suggests the possibility of iron (III)-siderophore utilisation by *P. tricornutum*. Furthermore, it was shown that *P. tricornutum* was able to uptake siderophores ferrioxamines B and E (Soria-Dengg and Horstmann, 1995). **Vitamins.** Prokaryotes are thought to be the main producers of B vitamins (Provasoli, 1963; Provasoli and Carlucci, 1974). Although *P. tricornutum* does not require cobalamin, thiamine and biotin (Croft *et al.*, 2006), production of organic compounds such as EPA can by considerably enhanced by the bioavailability of co-factors such as cobalamin (Yongmanitchai and Ward, 1991). This provides the basis for potential mutualistic interactions. For example, Alteromonadales, dominant in our cultures, are thought to be capable of producing B vitamins (Sañudo-Wilhelmy *et al.*, 2014). **Dissolved Organic Carbon (DOC).** It is estimated that up to 50% of carbon fixed via phytoplankton-mediated photosynthesis is utilised by marine bacteria (Azam *et al.*, 1983), mainly as DOC compounds, defined as the organic material <0.7µm in size (Stocker, 2012). DOC from diatoms originates either from live cells or recently lysed or grazed cells, which determines the type of DOCs available, and therefore likely determining the bacterial consortia associated with the diatom (Amin *et al.*, 2012). **Dissolved Organic Phosphate (DOP).** Both diatoms and bacteria primarily utilise orthophosphate as a source of phosphorus. However, to access phosphate from DOP compounds, both diatoms and bacteria developed mechanisms such as the excretion of enzymes, including phosphatases, to release orthophosphate (PO_4^{3-}) from DOP. The mechanism is not species-specific, which consequently means the 'free' orthophosphates can be acquired by any organism (Persson *et al.*, 1988).

4.1.1 Pseudoalteromonadaceae

The presence of Pseudoalteromonadaceae species is not unexpected as members of this family have been isolated from coastal, open and deep-sea waters, sediments, marine invertebrates, as well as marine fish and algae (Ivanova *et al.*, 2004). The Pseudoalteromonadaceae family has three genera, namely *Pseudoalteromonas*, *Algicola* and *Psychrosphaera* (Rosenberg *et al.*, 2014, 28). Several species of Pseudoalteromonadaceae are reported to possess antibiotic properties with bactericidal effects (Bowman, 2007). For example, concentrated supernanant of a marine bacterium *Pseudoalteromonas sp.* strain A28 contained various enzymes including proteases, DNases, cellulases, and amylases, capable of causing the lysis of the diatom *Skeletonema costatum* (Lee *et al.*, 2000). Species of Pseudoalteromonadaceae are also capable of producing cold-adapted enzymes (Venkateswaran and Dohmoto, 2000; Chen *et al.*, 2007; Khudary *et al.*, 2010; Lu *et al.*, 2010; Albino *et al.*, 2012; He *et al.*, 2012). Pseudoalteromonadaceae species can produce extracellular polymeric substances allowing them to colonise surfaces, enhancing nutrient uptake whilst limiting diffusion of particular substances across the cell membrane (Holmström and Kjelleberg, 1999). The ability of Pseudoalteromonadaceae species to suppress the growth of competing bacteria could explain the dominance of Pseudoalteromonadaceae in almost all cultures irrespective of media composition, particularly when *P. tricornutum* abundance is limited (Figure 9, Days 1 and 22). One could hypothesise that *P. tricornutum* may protect the other bacterial community members from the bacteriolytic ability of Pseudoalteromonadaceae by producing antibacterial compounds themselves. Desbois *et al.* showed that *P. tricornutum* excreted bacteriolytic

fatty acids such as eicosapentaenoic acid (EPA; 20:5n-3), nucleotides, peptides, and pigment derivatives that would eliminate unwanted competition for nutrients such as organic phosphates from certain bacteria (Desbois *et al.*, 2009).

4.1.2 Alteromonadaceae

The Alteromonadaceae family consists of 16 (yet annotated) named generafound predominantly in marine environments (http://www.bacterio.net/-classifgenerafamilies.html#Alteromonadaceae) (Rosenberg *et al.*, 2014, 5). Members of this family were isolated from nutrient-rich environments such as coastal, open, and deep-sea waters, sediments, marine invertebrates and vertebrates, algae, and temperate and Antarctic marine environments (Ivanova and Mikhaĭlov, 2001). They are able to utilise a vast array of compounds as carbon sources; from glucose to glycerol (Rosenberg *et al.*, 2014, 5). Members of this family are known siderophore producers (Reid and Butler, 1991; Holt *et al.*, 2005; Amin *et al.*, 2009). Greek for 'iron carrier', siderophores are a group of iron scavengers that act by chelating iron (III) that are produced and excreted by bacteria, and some cyanobacteria, which then reuptake the siderophores with bound iron (III) via outer-membrane transporters that are siderophore-specific (Vraspir and Butler, 2009). Iron acquisition is essential for biological processes such as photosynthesis, respiration and nitrogen fixation. Most bioactive trace metals, including iron, exist at nanomolar (10^{-9} M) to picomolar (10^{-12} M) concentrations in our oceans, approximately one-millionth of the intracellular concentration in diatoms (Bruland *et al.*, 1991; Morel and Price, 2003). Diatoms are not known to produce siderophores (Soria-Dengg and Horstmann, 1995; Amin *et al.*, 2009). However, based on

genome sequence analyses, the presence of a gene orthologue of a bacterial ferrichrome-binding protein suggests the possibility of iron (III)-siderophore utilisation by *P. tricornutum* (Soria-Dengg and Horstmann, 1995). No trace metals, including iron (III), were provided to minimal media cultures. However, natural seawater may contain minute traces of bioactive trace metals. The high abundance of Alteromonadaceae in the minimal media suggests a potential supportive role in sequestering traces of iron (III) that may be present in the sterile natural seawater to the *P. tricornutum* (Figure 9). This is further supported by the very low level of Alteromonadaceae in the complete media (11% in complete media compared to 55% in minimal media, both on Day 15) where the culture has been supplied with 11.7 µM of iron (III) chloride hexahydrate.

4.1.3 Flavobacteriaceae

Flavobacteriaceae are members of the Bacteroidetes phylum and include over 120 genera found in soil, sediments and seawater (http://www.bacterio.net/-classifgenerafamilies.html#Flavobacteriaceae) (see Yoon *et al.* 2015 for further references). Flavobacteriaceae belong within the Cytophaga-Flavobacterium cluster which has been shown to account for more than 10% of the total bacterial community in coastal and offshore waters (Glöckner *et al.*, 1999; Abell and Bowman, 2005; DeLong *et al.*, 2006). Members of Flavobacteriaceae are proficient degraders of various biopolymers such as cellulose, chitin and pectin (Manz *et al.*, 1996; Kirchman, 2002). They were shown to be omnipresent during phytoplankton blooms, and their preference for consuming more complex polymers rather than monomers suggests an active role in the processing of organic matter during these blooms (Cottrell and Kirchman, 2000; Pinhassi *et al.*, 2004).

Although the exact mechanisms behind them are not perfectly understood, algal blooms are a consequence of exponential growth of phytoplankton (Smayda, 1997). In this respect, the phase of exponential growth of *P. tricornutum* in complete media, when our results showed highest abundance of Flavobacteriaceae, can be interpreted as an 'artificial bloom' of *P. tricornutum* (Figure 9). In the minimal media, the abundance of Flavobacteriaceae remains very low; at its maximum on Day 8 it only accounts for 9% of the total bacterial community. Members of the Flavobacteriaceae family could be more demanding than other bacteria that require lower nutrient levels to thrive. It is estimated that up to 50% of carbon fixed via phytoplankton-mediated photosynthesis is utilised by marine bacteria (Azam *et al.*, 1983), mainly as Dissolved Organic Carbon (DOC) compounds, defined as the organic material <0.7µm in size (Stocker, 2012). DOC from diatoms originates either from live cells or recently lysed or grazed cells, which determine the type of DOCs available, and therefore are likely to influence the bacterial consortia associated with the diatom (Amin *et al.*, 2012). This suggests a dynamic complexity within the bacterial consortia based solely on the type of DOC available. Members of the Flavobacteriaceae family might possess the genetic ability to utilise specific DOC produced by *P. tricornutum* grown in complete media.

4.1.4 Pseudomonadaceae

Pseudomonadaceae are an extraordinarily diverse family of bacteria found in almost all habitats on Earth; in soils, freshwater as well as marine environments, as well as plant and animal-associated pathogens (Starr *et al.* 1981, chap. 58). Species from the *Pseudomonas* genus are the best studied of the Pseudomonadaceae family, whose sheer genetic diversity explains

the ability to thrive in such a wide range of environments (Anzai *et al.*, 2000). Marine isolates from the *Pseudomonas* genus have been shown to produce a wide range of bioactive compounds, many of which exhibit antibacterial as well as antiviral properties (see Isnansetyo & Kamei 2009 for further references). Based on the results, there is an abundance of Pseudomonadaceae evident on Day 22 of the complete media cultures, and on Days 8 and 22 of the minimal media cultures. The increased presence of Pseudomonadaceae when *the P. tricornutum* culture has 'crashed' could be attributed to its ability to produce antibacterial compounds allowing members of this family to begin to thrive in the community through inhibition of its competitors. Given its exceptional genetic diversity, and thus, its metabolic versatility, allows for members of Pseudomonadaceae to be truly saprophytic; providing a hypothetical explanation of its abundance when the *P. tricornutum* cultures crash (Figure 9. Day 22 in both media conditions).

4.2 Modelling approach

Work is currently on-going to prove that the experimental data in Chapter III are consistent with population evolution dynamics. The approach implemented is the use of Ordinary Differential Equations (ODE) modeling to simulate the development in time of the different bacterial species as well as the *P. tricornutum*. The model is based on the following working hypotheses: the growth (μ) of each population follows a standard Verhulst equation, scaled by Michaelis-Menten terms for necessary nutrients; the death of each population is inversely proportional to (1+ μ), meaning that cells during replication are healthier. An additional contribution to population death is given by the presence of noxious elements such as

bactericidal substances in the environment. Consumption and production rates of metabolites are in general directly proportional to the growth (μ) except in the case of the Dissolved Organic Carbon (DOC) and Dissolved Organic Phosphates (DOP) metabolites: here we introduce the hypothesis that, in the event of micronutrient scarcity, the diatom will secrete more organic carbons favorited by those bacteria able to provide the needed micronutrients (iron and vitamins in our model).

4.3 Conclusion

Bacterial communities associated with *Phaeodactylum tricornutum* cultures change over time, correlating with the growth and subsequent crashing of the diatom cultures. The bioavailability or absence of vitamins, trace metals and silicon alters the bacterial community.

We hypothesise that a role within the community can be filled, not by one specific species of bacteria, but rather a number of bacterial species capable of carrying out said role. Which bacteria fill the role is dependent upon the environmental characteristics and the prevailing needs of the community as a whole at any given time. If a niche is unfilled, bacteria with the ideal metabolic functionality will seize the opportunity and thrive within that niche. The absence of certain micronutrients creates a new niche that can be filled by a certain unique bacterial faction.

Further work is necessary to explore the hypotheses postulated in the Discussion section. This can be achieved by carrying out systematic co-culture experiments with culturable members of the bacterial families of interest. The role of each representative of the bacterial families can be identified by carrying out subsequent –omics studies, which provide a

holistic view of the genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a specific biological sample in a non-targeted and non-biased manner (Horgan and Kenny, 2011).

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4

Diatom-bacteria co-cultivation

preliminary investigation of controlled ***diatom-bacterial co-culture growth***

"Panapo wengi hapaharibiki neno."

(translated from Kiswahili: *"Where there are many, nothing goes wrong."*)

– **Kiswahili proverb**

1 Introduction

Previous work identified four main families, Alteromondaceae, Pseudoalteromonadaceae, Flavobacteriaceae and Pseudomonadaceae, as major players within the microbiome of *Phaeodactylum tricornutum* cultures. From our results, we proposed a network of putative interactions between *P. tricornutum* and each of the bacterial factions, thus providing a framework to understanding the dynamics of diatom-associated microbial communities. Preliminary co-culture experiments were carried out at Heinrich-Heine-University Düsseldorf with suitable members of the Alteromonadaceae, Flavobacteriacea and Pseudoalteromonadaceae families. We did not carry out preliminary studies with Pseudomonadaceae strains as they are an extraordinarily diverse family of bacteria found in almost all habitats on Earth (Starr *et al.* 1981, chap. 58), and decided to focus our research efforts on the Top Three members.

2 Experimental procedure

2.1 Phaeodactylum tricornutum strain selection

All *Phaeodactylum tricornutum* cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP) based in Oban, Scotland (http://www.ccap.ac.uk/our-cultures.htm). All cultures are obtained non-axenic. The *P. tricornutum* strains were grown on complete F/2 medium with the addition of sodium metasilicate as the source of silicon, as per (Ryther and Guillard, 1962; Guillard, 1975). Artificial seawater was prepared using Ultramarine synthetic sea salts from Waterlife Research Industries Limited (http://www.waterlife.co.uk/). Recipe for F/2 + Si was obtained from the Culture Collection of Algae and Protozoa website (see http://www.ccap.ac.uk/pdfrecipes.htm).

