EXPLORING THE POTENTIAL OF ALGAE-BACTERIA COMMUNITIES FOR BIOTECHNOLOGY

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This dissertation is submitted for the degree of Doctor of Philosophy

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DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the preface and specified in the text. The engineered strains of *Anabaena* sp. PCC 7120 in Chapter 4 were created by David Malatinszky, Imperial College London, with some contribution from myself. In Chapter 5, I worked in collaboration with Freddy Bunbury, University of Cambridge, for the 50 mL diurnal laboratory scale experiment and with Dr. Tiago Guerra and Celina Parreira of A4F, Portugal, for the 60 L pilot scale experiment.

This dissertation is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other university or similar institution except as declared in the preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other university or similar institution.

Finally, it does not exceed the prescribed word limit for the relevant Degree Committee.

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ABSTRACT

Microalgae are a large and diverse group of photosynthetic organisms ranging from prokaryotic cyanobacteria to eukaryotic algae spread across many phyla. Traditional algal biotechnology approaches have focused on growing algae in monoculture, in contrast to nature, where algae live in association with many other organisms. One association of interest is between the bacterium *Mesorhizobium loti* (Rhizobiales) and the green alga *Lobomonas rostrata,* deficient in the production of vitamin B_{12}. The alga provides fixed carbon to the bacterium whereas the bacterium supplies vitamin B_{12} to the alga. In the course of a screen for bacterial mutants altered in the interaction, I serendipitously discovered a novel symbiosis involving the non-Rhizobiales bacterium *Rhodococcus erythropolis* and *L. rostrata*. I characterised this novel interaction, together with interaction involving the more industrially relevant *Chlamydomonas reinhardtii* strain.

Nitrogen is a major limiting nutrient for industrial scale algal production. An alternative option to the Haber-Bosch process of synthesising and supplementing fixed nitrogen into media is to utilise nitrogen-fixing bacteria capable of secreting fixed nitrogen into the media, an approach known as biofertilisation. *Anabaena* sp. PCC 7120 is a filamentous cyanobacterium that can fix its own nitrogen. I cultured engineered strains capable of releasing fixed nitrogen in the form of amino acids and ammonium with the industrially relevant *Chlamydomonas reinhardtii metE*$^{-}$+ *M. loti* consortium and *Chlorella vulgaris* in nitrogen-free media, and I also measured growth and ammonium ion concentrations.

There are relatively few published studies investigating and outlining the challenges involved in scaling algal production from the laboratory through to pilot scale. Furthermore, these studies have typically focused on growing axenic cultures. I grew the vitamin B_{12}-dependent strain of *C. reinhardtii metE*$^{-}$in the presence of supplemented vitamin B_{12} or *M. loti* producing vitamin B_{12}, at a range of scales from lab scale (50 mL) to pre-pilot scale (10 L) and pilot scale (60 L). I measured and compared the growth efficiency, as determined by growth rate, and levels of bacterial contamination for both cultures at all scales. Additional measurements including vitamin B_{12} concentrations were made for the pilot scale culture.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
A_x	absorbance at X (nm) wavelength
B_{12}	vitamin B_{12} (cobalamin)
bp	base pairs
CFU	Colony Forming Unit
CO_2	carbon dioxide
DAPA	Diaminopimelic acid
DCW	dry cell weight
DI	Deionised water
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'deoxyribonucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
gDNA	genomic DNA
GGW	green glass wall
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HPLC	high-performance liquid chromatography
kb	kilo base pairs
Log	logarithmic
METE	B_{12}-independent methionine synthase
METH	B_{12}-dependent methionine synthase
M	molar
$NADP^+$	oxidised β-nicotinamide adenine dinucleotide phosphate

NADPH	reduced β-nicotinamide adenine dinucleotide phosphate
O_2	oxygen
OP	open pond
ORP	open raceway pond
OD	optical density
PBR	photobioreactor
PCR	polymerase chain reaction
RPM	rounds per minute
RT	room temperature
RuBisCO	ribulose 1,5-biphosphate carboxylase/oxygenase
SD	standard deviation
TBE	tris-borate-EDTA buffer
T_d	doubling time
TES	N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
TRIS	2-amino-2-hydroxy-methylpropane-1,3-diol
UV	ultraviolet

1 GENERAL INTRODUCTION

1.1 Algal history and diversity

Algae represent a highly diverse collection of photosynthetic microorganisms. Algae, from the point of view of this thesis, is a generic term for macroalgae (eukaryotic photosynthetic multicellular organisms colloquially known as 'seaweed'), microalgae (eukaryotic photosynthetic microorganisms) and cyanobacteria (prokaryotic photosynthetic microorganisms colloquially known as 'blue-green algae'). The first oxygenic photosynthetic prokaryotes are thought to have originated around 3 billion years ago (Nisbet et al., 2007; Planavsky et al., 2014), although anoxygenic photosynthesis may have arisen earlier. Approximately 250 million years later, multicellular cyanobacteria are believed to have arisen (Figure 1.1A) (Sanchez-Baracaldo, 2015). The ability of cyanobacteria to fix atmospheric CO_2 into organic compounds powered by oxygenic photosynthesis (the harnessing of light energy to utilise H_2O as an electron donor while releasing O_2 as a by-product) led to the decline of atmospheric CO_2 and a consequential increase in O_2 to the levels we see today (Figure 1.1B) (Price et al., 2013). The dramatic change around 2.4 billion years ago is arguably the single greatest terraforming event (also known as the 'great oxygenation event') we know of, for without this, much of life as we know it today would not exist.

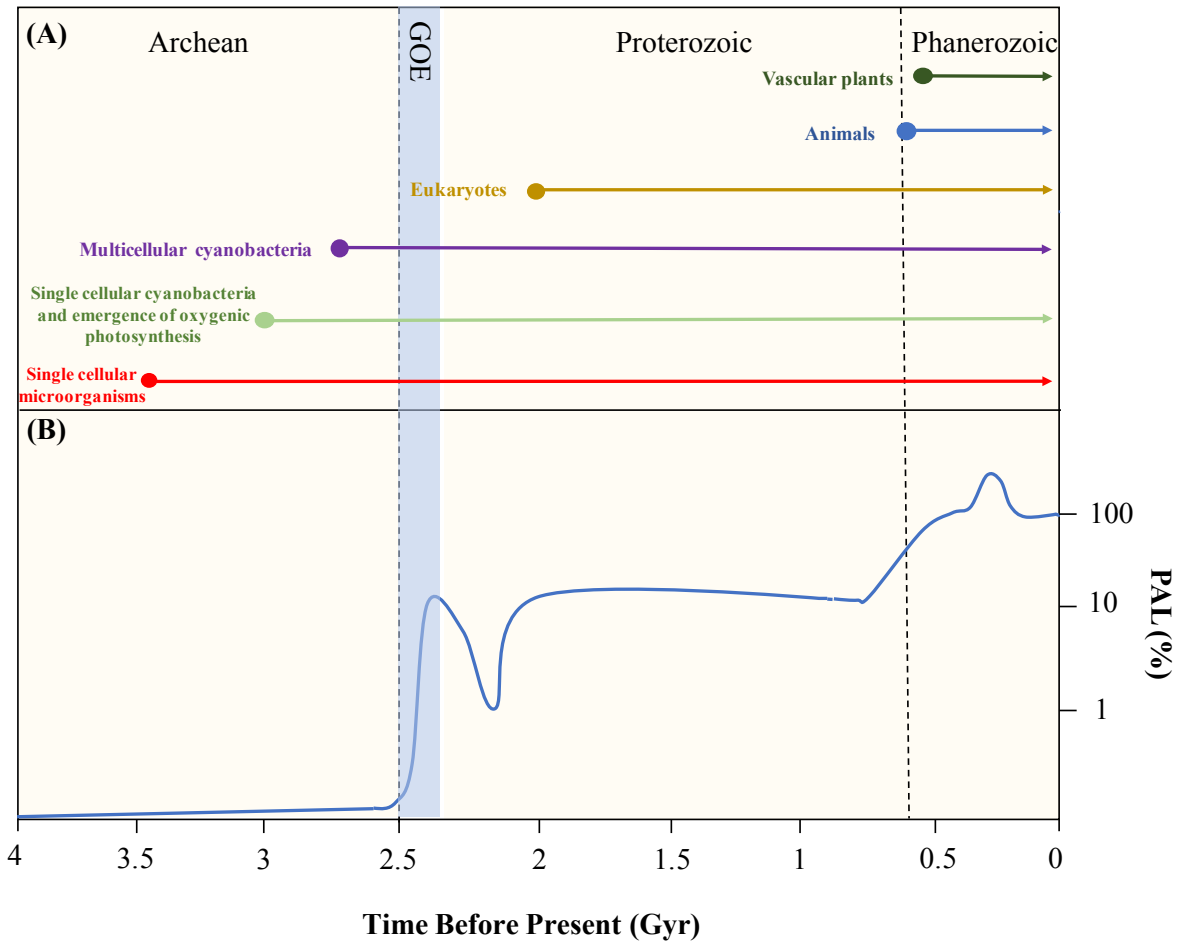


Figure 1.1 Timeline of oxygen and biological changes spanning the Archean, Proterozoic and Phanerozoic Eons.

A) Emergence of life though time in billions of years (Gyr). B) Atmospheric O_2 content through time in billions of years (Gyr), expressed as percentage of present atmospheric level (PAL) of O_2. Blue vertical bar represents the Great Oxygenation Event (GOE). Figure adapted from the following publications: Payne et al., (2008); Schirrmeister et al., (2012); Och and Shields-Zhou, (2012) and; Sanchez-Baracaldo, (2015).

Collectively, algae live in a wide range of environments, from saltwater oceans and freshwater lakes to the hot deserts of the Sahara and cold deserts of Antarctica. This adaptation to a wide range of environments led to a rich genetic, biochemical and morphological diversity among these organisms (Gimple et al., 2013). For example, algae range from microscopic motile or non-motile species (for example *Chlamydomonas reinhartdii* and *Dictyochloropsis reticulata* respectively) to macroscopic species such as *Ecklonia radiata* (Tatsumi and Wright 2016). Algae can also have unicellular, filamentous or branching morphologies (Lewis and McCourt, 2004).

1.2 Biotechnological potential of microalgae and cyanobacteria

Microalgae have several natural advantages that make the organisms desirable for industrial cultivation of compounds. 1) They are capable of year-round production in certain geographic locations (Beer et al., 2009). 2) Algae can be cultivated with brackish water (i.e. water that has more salinity than fresh water but less than sea water) on non-arable land, limiting environmental impacts while not compromising on food production (Gan et al., 2016). 3) They have much higher growth rates and require less land area than other conventional agricultural biodiesel feedstocks such as rapeseed or soybean crops (Mata et al., 2010). 4) Certain microalgal species have a high concentration of protein ranging from 50-70% of dry weight while others can have high oil content ranging from 20-70% dry weight which is beneficial depending on the application (Spolaore et al., 2006). 5) Microalgae can be grown using waste CO_2 from industrial manufacturing (Wang et al., 2008). 6) Microalgae can be grown on wastewater thus recycling nutrients such as nitrogen and phosphorus (Christenson and Sims, 2011). 7) Some algal species are amenable to genetic manipulation. 8) Several algae are also classified as "Generally Regarded As Safe" (GRAS) which is important for industrial production intended for human and animal consumption or expression and purification of recombinant proteins or metabolic compounds (Gangl et al., 2015).

1.3 Commercially valuable products from microalgae and cyanobacteria

Microalgae can produce a range of bioactive compounds such as carotenoids, omega-3 fatty acids and phycobilins (Borowitzka, 2013). Species such *as Arthrospira platensis* (otherwise known as *Spirulina platensis* or simply '*Spirulina'*) and *Chlorella vulgaris* are consumed as feed and dietary supplements due to their high protein (55-70%) and nutrient concentrations (Yakuub et al., 2014).

Not only is *Spirulina* produced for food or feed but it is a rich source of phycocyanin which is estimated to constitute 14% of the dry weight of the organism (McCarty, 2007). Phycocyanin is typically used as a natural food colourant. For example 'Nestlé' uses phycocyanin for their blue 'Smartie' (Morais et al., 2010). Furthermore, the tetrapyrrole chromophore of phycocyanin known as phycocyanobiliprotein, as well as other phycobiliproteins, is used as a fluorescent tag for labelling antibodies in scientific techniques such as immunofluorescence and flow cytometry (Erickson, 2008). Phycocyanin can also act as an antioxidant in mammals when it is enzymatically reduced to phycocyanorubin which inhibits the activity of NADPH oxidase, and consequently reduces the concentration of reactive oxygen species (Zheng et al., 2012).

Carotenoids are organic pigments which can be extracted from microalgae. For example, *Dunaliella salina* can produce high concentrations of β-carotene which has been shown to inhibit artherogenesis and fatty liver formation in mice (Harari et al., 2008). Under stressful conditions, such as high salinity, *Haematococcus pluvialis* can produce astaxanthin which is used as a food colourant in feed for salmon, trout and shrimp and is also consumed by humans for its health benefits (such as its antioxidant and anti-inflammatory effects, and cardiovascular disease prevention) (Ambati et al., 2013).

Omega-3 long chain polyunsaturated fatty acids (omega-3 LC-PUFA), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are commercially valuable fatty acids due to their health benefits in preventing cardiovascular disease. The primary commercial source of omega-3 LC-PUFA is from fish. However, consumers are increasingly turning away from fish-derived omega-3 LC-PUFA due to concerns over mercury contamination and environmental degradation from intensive fish farming. Fortunately, microalgae such as *Nannochloropsis oculata* and *Pavlova lutheri* are rich in EPA and DHA respectively. Furthermore, algal oils provide the added health benefits of containing carotenoids and polyphenols (Ryckbosch et al., 2014).

Poly-β-hydroxybutyrate (PHB) is a common prokaryotic intracellular storage compound that is found in cyanobacteria such as *Spirulina sp.*, *Aphanothece sp*., *Gloeothece sp*. and *Synechococcus sp*. PHB has been a focus of attention to replace fossil oil derived plastics due to its properties that mimic those of plastic with the added benefit of being non-

toxic, biocompatible (i.e. inert to the human immune system) and biodegradable. PHBs are degraded upon exposure to soil, compost or marine sediment where factors such as microbial activity, pH and moisture play an important role in the speed of degradation (Balaji et al., 2013). Unfortunately, yields are not high enough to validate commercial production of this compound but it is possible that higher concentrations of PHBs could be produced through utilising genetic modification technologies (Khetkorn et al., 2016).

Another area of interest in algal biotechnology is the production of biofuels, to replace non-renewable and environmentally damaging fossil fuels. Biofuels are any fuels that are derived from biological organisms and can include any variant such as bioethanol, biodiesel or biogas (Liette et al., 2013). Triacylglycerides (TAGs) are storage lipids found in algae that can be used as a source of oil for biodiesel production, and some species are reported to accumulate TAG up to 70% (Scott et al., 2010). Microalgae can also be grown to produce biogas in the form of hydrogen. For example, *Chlamydomonas reinhardtii* can produce H_2 in the presence of light (via photosynthesis, although the accumulation of oxygen inhibits the hydrogenase) or in the dark (via fermentation) (Oey et al., 2016). Cyanobacteria are also considered promising biological platforms to produce ethanol as well as isobutyraldehyde, isobutanol, ethylene, volatile isoprene hydrocarbons and alkanes with the help of genetic modification technologies (Dienst et al., 2014). Unfortunately, biofuel production in any of its forms from algae has not yet been able to achieve economic viability (Ridley, 2016).

Another application for large scale algae biotechnology is in carbon capture (Ou-Yang et al., 2018). As energy demand increases so too does the concentration of CO_2 in our atmosphere (Zamen and Moemen, 2017). Algae are being used today to sequester CO_2 into biomass to alleviate pollution levels and global warming. However, this use is limited by high capital requirements and limited profits (Ou-Yang et al., 2018). Nonetheless, examples exist whereby high-polluting companies (such as Secil, a concrete producer in Portugal) are incentivised to utilise algae to minimise their impact on the environment (Secil, 2016). Furthermore, if the flue gases emitted are not too toxic (or filtered prior to injection), the algae grown off the CO_2 could also be producers of the high-value compounds mentioned above, adding further value to the pipeline (Ou-Yang et al., 2018).

According to a report by Transparency Market Research , the global algae market was valued at USD $608.0 million in 2015 (Transparency Market Research, 2016). The total market

was projected to climb to USD $1,143.0 million by 2024. However, at approximately the same time of publication of this report, the crude oil prices were the lowest they have been in 15 years (USD $30 per barrel) likely as a result from increased supply from oil shale in the United States (Melek et al., 2017). This has had a significant impact on biofuel production from algae as they struggle to remain competitive in their pricing. As a result, many algae biofuel companies have either shut down or pivoted their business model to the production of high-value products. For example Algenol is now focussing on carbon capture and nutraceuticals; Algix now develops fish farms and algae harvest equipment,and Cellana now produces DHA and Omega-3 EPA (Wesof, 2017). In contrast, there is increased attention to the use of algae as a carbon capture mechanism which could be in great demand, especially since governments (and their citizens) are becoming increasingly concerned about air quality and global warming. Only time will tell as to the real valuation of the global algae market in 2024.

1.4 Genetic modification techniques of microalgae and cyanobacteria

Genetic modification technologies offer vast potential not only for improving the concentrations produced of the compounds outlined above but also in enabling algae to produce a wider range of novel products such as recombinant proteins for therapeutics, *e.g.* antibodies (Maliga and Bock, 2011), and industrial enzymes for polysaccharide degradation (Georgianna et al., 2013). With the increasing availability of algal genome data, genetic modification techniques have been developed for several microalgal and cyanobacterial species. However, the most advanced genetic toolkits have been developed for the microalgae *C. reinhardtii* and *Phaeodactylum tricornutum*, and for the cyanobacteria *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC7942 and *Anabaena* sp. PCC 7120.

1.4.1 Overview of DNA delivery methods

The DNA delivery methods for microalgae and cyanobacteria include ‘microparticle bombardment’ (also known as ‘biolistic delivery’), ‘electroporation’, ‘*Agrobacterium*-based transformation’, ‘glass bead method’, ‘silicon carbide whisker method’, ‘aminoclay nanoparticles’, ‘natural transformation’, and ‘conjugation’ (Heidorn et al., 2011; Gangl et al., 2015; Doron et al., 2016). Several microalgae and cyanobacteria have been shown to take up and integrate exogenous DNA with these methods (Table 1.1).

Table 1.1 DNA delivery methods for microalgae and cyanobacteria

DNA Delivery method	Species	References
Microparticle bombardment	*C. reinhardtii, P. tricornutum, Dunaliella tertiolecta, Euglena gracilis, H. pluvialis, Porphyridium sp., and Tetraselmis cordiformis*	Boynton et al., 1988; Randolf-Anderson et al., 1993; Apte et al., 1996; Purton et al., 2013; Cui et al., 2014
Electroporation	*C. reinhardtii*, *P. tricornutum, Anabaena* sp. PCC 7120, *Chlorella sp*., *Neochloris oleoabundans*	Brown et al., 1991; Miyahara et al., 2013; Thiel and Poo, 1989; Liu et al., 2014; Zuo et al., 2014; Chungjatupornchai et al., 2016
Agrobacterium tumefaciens	*C. reinhardtii, Isochrysis* sp., *H. pluvialis*, *Schizochytrium, Chlorella vulgaris*, *Nannochloropsis, Scenedesmus almeriensis, Dunaliella salina., Symbiodinium sp.*	Kumar et al., 2004; Prasad et al., 2014; Simon et al., 2015; Ortiz-Matamoros et al., 2015
Glass beads	*C. reinhardtii, Chlorella ellipsoidea*	Kindle et al., 1990; Jarvis and Brown, 1991
Silicon carbide whiskers	*C. reinhardtii*	Dunahay et al., 1993
Aminoclay nanoparticles	*C. reinhardtii*	Kim et al., 2014
Natural transformation	*Synechocystis* PCC 6803, *Synechococcus* PCC 7942, *Synechococcus* PCC 7002, *Thermosynechococcus elongatus* BP-1	Grigorieva and Shestakov, 1982; Shestakov and Khyen, 1970; Stevens and Porter, 1980; Onai et al., 2004
Conjugation	*Synechocystis* PCC 6803, *Anabaena* sp. PCC 7120, *Nostoc punctiforme* ATCC 29133, *Anabaena variabilis* ATCC 29413, *Synechococcus* PCC 7942 and PCC 6301	Wolk et al., 1984; Marraccini et al., 1993; Lindberg et al., 2002; Murry and Wolk, 1991, Tsinoremas et al., 1994

1.4.2 DNA delivery methods for microalgae

The following paragraph will briefly summarise the techniques mentioned above that are applied to microalgae. 'Microparticle bombardment' or 'biolistic delivery' is the most frequently used delivery method. The method utilises DNA-coated gold or tungsten microparticles that are delivered through a particle delivery system at high velocity into algal cells. 'Electroporation' applies an electric pulse to the algal cells which increases the permeability of the cells to foreign DNA. It is generally found that efficiency of transformation improves as the cell wall thickness decreases. '*Agrobacterium*-based transformation' was first used on higher plants but has since found to be useful for transforming not only algae but also fungal and human (HeLa) cells (Doron et al., 2016). *Agrobacterium tumefaciens* is a natural plant engineer that introduces DNA into plant hosts that then reprogrammes the plant host cells

to produce novel amino acid-sugar conjugates called opines which are metabolised by the *A. tumefaciens* as a carbon and nitrogen source. The integrated DNA also contains genes for the synthesis of plant growth regulators, and the consequence of this process is the formation of tumours or 'crown galls'. The engineering technique takes advantage of this biology by introducing recombinant DNA in lieu of the genes required for opine synthesis and tumour formation into the recipient cell (Pitzschke and Hirt, 2010). The 'glass bead method' is a simple method whereby cell wall-deficient algal cells are agitated in the presence of glass beads, polyethylene glycol (PEG) and DNA (Enonomou et al., 2014). The 'silicon carbide whisker method' is an alternative, albeit similar to the glass bead method, which involves the agitation of the cells with silicon carbide whiskers to allow the introduction of foreign DNA. This method is gentler than the 'glass bead method', resulting in little loss in cell viability therefore allowing flexibility for the use in a range of algal species (Dunahay et al., 1993). However, the silicon carbide whiskers can be difficult to obtain and can pose a health hazard if inhaled. The 'aminoclay nanoparticles method' utilises positively charged nanoparticles based on 3-aminopropyl functionalized magnesium phyllosilicate. The algal cells are vortexed in the presence of aminoclay nanoparticles and foreign DNA. It has the benefit of giving high transformation rates and it can be used on algal strains with cell walls (Kim et al., 2014).

1.4.3 DNA delivery methods for cyanobacteria

The following techniques mentioned are typically applied on cyanobacteria. 'Natural transformation' is a method of exploiting the ability of some cyanobacterial species to take up exogenous plasmids or linear DNA from the medium. For those cyanobacterial species that do not naturally take up DNA, the 'conjugation method' is an alternative option. The method involves the use of two *Escherichia coli* strains, a helper strain which carries a conjugative plasmid (carrying genes for conjugation and DNA transfer) and a donor strain, which carries a mobilisable plasmid carrying the genes of interest that are to be inserted into the cyanobacterium. Finally, the 'electroporation method' has also been shown to be successful with *Anabaena* sp. PCC 7120, although it is not commonly used due to low efficiency (Heidorn et al., 2011; Brown et al., 2013).

1.5 Industrial scale microalgae and cyanobacteria production

1.5.1 Cultivation conditions

The industrial scale cultivation of microalgae and cyanobacteria is a complicated feat whereby several variables need to be considered. The first major consideration that heavily affects the growth characteristics and metabolic composition of microalgae is the cultivation conditions. The conditions include photoautotrophic growth, heterotrophic, mixotrophic and photoheterotrophic cultivation (Chen et al., 2011).

Photoautotrophic growth is where microalgae use light (e.g. sunlight) as the energy source and inorganic carbon (e.g. CO_2) as the carbon source and is the most commonly used cultivation condition (Figure 1.2A). It has the advantages of being relatively cheap in its energy inputs (e.g. CO_2 and light) and suffering from relatively low levels of contamination allowing for use in open ponds or raceway ponds. However, disadvantages include relatively low biomass yields and the requirement for a high surface area to volume ratio for maximum light penetration, which can be expensive as large amounts of land are required for large-scale cultivation. Furthermore, the requirement for sunlight and large land areas for photoautotrophic growth of microalgae limit production to specific geographical locations (Chen et al., 2011). Heterotrophic growth is the use of an organic source for both energy and carbon in biomass (Figure 1.2B). This cultivation method has the advantages of higher biomass and lipid productivity than in photoautotrophic conditions, as well as a low surface area to volume ratio allowing production of algal biomass in a smaller space. However, the disadvantages are the input costs for the carbon substrate and the costs accompanying the frequent contamination that occurs given the presence of organic compounds in the growth medium. Various microalgal compounds from a range of different species can nevertheless be produced from this growth regime such as lipids, astaxanthin, DHA (docosahexaenoic acid), general biomass, and even hydrogen (Chen et al., 2011; Morales-Sanchez et al., 2015). Mixotrophic growth of algae uses both light and an organic source for energy and organic carbon and inorganic carbon for its carbon source (Figure 1.2C). This cultivation method has the advantage of higher biomass productivity than growth in photoautotrophic conditions although productivity is not quite as high as in heterotrophic growth. This method of cultivation is rarely used in algal production due to the cost of contamination, cost of equipment (requirement for an enclosed photobioreactor with a reasonably high surface area to volume ratio) and cost of carbon

substrate (Liang et al., 2009; Chen et al., 2011). Photoheterotrophic growth of microalgae uses light for its energy source and organic carbon for its carbon source (Figure 1.2D). However, much like mixotrophic growth, the culture requires both sugars and light for growth. The advantages and disadvantages for this method are similar to mixotrophic growth and again this method is rarely used due to the costs involved (Chen et al., 2011; Hwang et al., 2014).

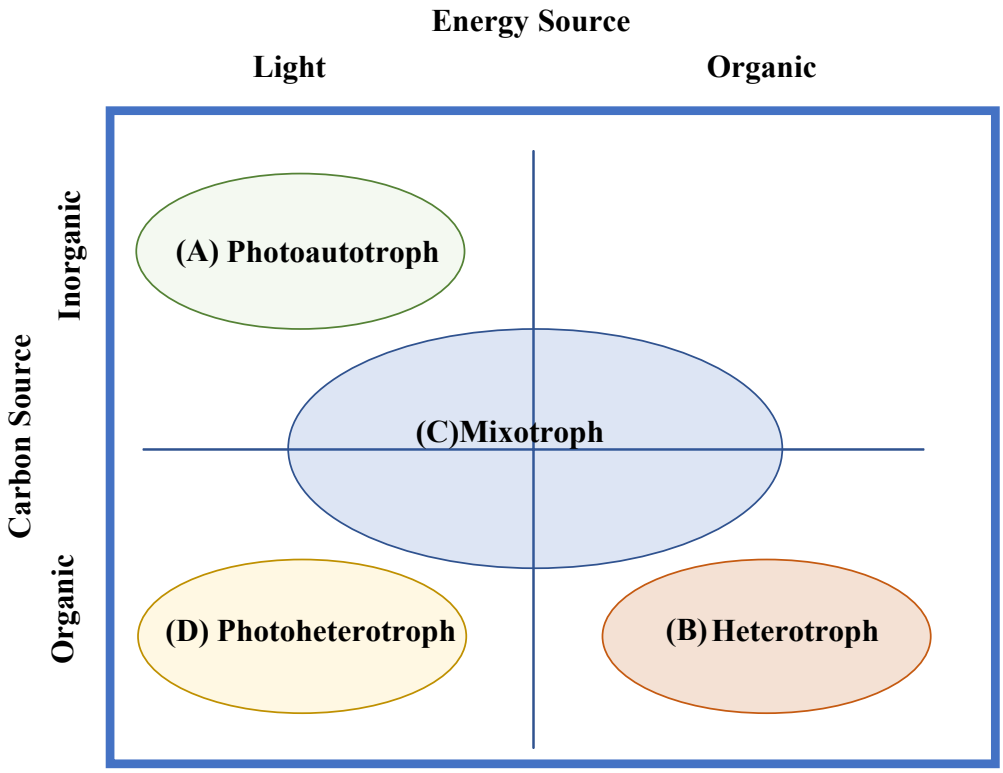


Figure 1.2 Cultivation conditions for algal production.

A) Photoautotrophic growth utilises light as an energy source and an inorganic source of carbon. B) Heterotrophic growth utilies an organic source for both energy and carbon. C) Mixotrophic growth utilises both light and an organic source for energy and organic carbon and inorganic carbon for its carbon source. D) Photoheterotrophic growth utilises light as an energy source and an organic source of carbon.

1.5.2 Industrial scale bioreactors

Successful cultivation of microalgae on the industrial scale also requires a thorough understanding of the various culturing systems available. These include vats or fermenters, photobioreactors (closed systems), open raceways and ponds (open systems). The choice of system to use largely depends on which algal species are to be cultured and for what purpose.

The use of vats or fermenters is required for heterotrophic cultivation of microalgae (Figure 1.3A). Vats or fermenters are closed system reactors that are not illuminated by light. They can be stirred or bubbled and allow the controlled addition of an organic source of carbon and energy. Use of this method is similar to procedures to cultivate bacteria or yeast at large scale in closed tanks. Microalgae have been grown in these systems for high value compounds; for example, *Nannochloropsis sp.* has been grown for polyunsaturated fatty acids (PUFAs), pigments and antioxidants (Bumbak et al., 2011; Marudhupandi et al., 2016). The advantages are a low surface area to volume ratio (low surface space requirement), the option for continuous culture of microalgae and high levels of biomass density. However, disadvantages include limitation of algal species capable of growth in this environment, variation in chemical composition of algae, as well as the cost of maintaining sterility (Bumbak et al., 2011).