Figure 1A-D are Scanning Electron Microscope (SEM) photographs of a non-axenic culture of *P. tricornutum*. Future experimental approaches would include SEM photographs of species-specific co-cultures.

Figure 1A-D (next pages): Scanning Electron Microscope photographs of a non-axenic culture of *P. tricornutum* taken at the Centre for Advanced Imaging (CAi) at Heinrich-Heine-University Düsseldorf and were taken by Steffen Köhler.

Figure 1A

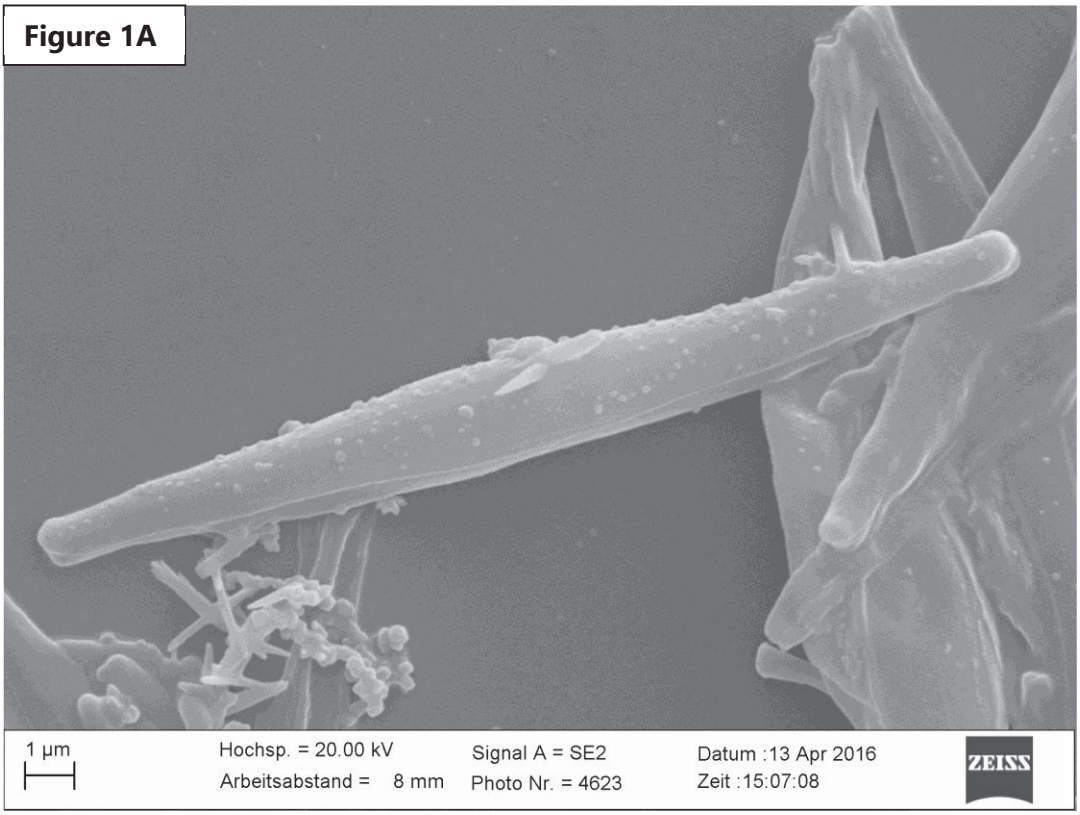
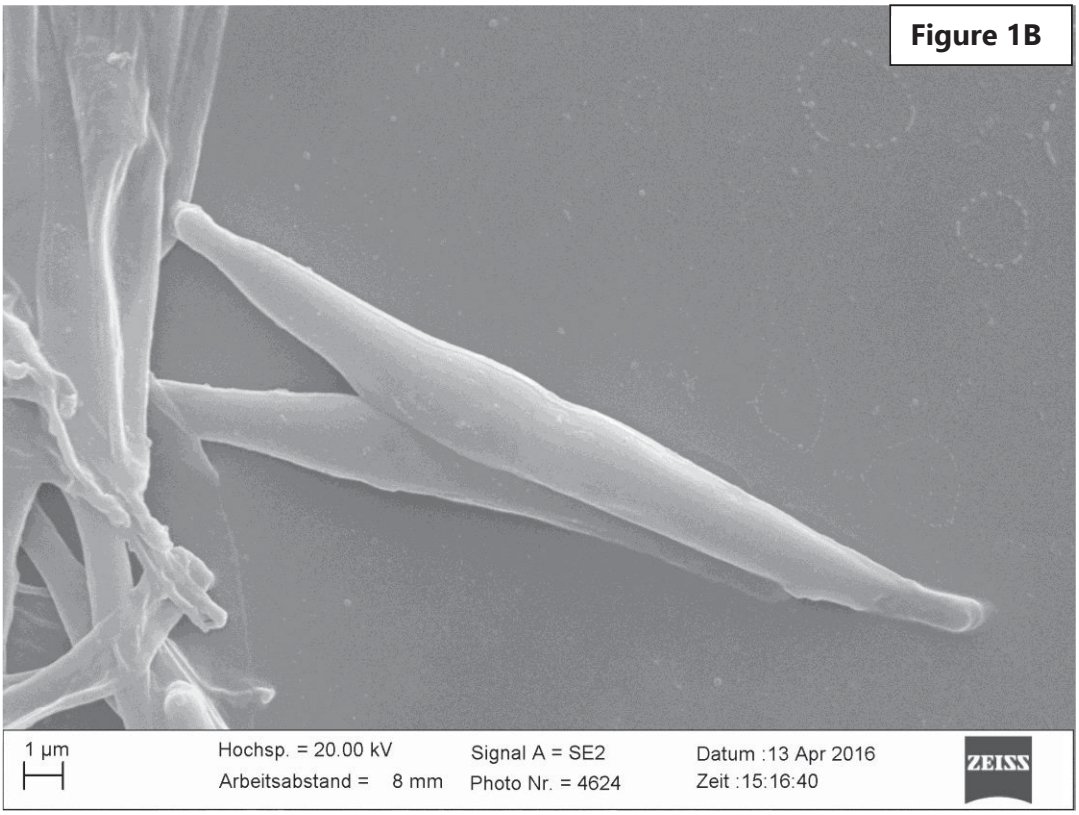


Figure 1B



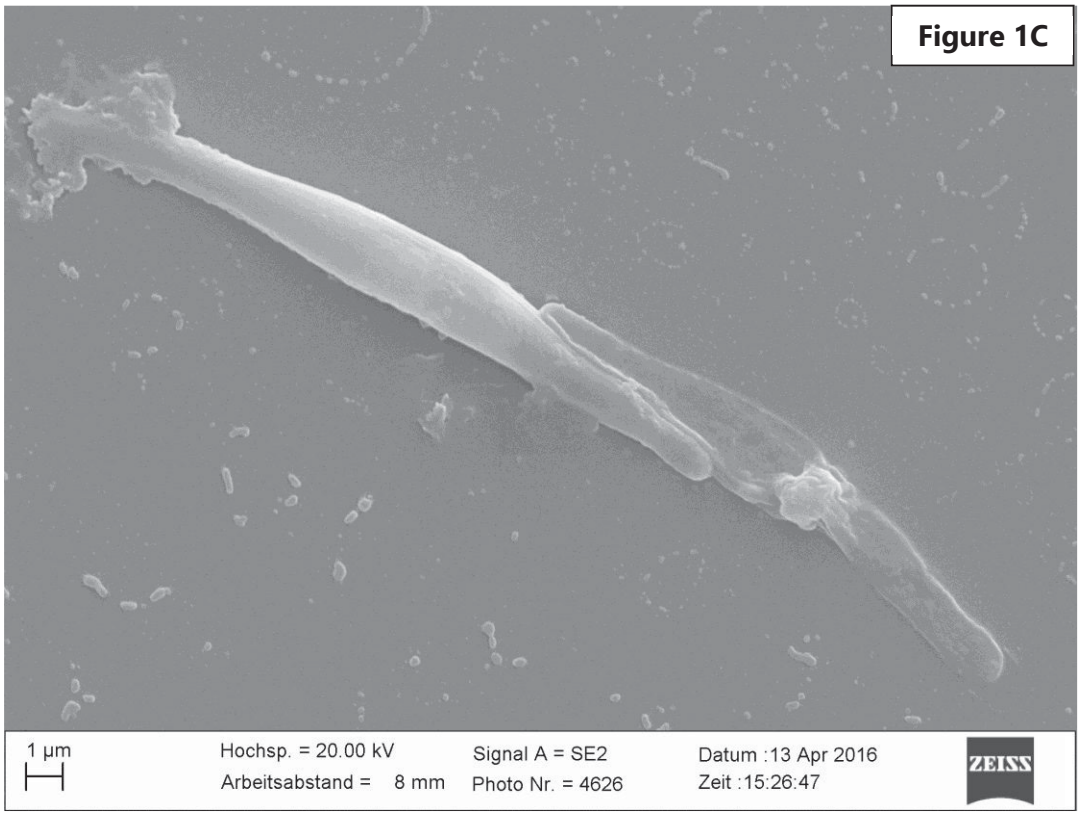


Figure 1C
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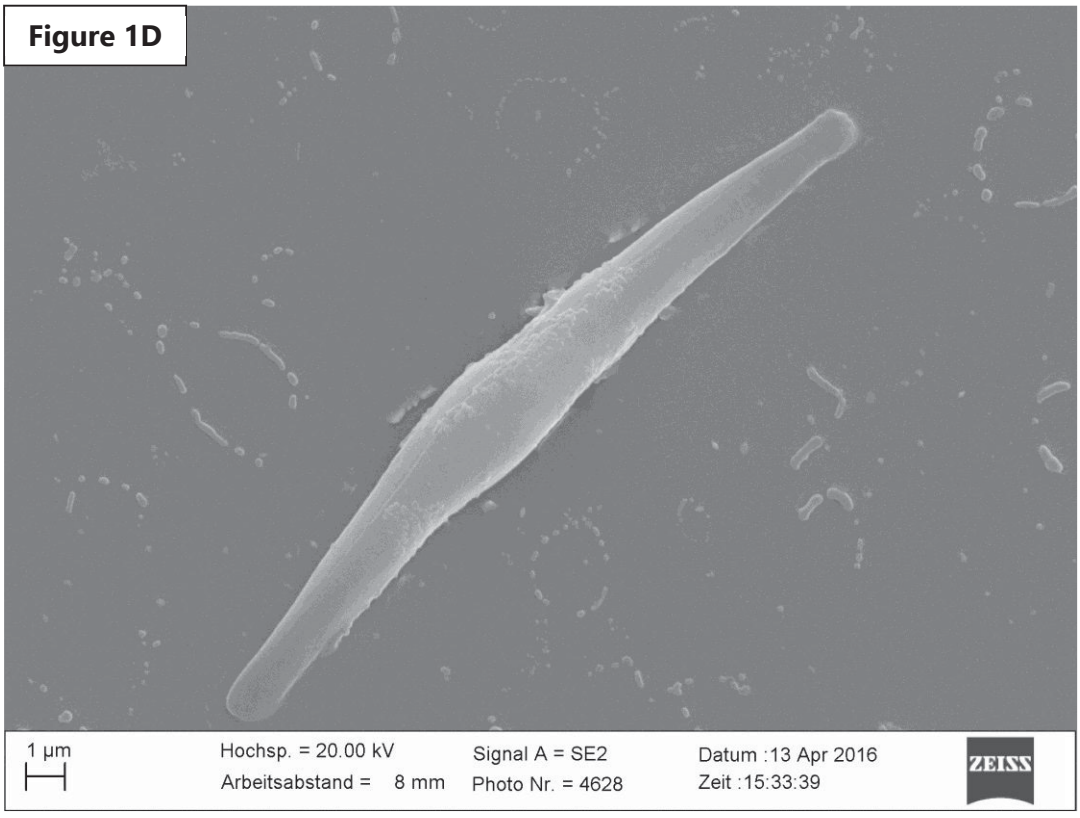
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2.2 Bacterial strain selection

Parameters for the selection of suitable bacterial strains for the co-culture experiments were set based on isolation location and culture conditions. Strains of *P. tricornutum* used in previous experiments were:

* CCAP 1055/1 which was isolated off the coast of Blackpool, UK
* CCAP 1052/1B which was isolated off the coast of Plymouth, UK

Therefore, the isolation location was limited to bacterial strains found in coastal areas in the northern temperate zone, more specifically, the British Isles. *P. tricornutum* is cultured at 18-20°C and in a marine-based medium (F/2 medium with added sodium metasilicate as the source of silicon, as per Ryther and Guillard (1962) and Guillard (1975)). Therefore, the suggested media conditions should be similar to this, with the media being either seawater agar, marine broth, or derivatives of these. Based on out geographical location parameter, we limited our search to the National Collections of Industrial, Marine and Food Bacteria (NCIMB) culture collection, located in Aberdeen, Scotland (http://www.ncimb.com/). Our search yielded the following suitable bacterial strains:

1. *Pseudoalteromonas sp.* NCIMB 399; found in seawater off the coast of Aberdeen, UK. A literature hit identified this strain in a marine hatchery in Canada (still within this temperate zone); cultured at 20°C in sea water agar

2. *Alteromonas sp.* NCIMB 2024; found in the largest and deepest coastal waters in the British Isles – the Firth of Clyde off the coast of Scotland; cultured at 20°C in sea water agar

3. Flavobacteriaceae NCIMB 14028; found in Hope Cove coastal waters near Plymouth, UK; cultured at 20°C in Bacto™ marine medium

2.3 Antibiotic treatment of *Phaeodactylum tricornutum* cultures

Axenic *P. tricornutum* was prepared using an antibiotic treatment adapted from Amin *et al.* (2015) and personal communication with Prof. Wim Vyverman from Ghent University, Belgium. The protocol implemented by Ghent University includes kanamycin (200 µg/mL), spectinomycin (50 µg/mL), and gentamycin (50 µg/mL). Amin *et al.* used streptomycin (50 µg/mL), gentamycin (67 µg/mL), ciprofloxacin (20 µg/mL), chloramphenicol (2.2 µg/mL), and ampicillin (100 µg/mL). We used an antibiotics cocktail with gentamycin (50 µg/mL), spectinomycin (50 µg/mL), kanamycin (200 µg/mL), ampicillin (100 µg/mL), and chloramphenicol (2.2 µg/mL). 1 mL of non-axenic *P. tricornutum* cultures in the exponential-phase were treated with the antibiotics cocktail in 9 mL sterile F/2 + Si media for 24 h. 1 mL of the antibiotic-containing culture was then transferred to 25 mL antibiotic-free sterile F/2 + Si media. After 5 to 7 days, cultures were monitored under the microscope and by plating aliquots on solid seawater agar.