Photobioreactors (PBRs) are typically enclosed systems, that are illuminated by artificial light, sunlight or both (Figure 1.3B). PBRs come in a range of various styles including vertical column photo-bioreactors, flat-plate photobioreactors, tubular photobioreactors and flexible bag cultures. PBRs can be used for the cultivation of algae in photoautotrophic, mixotrophic, or photoheterotrophic conditions. Microalgae have been grown in PBRs for various products such as astaxanthin, EPA and β-carotene (Guedes et al., 2011). Overall, PBRs minimize contamination and allow axenic cultivation of monocultures, prevent loss of water and CO_2, and provide reasonable control over conditions such as pH, temperature, light and CO_2. However, PBRs are prone to fouling (i.e. accumulation of algae on light transmitting surfaces) which leads to the requirement for regular cleaning, and also results in poor mass transfer and formation of gradients of pH, CO_2 and oxygen. Tubular bioreactors are the most commonly used reactors due to efficient use of sunlight, good biomass productivities and relatively low cost of maintenance (Ugwu et al., 2008; Singh et al., 2012).

The two most common open systems for algal cultivation are open ponds (OPs) or open raceway ponds (ORPs) whereby photoautotrophic cultivation of microalgae or cyanobacteria is carried out (Figure 1.3C). An OP is a pond that is exposed to the weather and is not mixed or stirred. In contrast, ORPs are closed loop channels whereby the culture is mixed by a paddlewheel and are typically used where land costs and site preparation costs are higher and where climate may not allow for year-round cultivation. OPs and ORPs are both beneficial due to the low cost of algal production such as utilisation of natural sunlight and minimal

requirement of equipment maintenance. However, OPs and ORPs are limited by the small number of algal species that can be grown successfully in these open systems. OPs have an advantage over ORPs in that energy inputs are lower as the cultures are not mixed other than by wind and convection. An example of a successful OP is in Australia, where Betatene Ltd cultivate *D. salina* for β-carotene, on land that is low in value and where algal production can occur all year round. However, ORPs have an advantage over OPs in the gain in algal productivity from paddlewheel mixing, and which is required for commercial production of *Spirulin*a and *Chlorella*. The disadvantage for open systems is the inability to control the surrounding environment which leads to contamination, losses in productivity and CO_2 limitation. Furthermore, depth of the OPs (typically around 20-30 cm deep) or ORPs (up to 50 cm deep) also plays an important part in the productivity of algae as the need for sufficient exposure to light must be weighed with the requirement for adequate depth for mixing and changes in salt concentration due to evaporation (Borowitzka, 1999; Kumar et al., 2015).

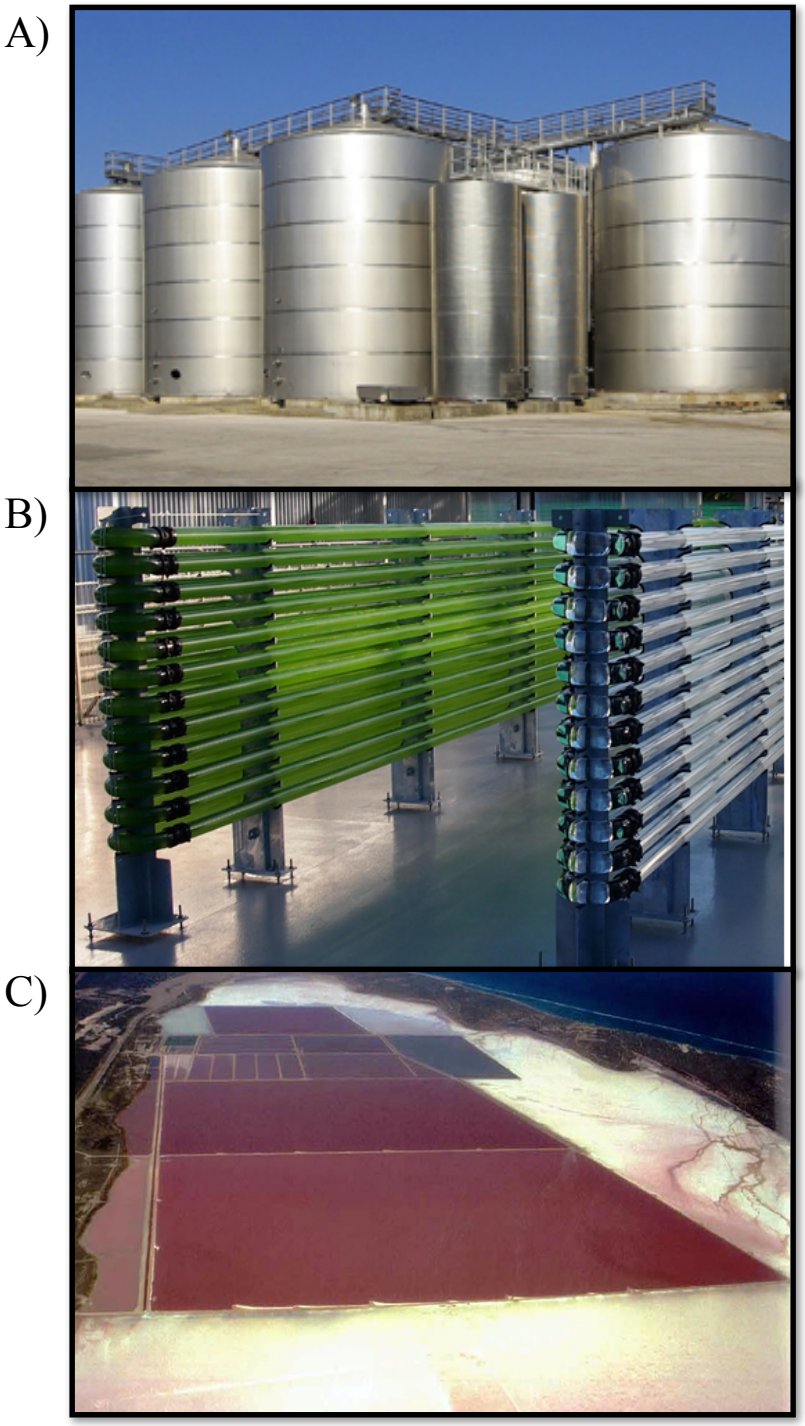


Figure 1.3 Major type of industrial scale bioreactors

A) Microalgal fermenting vats (Photo courtesy of 'Fermentalg', France). B) Horizontal tubular photobioreactors (photo courtesy of 'A4F', Portugal). C) Open Ponds (OPs) (photo courtesy of 'Betatene Ltd', Australia).

1.5.3 Challenges of industrial scale cultivation

Although microalgae and cyanobacteria are commercially grown for a wide range of useful products, the use of these organisms for biofuels still represents a significant challenge. The challenges of efficient algal production include biotic factors such as algal growth rate, cell population density and product output (i.e. low lipid, bioethanol or biohydrogen production) as well as biological contamination. Abiotic factors include the costs of maintaining cultivation systems (i.e. cleaning) and energy costs of downstream processing and production such as biomass collection, dewatering and rupturing (Scott et al., 2010; Singh and Olsen, 2011; Davis et al., 2011). Although there are studies that conclude the theoretical potential of biofuel production from microalgae could be competitive with fossil fuels, there is a limited amount of experimental data, and few commercial applications of this technology (Liu et al., 2012; Rogers et al., 2014).

When comparing the energy efficiency of open ponds (including open raceway ponds) versus PBRs, open ponds were found to be more efficient due to lower energy requirements for cultivation, although it should be noted that energy costs were higher in harvesting and drying (Lardon et al., 2009; Kadam et al., 2002; Richardson et al., 2012; Slade and Bauen, 2013). The major costs of PBRs were identified in construction and culture circulation (Stephenson et al., 2010; Jorquera et al., 2010). There are plenty of areas to improve in algae farming which could help to reduce the costs of cultivation and processing. Possible options include use of recycled CO_2 (from a manufacturing facility) as well as fertilisers and water (from a wastewater treatment facility). Furthermore, microalgal production of biofuels could be coupled to the production of high-value products or animal feed to maximize revenue streams (Davis et al., 2011; Slade and Bauen, 2013).

1.6 Microalgal and cyanobacterial communities

In their natural environment, algae live intimately with many other organisms. These communities exhibit complex interactions including metabolite exchange, cell aggregation, and biofilm formation (Jagmann and Philipp, 2014). Depending on the circumstances, these interactions can be described as mutualisms, parasitisms or commensalisms (Figure 1.4).

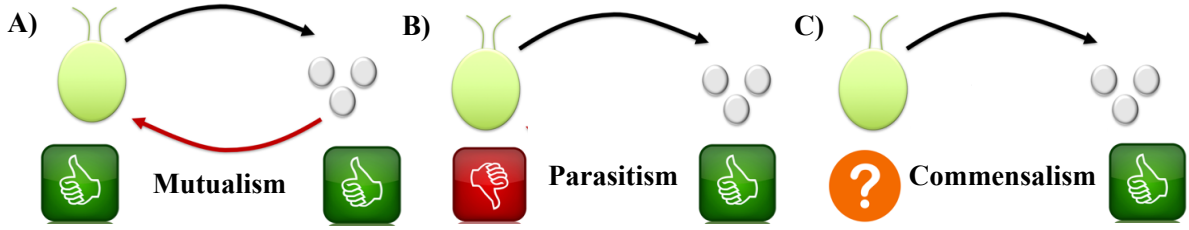


Figure 1.4 Three major types of microbial interactions

A) Mutualism: where interactions in which all the organisms involved benefit from the interaction. B) Parasitism: where one organism benefits from an interaction which negatively affects the other. C) Commensalism: where only one partner benefits while the other partner obtains neither benefit nor detriment from the interaction.

Mutualisms are interactions in which all the organisms involved benefit from the interaction. For example, at least half of all algal species require an exogenous supply of vitamin B_{12} (hereafter B_{12}), and bacteria can be a source for this co-factor in return for a carbon source, thus forming a tightly regulated symbiosis (Croft et al., 2005; Kazamia et al., 2012). This mutualism was found to be obligate or facultative depending on the presence of the B_{12}-dependent (METH) and B_{12}-independent (METE) methionine synthases of the algal species tested and many algal species can be involved in relationships dependent on B_{12}. For example, *Lobomonas rostrata* only contains METH (therefore requires an exogenous source of B_{12}) and enters an obligate symbiosis with the B_{12}-producing bacterium *Mesorhizobium loti*. In contrast, the model organism *Chlamydomonas reinhardtii*, which contains both METE and METH, represses its METE gene expression and utilises the exogenous B_{12} available when in association with *M. loti.* Further studies from Alison Smith's group, who studied these relationships, provided evidence of the possible evolutionary adoption of this mutualism (Helliwell et al., 2011; Helliwell et al., 2015).

Parasitism is where one organism benefits from an interaction which negatively affects the other. Algal parasitism has been identified between the dinoflagellate *Symbiodinium microadriaticum* and the jellyfish, *Cassiopea xamachana* where the dinoflagellate feed and replicate inside the jellyfish, at the cost of jellyfish growth and reproduction (Sachs and Wilcox, 2006). Red algae are an intriguing parasitic model as 80% of their parasitic interactions occur on hosts that are closely related i.e. having been recently derived from a common ancestor. In general, Red algae such as *Gracilariophila oryzoides* and *Plocamiocolax pulvinata,* infect the host (by introducing organelles such as the nucleus and mitochondria etc.) which, after

numerous rounds of replication, give rise to red algal gametes and are eventually released into the environment. Red algal parasitism has arisen multiple times and the degree of parasitism ranges from entirely obligate to more facultative, as well as from being highly specific in their interactions to having multiple hosts (Hancock et al., 2010).

A commensalism is where only one partner benefits while the other partner obtains neither benefit nor detriment from the interaction. Identifying a commensal relationship is quite difficult as the more that is understood about an interaction, the more commonly a cost or a benefit is identified, therefore rendering the interaction a parasitism or mutualism respectively. However, as Zapalski (2011) discusses, commensals could be often overlooked as they could be considered non-interacting partners. Cho et al. (2015) claim to have found evidence of bacteria harboured in the *Ca. vulgaris* sheath receiving carbon and shelter with no apparent benefit identified toward the alga. A more recent example of a potential commensal relationship included two of the most abundant cyanobacteria on the planet, *Prochlorococcus* and *Synechococcus*, which could produce and accumulate hydrocarbons in the form of C15 and C17 alkanes. It was demonstrated that both obligate and facultative marine hydrocarbon-degrading bacteria such as *Alcanivorax borkumensis* SK2, *Acinetobacter baylyi* ADP1 and *Marinobacter aquaolei* VT8 could consume the cyanobacterial alkanes (Lea-Smith et al., 2015). However, it must be noted there could be indirect benefits to the cyanobacteria that are difficult to measure or have not yet been measured, such as experiencing an increased concentration of dissolved CO_2 (released from bacteria undertaking respiration) for cyanobacterial carbon fixation or other nutrients such as vitamins.

Algal interactions with other organisms can be found in a wide range of habitats. The lichen is one example whereby fungus (mycobiont) provides a habitat for an alga or cyanobacteria (photobiont) in return for fixed carbon in the form of complex sugar alcohols or glucose (Hodgkinson et al., 2012) while other species such as *Anabaena azollae* provide fixed nitrogen to the plant host *Azolla caroliniana* (a small water fern) in return for fixed carbon and a protected environment (Peters and Meeks, 1989). Similarly, corals also provide a habitat to algae and cyanobacteria in return for fixed carbon. Furthermore, recent evidence has shown that corals help the algae with fixing carbon by establishing a type of carbon concentrating mechanism in the symbiosome, which surrounds the alga (Barott et al., 2015). Algae and bacteria have also been shown to adapt to extreme environments by working together to survive high salinity, low temperatures, low light or inorganic carbon and high UV radiation found in

Antarctic sea-ice (Thomas and Dieckmann, 2002). The 'Phycosphere' is a term used to describe a zone containing algal extracellular products that extends outwards from an algal cell or colony in which bacterial growth is stimulated (Figure 1.5). The products have been identified as carbon sources for various bacterial species (however, not all bacteria) and has been argued as one of the more ignored or overlooked habitats for bacteria (Granam et al., 2002; Sapp et al., 2007; Ramanan et al., 2016).

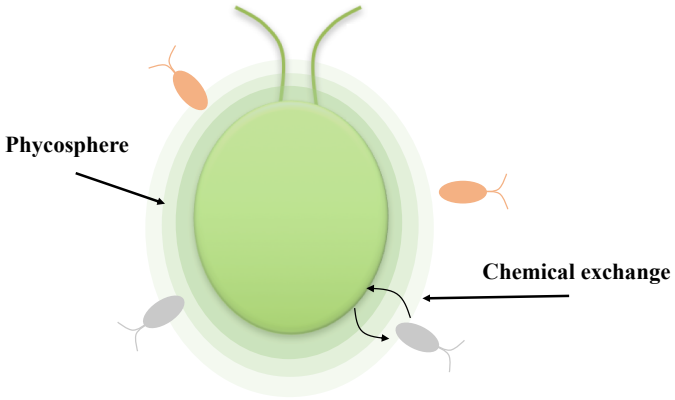


Figure 1.5 The Phycosphere

The phycosphere is the zone that surrounds algal cells. The phycosphere contains extracellular carbon products that surrounding bacteria can feed off. The phycosphere provides an environment where chemical exchange between the bacteria and the algae can occur (Seymour et al., 2017).

1.7 Potential advantages of community cultivation

Most biotechnological studies have focused on maximising the productivity of single species or monocultures. However, the use of monocultures comes with several challenges such as being prone to perturbation and culture instability (e.g. fluctuations in biomass production) (Kazamia et al., 2014). Furthermore, monocultures have been hypothesized to encourage the proliferation of contaminants in accordance with basic principles of community ecology which describe natural systems as increasing in complexity over time (Elton, 1958; Smith and Crews, 2014). The current knowledge of algal interactions in nature and the ecological principles underlying these communities may allow us to utilize communities successfully to make algal biotechnology a more commercially viable industry. For example, it has been predicted that culturing algae in communities could have several advantages such as 1) improved productivity due to maximising resource-use complementarity, 2) greater crop protection by taking advantage of the competitive exclusion principle (summarised below), 3)

reduction of energy inputs through trading metabolites and 4) greater stability and resilience through increased algal diversity and richness (Ortiz-Marquez et al., 2013; Jagmann and Philipp, 2014; Kazamia et al., 2014; Nalley et al., 2014; Pandhal and Noirel, 2014; Smith and Crews, 2014).

1.7.1 Resource-use complementarity

The first community ecological principle that could be taken advantage of is 'resource-use complementarity'. 'Resource-use complementarity' says that when species with different growth requirements are cultivated together the competition for resources between the organisms is lowered, allowing the individual species to cohabit, thus increasing net biomass of the culture. An example of resource use complementarity includes making use of a diverse portfolio of accessory light harvesting photosynthetic pigments to maximise light absorption such as chlorophyll *b* found in Chlorophyta, chlorophyll *c* found in Chromalveolata and phycocyanin and phycoerythrin found in cyanobacteria. Behl et al. (2011) conducted a laboratory experiment with 85 artificially assembled phytoplankton communities composed of species from four phylogenetic groups. Carbon uptake, photosynthetic pigments and light absorbance were measured for each community. As phylogenetic diversity of the communities increased, carbon uptake and light absorption also increased. It was found that all algal communities consisting of species from two, three, or four different groups showed non-transgressive overyielding (Equation 1.1) compared to cultures of individual species, with transgressive overyielding (Equation 1.2) identified in more than half of the total assemblages studied. It is possible the diverse photosynthetic pigments (maximising the use of sunlight) could be the reason for the overyielding measured in the communities. Previous studies have also found similar gains in productivity with communities (Striebel et al., 2009; Cardinale, 2011; Olli et al., 2014). Stockenreiter et al., (2016) studied the effect of microalgal communities grown in wastewater and it was found that in terms of biomass, the communities with the strongest growth exhibited non-transgressive overyielding but in terms of lipid accumulation some communities exhibited transgressive overyielding compared single species cultures. Overall it was concluded that gains in algal productivity may not simply be achieved by increasing algal diversity through random choice but by careful selection of communities based on physiological and ecological knowledge.

Equation 1.1

Non-Transgressive Overyielding = X > Y

Where:

X= Total biomass yield from a community of algal species.

Y= Average biomass yield achieved of individual algal community members grown in monoculture.

Equation 1.2

Transgressive Overyielding = X > Y

Where:

X= Total biomass yield from a community of algal species.

Y= Highest biomass yield achieved of any individual algal community member grown in monoculture.

1.7.2 Competitive exclusion principle

As previously mentioned, growth of algae at industrial scale exposes the culture to contamination. The contamination can come in the form of pathogenic organisms such as bacteria or fungi, photosynthetic organisms (algae or cyanobacteria) or predators such as rotifers (Benemann and Oswald, 1996). This can lead to lower efficiencies of biomass production and at worst lead to culture 'crashes' (i.e. death of the algal population) (Sommer et al., 2001; Letcher et al., 2013). The use of algal communities has been predicted to provide an element of crop protection though the 'competitive exclusion principle'. The 'competitive exclusion principle' applies to a scenario where a diverse community of organisms occupy all available resources or niches leading to an increased ability to exclude invaders competitively. Shurin et al. (2013) set up an experiment in which one, two, five or ten species of algae in various combinations were inoculated with the grazer *Daphnia pulex*. It was found that survival of *Da. pulex* declined as the algal diversity increased. Sharifah and Eguchi (2011) found that when *Nannochloropsis oculata* was co-cultivated with its natural bacterial symbiont of the clade *Roseobacter*, this exhibited a greater antibacterial effect against the fish pathogen, *Vibrio anguillarum,* than if *Roseobacter* was cultivated alone.

1.7.3 Reduction of energy inputs

Bacteria have a large metabolic capacity that can be exploited not only to benefit the growth of algae but also to replace external inputs of scarce or expensive resources. For example, the bioavailability of iron for algae is limited due to poor solubility at neutral pH conditions. However, it has been shown that in low iron environments the growth of the alga *Dunaliella bardawil* improves in the presence of the halophilic and oligotrophic bacterium *Halomonas* sp. This is because the bacteria increased the solubility of iron thereby enhancing its availability to the algae (Keshtacher-Liebson et al., 1995). It has also been reported that several clades of *Marinobacter* produce siderophores, small iron chelating organic molecules, that increase the solubility of iron and consequently increase algal uptake of iron by more than 20 times its normal rate (Amin et al., 2009). Phytohormones are a class of small molecules that are known to regulate plant growth and development. Indole-3-acetic acid (IAA) is one such phytohormone, and its production by bacterial species *Azospirillum brasilense* and *Azospirillum lipoferum* has been shown to enhance the growth of *Chlorella sp.* by as much as 11-19 fold (De-Bashan et al., 2008; Tate et al., 2013). Approximately half of all algae are known to require B_{12} for growth and the supply of B_{12} must come via prokaryotes as they are the only known producers (Croft et al., 2005). *M. loti* has been shown to provide B_{12} to *L. rostrata* in return for fixed carbon, and the marine dinoflagellate *Lingulodinium polyedrum* has been reported to receive vitamin B_1 and B_{12} from its naturally associated bacterial community (Kazamia et al., 2012; Lopez and Maske, 2016). Given their inability to synthesise it, microalgae must obtain the vitamin either directly through mutualisms with bacteria or indirectly by scavenging from the environment. Nitrogen is another major limiting nutrient for algal growth and nitrogen-fixing cyanobacteria have been identified to provide fixed nitrogen to algae. For example, the cyanobacterium *Candidatus* sp. (or UCYN-A) was found to enter a mutualistic symbiosis with a prymnesiophyte whereby the prymnesiophyte receives fixed nitrogen in exchange for fixed carbon (Thompson et al., 2012; Newby et al., 2016).

1.7.4 Increased stability and resilience

The use of communities for microalgal biotechnology has the potential to introduce stability and resilience into the cultures. According to Nalley et al. (2014), stability of a culture is defined as the ability of communities not to fluctuate widely in the face of disturbance, and

resilience is defined as the ability of a culture to rebound after a disturbance. Disturbances can include contamination (i.e. grazers such as rotifers), and high variation in temperatures which particularly affect species assemblages in outdoor ponds. Indeed, several studies have provided evidence of the improvement of culture stability using communities (Cardinale, 2011; Stockenreiter et al., 2012; Stockenreiter et al., 2013). Corcoran and Boeing (2012) found that species-rich cultures were the most stable over time due to their ability to recover from rotifer grazing. This stability and resilience were hypothesised to be due to an "interference effect" i.e. diverse phytoplankton assemblages interfered with efficient rotifer grazing leading to the decline of rotifer populations. A similar result was identified by Shurin et al. (2013) as outlined earlier, where increased algal diversity led to the population decline of the grazer *Daphnia pulex* and survival of the algal cultures. This ability of polycultures to resist the disturbance of contaminating organisms is hypothesised to be because most known pathogens or grazers have specifically evolved to infect or feed on particular organisms. Therefore, algal communities would be largely immune to infection as only a subpopulation would be affected by a contaminant and so in the event of losing a single species, a complementary species could take up the vacant niche (Stockenreiter et al., 2016). However, minimal evidence exists to show communities provide an advantage for providing stability and resilience against fluctuating temperature. For example, Schabhüttl et al. (2012) carried out an experiment on 25 mixed algal communities with varying species richness (2-12 species). The communities were exposed to constant or diurnal temperature conditions. The results found that regardless of whether temperature was constant or diurnal, growth rates increased with increased species richness. However, exposure to diurnal temperatures led to a decline in species diversity with a selection for cyanobacterial species. This decline in species diversity and selection for cyanobacterial growth may be problematic for industrial production of algal cultures for a specific product such as lipid, which is primarily obtained from eukaryotic algae.

1.8 The potential of engineering algae–bacteria communities

We are not limited to the use only of naturally occurring algal communities but we can also use artificial communities, which have the potential to overcome some of the challenges faced in algal biotechnology. For example, Le Chevanton et al. (2013) screened algal-bacterial cultures with a high-throughput optical technique to identify interactions that gave the highest chlorophyll *a* fluorescence. The authors found two bacterial strains enhanced biomass accumulation and nitrogen provision for *Dunaliella* sp. (Le Chevanton et al., 2013). In other

examples, the nitrogen-fixing bacterium *Azotobacter vinelandii* was engineered to secrete fixed nitrogen into the growth medium, replacing the need for addition of synthetic nitrogen inputs for algal growth (Ortiz-Marquez *et al.*, 2012; Ortiz-Marquez *et al.,* 2014), and the cyanobacterium *Synechocystis sp.* PCC 6803 was engineered to secrete a carbon source for consumption by *E. coli* which could then be grown to produce low and high-value compounds (Niederholtmeyer *et al.,* 2010)

1.9 Project Aims

There are therefore a number of ways in which co-culture of algae with bacteria rather than axenically may be advantageous for enhancing productivity in biotechnological applications. For example, co-culture may reduce the number or amounts of nutrients that must be provided, or protect against undesirable contaminants. My project aims to study three important areas of bacterial-algal co-culture. The first area is the interactions underpinning algae-bacteria mutualisms, with the trading of B_{12} and fixed carbon as a model. The discovery of these mutualisms is relatively new, so there is a limited understanding of their molecular basis. In addition, if more instances of these interactions could be identified it would help in identifying the general principles governing the interactions. The second important area is to test the feasibility in principle of using bacteria to provide fixed nitrogen for algal growth. Large-scale growth of microalgae requires the addition of this nutrient. The Haber Bosch process is the primary means of providing this nitrogen, but it is energy and carbon intensive. Biofertilisation using nitrogen fixing bacteria would be an attractive alternative to the Haber Bosch process. Work on biofertilisation for algae production to date has mainly focused on the heterotrophic bacterium *Azotobacter vinelandii*, although the nitrogen-fixing cyanobacterium *Anabaena* is also an attractive possibility. A third area of importance for developing biotechnological co-culture of algae and bacteria is to identify the problems likely to be encountered in scale-up of co-cultures from bench to larger scales.

There are three specific objectives for the project, one from each of the interlinked areas presented above. My first objective was to attempt to characterise the underlying genetic control of the *L. rostrata – M. loti* interaction by carrying out a screen of *M. loti* mutants obtained from a publicly-available collection as well as generating and screening a library of

transposon *M. loti* mutants. Although the project did not succeed in isolating mutants affected in the interaction, I identified a novel symbiosis, and this was studied in more detail.

The second objective was a proof-of-concept test of whether engineered strains of *Anabaena,* capable of secreting nitrogen in the form of amino acids and ammonium ions, could be grown as a consortium with eukaryotic algae and provide enough fixed nitrogen to the medium to provide enhanced growth for the microalgae. The organisms I chose to test the potential for nitrogen provision from *Anabaena* were *Chlamydomonas reinhardtii metE*$^{-}$ grown in conjunction with *M. loti,* and *Chlorella vulgaris.* Particularly promising results were seen with *Ca. vulgaris.*

The third objective was to test the consequences of progressing *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* co-culture from 50 mL laboratory scale, to 10 L pre-pilot scale and then 60 L pilot scale. I aimed to test whether the co-culturing of the B_{12}-dependent alga with B_{12}-producing bacteria proved to be as efficient in terms of growth as supplementing the culture directly with B_{12} at 60 L pilot scale. I also wished to determine whether co-culturing with *M. loti* could provide protection from contaminant/invading bacteria. I found that, although *M. loti* was capable of providing B_{12}, contaminant/invading bacteria were still present.

2 MATERIALS AND METHODS

2.1 Chemicals, reagents and equipment.

Analytical grade laboratory reagents were used and were obtained from Fisher Scientific (Loughborough, UK) or Sigma Aldrich (Dorset, UK) unless otherwise stated. Oligonucleotide primers were synthesized by Sigma Aldrich (Dorset, UK). Vitamin B_{12} (as cyanocobalamin) and amino acids were obtained from Sigma Aldrich (Dorset, UK). Bacto-tryptone, yeast extract and bacto-agar were from Oxoid (Basingstoke, Hampshire, UK). GoTAQ DNA polymerase was from Promega (Southhampton, UK), Hyperladder DNA markers (I and IV) and dNTPs were from BioLine (London, UK). Restriction enzymes used were from New England Biolabs or Fisher Scientific. UltraClean DNA Purification kits were obtained from MO BIO Laboratories (CA, USA), and Miniprep kits were obtained from Thermo Fisher Scientific (Loughborough, UK). For centrifugation, an Eppendorf Minispin (Stevenage, UK) was used at room temperature for sample volumes up to 2 ml. For centrifuging sample volumes up to 50 ml, a Hettick Universal 32R was used. PCR reactions were carried out using a Eppendorf Mastercycler (Stevenage, UK). Visualization of DNA in gels was carried out using a Syngene U:Genius Gel Imager (Cambridge, UK). Spectrophotometry was carried out using a Thermo Spectronic Helios and weights were measured using a Precisa 100M-300C. pH determination was carried out using a Hannah Instruments pH Meter (Bedfordshire, UK). Sterilisation was carried out using a Prestige Medical, Omega Media Autoclave (Blackburn, UK) and incubation of bacteria and algae was carried using New Brunswick, Innova 4230 and Infors HT Multitron (Basel, Switzerland) incubators. Ultrapure water was obtained using a Milli-Q Integral Water Purification System, Merck Millipore (Hertfordshire, UK).

2.2 Biological materials and maintenance routines

2.2.1 Microalgae species

Lobomonas rostrata (SAG 45-1) (Table 2.1) was kindly donated by Professor Alison Smith's Lab in the Department of Plant Sciences, University of Cambridge, UK. The Smith lab originally obtained the algal species from the Experimental Phycology and Culture Collection of Algae at the University of Goettingen (EPSAG), Germany. *L. rostrata* was cultured in TAP or TP+ medium (Appendix 8.1) supplemented with vitamin B_{12}, in the form of cyanocobalamin, at a concentration of 100 ng L^{-1} (Kazamia et al., 2012). Laboratory cultures were grown at 25°C with shaking at 120 rpm and illuminated by cool white fluorescent lamps with a photon flux density of 40 μmol $m^{-2}s^{-1}$ at constant light unless otherwise mentioned.