2.4 Co-culture experimental set-up

The co-culture experiments were carried out in a multi-cultivator MC 1000-OD purchased from Photon Systems Instruments (PSI, Czech Republic).

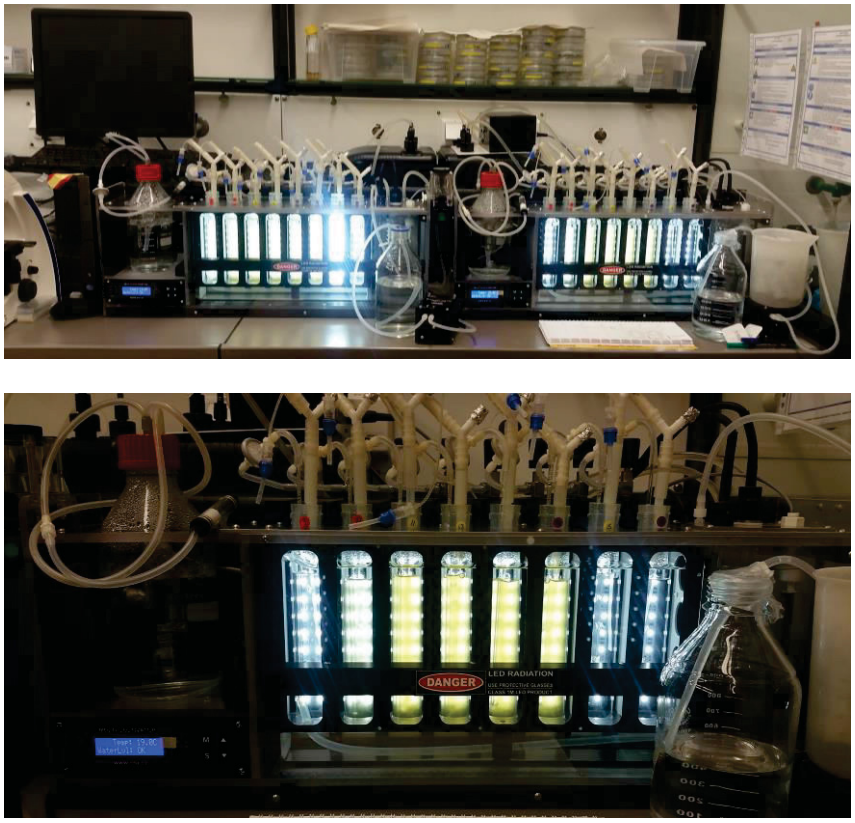

Figure 2: Photographs of the multi-cultivator (PSI, Czech Republic) set-up

Each 80mL culture is immersed in an automated temperature-controlled water bath set at 19°C, and each vessel is individually illuminated with cool white LEDs with a light intensity of 100 µmol m^{-2} s^{-1}. Each vessel is also supplied pressurised atmospheric air with the flow rate set manually.

Based on previous co-culture experiments carried out by Amin *et al.* (2015), Suminto and Hirayama (1997) and Paul *et al.* (2013), an inoculation ratio of 1:50 diatom:bacterial cells was used, with a starting cell count of 3 000 diatom cells to 150 000 bacterial cells. Culture media used was F/2 + Si medium. Traditional plating techniques on solid seawater agar were used to verify the identity of each bacterial strains as well as the axenicity of the *P. tricornutum* culture.

Two separate experiments were run. The first experimental run contained cultures of axenic *P. tricornutum*, axenic *Alteromonas sp.*, and the *P. tricornutum-Alteromonas sp*. co-culture in one multicultivator. The second multicultivator contained cultures of axenic *P. tricornutum*, axenic *Pseudoalteromonas sp.*, and the *P. tricornutum-Pseudoalteromonas sp.* co-culture. The second multicultivator experienced a number of technical issues and was therefore repeated in the second experimental run which included cultures of axenic *P. tricornutum*, axenic *Pseudooalteromonas sp.*, axenic Flavobacteriaceae *sp.* and the co-cultures of *P. tricornutum-Pseudoalteromonas sp*. and *P. tricornutum*-Flavobacteriaceae *sp*. co-culture equally distributed across both multicultivators.

2.5 Growth measurements

The multi-cultivator MC 1000-OD has the in-built capacity to record optical density measured at 680 nm and 720 nm. The optical density is read at 30 min intervals and the stored data downloaded onto a computer.

Growth was also monitored manually every 24 to 48 h using a light microscope and carrying out cell counts using a Neubauer counting chamber of each culture in quadruplicate. During the cell counts the ratios of the three different morphotypes (oval, fusiform, and triradiate) were recorded, and descriptions of the nature of each culture noted.

3 Results

3.1 Effect of the presence of bacterial species on the growth efficiency of *Phaeodactylum tricornutum*

Figures 3-5 illustrate the growth characteristics of P. tricornutum when it has been cultivated axenically and in co-culture with the three bacterial species, *Pseudoalteromonas sp.* (NCIMB 399), *Alteromonas sp.* (NCIMB 2024) and a Flavobacteriaceae *sp.* (NCIMB 14028).

The main graphs represents growth of axenic *P. tricornutum* and in co-culture with the respective bacterial species based on optical density measurements (OD680). Inserted graph represents bacterial growth of axenic bacterial species and in co-culture with *P. tricornutum* based on bacterial cell counts (x 10^6 cells/mL).

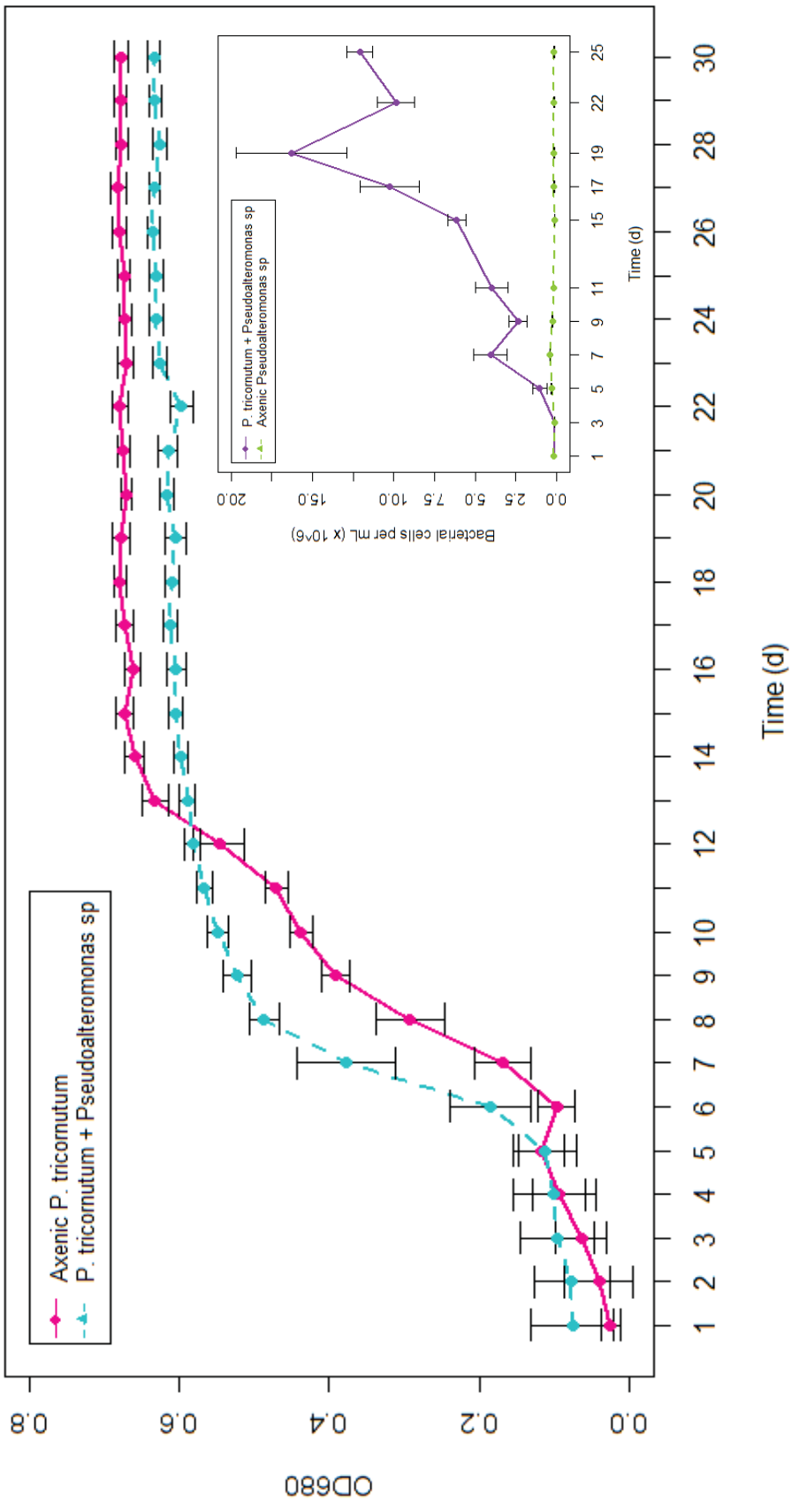

Figure 3: Growth of *P. tricornutum* in co-culture with Pseudoalteromonas sp (NCIMB 399)

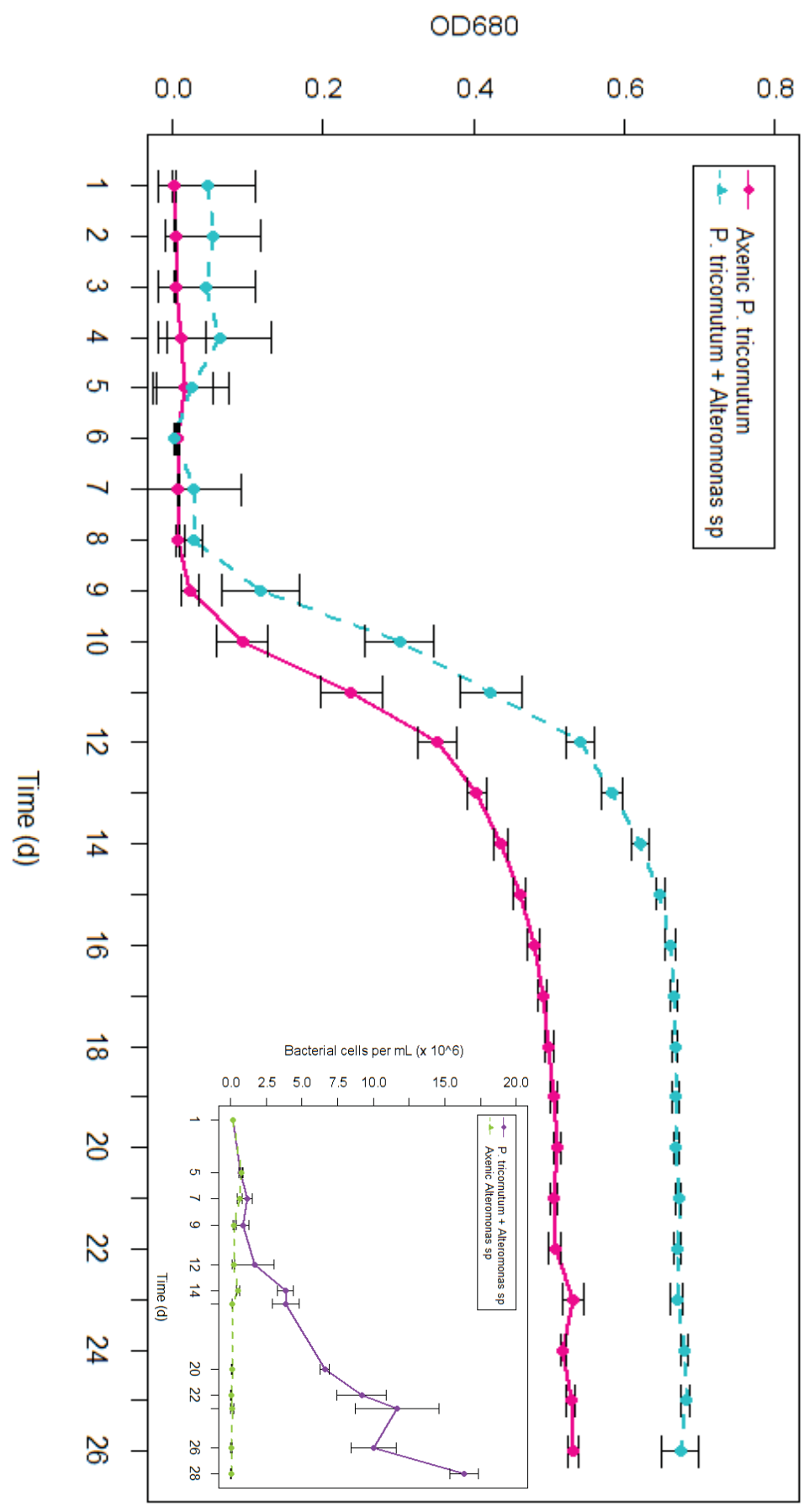

Figure 4: Growth of *P. tricornutum* in co-culture with Alteromonas sp (NCIMB 2024)

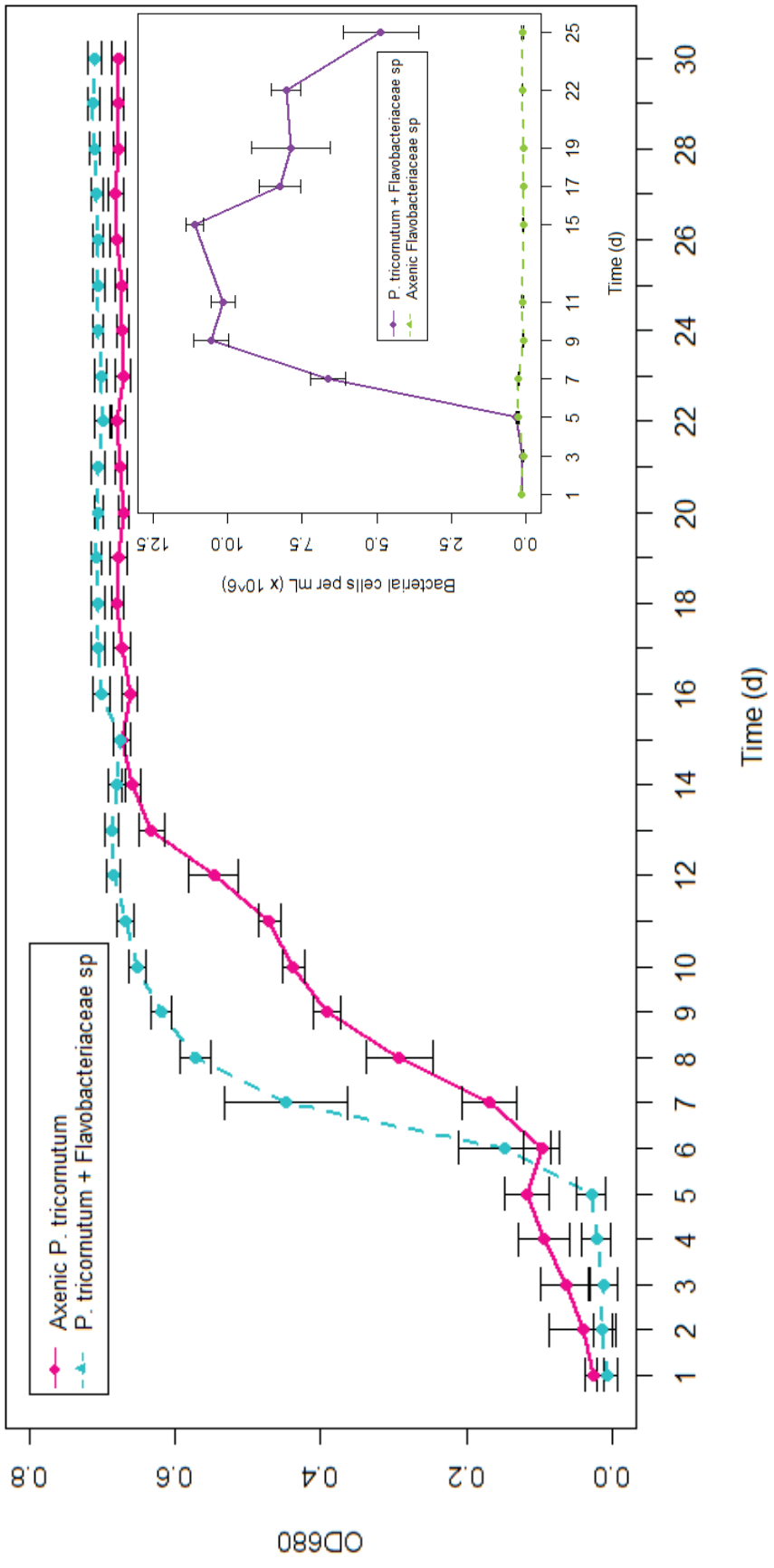

Figure 5: Growth of *P. tricornutum* in co-culture with Flavobacteriaceae sp (NCIMB 14028)

Table 1: Maximal cell densities of *P. tricornutum* in co-culture

	Cultivation scale (cells/mL)		
	Pseudoalteromonas sp	**Alteromonas sp**	**Flavobacteriaceae sp**
Co-culture	16.17 x 10^6 ± 1.26	16.83 x 10^6 ± 0.93	15.5 x 10^6 ± 1.16
Axenic	14.88 x 10^6 ± 2.16	10.13 x 10^6 ± 0.93	14.88 x 10^6 ± 2.16

Table 2: Specific growth rates (μ d^{-1}) of of *P. tricornutum* in co-culture

	Specific growth rate (μ d^{-1})		
	Pseudoalteromonas sp	**Alteromonas sp**	**Flavobacteriaceae sp**
Co-culture	0.91 ± 0.07 d^{-1}	1.10 ± 0.12 d^{-1}	1.32 ± 0.39 d^{-1}
Axenic	0.86 ± 0.05 d^{-1}	0.89 ± 0.28 d^{-1}	0.86 ± 0.05 d^{-1}

Tables 1 and 2 above contain the maximal cell densities and specific growth rates achieved by axenic *P. tricornutum* as well as in co-culture.

3.2 Effect of the presence of bacterial species on the morphology of *Phaeodactylum tricornutum*

Figures 6-8 illustrate the ratio of the fusiform, triradiate and oval morphotypes of *P. tricornutum* in co-culture with three bacterial species over time.

Figure 6: Morphology dynamics of axenic *P. tricornutum* and in co-culture (Pseudoalteromonas sp.)

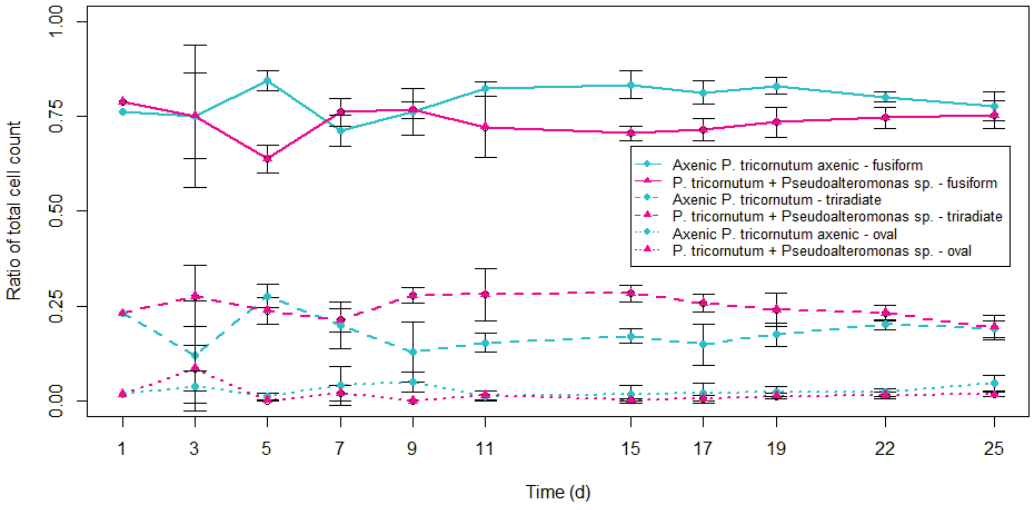

Figure 7: Morphology dynamics of axenic *P. tricornutum* and in co-culture (Alteromonas sp.)

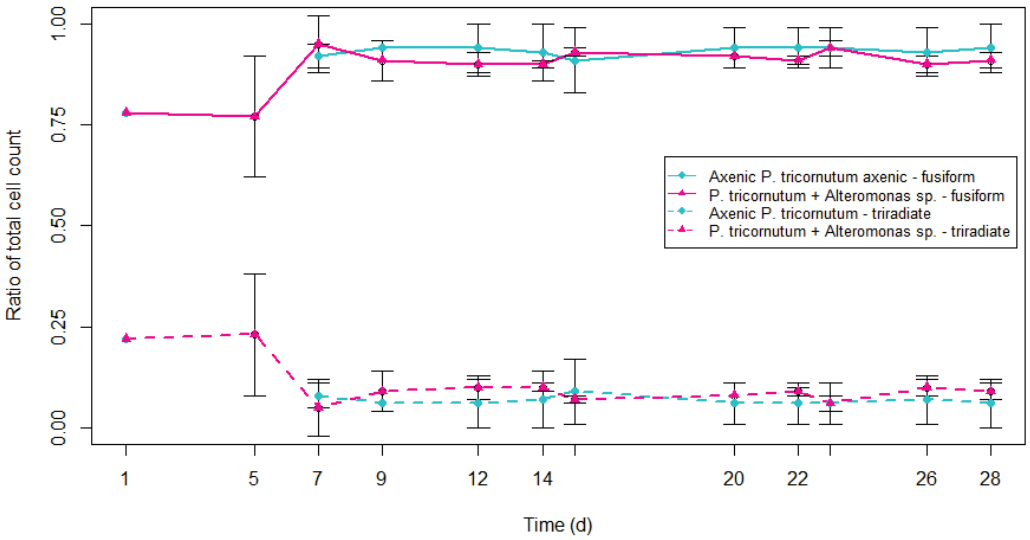

Figure 8: Morphology dynamics of axenic *P. tricornutum* and in co-culture (Flavobacteriaceae sp.)

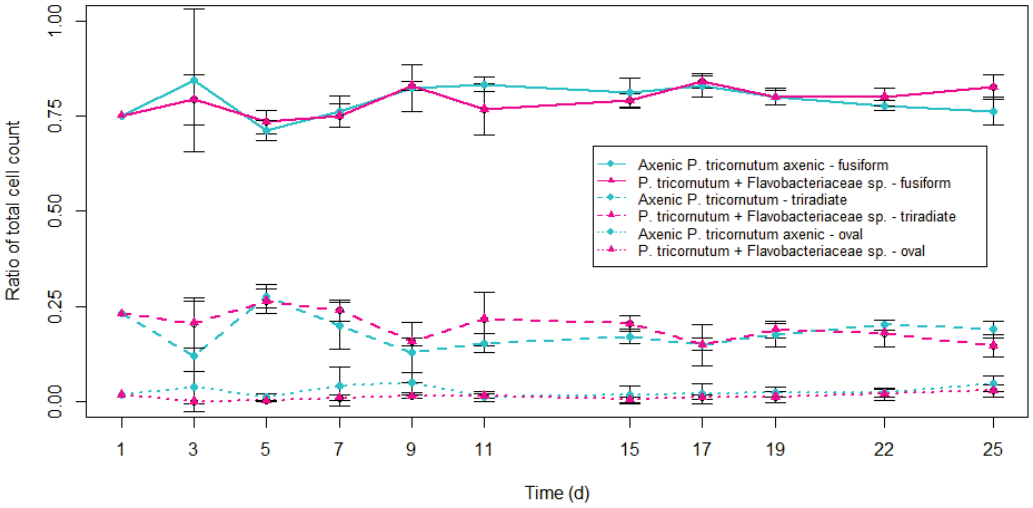

Figure 6 indicates a change in the morphology between Day 9 and Day 19. Reverting back to Figure 3, these days cover the end of the exponential phase and the first part of the stationary phase. A simple statistical analysis on the average ratio of triradiate cells during this period for all co-culture experiments have been represented in Table 3.

Table 3: Student's t-test for average percentage of trirdiate morphology of *P. tricornutum* CCAP 1052/1B in co-culture with different bacterial species

			***p* = < 0.05**
	***p*-values**		
	Pt+Pseudo	**Pt+Altero**	**Pt+Flavo**
Axenic	0.0002	0.4818	0.3097

The experiments would need to be repeated with emphasis on the morphology during the days covering the end of the exponential phase and the first part of the stationary phase to concretely state that *Pseudoalteromonas sp.* elicits a morphological change in *P. tricornutum* by increasing the number of triradiate cells when compared to axenic cultures of *P. tricornutum*.