Chlamydomonas reinhardtii WT12 (CCAP 11/32A) was kindly donated by Professor Alison Smith's Lab in the Department of Plant Sciences, University of Cambridge, UK. The Smith lab originally obtained the algal strain from The Culture Collection of Algae and Protozoa (CCAP) within the Association for Marine Science Campus, Scotland. Laboratory cultures were grown as outlined for *L. rostrata* without the addition of vitamin B_{12}.

Chlamydomonas reinhardtii metE⁻ (derived from CCAP 11/32A) was kindly donated by Professor Alison Smith's Lab in the Department of Plant Sciences, University of Cambridge, UK (Helliwell et al., 2015). Laboratory cultures were grown as outlined for *L. rostrata.*

Chlorella vulgaris (CCAP 211/11B) was kindly donated by Professor Alison Smith's Laboratory in the Department of Plant Sciences, University of Cambridge, UK. Laboratory cultures were grown in BG11 medium unless otherwise stated (Appendix 8.1.3).

2.2.2 Bacterial species

Anabaena (Nostoc) sp. PCC 7120 was obtained from the Pasteur Culture Collection of Cyanobacteria (PCC), Paris, France. *Anabaena* (Nostoc) sp. PCC 7120 CSX60-R10 was kindly donated by Professor Enrique Flores, Institute of Plant Biochemistry and Photosynthesis, University of Sevilla, Spain. *Anabaena* (Nostoc) sp. PCC 7120 strains 160-167 (outlined in section 4.2.5) were engineered by David Malatinszky at Imperial College London (with some assistance from the author of this dissertation). Laboratory cultures were grown in BG11 + HEPES buffer (Appendix 8.1.3) at 30°C with shaking at 120 rpm and illuminated by cool white

fluorescent lamps with a photon flux density of 40 μmol $m^{-2}s^{-1}$ at constant light unless otherwise stated.

Mesorhizobium loti (MAFF 303099), *Delftia acidovorans* and *Pseudomonas putida*, were kindly donated by Professor Alison Smith's Lab in the Department of Plant Sciences, University of Cambridge, UK. *Rhodococcus erythropolis* arose as a contaminant during this study. Cultures were grown in TY medium (Appendix 8.1.5) at 30°C with shaking at 120 rpm.

Escherichia coli strain β2163 was kindly donated by Professor George Salmond and Dr. Rita Monson in the Department of Biochemistry, University of Cambridge, UK. Cultures were grown in LB or TY medium (Appendix 8.1.5 and 8.1.6) with appropriate antibiotics (Table 2.2) at 30°C with shaking at 120 rpm.

Table 2.1 Complete strain list for this study

Organisms (strain)	Primary Growth Medium (in this study)	Description	Notes	Reference
Anabaena sp. PCC 7120	BG11	Wild type	N/A	N/A
Anabaena (CSX60-R10)	BG11	Δ*natA* and Δ*bgtA*	Contains a knockout of *natA* and *bgtA (*both genes expressing ATP-binding subunits of ABC transporter systems. In particular, this affects the function of N-I, N-II and Bgt transporter systems preventing the uptake of amino acids such as alanine.	Pernil et al. (2008)
Anabaena sp. PCC 7120 (160)	BG11	P*nifHDK-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Nitrogenase gene promoter (P*nifHDK*); Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*). Predicted to release ammonium.	This study
Anabaena sp. PCC 7120 (161)	BG11	P*gifA-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Native *gifA* gene promoter; glutamine synthetase inactivation factor (*gifA*); native terminator from *gifA* (T*gifA*). Control strain.	This study
Anabaena sp. PCC 7120 (162)	BG11	P*rbcLS-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Promoter for the large and small subunits of the Ribulose -1,5 –bisphosphate carboxylase/oxygenase (RuBisCO) operon (P*rbcLS*); glutamine synthetase inactivation factor (*gifA*); native terminator from *gifA* (T*gifA*). Control strain.	This study
Anabaena sp. PCC 7120 (163)	BG11	P*nifHDK-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Nitrogenase gene promoter (P*nifHDK*); glutamine synthetase inactivation factor (*gifA*); native terminator from *gifA* (T*gifA*). Predicted to release ammonium.	This study
Anabaena sp. PCC 7120 (164)	BG11	P*gifA-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Native *gifA* gene promoter; glutamine synthetase inactivation factor (*gifA*); native terminator from *gifA* (T*gifA*). Control strain.	This study
Anabaena sp. PCC 7120 (165)	BG11	P*petE-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Copper inducible plastocyanin gene promoter (P*petE*): glutamine synthetase inactivation factor (*gifA*); native terminator from *gifA* (T*gifA*). Predicted to release ammonium.	This study
Anabaena sp. PCC 7120 (166)	BG11	P*rbcLS-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Promoter for the large and small subunits of the Ribulose -1,5 –bisphosphate carboxylase/oxygenase (RuBisCO) operon (P*rbcLS*); glutamine synthetase inactivation factor (*gifA*); native terminator from *gifA* (T*gifA*). Control strain.	This study
Anabaena sp. PCC 7120 (167)	BG11	P*petE-gifA*-T*gifA*::RSF1010-SmR	Contains a plasmid with the following genes: Copper inducible plastocyanin gene promoter (P*petE*): glutamine synthetase inactivation factor (*gifA*); native terminator from *gifA* (T*gifA*). Predicted to release ammonium.	This study
Chlamydomonas reinhardtii metE-	TP^+	*metE-*	Contains a 9 base pair insertion into the B_{12}-independent methionine synthase (*metE)* gene rendering the gene non-functional. The strain is unable to grow in absence of supplemented B_{12}.	Helliwell et al. (2015)
Chlamydomonas reinhardtii WT12 (CCAP 11/32A)	TP^+	Wild type	N/A	N/A
Delftia acidovorans	TY	Wild type	N/A	N/A
Escherichia coli (β2163)	TY	F− RP4-2-Tc::Mu 1*dapA*::(erm-pir) + pKRCPN1	Contains the transposon plasmid pKRCPN1. pKRCPN1 carries Tn5 transposon sequences which flank a kanamycin resistance cassette and there are also a tetracycline resistance cassette and a gene encoding a transposase on the backbone. The strain is also unable to synthesise its own diaminopimelic acid (DAPA), as the *dapA* gene is knocked out with an erythromycin cassette inserted in its place.	Monsen et al. (2015)
Lobomonas rostrata	TP^+	Wild type	N/A	N/A
Mesorhizobium loti	TY	Wild type	N/A	N/A
Pseudomonas putida	TY	Wild type	N/A	N/A
Rhodococcus erythropolis	TY	Wild type	N/A	N/A
Salmonella typhimurium metE cysG (AR3612)	M9-based medium	Δ*metE* and Δ*cysG*	Contains a knockout of B_{12}-independent methionine synthase (*metE*) and uroporphyrinogen-III C-methyltransferase (*cysG*) genes. The strain is unable to grow in the absence of vitamin B_{12} and methionine.	Raux et al. (1995)

2.2.3 Maintenance

All algal and bacterial strains were maintained on agar plates (with appropriate media or antibiotics) by streaking a small number of colonies with a sterile bacteriological loop onto a new plate every 2-3 weeks. The plates were sealed in plastic wrap (to prevent water loss) and incubated agar side down in a growth cabinet. Algal strains (*C. reinhardtii* WT12, *C. reinhardtii metE⁻, L. rostrata* and *C. vulgaris*) were incubated at 25°C and all bacterial strains (including *Anabaena*) were incubated at 30°C. Photosynthetic strains (including *C. reinhardtii* WT12, *C. reinhardtii metE⁻, L. rostrata*, *C. vulgaris* and *Anabaena*) were illuminated at 40 μmol $m^{-2}s^{-1}$. Axenity of algal and cyanobacterial cultures were confirmed by serially diluting 0.5 ml of culture on to LB and TY agar plates (Appendix 8.1.5 and 8.1.6) and incubating for 2 days at 30°C.

2.2.4 Storage

For long term storage at -80°C, *Anabaena* was prepared by harvesting a 20-30 ml culture at mid-log growth by centrifugation at 4250 x g for 5 minutes. The pellet was then resuspended in 2 ml of BG11 medium and split into one aliquot with DMSO (930 μL cells, 70 μL DMSO) and one with glycerol (800 μL cells, 200 μL filter sterilised 80% glycerol in water), mixed well and snap frozen with liquid N_2. Strains were revived by streaking a small amount of frozen cells onto a BG11-agar plate with appropriate antibiotics and incubating at 30°C with continuous light.

For long term storage of non-photosynthetic bacterial strains, cultures were grown overnight in 2 mL of LB broth. Cells were harvested from 1 mL of culture and washed them with TY or LB (without antibiotics) and then resuspended them in 1 mL of filter sterile 25% glycerol LB or TY, they were then mixed well and snap frozen with liquid N_2. Strains were revived by streaking a small amount of frozen cells onto an LB or TY-agar plate with appropriate antibiotics and were incubated at 30°C or 37°C.

2.2.5 Buffers, antibiotics and additives in liquid and agarose media

HEPES buffer was added to BG11 at a final concentration of 25 mM for growth of *Anabaena* strains in order to maintain the pH at around pH 7.5. Antibiotics were used as a selection tool to maintain cells containing the specific antibiotic cassette in both liquid and solid media. Antibiotics were added after autoclaving and cooling the broth to 55-60°C in a

water bath to prevent antibiotic inactivation. For axenic cultures of *L. rostrata*, vitamin B_{12} was added. For axenic cultures of *M. loti*, I added 0.1% glycerol (Table 2.2).

Table 2.2 Antibiotics and additives used in this study

Antibiotic/Additive	Organism (strain)	Stock Concentration	Final Concentration	Solvent and Storage
Ampicillin	*E. coli* (ED8654)	100 mg/mL	100 µg/mL	H_2O, filter sterilised, -20°C
Chloramphenicol	*E. coli* (HB101)	35 mg/mL	35 µg/mL	Ethanol, filter sterilised, -20°C
Diaminopimelic Acid (DAPA)	*E. coli* (β2163)	0.3 M	0.3 mM	H_2O, filter sterilised, 4°C
Erythromycin	*E. coli* (β2163)	50 mg/mL	200 µg/mL	2 M HCl, filter sterilised, 4°C
Glycerol	*M. loti, D. acidovorans, P. putida, R. erythropolis*	100%	0.1%	H_2O, filter sterilised, 4°C
Kanamycin	*Synechocystis*, *M. loti, E. coli* (β2163)	25 mg/mL	25 µg/mL	H_2O, filter sterilised, -20°C
Neomycin	*Anabaena* (CSX60-R10)	50 mg/mL	40 µg/mL	H_2O, filter sterilised, 4°C
Phosphomycin	*M. loti*	50 mg/mL	100 µg/mL	1M HCl, filter sterilised, 4°C
Spectinomycin	*M. loti, Anabaena* (CSX60-R10), *Anabaena* strains 160-167	50 mg/mL	*M. loti* (100 µg/mL), CSX60-R10 (5 µg/mL), *Anabaena* strains 160-167 (2.5 µg/mL)	H_2O, filter sterilised, -20°C
Streptomycin	*M. loti, Anabaena* (CSX60-R10), *Anabaena* strains 160-167	50 mg/mL	*M. loti* (100 µg/mL), CSX60-R10 (5 µg/mL), *Anabaena* strains 160-167 (2.5 µg/mL)	H_2O, filter sterilised, -20°C
Sucrose	*Synechocystis*	50% (w/v)	5% (w/v)	H_2O, filter sterilised, 4°C
Tetracycline	*E. coli* (β2163)	15 mg/mL	15 µg/mL	70% EtOH, filter sterilised, -20°C
Vitamin B_{12}	*L. rostrata, C. reinhardtii metE*$^-$	0.05 mg/mL	100 ng/L	H_2O, filter sterilised, 4°C.

2.3 Growth Experiments

2.3.1 Measurement of population density

2.3.1.1 Cell counts

Microalgal cell counts were primarily gathered using a Beckman Coulter Z2 cell counter (High Wycombe, UK), as described by Evans and McGill (1969), unless otherwise specified. This was performed using a 70 μm aperture and the software was set to measure all particles greater than 4 μm in size. Because of this size gate, bacteria present in co-cultures or as contaminants did not affect the algal cell counts.

2.3.1.2 Spectrophotometry

Spectrophotometry was occasionally used to measure population density for microalgae, cyanobacteria and non-photosynthetic bacteria. The optical density of 750 nm was used to determine growth for microalgae and cyanobacteria. Occasionally, the population density of non-photosynthetic bacteria were determined by using spectrophotometry at an optical density at 600 nm.

2.3.1.3 Colony Forming Units (CFUs)

Microalgal and bacterial populations were quantified through counting colony forming units (CFUs). Aliquots (180 μL) of growth medium (e.g. TY medium for *M. loti* or BG11 medium for *Ca. vulgaris*) were placed into each of 6 wells of a 96 well plate. Twenty μL of sample was introduced into the first well to create a 1.0 $x10^1$ dilution. Twenty μL of the 1.0 $x10^1$ dilution were then added to the next well to create a 1.0 $x10^2$ dilution. The process was repeated until a 1.0 $x10^6$ dilution was reached. Ten μL of each of the dilutions was spread onto solid growth medium (e.g. TY agar for *M. loti* or BG11 agar for *Ca. vulgaris*) and left to dry in the sterile flow hood. The plates were incubated for however long it took for individual microscopically visible colonies to appear (e.g. 2 days at 30 ^oC for *M. loti* and 7 days at 25 ^oC for *Ca. vulgaris*). The colony numbers were counted at dilutions which contained approximately 10-100 colonies under a microscope at 20-40 times magnification. The CFUs obtained were multiplied by the dilution factor and the final number was converted to CFUs per mL.

2.3.2 Establishment

Liquid cultures of eukaryotic algae (*L. rostrata* and *C. reinhardtii*) were initiated by inoculating axenic cells from a plate into liquid TAP (with appropriate antibiotics and additives) and incubated until they reached OD_{750} 0.4-0.6. Cell populations were calculated by using a Beckman Coulter Z2 cell counter. The starter culture was split into three or four 40 mL cultures at a cell density of 25,000 cells/mL. The cell density of a 1 mL sample was recorded daily and growth was followed for at least 10 days.

Liquid cultures of non-photosynthetic bacteria (*M. loti* and *R. erythropolis*) were initiated by inoculating axenic cells from a plate into liquid TY (with appropriate antibiotics) and were incubated until OD_{600} reached 0.4-0.6. The cultures were split into three or four 40 mL cultures at OD_{600} 0.005. The optical density (600 nm) of a 1 mL sample was recorded daily. The growth was followed for at least 2 days.

Liquid cultures of *Anabaena* were initiated by inoculating axenic cells from a plate into liquid BG11 (with appropriate antibiotics) and were incubated until OD_{750} reached 0.4-0.6. The cultures were then split into three or four 40 mL cultures at OD_{750} 0.05. The absorbance at 680 and 750 nm of a 1 mL sample was recorded daily. The growth was followed for at least 7 days.