4 Discussion

The null hypothesis adopted for this experiment was that the growth efficiency of *P. tricornutum* is not affected by the presence of a bacterial species. Tables 4 and 5 below show that only in the case of the maximal cell densities achieved by the *P. tricornutum-Alteromonas sp.* co-culture can we reject the null hypothesis. The presence of *Alteromonas sp.* has a statistically significant effect on the maximal cell density achieved by *P. tricornutum*.

Table 4: Student's t-test *p*-values comparing maximal cell densities of axenic *P. tricornutum* to growth in co-culture

			***p* = < 0.05**
Co-culture	**Pseudoalteromonas sp**	**Alteromonas sp**	**Flavobacteriaceae sp**
***p*-value**	0.342	0.0009	0.6311

Table 5: Student's t-test *p*-values comparing specific growth rates (μ d-1) of of axenic *P. tricornutum* to growth in co-culture

			***p* = < 0.05**
Co-culture	**Pseudoalteromonas sp**	**Alteromonas sp**	**Flavobacteriaceae sp**
***p*-value**	0.2892	0.2984	0.0579

With regards to the specific growth rate of *P. tricornutum* when in co-culture, the presence of Flavobacteriaceae *sp.* seems to have the greatest effect on the specific growth rate of *P. tricornutum* compared to the other two bacterial species.

Looking closely at the bacterial growth in Figures 3-5 show that no growth was observed when the bacterial species were in axenic conditions. This can be attributed to the lack of a carbon source in the F/2 + Si media

composition. When cultivated in co-culture, the bacterial species thrive. The *Pseudoalteromonas sp.* and *Alteromonas sp.* began growing exponentially after the *P. tricornutum* had already entered its stationary growth phase. However, the Flavobacteriacaea *sp.* begins its exponential growth when *P. tricornutum* enters its exponential growth phase. Flavobacteriaceae species have been shown to be omnipresent during phytoplankton blooms, and their preference for consuming more complex polymers rather than monomers suggests an active role in the processing of organic matter during these blooms, which explains their ability to grow exponentially with the *P. tricornutum* (Cottrell and Kirchman, 2000; Pinhassi *et al.*, 2004).

The highest maximal cell densities reached was when *P. tricornutum* was co-cultured with *Alteromonas sp*. (16.83 x 10^6 ± 0.93 cells/mL). Repetition of these experiments will further allow to conclusively state the positive influence of *Alteromonas sp*. on the *P. tricornutum* cell densities. In Chapter III, we found Alteromonadaceae species to be highly abundant in cultures of *P. tricornutum* cultivated using minimal media containing just nitrate and phosphate. Further experiments in minimal media will allow us to support the hypotheses discussed in Chapter III.

These experiments must be replicated in order to concretely elucidate the effect the presence of certain bacteria have on the growth efficiency of *P. tricornutum*.

Future experiments will have to be carried out with other bacterial species that were not highly abundant in the samples examined in Chapter III. This will allow is to determine whether *P. tricornutum* has a specific preference of bacterial species, or whether the bacteria fill a specific role, and whether

the absence of any other bacterial species allows them to thrive due to lack of niche competition.

Metabolic profiling of the samples at specific time points will further allow us to pin-point what the role of the bacterial species is in the artificial ecosystem based on which metabolites are being used as a form of currency in the system.

Coupling the resulting experimental results from further growth data and metabolic data with the modelling approaches introduced in Chapter III will allow the dynamic model to develop from qualitative to quantitative, allowing for the planning of future combinatorial experiments as well as providing a powerful predictive tool for culture monitoring such as predicting harvesting point based on the bacterial community.

5 References

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Future perspectives

"The future cannot be predicted, but futures can be invented."

– **Dennis Gabor** in 'Inventing the future'
New York: Alfred A, Knopf
1963

1 Algal-bacterial co-culture systems for optimising the algal-based bioeconomy

To realise the industrial potential of microalgal derived products, large quantities of microalgal biomass are required. However, large-scale cultivation methods are susceptible to contamination by organisms such as amoeba, ciliates, rotifers, bacteria, viruses, and other photosynthetic organisms (Day *et al.*, 2012; Wang *et al.*, 2013). These organisms can cause the culture to 'crash' which leads to loss of biomass, and therefore, loss of revenue. Rather than looking at these organisms as contaminants, understanding them could allow for the exploration of 'synthetic ecology' as a novel scaling up technique (Kazamia *et al.*, 2012).

The current state of diatom-bacterial interactions research, or algal-bacterial interactions in general, is solely based on applying laboratory and ecological data to create synthetic ecologies which, in theory, have the potential to optimise scaling up techniques. Further research is required to explore the full potential of applied microbial ecosystem management for a sustainable bio-economy. The scope of algal-bacterial co-cultivation approaches should look beyond just scale-up but we should identify further potential applications of this technology. After all, co-culturing in biotechnology is not a novel idea (Bader *et al.*, 2010), although much of the focus has been on bacterial-bacterial interactions (Goers *et al.*, 2014). Could algal-bacterial co-cultures potentially be a practical and profitable technology with applicability to more than just large-scale cultivation of

algal biomass? Would such co-cultures have the capabilities to produce industrially-relevant molecules at a lower production cost compared to current production methods? After all, the photoautotrophic nature of microalgae would have the advantage that no external carbon source is required, which could make such an approach more cost-effective.

The genesis of such an approach could begin by identifying, based on available laboratory as well as ecological data, which compounds naturally synthesised by microalgae are affected by the presence of bacteria and *vice versa*. This would allow us to identify industrially-relevant products gaining from a co-culture. Secondly, technically feasible cultivation methods and harvesting protocols required to deliver a final suitable product. Finally, investigation of the supply chain, and subsequently, a life-cycle analysis, will be necessary to convincingly show that co-culture of microalgae and bacteria is a potentially profitable novel biotechnology.

We could base this pioneering field on the following questions:

1. To what extent is the knowledge of plant-microbe interactions for the bioeconomy applicable to algae-bacteria interactions?
2. Development of microorganisms as biocatalysts for the production of industrially or pharmaceutically relevant products; what molecules of interest are promising products of algae-bacterial co-cultures?
3. Development of new methods and apparatus for efficient bioprocessing, and development of efficient separation of substances; which scale-up approaches would be economically feasible for our co-cultures? Are current scale-up techniques sufficient or do we need to explore novel techniques? Harvesting and

downstream processing are a current bottleneck – are current harvesting protocols sufficient?

4. Commercialisation of new technologies and qualitative and quantitative analysis of the value chains of products – is there a gap in the market for our molecules of interest? Is there a trend for these kinds of molecules? Life cycle assessments must be carried out.

2 The role of chitin synthase in stress response of Phaeodactylum tricornutum cultures

Although not directly related the main premise of the PhD project, one question that kept resurfacing is the role of chitin synthase in *P. tricornutum*.

Chitin is the most abundant polymer in the ocean and the second most abundant polymer on Earth, exceeded only by cellulose (see (Durkin *et al.*, 2009) for further references). Several diatom strains are able to produce chitin and have the genes necessary to encode chitin synthase. Chitin synthase is used by all chitin-producing organisms to generate the beta-(1-4)-linked N-acetylglucosamine polymers (Durkin *et al.*, 2009).

All genes necessary for chitin synthesis were found in *P. tricornutum* but there is no experimental evidence that it actually produces chitin fibres. Durkin *et al.* suggest a broad distribution of chitin in diatoms due to a fundamental structural function in the cell wall with cases of additional functional diversification such as fibre production (Durkin *et al.*, 2009). Furthermore, the discovery of chitin as the major carbohydrate component in silica frustules supports this hypothesis regarding the role of chitin in diatoms (Tesson *et al.*, 2008).

Unlike diatoms such as *Thalassiosira pseudonana* that synthesise silica frustules, *P. tricornutum* does not have an obligate requirement to synthesise silica frustules. Studies showed that *P. tricornutum* cultures

lacking silica showed no significant difference in growth when compared to silica-rich cultures (Zhao *et al.*, 2014). However, when silica-lacking and silica-rich *P. tricornutum* cultures were cultivated under light and temperature stresses, silica-rich cultures showed significantly better growth than silica-lacking cultures. This was especially true when placed under green light and at 10˚C (Zhao *et al.*, 2014).

Could the use of methods such as qPCR in silica-lacking and silica-rich cultures under normal growth conditions (40µE m^{-2} s^{-1}; 18˚C), green light stress, temperature stress (10˚C), and both green light and temperature stress (10˚C) stimulate chitin synthase expression in *P. tricornutum*?

Quantifying mRNA expression requires the precise extraction of the relatively volatile RNA from the cultures, followed by the creation of cDNA. The subsequent steps are included in the reverse transcription RT-qPCR protocol. RT-qPCR allows us to simultaneously analyse transcript levels for many genes in many different samples, making it especially suitable when biological material is only available in limited amounts (Heid *et al.*, 1996). Relative quantification is the most common method to analyse RT-qPCR data where the normalised expression of a target gene is compared to an internal stable reference gene (Siaut *et al.*, 2007). Housekeeping genes have widely been used as references but some were shown to be unstable under changing environmental conditions. For example, the commonly used GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was shown to be light-sensitive, making it unsuitable for our experiments (Siaut *et al.*, 2007). Siaut *et al.* tested the mRNA expression levels of a number of housekeeping genes under changing light conditions and showed that the most stable expression levels were displayed by histone *H4*, 30S ribosomal protein subunit *RPS*, cyclin dependent kinase *CdkA* and TATA box binding

protein *TBP* (Siaut *et al.*, 2007). *RPS* showed the lowest levels of mRNA, whereas *H4* showed the highest. Transcript levels of *TBP* were shown to be relatively stable under various test conditions including growth stages, light-dark cycle phases, and nutrient stresses in the diatoms *Skeletonema costatum* and *Chaetocerus affinis* (Kang *et al.*, 2012). For these reasons, we would choose TBP as our reference housekeeping gene.

Such a study would allow us to elucidate the role of chitin synthase in *P. tricornutum* by addressing the following questions:

1. Is chitin synthase even expressed?
2. If so, does the expression level of chitin synthase change under different stress conditions?
3. Does the lack or presence of silica affect stress response?
4. Does the lack or presence of silica affect chitin synthase expression?
5. Could chitin be involved in diatom-bacterial interactions?

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Special chapter: Algae for Africa

microalgae as a source of ***food and fuel in Kenya***

"Sustainable development is the pathway to the future we want for all. It offers a framework to generate economic growth, achieve social justice, exercise environmental stewardship and strengthen governance."

– **Ban Ki-moon** remark at G20 working dinner
"Sustainable Development for All" in St Petersburg, Russia
5 September 2013
(excerpt from United Nations Statements)

1 Introduction

Microalgae can inhabit diverse ecological habitats ranging from freshwater, brackish water, or seawater, they are equipped to thrive in various extreme temperatures and pH conditions. They are sunlight-driven cell factories that convert CO_2 into potential biofuels, foods, feeds and high-value bio-actives (Chisti, 2007). These peculiarities make microalgae the most abundant organisms on Earth.

In the early 1950s, the increase in the world's population and possibility of an insufficient protein supply eminent, research for new alternative and unconventional protein sources was carried out, and microalgal biomass appeared to be a possible candidate (Spolaore *et al.*, 2006). Meanwhile, extensive research into algae for biologically active substances, particularly antibiotics, began (Borowitzka, 1995). Further researched focused on environmental technologies aimed at the improving the quality of wastewater, and with the onset of the energy crisis of the 1970s, microalgae were then suggested as a source of biomass for methane (Chaumont, 1993).

Fast-forward a few decades and the microalgal biotechnology field includes applications such as; in aquaculture as feed in e.g. bivalve, echinoderm, crustacean and fish hatcheries, as potential biomass for biofuels, pharmaceuticals, nutraceuticals, nanotechnology, and bioremediation industries.

But to what extent is microalgal biotechnology applicable to developing countries? This Review attempts to identify issues faced by developing countries and how microalgae could be utilised in sustainable development approaches to address these issues. Kenya is still considered a developing country according to the UN Human Development Report (UNDP, 2015), and was chosen as the focus of this Review.

2 The cultivation of microalgae

There are five main parameters required for the successful cultivation of microalgal biomass; light, CO_2, water and inorganic salts, and an ideal temperature of between 20°C and 30°C.

2.1 Light

Photosynthesis converts light energy, usually sunlight from the Sun, into chemical energy. When light is the only limiting factor, microalgal productivity becomes proportional to the light conversion efficiency (Richmond *et al.*, 2003; Kumar *et al.*, 2010). At night, or other dark conditions, photosynthesis cannot occur, and the microalgae utilise stored energy for respiration. Depending on the temperature and other conditions, up to 25% of the biomass produced during the day may be lost again at night (Chisti, 2007).

Approximately 45% of the total light spectrum from the Sun is Photosynthetically Active Radiation (PAR). Only the irradiation spectral range of 400 to 700 nanometres can be utilised by photosynthetic organisms (Thimijan and Heins, 1983). The maximum efficiency of photosynthesis by microalgae is approximately 27%, and multiplying these two factors gives the maximum theoretical conversion of light energy to chemical energy by photosynthesis of approximately 11% (Gao *et al.*, 2007). The climatic zones with optimum growth conditions, based on the average global temperatures and average global solar radiation in Figures 1 and 2, are located between 40° north and 40° south latitude. Kenya lies perfectly

on the equator and has an average horizontal irradiance of 2000 kWh/m^2 (Figure 3).

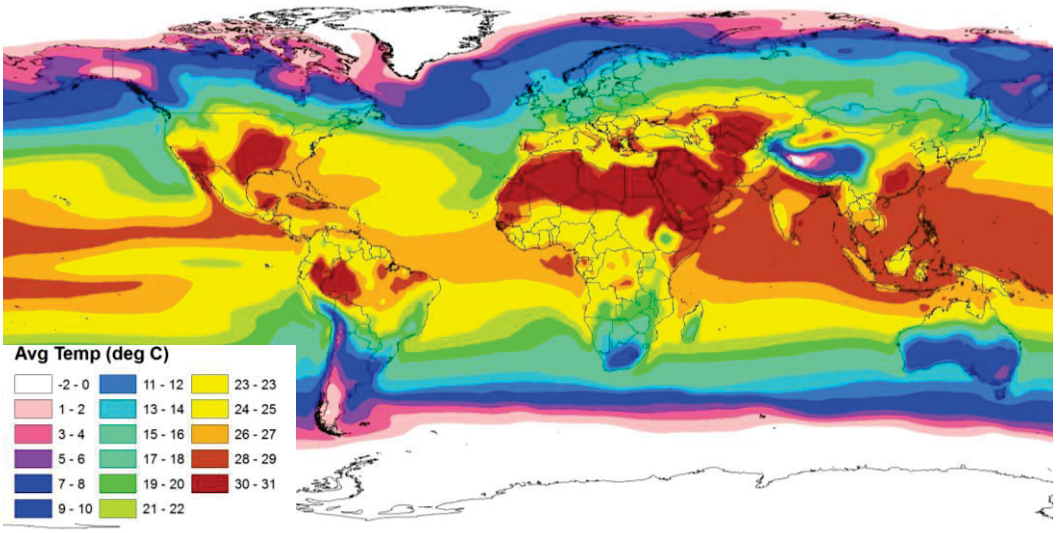

Figure 1: Global map of average temperatures (July 2000) (courtesy of Oak Ridge National Laboratory, U.S. Dept. of Energy)

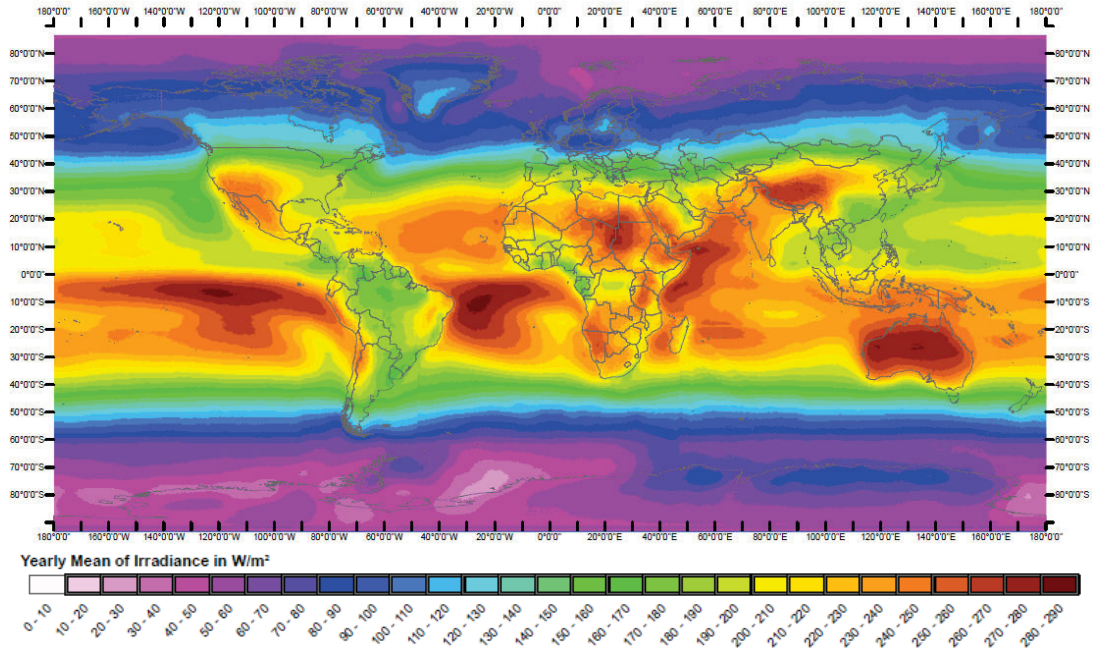

Figure 2: Global map of average solar radiation (1990-2004) (reprinted with permission from SoDa Services, Copyright Mines ParisTech / Armines 2006)

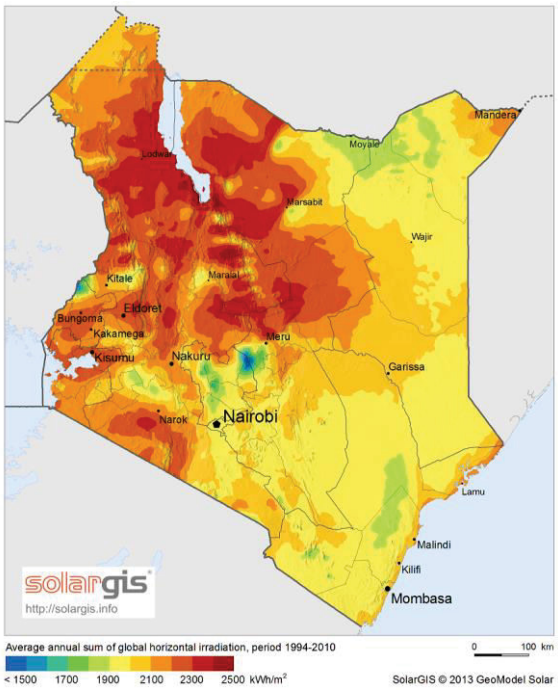

Figure 3: Global horizontal irradiation in Kenya (1994-2010) (SolarGIS© 2016 GeoModel Solar)

2.2 Temperature

Temperature is one of the major factors that regulate cellular, morphological and physiological responses of microalgae: higher temperatures generally accelerate the metabolic rates of microalgae, whereas low temperatures lead to inhibition of microalgal growth (Kumar *et al.*, 2010). Towards the North and South poles there is a lower light intensity and temperature, which results in lower biomass productivity, therefore the equator is considered an ideal location due to the temperature stability (Figure 1). Nonetheless, this does not necessarily mean the highest biomass yields as there are other climatic occurrences that can affect growth (Moody *et al.*, 2014).

Ideal sites are those that do not close due to cold conditions and are able to operate throughout the year as well producing a high annual lipid yield. High variability results in increased infrastructure which would create further operation costs. A study of the global biofuel potential from microalgae by Moody *et al.* (2014) showed the western Kenyan city of Kisumu to maintain an average annual temperature close to the optimum and would, therefore, be able to maintain high biomass yields, making it an ideal location for microalgae cultivation (Moody *et al.*, 2014).

2.3 Carbon dioxide (CO_2)

As with all photosynthetic organisms, microalgae predominantly use CO_2 as a carbon source. No growth can occur in the absence of CO_2, and an insufficient supply of CO_2 is often the limiting factor in productivity. Natural dissolution of atmospheric CO_2 into the water is not enough; atmospheric CO_2 levels are at approximately 0.0387%, which are not sufficient to support the high microalgal growth rates and productivities needed for large-scale biofuel production. Usual sources of CO_2 for microalgae are atmospheric CO_2, CO_2 from industrial exhaust gases (e.g. flue gas and flaring gas, which typically contain about 4% to 15% of CO_2), and CO_2 chemically fixed in the form of soluble carbonates (e.g. $NaHCO_3$ and Na_2CO_3) (Kumar *et al.*, 2010). Uptake of these inorganic forms of carbon also has the potential to increase the pH within the cultures (Hansen, 2002). Oxygen (O_2) is a product of photosynthesis. Oxygen levels above atmospheric O_2 levels (0.2247 mol O_2 m^{-3}) can inhibit photosynthesis in a number of microalgal species, even when the CO_2 levels remain elevated (Aiba, 1982). Furthermore, elevated levels of O_2 coupled with high irradiance can lead to photo-oxidation (Richmond, 1990; Camacho Rubio *et al.*, 1999).

In 2015, Kenya signed the United Nations Framework Convention on Climate Change (UNFCCC) Paris Agreement, a daughter treaty of the 1997 Kyoto Protocol, which aims to empower all countries to act to prevent average global temperatures from rising by more than 2°C (UNFCCC, 2015). Increasing global temperatures can be largely attributed to elevated CO_2 (Solomon *et al.*, 2009). Theoretically, microalgae are capable of utilising up to 9% of the incoming solar irradiance producing 280 tonnes of dry biomass ha^{-1} $year^{-1}$ whilst sequestering roughly 513 tonnes of CO_2 (Bilanovic *et al.*, 2009). Implementing microalgal cultivation strategies in Kenya will allow the country to be an active participant in the global efforts to reduce atmospheric CO_2 levels.

2.4 Water

Drought is a prevalent natural catastrophe that affects the 10 million mostly livestock-dependent people in the arid and semi-arid lands in the north and north-east of Kenya (Zwaagstra *et al.*, 2010). Therefore, the use of freshwater is not sustainable. However, microalgae are capable of thriving in saline, brackish and wastewater.

2.4.1 Seawater

With a coastline stretching 1420km, Kenya has every possibility to utilise this a source of water for algae culture. Kibuyuni, in the south coast of Kenya, has found success in the cultivation of macroalgae (seaweed). Kenya Coastal Development Project (KCDP) facilitated in the construction of seaweed farms and supplied tools, training and seeds. The project has created new livelihood opportunities, particularly for women and income generated has allowed farmers to send their children to school and create

better housing for themselves. The community has managed to produce 10 tonnes of dried seaweed since February 2015 worth 450,000 KSH (£3,064) by exporting to China, Ireland and Malaysia (KCDP, 2015). The implementation of microalgae cultivation farms in the coastal region of Kenya could also be realised.

Moreover, utilising seawater would provide trace elements beneficial to microalgal growth. Most bioactive trace metals, including iron, exist at nanomolar (10^{-9} M) to picomolar (10^{-12} M) concentrations in our oceans, approximately one-millionth of the intracellular concentration in diatoms (Bruland *et al.*, 1991; Morel and Price, 2003).

2.4.2 Wastewater

Nairobi is one of largest cities in Africa and with a growing urban population, the wastewater generated could be exploited for culturing microalgae. Microalgae grow abundantly in wastewater as it is rich in organic carbon and inorganic nitrogen and phosphorus and so, algae is able to efficiently remove the nitrogen and phosphorus. Oxygen produced by the photosynthetic microalgae would allow treatment of wastewater via oxidation as well as aeration of treatment ponds, without the need of mechanical aeration and thus reducing operational costs. This type of integrated system allows for an economically viable method to both treat wastewater as well as culturing microalgae for biofuel (Mahapatra *et al.*, 2013).

Olguín *et al.*, (2003) describe a system where 84-96% of nitrogen and 72-87% of phosphorus was removed from the anaerobic effluent of piggery wastewater by growing algae, thereby reducing eutrophication in the environment.

Nevertheless, only 30% of published work on microalgae used wastewater as a source of nutrients, regardless of its promising benefits. The vast majority of laboratory based experiments use chemical fertilisers as it is much more readily available (Lam and Lee, 2012). However, this becomes an issue when up-scaling as the production of chemical fertilisers is energy intensive and not sustainable; 1.2kg of carbon dioxide is released for every 1kg ammonia produced (Kim and Dale, 2005).

2.5 Inorganic salts

Apart from the essential need of a carbon source, microalgae require a nitrogen source. Nitrogen is an important constituent of both nucleic acids and proteins; crucial for the primary metabolism of microalgae (Becker, 1994). Phosphorus is another important nutrient required by microalgae for growth. It is indeed the 'staff of life'; the scaffolding on which all biomass is built (Karl, 2000).

Sources of nitrogen and phosphorus include agricultural fertilisers, which are easily available but can be a significant cost factor at a large-scale cultivation level. More cost-effective sources of nitrogen and phosphorus have been explored such the use of wastewater mentioned above.

2.6 Scaling up techniques

The production of microalgal biomass requires relatively simple photosynthetic growth conditions: light, carbon dioxide, water and inorganic salts. The system must also include a means of agitation or mixing of the culture to prevent the settling of the microalgal cells, the elimination of thermal stratification (temperature layering effect that occurs in water),

distribution of carbon dioxide and inorganic salts, removal of the photosynthetically produced oxygen, and the enhancement of light utilisation efficiency (Terry and Raymond, 1985). There are two main land-based cultivation methods implemented for the large-scale cultivation of microalgae; raceway ponds and photobioreactors. A comparison summary can be found in Table 1.