2.3.3 Initiation of algae-bacteria co-cultures

Algal-bacterial co-cultures were initiated by picking axenic algal colonies with a sterile bacteriological loop and inoculating them in 100 mL TAP medium supplemented with 100 pg/mL vitamin B_{12}. The cultures were then incubated in standard conditions (25°C with shaking at 120 rpm with constant illumination by cool white fluorescent lamps with a photon flux density of 40 μmol $m^{-2}s^{-1}$) until mid-log phase (OD_{750} 0.4-0.6). The cells were harvested by centrifugation (approximately 3000 x g) and were washed 3 times, suspended in fresh 100 mL TAP medium and then incubated again until the cell density reached a plateau, indicating the alga had depleted all its B_{12} reserves. The bacterial colonies were inoculated from plates in 100 mL TP^+ medium supplemented with 0.1% glycerol and incubated at 30°C until the culture reached OD_{600} 1.0-1.2. The bacterial culture was harvested, washed in TP^+ medium three times and inoculated at 0.5% of the final volume of the algal-bacterial culture. For example, 0.2 mL of bacterial culture (at OD_{600} 1.0-1.2) was added to 40 mL. The co-culture was incubated in standard conditions (as indicated above) for 7 days to allow the mutualism to establish. The identity of *M. loti* was confirmed by colony PCR and sequencing of the 16S rDNA, by utilising the 16S rDNA forward and reverse primers (Table 2.3), as described in section 2.4.

2.3.4 Data entry, analysis and statistics

Data entry, growth curves and statistical analysis were all carried out in Microsoft Excel (Mac 2016, Microsoft, WA, USA). The mean was calculated as the sum of the three independently replicated samples at each time point divided by the number of samples (Equation 2.1). The standard deviation was calculated via Excel. Statistical significance was determined by One-Way ANOVA as described by Adbi and Williams (2010), and significance was inferred if $p \leq 0.05$.

Equation 2.1

$$Mean = \frac{(sample\ 1 + sample\ 2 + sample\ 3)}{3\ (No.\ of\ samples)}$$

2.3.5 Maximum growth rate

Algal or bacterial maximum growth rate was calculated using Equation 2.2:

Equation 2.2

$$GR = \frac{(N2 - N1)}{(T2 - T1)}$$

Where:

GR = Maximum growth rate (expressed as units* per day)

*N*1 = Initial Population (cells per mL or OD_{600}/OD_{750})

*N*2 = Final Population (cells per mL or OD_{600}/OD_{750})

*T*1 = Initial Time

*T*2 = Final Time

* insert the units of measurement for the particular experiment for example, OD_{600}/OD_{750} or cells.

2.3.6 Doubling time

Algal or bacterial doubling time is defined as the time it takes for a doubling of cells to occur. In calculating the doubling time, it was assumed that cells were growing exponentially over the period for which the doubling time was calculated. Although this was not necessarily

the case, it allowed semiquantitative comparisons to be drawn. The doubling time was calculated by first determining the specific growth rate constant of strains as outlined in Equation 2.3:

Equation 2.3

$$k = \frac{\ln (N2/N1)}{(T2 - T1)}$$

Where:

k = Specific Growth Rate Constant (expressed as hr^{-1})

$N1$ = Initial Population

$N2$ = Final Population

$T1$ = Initial Time

$T2$ = Final Time

The specific growth rate constant was then used to calculate the doubling time for the organism as outlined in Equation 2.4:

Equation 2.4

$$Td = \frac{\ln (2)}{(k)}$$

Where:

T_d = Doubling Time (expressed in hours)

k = Specific Growth Rate Constant

2.4 Identification of bacteria

Bacterial species were identified by serially diluting cultures and streaking onto 1.5 % agar plates of a suitable medium (i.e. LB or TY), and grown at 25°C for 3-4 days. Plates were visually assessed, and single colonies picked, transferred to fresh plates and incubated again. From these plates, single colonies were picked and inoculated into a GoTAQ PCR reaction (as outlined below in section 2.4.2) using universal 16S ribosomal DNA (rDNA) bacterial primers (Nielson et al., 1999; Martin-Laurent et al., 2001) (Table 2.3). The PCR reaction products were run on an agarose gel (section 2.4.3), then purified (section 2.4.4), quantified (section 2.4.5) and sequenced (section 2.4.6). The sequence obtained was compared to the sequence database on NCBI (NCBI Resource Coordinators, 2016).

2.4.1 Primers

Primers used for determining the identity of species were the 16S rDNA forward and reverse primers (Table 2.3). Primers ranged from 18-20 nucleotides in length and were purchased from Sigma-Aldrich (Dorset, UK). Primers were resuspended in sterile milli-Q water to 100 μM and a working concentration of 2 μM.

Table 2.3 Oligonucleotide primers used in this work

Name	Description	Sequence
16S rDNA FWD	Forward primer for amplifying universal 16S rDNA	AGA GTT TGA TCM TGG CTC AG
16S rDNA REV	Reverse primer for amplifying universal 16S rDNA	ACG GGC GGT GTG TRC AAG

2.4.2 Polymerase Chain Reaction (PCR)

GoTaq polymerase (Promega, Southampton, UK) was used for identification of bacterial species. Bacterial template DNA for colony PCR was obtained by picking a single colony of cells (approximately 1 µL) and then suspending the cells in the PCR reaction. The cells were broken and DNA released by incubating the first step of the PCR reaction at 95 ^{o}C for 10 minutes (Table 2.4).

Table 2.4 GoTAQ PCR Reaction:

Volume	Reagent
10 µL	5x Green GoTaq Reaction Buffer
1 µL	100 µM Forward Primer
1 µL	100 µM Reverse Primer
1 µL	dNTP mix, 10 mM each
0.25 µL	GoTaq DNA Polymerase (5 U μL^{-1})
2 µL	MgCl2
To 50 µL	Sterile Milli-Q Water

The PCR program was:

1) an initial denaturing step at 95 ^{o}C for 10 minutes to lyse the cells and release the DNA);

2) 35 cycles at 95 ^{o}C for 1 minute, annealing at temperature at 50 ^{o}C, extension at 72 ^{o}C for 1 minute per kb of DNA;

3) and then a final extension at 72 ^{o}C for 5 minutes.

2.4.3 Agarose gel electrophoresis

Gel electrophoresis was used to separate PCR products according to their size. Agarose gel was made at 1% w/v concentration with 1x TBE buffer (Tris-Boric acid-EDTA) (Appendix 8.1.7). 7 µL of 1 kb Hyperladder (Bioline) was added to one of the wells as a size marker. All samples were accompanied with a DNA loading buffer containing 0.25% bromophenol blue and 30% glycerol. The gels were run in 1 x TBE buffer at 100 V for 45-75 minutes. DNA was visualised via ethidium bromide staining (at a final concentration of 0.5 µg/ml, Sigma-Aldrich) and UV illumination at 365 nm.

2.4.4 DNA purification

It was necessary to purify specific DNA fragments away from the agarose gel for sequencing. DNA from the gels were purified using the Mo Bio UltraClean 15 (CA, USA) Purification kit as per manufacturer's instructions.

2.4.5 Quantifying DNA concentration

The amount of PCR or plasmid DNA isolated was calculated using a Nanodrop spectrophotometer (Thermo Fisher Scientific) as per the manufacturer's instructions.

2.4.6 DNA sequencing

Sequencing was carried out by the DNA sequencing facility, Department of Biochemistry, Cambridge. Each sequencing sample was prepared to a final volume of 10 µL at a concentration of 5-20 ng/µL. Sequence was read using Geneious DNA sequencing analysis software (Kearse et al., 2012).

2.5 Determining Chlorophyll *a* concentration

2.5.1 Chlorophyll *a* analysis via methanol extraction

Chlorophyll *a* was measured using the method outlined by Porra et al. (1989). 10 mL of culture was pelleted and resuspended in 10 mL of 100 % ice-cold methanol and vortexed for 10 minutes. The suspension was centrifuged for 10 min, 3000 rpm at 4°C. The chlorophyll *a* was directly analysed by recording absorbance at OD 665 and 652 nm using a Thermo Spectronic Helios for work in Cambridge and GENESYS UV-Vis spectrophotometer (Thermo Scientific, USA) for work in Portugal (i.e. 60 L pilot scale growth, Chapter 5). The concentration of chlorophyll *a* (in units nmol chl *a* per mL) was then determined by substituting the OD readings into Equation 2.5. For baseline correction, pure 100 % methanol was measured prior to the samples.

Equation 2.5

$$\text{Chl } a = 18.22 \times A^{665} - 9.55 \times A^{652}$$

Where:

Chl *a* = Concentration of chlorophyll *a* (nmol chl *a* per mL)

A^{665} = Absorbance reading at 665 nm

A^{652} = Absorbance reading at 652 nm

2.5.2 Chlorophyll *a* conversion factor

Chlorophyll *a* conversion factors were calculated for *Anabaena* so chlorophyll *a* could be accurately estimated from absorbance readings without having to measure it directly using the technique outlined in section 2.5.1 (Porra et al., 1989). In order to calculate the conversion factor, a number of steps had to be taken. Thirty 2 mL samples of *Anabaena* were set up, each with a different cell concentration i.e. from 1/30 dilution up to 29/30 dilution as well as a sample that was not diluted. The larger the spread of dilution the better, therefore the concentration of cells to be diluted started at OD_{750} 3.0. Each of the samples was then split in two (2x 1 mL), one for direct readings at absorbance 750 nm and 680 nm and one for methanol extraction as outlined in section 2.5.1. Care was taken to ensure cell pellet was white before absorbance readings (i.e. chlorophyll was completely extracted from cells). The absorbance

value for 750 nm was then subtracted from the absorbance value for 680 nm. The absorbance values obtained for methanol extracted chlorophyll *a* were substituted into Equation 6 and the concentration of chlorophyll *a* determined. The chlorophyll *a* concentration was plotted against the OD_{680} - OD_{750} value (Figure 2.1). The R^2 value was calculated at 0.944, indicating a high correlation between the readings and the equation for the slope of the line was y=20.224x, indicating the conversion factor for *Anabaena* OD_{680} - OD_{750} readings to nmol chl *a* per mL was 20.224.

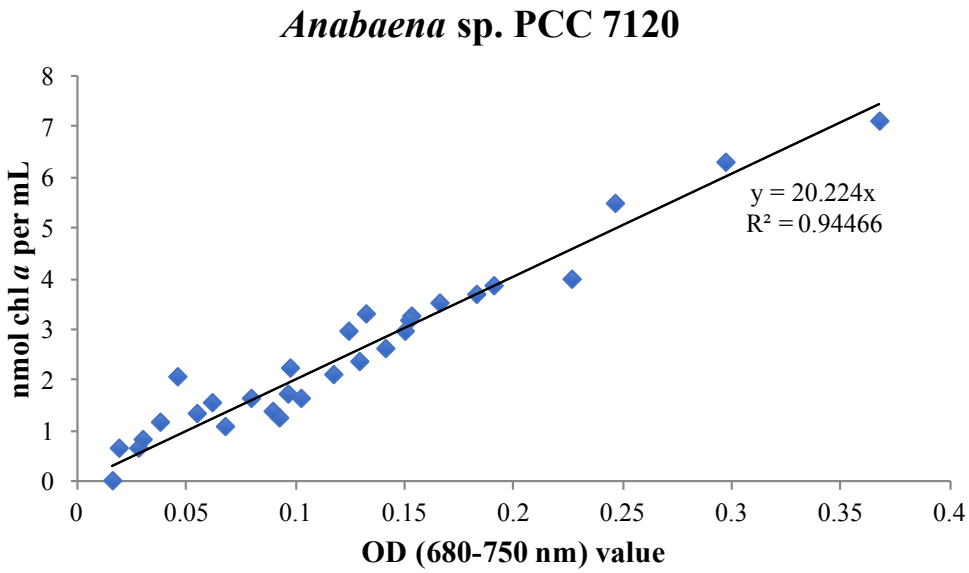


Figure 2.1 Correlation between the OD_{680} - OD_{750} value and amounts of chlorophyll *a* measured following methanol extraction.

2.6 Vitamin B_{12} Bioassay

The vitamin B_{12} concentration in samples was assayed using *Salmonella typhimurium metE cysG* (AR3612) as a B_{12} indicator strain. *S. typhimurium metE cysG* (AR3612) does not contain the B_{12}-independent methionine synthase (MetE) and only contains the B_{12}-dependent methionine synthase (MetH). To block the B_{12} synthesis pathway, the strain also carries a knockout of *cysG* (encoding uroporphyrinogen-III C-methyltransferase), the enzyme which catalyses the first reaction specific to the B_{12} synthesis pathway. The strain is unable to grow in the absence of vitamin B_{12} or methionine. Therefore, comparison of *S. typhimurium metE cysG* (AR3612) growth in the presence of a sample containing B_{12} with growth in the presence of known amounts of B_{12} provides a measure of the amount of the vitamin present in the sample.

2.6.1 Preparing *S. typhimurium*

A glycerol stock of *S. typhimurium* was stabbed with a pipette tip containing 80 µL of sterile water. The water and *S. typhimurium* (AR3612) were released onto minimal agar (containing L-methionine) and spread evenly (Appendix 8.1.8.2). The agar plate was sealed with Parafilm and incubated at 37˚C overnight. The plate was then checked for contamination and 1 mL of sterile 0.9% NaCl in water was added to the lawn of *S. typhimurium* (AR3612) cells. The cells were scraped, using a spreader, into suspension with the 0.9% NaCl solution. This solution was then aliquoted into a 1.5 mL microcentrifuge tube. The solution was washed with 0.9% NaCl three times by centrifuging at 20,000 g for 1 minute. This was carried out to wash away any methionine derived from the minimal medium plates. 50-100µl of washed *S. typhimurium* (AR3612) was added to 250ml of 2x M9-based medium (Appendix 8.1.8.3). The *S. typhimurium* (AR3612)-inoculated 2x M9-based medium was stored at 4˚C during the time the experiments were conducted. Any unused medium containing *S. typhimurium* (AR3612) was discarded within 2 weeks.

2.6.2 Preparing culture samples for the B_{12} bioassay

Sample volumes required varied depending on concentrations of B_{12} in the sample. However, approximately 1 ml of *M. loti* sample at 10^6 cells/ml was at the lower limit of detection. 1.1 ml of sample was placed into a 1.5 ml Eppendorf tube and boiled for 15 minutes. The samples were then centrifuged at 13,000 rpm/17,000 g for 2 minutes. 1ml of supernatant (or diluted supernatant if bacterial density was high e.g. *M. loti* cultures between 10^8 and 10^9 cells/ml) was added to a 24 well plate. 1ml of M9-based medium containing *S. typhimurium*

(AR3612) was added to the same well. The 24 well plate was sealed with micropore tape, and incubated at 37°C in a shaking incubator for 16-20 hours alongside the standard curve plate.