Table 1: Comparison of raceway ponds and photobioreactors (derived from Pulz, 2001)

CULTURE SYSTEM	RACEWAY PONDS	PHOTOBIOREACTORS
Required space	High	For photobioreactor itself, low
Water loss	Very high, may also cause salt precipitation	Low
CO_2 loss	High, depending on pond depth	Low
Oxygen concentration	Usually low enough because of continuous spontaneous outgassing	Build-up in closed system requires gas exchange devices (O_2 must be removed to prevent inhibition of photosynthesis and photo oxidative damage)
Temperature	Highly variable, some control possible by pond depth	Cooling often required (by spraying water on photobioreactor or immersing tubes in cooling baths)
Shear	Usually low (gentle mixing)	Usually high (fast and turbulent flows required for good mixing, pumping through gas exchange devices)
Cleaning	No issue	Required (wall-growth and dirt reduce light intensity), but causes abrasion, limiting photobioreactor life-time
Contamination risk	High (limiting the number of species that can be grown)	Medium to low
Biomass quality	Variable	Reproducible
Biomass concentration	Low, between 0.1 and 0.5g/l	High, generally between 0.5 and 8g/l
Production flexibility	Only a few species possible, difficult to switch	High, switching possible
Process control and reproducibility	Limited (flow speed, mixing, temperature only by pond depth)	Possible within certain tolerances
Weather dependence	High (light intensity, temperature, rainfall)	Medium (light intensity, cooling required)
Start-up	6 – 8 weeks	2 – 4 weeks
Capital costs	High ~ US$100000 per hectare	Very high ~US$250000 to US$1000000 per hectare (photobioreactor plus supporting systems)
Operating costs	Low (paddlewheel, CO_2 addition)	Higher (CO_2 addition, oxygen removal, cooling, cleaning, maintenance)
Harvesting costs	High, species dependent	Lower due to high biomass concentration and better control over species and conditions

2.6.1 Raceway ponds

Raceway ponds are simple open-air microalgal biomass cultivation systems which have been used for the large-scale cultivation of microalgae since the 1950s. They are typically made of a closed loop recirculation channel that is typically 0.3m deep. The mixing (and circulation) is achieved by the implementation of a paddlewheel (see Figures 4 and 5) which must operate continuously to prevent sedimentation. Broth is harvested behind the paddlewheel. The building materials utilized for raceway ponds are concrete, compact earth, and may be lined with white plastic (Chisti, 2007).

As carbon dioxide is the carbon source for algae, its sequestration from the air into the culture limits the growth rate. Other major bottlenecks of raceway ponds are the lack of temperature control (any cooling is only achieved by evaporation, and any heating will add to the production cost), and the susceptibility to contamination by other organisms including other algal strains that might out-compete the desired species (Borowitzka, 1999).

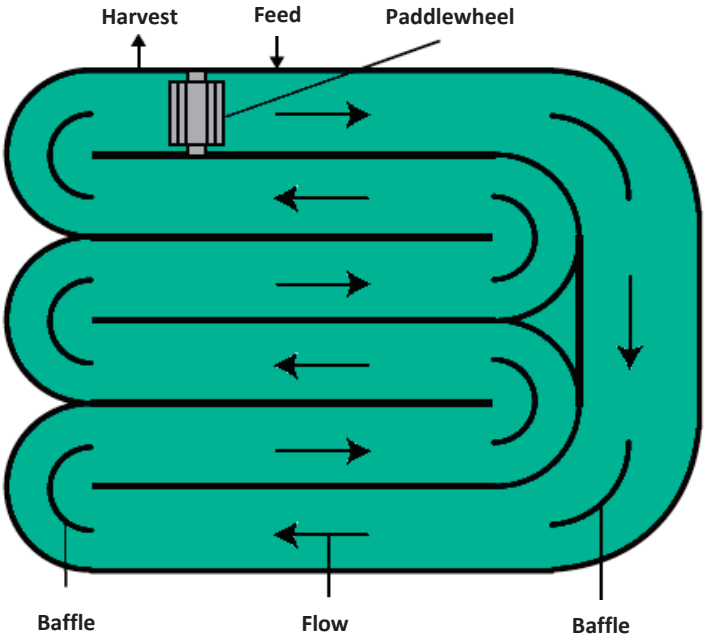

Figure 4: A schematic aerial view of a raceway pond (modified from Chisti, 2007)

Figure 5: Photograph of a 250m^2 open raceway ponds (taken November 2013; Application Centre for Renewable Resources (ACRRES; Wageningen University initiative) in Lelystad)

2.6.2 Photobioreactors

Photobioreactors are closed microalgal biomass cultivation devices that allow for the production of a monoseptic culture which is fully isolated from a potentially contaminating environment (Grima and Fernández, 1999). Although there are a number of photobioreactor configurations available, they all have the same basic principles and parameters. The main parameter that affects photobioreactor design is provision for light penetration; i.e. high surface-to-volume ratio, crucial if one wants to improve the photosynthetic efficiency. In order to achieve a high surface-to-volume ratio, several designs have been developed which can be grouped into three basic types; tubular, flat-plate and fermenter-type (Carvalho *et al.*, 2006). Tubular and flat-plate photobioreactors are specifically designed for efficient sunlight harvesting and discussed in Figures 6 and 7.

Figure 6: Photograph of a Horizontal Tubular Reactor (HTR) (taken in November 2013; AlgaePARC, Wageningen University). HTRs are composed of horizontal transparent glass or plastic (polyethylene) tubing. Gas transfer takes place in the tube connection or via a dedicated gas-exchange unit, and the angle toward sunlight is particularly adequate for efficient light harvesting (Carvalho *et al.*, 2006).

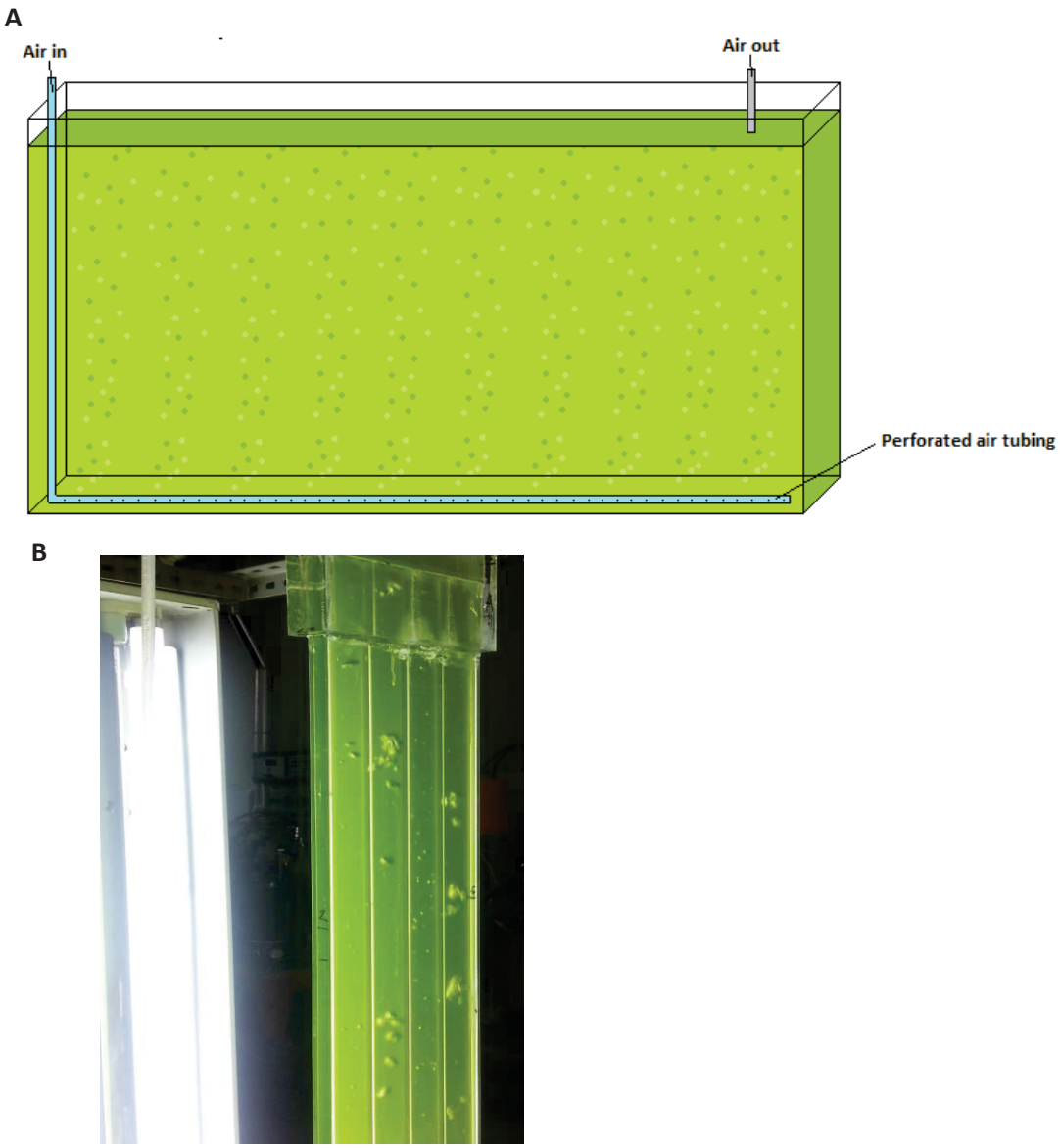

Figure 7: Schematic diagram of a flat-plate photobioreactor. Flat-Plate Reactors (FPRs) are designed to make efficient use of sunlight by constructing narrow panels so as to attain high area-to volume ratios **(A)**. Panels can be constructed from polyethylene bags supported by a metal framework, or from acrylic plastic walls. CO_2 is supplied via bubbling, which also provides mixing and the efficient removal of 0_2. They can either be placed outside and utilize natural sunlight or be placed indoors and utilize an artificial light source **(B)** (picture taken in November 2013, Laboratório Nacional de Energia e Geologia (LNEG), Lisbon, Portugal; schematic diagram based on designs seen at A4F AlgaeFarm).

3 Microalgae as feed

Microalgae contain an abundance of properties that have benefits in both animal feed and aquaculture. The growing production has seen a rise from 9.7 million tonnes in 2001 to 21 million tonnes of world aquaculture production of aquatic algae in 2011, with the African continent almost doubling its production from 0.08 million tonnes to 0.14 million tonnes in that time (FAO Fisheries and Aquaculture Department, 2013). These unicellular, but diverse organisms, have a wide range of applications within the animal feed and aquaculture markets.

3.1 Animal feed

Microalgae has shown great benefits in animal feed by providing vitamins, minerals and essential fatty acids to boost immune response; even very small amounts have proven to positively affect the animals physiology. This affect also creates a shiny coat and feathers on pets (Pulz and Gross, 2004). Feed for poultry are rationed to 5-10% and give the yellow colour seen in broiler skin and egg yolks (Spolaore *et al.*, 2006).

The yellow colour that microalgae can give comes from the carotenoids that they contain, of which the two most common are β-carotene and astaxanthin. Although most microalgae only contain about 1-2% concentration of carotenoids, species of *Dunaliella* can contain up to 14% β-carotene, if grown under the correct conditions. For this reason, *Dunaliella* species are successfully being grown on an industrial scale and sold for both animal and human use (Milledge, 2011).

3.2 Aquaculture

Aquaculture in Africa is on the increase; with Egypt leading the race with 987 tonnes produced in 2011 (FAO Fisheries and Aquaculture Department, 2014). The global aquaculture industry rakes in billions of dollars; 2012 saw a global aquaculture production of 90.4 million tonnes worth $144.4 billion (FAO, 2014).

Microalgae are a vital component in aquaculture as they are the primary producers of the food chain, particularly acting as a food source for larvae of molluscs, crustaceans and fish (Pulz and Gross, 2004). Just as in animal feed, microalgae is also exploited for its colouring capabilities such as the carotenoid astaxanthin which can pigment prawns, salmon and ornamental fish with a reddish colour. The market for this colourant alone was estimated at $200 million in 2004 costing around $2,500/kg (Spolaore *et al.*, 2006). Unlike β-carotene, the synthetic form of astaxanthin overshadows the natural in the market due to its high production costs (Milledge, 2011).

4 Microalgae as food

For centuries people have been using microalgae as a food source; from China where the cyanobacteria *Nostoc* was used to combat famine, to another cyanobacteria *Athrospira* (colloquially known as Spirulina) in Chad (Kay and Barton, 1991). Despite this, it is only very, recently (the 1950's) that we began large-scale cultivation and commercial application (Spolaore *et al.*, 2006). More than just nourishment, microalgae is now beginning to make gains in the health food sector with products like Spirulina being named as one of the greatest superfoods on Earth by the World Health Organisation (Chacón-Lee and González-Mariño, 2010). The baby food market also has potential for the inclusion of microalgae and finally, its use in therapeutic applications in HIV positive patients.

4.1 Health supplements

Spirulina contains 60-70% protein per weight and are a rich source for vitamins (particularly vitamin B_{12} and β-carotene which is a vitamin A precursor), minerals (principally iron) and a source of dietary γ-linolenic acid (GLA)) (Belay *et al.*, 1993). Of the microalgal biomass produced annually, 75% is used for powders, tablets, capsules and pastilles; most of which are *Chlorella* and Spirulina. However, functional foods that contain microalgae biomass are more convenient and attractive, such as in pasta, biscuits, bread and soft drinks. Spirulina supplemented nutraceuticals and in pure form have shown to support healthy intestinal bacteria such as *Lactobacilli*; at minimum of a 10-fold increase in growth rate of the bacteria was

revealed, showing the beneficial probiotic effects of the microalgae (Pulz and Gross, 2004).

Chlorella is another key species that is produced by over 70 companies as it is an significant source for β-1,3-glucan; an active immunostimulator, free-radical scavenger and can reduce blood lipids (Spolaore *et al.*, 2006).

The pennate marine diatom *Phaeodactylum tricornutum* has the ability to produce the poly-unsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in high proportions of the total fatty acid content (Fajardo *et al.* 2007; Siron *et al.* 1989; Rebolloso-Fuentes *et al.* 2001). Marine-derived EPA and DHA, colloquially known as omega-3 PUFAs, are important in human nutrition with health benefits such as reduced cardiovascular morbidity and mortality, reduced risk of premature births and improved cognitive and behavioural development of the foetus, as well as benefiting patients with atherosclerosis, hypertension, and neurological and neuropsychiatric diseases (Yashodhara *et al.*, 2009).

4.2 Infant and young child feeding

In poor areas within developing countries, baby formulas are often of a low nutritional value that do not provide sufficient protein, lipid or micronutrients to the infants. Babies need adequate calories for normal and healthy development. Algae species such as Spirulina have proven to be an appropriate addition to baby food; it has the ability to help tissue growth, vision and boost the immune system (Sharoba, 2014).

More specifically, is the addition of docosahexanoic acid (DHA), a long-chain omega-3 fatty acid that is a major component in brain and visual

development allowing for improved cognitive skills and vision (Beelen *et al.*, 2007). Higher plants and animals cannot synthesise polyunsaturated fatty acids (PUFAs) and thus get it from their food; fish and fish oils, which in turn uptake PUFAs from microalgae. So going straight to the source (microalgae) and by passing the risk of accumulating toxins and smelly products. DHA occurs naturally in breast milk, but not in cow's milk and since 1990, DHA has been recommended as an addition into infant formula (Spolaore *et al.*, 2006).

4.3 HIV and microalgae

Kenya has around 1.6 million people living with HIV, of which women account for about 57%. Although the country has universal testing for pregnant women where 90% of women who attended antenatal care received their results. Only half of pregnant women who were infect received antiretroviral (ARV) treatment during breastfeeding to reduce transmission risks (National AIDS Control Council (Kenya), 2014). Breastfeeding is crucial for an infant's development and the need for ARVs in HIV infected mothers during this period is imperative to reduce mother to child transmission (Thomas *et al.*, 2011). However, it is evident that resources and availability of ARVs could hinder this and place infants at risk. The use of DHA from algae could help to overcome such a barrier.

The effects of long term use of highly active antiretroviral therapy (HAART) can lead to insulin resistance and then to dyslipidemia and weight and fat changes (Azabji-Kenfack *et al.*, 2011a). Although dietary supplements have proven successful in treatment of insulin resistance, the WHO states that the cheap and easily available, Spirulina could be highly beneficial as it has shown to better glycaemic control, lower cholesterol and reduce blood

pressure in diabetics. It was unequivocally shown in a study by (Azabji-Kenfack *et al.*, 2011b) the study that Spirulina drastically increased insulin sensitivity by 224.7% compared to 60% in the group using soybeans . This effect is likely due to the immune modulating effect of Spirulina, however, further study is still needed.

Other studies on HIV and/or severe immune deficient patients showed an increase in body weight when supplemented with Spirulina as well as increased haemoglobin levels, which in turn reduced anaemia (Azabji-Kenfack *et al.*, 2011a).

These HIV studies therefore shed light on the use of microalgae to tackle malnutrition, particularly in children whose development can be stunted by it. In Kenya, 35% of children under the age of 5 have stunted growth, with 42% of these children living in Kenya's Eastern Province where they survive off a low energy maize based diet (Tomedi, 2012). These children would greatly benefit from the addition of a microalgae supplement in order to better their physical and neurological development as well as survival.

5 Microalgae as biofertilisers

Phosphorus (P) lies at the heart of modern agriculture as it is a major source of nutrients for plants. However, P is a finite non-renewable resource and reserves for phosphate rock could be depleted in 50-100 years. In fact, Morocco has a near monopoly on the reserves in Western Sahara while China has reduced exports to secure their own supply. On the other hand, the US only has about 30 years in supplies left as Western Europe and India are already completely dependent on exports. The issue is that the P is not efficiently used as it usually ends up in wastewater or as runoff into our rivers and oceans, leading to toxic algal blooms (Cordell *et al.*, 2009).

Globally, humans physically consume about 3 tonnes of P and since virtually 100% of consumed P is excreted, the same 3 tonnes finds its way out into our wastewater. Usually this would then be removed via waterways. Currently, only 10% of human excreta is intentionally or unintentionally recirculated, however urban sites can be considered P hotspots and be exploited to better recycle the finite resource (Cordell *et al.*, 2009).

Microalgae have the unique capacity for 'luxury P uptake', essentially sequester high amounts of P, and wastewater has an excess of P required for microalgae growth. This simple idea is used in small communities globally to treat local wastewater systems (Powell and Shilton, 2008), however, this can then be taken a step further by employing this microalgae as a biofertiliser.

6 Biofuels

Biofuels are generally divided into "first-generation" and "second-generation" biofuels, with microalgal-based biofuels considered "third-generation". Any biofuel production process, which can successfully replace an equivalent conventional fuel, needs to fulfil three basic requirements; first, a sufficient feedstock to produce fuel at a commercial scale should be produced, secondly it should cost less than conventional fossil fuel, and thirdly, it should match standard specification of fuel quality (Singh and Gu, 2010).

First-generation biofuels are generally the product of the often edible and above-ground biomass (usually sugars, grains or seeds) produced by crop feedstock, and relatively simple processing of the biomass is required to produce a finished fuel (Naik *et al.*, 2010). Second-generation biofuels are produced from biomass in a more sustainable fashion, which is truly carbon neutral or even carbon negative in terms of its impact on CO_2 concentrations (Gomez *et al.*, 2008). The biomass utilised is known as lignocellulosic (LC) biomass, and makes up the majority of the cheap and abundant non-food materials available from plants, including residues of forest management or food crop production (such as corn stalks or rice husks) or whole plant biomass, such as grasses or trees grown specifically for biofuel purposes (Naik *et al.*, 2010).

Microalgae derived biofuels have three major advantages over first and second-generation biofuels; overcoming food-versus-fuel predicament, a higher potential biofuel yield per hectare and the ability to be harvested

throughout most of the year, thus giving a regular supply of biomass (Chisti, 2007, 2008).

Table 2: Comparison of some sources of biodiesel (from Chisti, 2007)

Crop	Oil yield (L/ha)	Land area needed (M ha)[a]
Corn	172	1540
Soybean	446	594
Canola	1190	223
Jatropha	1892	140
Coconut	2689	99
Oil palm	5950	45
Microalgae[b]	136900	2
Microalgae[c]	58700	4.5

[a] For meeting 50% of all transport fuel needs of the United States
[b] 70% oil (by wt) in biomass
[c] 30% oil (by wt) in biomass

However, as it stands, microalgal-derived biofuels are not a commercial reality simply because they are not economically feasible. The cost of production and thus the market price of algal biofuels simply cannot compete with the comparably low cost of fossil fuel prices (Norsker *et al.*, 2011).

6.1 The food-versus-fuel predicament

In a country such as Kenya, food security remains a major issue and therefore using crops as a source of biomass for biofuel is not feasible. The ability for microalgae to use non-arable land is another characteristic that makes it so favourable as a biofuel source; Kenya has 45.8% non-arable land that would not compete for land needed for food crops (CIA, 2016). In 2009, Bedford Biofuels, a Canadian company, began talks with local ranch owners in Kenya's Tana Delta District on establishing a large Jatropha plantation. However, they were met with resistance by local NGOs even though the

project could have brought in international investment and secured jobs for the local community. A year after planting the first seedlings their operations were closed down and the company filed for bankruptcy in 2013. The collapse of the project was down to a combination of an 'anti-BB' campaign by NGOs, local residents who were afraid of eviction or losing access to grazing land and the eruption of ethnic violence (Krijtenburg and Evers, 2014). Fortunately, microalgae cultivation would not face the same issues as it will not take up grazing or arable land therefore reducing the resistance from both local NGOs and residents.

6.2 Theoretical maximum annual average lipid yields

A study carried out by Moody *et al.* (2014) used large-scale, validated, outdoor photobioreactor microalgae growth model based on 21 reactor- and species-specific inputs to model the growth of *Nannochloropsis*. The model accurately accounts for biological effects such as nutrient uptake, respiration, and temperature and uses hourly historical meteorological data to determine the current global productivity potential. Table 3 contains the results of the model in terms of average microalgae lipid yields from various regions around the world.

Table 3: Average microalgae lipid yields in cubic metres per hectare^{-1} per metre^{-1} (corresponding biomass yields in grams per metre^{-1} per day^{-1}) of various regions around the world with respective high and low monthly lipid yields (Moody *et al.*, 2014)

Location	Maximum monthly	Average monthly	Lowest monthly
Kisumu, Kenya	2.47 (15.9)	2.28 (14.8)	2.07 (13.3)
Learmonth, Australia	2.61 (18.0)	2.16 (14.0)	1.49 (9.64)
Trivandrum, India	2.42 (15.6)	2.08 (13.4)	1.75 (11.3)
Cali, Columbia	2.27 (14.6)	2.04 (13.2)	1.91 (12.3)
Hawaii, United States	2.36 (15.3)	1.97 (12.8)	1.50 (9.95)
Yuma, AZ, United States	2.68 (17.3)	1.80 (11.7)	0.68 (5.16)
Poltavka, Russia	2.30 (14.1)	1.06 (6.84)	0.46 (2.23)
Bagaskar, Finland	2.19 (14.1)	0.77 (5.00)	0.55 (3.86)
Punta Arenas, Chila	1.77 (11.9)	0.77 (5.07)	0.51 (3.25)

Table 3 shows that Kisumu, a city in the west of Kenya, would be the optimal location for cultivation of the microalgae *Nannochloropsis* in an outdoor bioreactor.

7 Concluding remarks

Geographically speaking, Kenya is an ideal location for culturing algae as it lies on the equator and therefore receives optimum light and temperature for high yields. Moreover, the Kenyan government has also set out a development policy termed 'Kenya Vision 2030' that aims to transform the nation into a newly industrialized country with a high quality of life for all its people by the year 2030 (http://www.vision2030.go.ke/). Microalgae has the capability to fulfil some of the Kenya Vision 2030 goals.

Native Spirulina is already being successfully cultured in Kisumu, Kenya by a company called Dunga Spirulina that sell the microalgae as a health supplement and to NGOs to support those with HIV (http://www.dungaspirulina.com/). The Kenyan government aims to enhance locally derived natural health products and the natural products industry. Using Dunga Spirulina as a small success, it is evident that high value products such as β-carotene and astaxanthin could be sold, and even exported, as colourants, animal feed, health food supplements and aquaculture in Kenya. As a healthy food source, microalgae could also have an impact in combating hunger, HIV and reducing child mortality; 3 of the 8 Millennium Development Goals (UN, 2015). Infant formula supplemented with DHA from microalgae allows for a more nutrient rich formula, necessary for healthy development. Furthermore, this principle would aid mothers with the HIV virus who are unable to access ARVs to avoid breastfeeding, thus reducing mother to child transmission. Using this natural product would also serve to treat malnourishment in Kenya, an

aspect that could in turn help individuals to fight off infection of other diseases prevalent in the region.

The Kenya Vision 2030 also aims to make fertilisers more affordable and accessible to further increase their agriculture as low rates in sub-saharan Africa mean 75% of the soils are nutrient deficient; the irony is that Africa is the biggest phosphate rock export, but has the greatest food shortages. Kenya Vision 2030 also aims to reduce solid waste and pollution is urban areas, and by utilising microalgae in wastewater management, they can tackle these goals simultaneously; recycling the finite resource, phosphorus, by culturing microalgae in wastewater and then using the biomass in fertilisers.

For the Kenyan people, particularly those in rural communities, sustainable developments such as culturing microalgae will offer a world of opportunity. Beyond health and economic benefits, microalgae farms would offer employment, particularly for low-skilled jobs. This would in turn improve their quality of life as they would be able to improve housing and education opportunities for their families. However, an important aspect would be to promote employment for women due to persistent gender-based inequalities in most developing countries (Rossi and Lambrou, 2009), another one of the Millennium Development Goals. These sustainable microalgae developments could allow for rural communities to work to together to attain, a food source, biofertiliser, health supplement and the opportunity to generate income from jobs and high-value products. All this could be possible thanks to tiny, microscopic algae.

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"When was ever honey made with one bee in a hive?"

– **Thomas Hood** in his poem "The Last Man".
In *Whims and Oddities in Prose and Verse*. London:
Lupton Relfe
1826

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This three-year PhD project focused on the pennate diatom *Phaeodactylum tricornutum,* a model organism able to synthesise a number of industrially relevant molecules. In nature, diatoms are not found as isolated entities but rather are active members of a complex ecosystem, which is poorly understood. Bacteria, which have co-existed with diatoms for more than 200 million years, form a crucial part of this ecosystem and have been shown to enhance the growth of diatoms. Increased understanding of the interactions could allow for the exploration of 'synthetic ecology' as a novel scaling up technique. The microbiome of batch grown non-axenic cultures of *P. tricornutum* revealed that the bacterial community associated with *P. tricornutum* cultures changes over time and a network of putative interactions between *P. tricornutum* and each of the bacterial factions was proposed, thus providing a framework to understanding the dynamics of diatom-associated microbial communities.

Cover is the artistic depiction of the dynamics of the bacterial community over time based on a taxa summary plot (landscape)

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