2.6.3 Preparing the standard curve

A standard curve was prepared with eight B_{12} concentrations within the range of 0-125 ng/L of B_{12}. Three replicates of each concentration were made. Four ml of growth medium (same as that being tested) was added to 8 Falcon tubes. The appropriate volume (in µl) from a 10 ng/ml stock of B_{12} (indicated in column 2 of Table 2.5) was added to the Falcon tubes. (Note that on the addition of these volumes of stock solution the concentration was double the stated final concentration; this was so that on adding the 1ml B_{12}-containing medium with 1ml of M9-based medium containing *S. typhimurium* (AR3612), the desired final concentration was achieved). The tubes were mixed by inversion several times and then 1 ml was added to a 24 well plate. 1 ml of M9-based medium containing *S. typhimurium* was then added to the 1 ml of B_{12}-containing medium, the plates were sealed with micropore tape, and placed in a 37°C shaking incubator for 16-20 hours at the same time as the test plates.

Table 2.5 Volumes of 10 ng/ml B_{12} added 4 mL of growth medium

Final concentration (pg/ml)	Volume (µl) of 10ng/ml B_{12} stock to add
0	0.0
10	8.0
20	16.1
30	24.1
50	40.4
75	60.9
100	81.6
125	102.6

2.6.4 B_{12} bioassay analysis

After 16-24 hours, the 24 well plates were removed from the incubator and optical density at 595nm of the samples was measured in a spectrophotometer. A logistic curve was fitted to the standards data (e.g. Figure 2.2) and the equation used to predict B_{12} concentrations in the unknown samples.

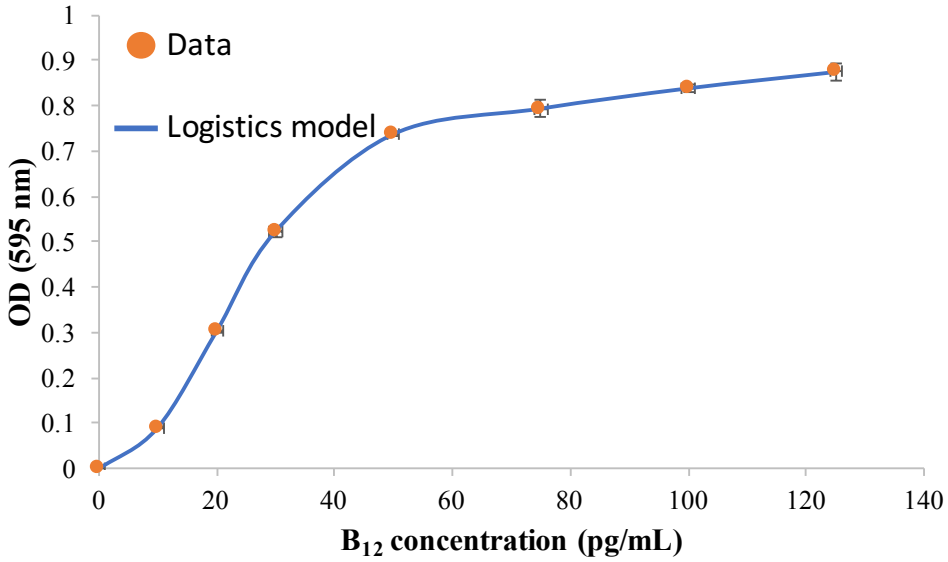


Figure 2.2 Example standard curve plotted by fitting a logistic model to the standard optical density data.

2.7 Bioinformatic and software tools

2.7.1 Gene databases

CyanoBase (Fujisawa et al., 2014) (http://genome.microbedb.jp/cyanobase)

NCBI Blast (NCBI Resource Coordinators, 2016) (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

2.7.2 Desktop programs

Geneious software (Kearse et al., 2012) (http://www.geneious.com/)

Microsoft Excel (Mac 2016, Microsoft, WA, USA)

Mendeley Desktop (https://www.mendeley.com/)

2.8 Methods used in Chapter 3

2.8.1 Transposon mutagenesis on *M. loti*

The following transposon mutagenesis technique has been adapted from Monson et al. (2015). *E. coli* strain β2163 contains the transposon plasmid pKRCPN1 (Figure 2.3). pKRCPN1 carries Tn5 transposon sequences which flank a kanamycin resistance cassette and there are also a tetracycline resistance cassette and a gene encoding a transposase on the backbone. Furthermore, *E. coli* strain β2163 is unable to synthesise its own diaminopimelic acid (DAPA). DAPA is an amino acid required for the cell wall and its biosynthesis requires the gene *dapA*. *E. coli* strain β2163 has *dapA* knocked out and an erythromycin cassette inserted in its place. The strain was grown in TY medium overnight containing diaminopimelic acid (DAPA), kanamycin, erythromycin and tetracycline. In parallel, WT *M. loti* was grown for 48 hours in 2 mL TY medium. The *E. coli* and *M. loti* were combined at a 2:1 ratio based on optical density (OD_{600}) and mixed. Aliquots (10 μL) of the co-cultures were dropped onto TY plates (containing DAPA to allow growth of *E. coli* strain β2163 but lacking antibiotics, to allow growth of *M. loti*), and incubated at 30°C overnight. The mixed colonies of *E. coli* and *M. loti* were then streaked off with a pipette tip and then used to inoculate 2 mL of TY medium (without any additives) in bijou bottles. The cultures were then diluted to 1/10, and a 100 μL sample was plated and spread on TY plates containing kanamycin but lacking DAPA (so only *M. loti* with a transposon inserted into its genome should grow and no *E. coli* strain β2163 should grow as DAPA is absent from the plates). Any colonies that grew on the kanamycin plates were then picked and patched on tetracycline plates as a negative control. It was expected that colonies should grow on kanamycin plates and not tetracycline plates as only the transposon (carrying a kanamycin resistance cassette) should be integrated into the genome and the plasmid backbone (carrying the tetracycline resistance cassette and gene encoding a transposase) should not be present (Demarre et al., 2005; Monson et al., 2015).

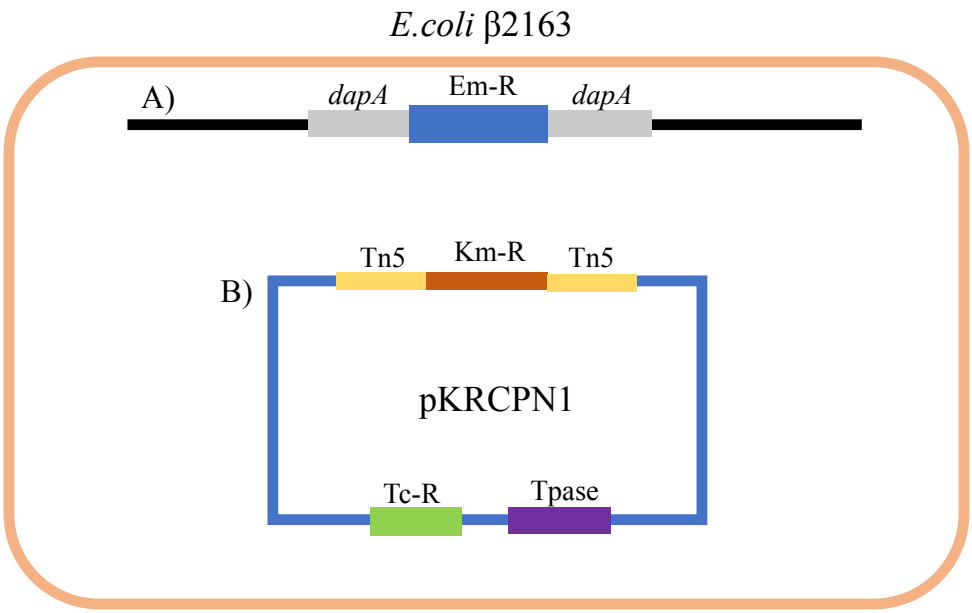


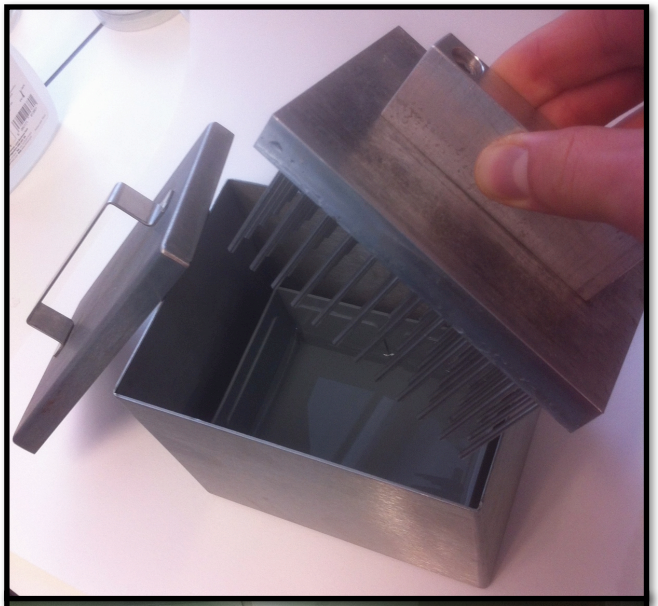
Figure 2.3 Illustration of *E.coli* β2163 and plasmid pKRCPN1.

A) *E.coli* β2163 genome highlighting the erythromycin resistance cassette (Em-R) inserted into the *dapA* gene. B) pKRCPN1 plasmid containing a kanamycin resistance gene (Km-R) flanked by Tn5 transposon sequences, a tetracycline resistance cassette (Tc-R) and a transposase gene (Tpase).

2.8.2 Screening *M. loti* mutants for genes involved in symbiosis

L. rostrata were grown axenically and inoculated at a concentration of 0.5 µg/mL chlorophyll *a* mixed in to TP^+ 0.6% agar plates. Single colonies from the *M. loti* transposon insertion library ('mutants') were picked and grown overnight in sterile 96 well plates containing TY medium. The cells growing in 96 wells were picked using a 96-pinned stainless steel 'hedgehog' (Figure 2.4A) and inoculated onto the TP^+ agar containing *L. rostrata* as well as TY + Kanamycin (positive control) and TY + tetracycline (negative control). The plate was incubated at room temperature in a continuously illuminated (40-60 µmol m^{-2} s^{-1}) custom-designed incubator tower (Figure 2.4B). *L. rostrata* growth could be seen after around one week. Putative mutants in the interaction would be identified by the absence/increase of growth of *L. rostrata*. The mutants would then be identified and picked from the mutant library (found on TY + kanamycin plates) and then subjected to PCR amplification and sequencing of the transposon insertion site. Figure 2.5 outlines the protocol in a flowchart.

A)



B)

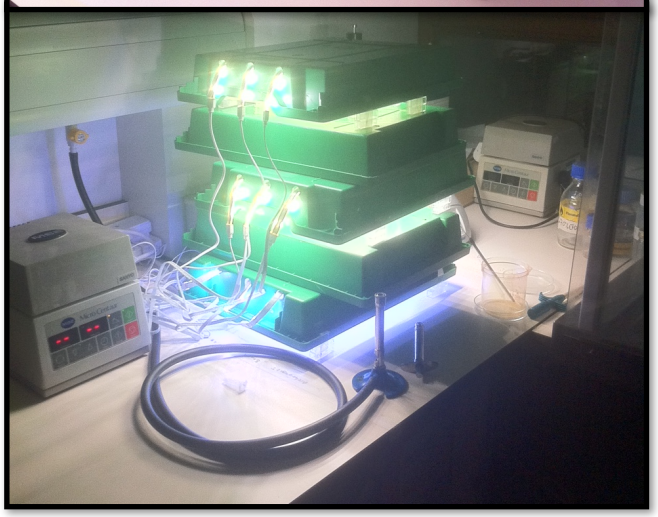


Figure 2.4 Custom-made equipment for the transposon screen

A) A 96-pinned stainless steel 'Hedgehog'. The 'Hedgehog' fits inside a bath filled with 70% ethanol for sterilization. The 'Hedgehog' and ethanol-filled bath fit inside a stainless-steel case with a lid to ensure suffocation of a fire if one were to accidentally be lit inside the bath. B) An algae incubator tower. Three inverted plastic drawers contained white LED strip lighting adhered to the base of the drawer. The drawers were drilled to create six circular holes (three on each end) to allow the ends and connections for LED strip lighting to protrude. These incubators were stacked three high and interspersed with spacer drawers (two for one tower) which gave space between each incubator so the heat produced from the LED lighting did not cause evaporation of water in the algae-inoculated TP^{+} agar plates positioned on the top of the spacer drawers.

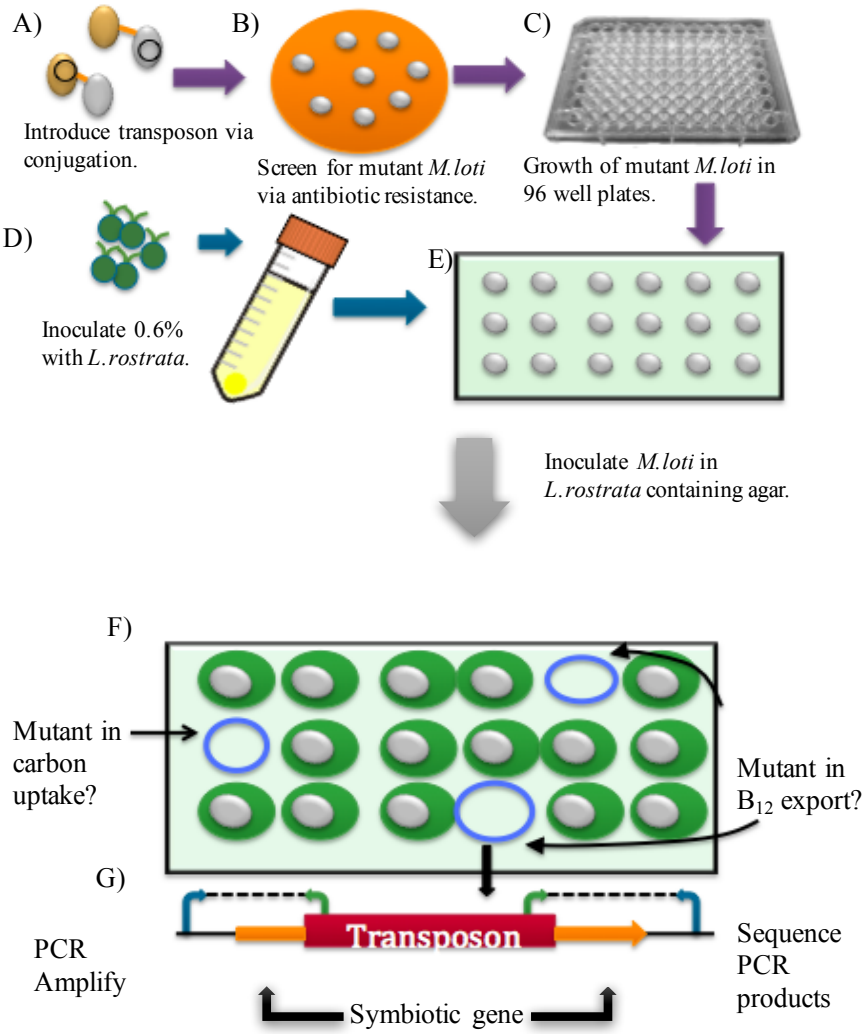


Figure 2.5 Screening *M. loti* mutants for symbiosis breakdown

Screening *M. loti* transposon mutants for symbiosis breakdown involved: A) Introducing the transposon via conjugation; B) Screening for *M. loti* cells with transposon insertions; C) Growth of these mutants on 96 well plates; D) Inoculating TP^+ agar with *L. rostrata* and pouring them in agar plates; E) Inoculating the *M. loti* in the *L. rostrata* containing agar; F) Allowing the plates to incubate for one week and observing whether any inoculation points are lacking *L. rostrata* growth and; G) If an inoculation point results in no *L. rostrata* growth, then the respective *M. loti* strain is isolated from the mutant library and screen repeated. If the phenotype is replicated then a growth curve with *L. rostrata* would be carried out. If the *M. loti* mutant proved again to be unable to undergo symbiosis with *L. rostrata*, the *M. loti* mutant strain would be sequenced across the site of transposon insertion. This would be carried out by using known sequences in the transposon for one set of primers and another set of randomly generated primers for the complementary primers.

2.8.3 *M. loti* STM Mutants

M. loti STM mutants were obtained from Legume Base (https://www.legumebase.brc.miyazaki-u.ac.jp/about.jsp). The mutants were generated by using a signature-tagged mutagenesis (STM) technique as outlined by Shimoda et al. (2008).

2.9 Methods used in Chapter 4

2.9.1 Haemocytometer for *Anabaena* cell number determination

Anabaena is a filamentous cyanobacterium and therefore it was not possible to count algal cells in the Coulter counter as outlined in section 2.3.1. Instead algal cell numbers were determined by using a haemocytometer (Improved Neubauer Haemocytometer Counting Chamber) and microscope.

2.9.2 Bath and probe sonication

Bath sonication was carried out by a 'Bioruptor' bath sonicator, Diagenode (Seraing, Belgium) which was kindly lent by Professor David Baulcombe's Laboratory. Probe sonication was carried out by a Soniprep 150, MSE (London, United Kingdom).

2.9.3 Filters for filament separation

The filters used for filament separation were 25 µm stainless steel mesh, Metal Mesh (Worcestershire, UK) and 25 µm polypropylene filters, 1.7 mm thick Sigma-Aldrich (Dorset, UK)

2.9.4 Spectroscopic analysis

Spectroscopic analysis was carried out with a Shimadzu UV-1800 UV spectrophotometer (Buckinghamshire, UK).

2.9.5 Measuring ammonium concentration

Ammonium (NH_4^+) levels in the culture supernatant were analysed using a Nutrafin Ammonium Test Kit (Hagen, Canada) following the manufacturer's instructions:

1) A 5 mL sample of supernatant to be tested was placed into a clean test tube/centrifuge tube.
2) A 70 µL aliquot of "Reagent #1" was added into the tube containing the supernatant and the tube was shaken for approximately 1 minute.
3) A 70 µL aliquot of "Reagent #2" was added into the tube containing the supernatant and the tube was shaken for approximately 1 minute.
4) A 70 µL aliquot of "Reagent #3" was added into the tube containing the supernatant and the tube was shaken for approximately 1 minute.
5) The solution was incubated for 20 minutes for the colour to develop so absorbance could be measured at OD_{640} nm.
6) The absorbance reading at OD_{640} nm was converted to ammonium concentration (mM) via the standard curve outlined in Figure 2.6.

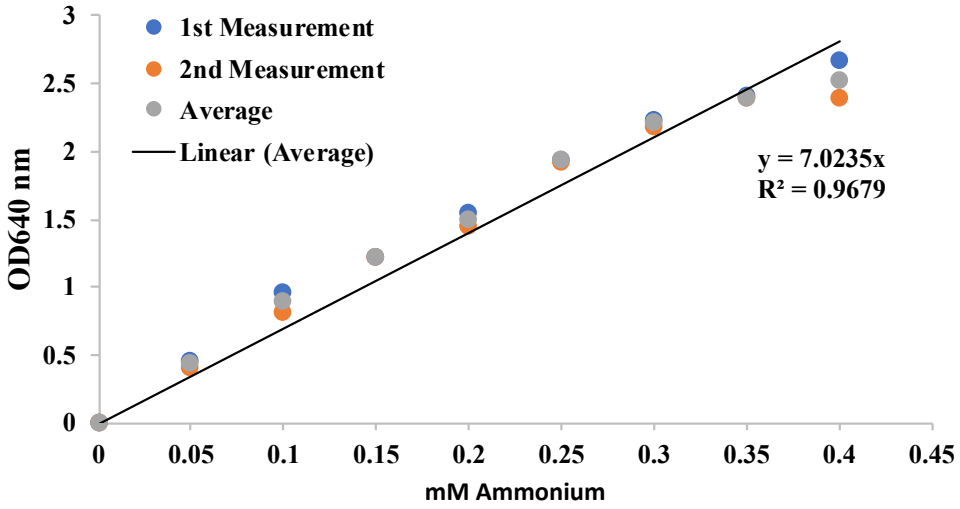


Figure 2.6 Correlation between the OD_{640} nm and concentration of ammonium (mM)

2.10 Methods used in Chapter 5

2.10.1 Pre-pilot scale growth

2.10.1.1 Starter cultures of *C. reinhardtii metE⁻* and *C. reinhardtii metE⁻* + *M. loti*

Prior to inoculation at 10 L pre-pilot scale, cultures were grown in 50 mL conical flasks for approximately 1-2 weeks. These cultures were then transferred to 500 mL conical flasks

for 1 week and then 2 L conical flasks for approximately 2 weeks until cell populations reached at least 2.0 x10^5 cells per mL.

2.10.1.2. Pre-pilot scale inoculation of *C. reinhardtii metE⁻* and *C. reinhardtii metE⁻* + *M. loti*

The 2 L cultures were then transferred to the 10 L vertical tubular bioreactors situated in the Algal Innovation Centre, University of Cambridge, UK. 7.9 L of non-sterile distilled water was added to the cultures as well as 100x TP^+ medium concentrate in 100 mL of water, to give the standard concentration of TP^+ ingredients for 10 L of TP^+ medium.

2.10.1.3 Monitoring of algal growth

Microalgal cell counts were gathered using a Beckman Coulter Z2 cell counter (High Wycombe, UK), as described in section 2.3.1.1. Dry cell weight was determined by filtration of 90 mL of culture through 1.2-µm glass fibre filters (Merck Millipore, USA) and drying for two days in a 50°C drying cabinet. Dry cell weight was determined using Equation 2.6 (Note: scales were calibrated to exclude the weight of the filter):

Equation 2.6

$$DCW = W2 - W1$$

Where:

DCW= Dry cell weight (mg/ml)

W1 = Weight of filter (before wet biomass added) prior to drying

W2 = Weight of biomass and filter after drying

2.10.2 Pilot scale cultivation setup and sampling

2.10.2.1 Starter cultures of *C. reinhardtii metE⁻* and *C. reinhardtii metE⁻* + *M. loti*

Prior to inoculation at 60 L pre-pilot scale, cultures were grown in 50 mL conical flasks of TP^+ medium for approximately 1-2 weeks. These cultures were then transferred to 500 mL conical flasks of TP^+ medium for 1 week and then 2 L conical flasks of TP^+ medium for approximately 1 week. The cultures were then transferred to 3x 6 L flasks, bubbling until cell

populations reached at least 3.0 x10^6 cells per mL.

2.10.2.2 Pilot scale inoculation of *C. reinhardtii metE⁻* and *C. reinhardtii metE⁻* + *M. loti*

Pilot scale cultivation was carried out in Glass Green Wall (GGW) PBRs installed inside a greenhouse of the Experimental Unit of A4F in Lisbon, Portugal. The GGWs were the same GGWs outlined by Zedler et al. (2016). The two GGWs were filled with 40 L of fresh water. The water-filled GGW PBRs were sterilized with 50 ppm sodium hypochlorite, and left overnight. The reactors were then boiled for 40 min to decontaminate the culture medium further and to boil off the sodium hypochlorite, and cooled to 25°C. 2 L of 30x concentrated TP^+ was added to the 60 L cultures. The two GGWs were then inoculated simultaneously with stationary phase cultures of *C. reinhardtii metE⁻* supplemented with B_{12} and *C. reinhardtii metE⁻* + *M. loti* co-culture to an initial OD_{750} of 0.14 to 0.15. The GGWs were maintained at a temperature of 25°C. In addition, they were bubbled with air supplemented with 0.5 % CO_2 to maintain the pH levels of the cultures between 7 and 8 and provide an ample source of carbon, and irradiated by natural sunlight. The cultures were initially shaded with plastic sheets to allow for adjustment to the new light intensities and were left on for the first eight days of the experiment. Samples from the cultures were taken daily in the morning between 09.00 and 10.00.

2.10.2.3 Monitoring of cell viability and growth

The cultures were observed daily using an Olympus BX53 upright microscope to monitor cell motility, autofluorescence, cell division/debris and bacterial presence in the cultures. Cell growth and viability were analysed by cell counting with a Muse® Cell Analyzer (Merck Millipore, USA). Cell viability was determined by the addition of 1.0 mg/mL of propidium iodide at a ratio of 10% of the final volume. For example 0.1 mL of 1.0 mg/mL propidium iodide would be added to 0.9 mL of sample. Optical density was measured at 750 nm with a GENESYS UV-Vis spectrophotometer (Thermo Scientific, USA). Dry weight was determined by filtration of 90 mL of culture through 1.2-µm glass fibre filters (Merck Millipore, USA) and subsequent drying at 180°C using an MS-70 moisture analyser (A&D Company, Japan) and calculation as outlined in section 2.10.1.3. Chlorophyll *a* concentration per algal cell was determined by dividing total chlorophyll *a* by algal cell counts.

2.10.2.4 Monitoring of culture medium parameters

The pH of the cultures was measured twice daily. Ammonium (NH_4^+) levels in the culture supernatant were analysed daily using a Nutrafin Ammonium Test Kit (Hagen, Canada) following the manufacturer's instructions.

2.10.2.5 Bacterial morphologies

To gauge the number of contaminating bacteria in the cultures, and their morphologies, a modified version of the replica plating technique as described by Jett et al. (1997) was used. Cultures were diluted by a suitable factor to allow the identification of single colonies on a plate. Aliquots (10 µl) were placed at one end of a Petri dish containing TY medium with 1.5 % agar, then the plate was angled so that the drops formed vertical lanes across the plate, and were then left to dry. Plates were grown for 3-5 days at 25°C, visually assessed and single colonies picked, transferred to fresh plates and incubated again. Note, it was acknowledged that not all bacterial species grow on TY media. Therefore, this technique was only used as en estimate for the range of species present in the culture.

3 EXPANDING OUR UNDERSTANDING OF ALGAE-BACTERIA INTERACTIONS

3.1 Introduction

3.1.1 The *L. rostrata* + *M. loti* consortium

Approximately half of all algal species require vitamin B_{12} (cobalamin, and hereafter referred to as B_{12}) for growth, and it was suggested that the vitamin is obtained by algae through direct symbiosis with bacteria (Croft et al., 2005). This hypothesis originated when a B_{12}-synthesising bacterium, *Halomonas sp*., was isolated from the blades of *Porphyra miniata* (a red alga). In order to test the hypothesis further, a model laboratory system was required that could lend itself easily to physiological and molecular manipulation - a difficult feat with the macroalga *Porphyra miniata.*

Kazamia (2012) chose the freshwater green alga *Lobomonas rostrata* (SAG 45A1) and *Chlamydomonas nivalis* as candidates to establish a model for studying symbiosis as both strains were known to be B_{12}-dependent (require an exogenous source of B_{12}). Interestingly, when a culture of *C. nivalis* was obtained from a culture collection, it was found to be contaminated with bacteria. The bacterial contaminant was isolated and identified by its 16S rDNA sequence as *Mesorhizobium loti* (MAFF 303099). Kazamia (2012) found that it was not possible to culture *C. nivalis* in the absence of the bacteria, and so all future work focussed on *L. rostrata* where axenic culturing was possible.

M. loti is a Gram-negative soil bacterium of the order Rhizobiales capable of symbiotic interactions with leguminous plants such *Lotus japonicus* (Yakota et al., 2009). *M. loti* cells establish themselves inside the root nodules of the plant and provide fixed nitrogen in return for a microaerobic, secure habitat and a source of fixed carbon for growth. Methods such as the construction and screening of a signature-tagged mutant (STM) *M. loti* library have helped to elucidate the bacterial genes involved in this interaction. Shimoda et al. (2008) created 3680 *M. loti* mutants that contained a transposon insertion in non-redundant genes. The authors were

then able isolate two *M. loti* mutants defective in the ability to form root nodules with *L. japonicus.*

Subsequent research carried out on *L. rostrata* and *M. loti* found that *M. loti* could provide B_{12} to *L. rostrata* and, in return, *L. rostrata* provided a carbon source to *M. loti* (Kazamia, 2012) (Figure 3.1). The consortium was investigated in detail by Kazamia (2012) and, importantly, found to be regulated and specific. The evidence for the regulation and specificity is summarised below:

(1) *L. rostrata* was co-cultured with *M. loti*, *Rhizobium leguminosarum* and *Sinorhizobium meliloti* over an initial 14-day period. All bacteria appeared to support the growth of the alga during this time. However, when the cultures were grown over the long term (through 6 sub-cultures) only the *L. rostrata* + *M. loti* co-culture persisted. This suggested an element of specificity in the *L. rostrata* + *M. loti* interaction.

(2) A qualitative experiment was carried out investigating whether live cells, boiled bacterial extract and bacterial supernatant of *M. loti* could stimulate the growth of *L. rostrata* inoculated into agar plates. The results showed that live cells of *M. loti* spread onto the *L. rostrata*-inoculated agar could stimulate the growth of *L. rostrata* but boiled bacterial extract and supernatant from *M. loti* cultures did not support the growth of *L. rostrata.* This suggested that *M. loti* upregulates its B_{12} production in the presence of the alga.

(3) B_{12} measurements indicated a higher concentration of B_{12} by a factor of 300 in the presence of *L. rostrata* compared to axenic *M. loti* culture. This result contrasts with similar experiments with *Rm. leguminosarum* where there was just over a two-fold difference in B_{12} production between axenic culture and co-culture with *L. rostrata*. It was also found that when *M. loti* was cultured axenically, the species had a lower basal rate of B_{12} production compared to that of *Rm. leguminosarum* (146-fold difference). This gives an indication of the possible magnitude of upregulation of B_{12} production in the presence of the alga.

(4) The ratio between *L. rostrata* and *M. loti* cell numbers in a co-culture reached an equilibrium between 1:10 to 1:30 (algae to bacteria) regardless of the starting inoculum ratio, which ranged from $1:10^5$ to $10^5:1$.

(5) It was also possible to perturb the established *L. rostrata* + *M. loti* interaction by supplementing the co-culture with either B_{12} or a source of carbon (glycerol). When B_{12} was added to co-cultures, algal growth was favoured without concomitant increase in bacterial densities. Conversely, when a source of carbon was added, bacterial cell numbers increased exponentially and did not lead to an increase in algal density. The author concluded this provided strong evidence of specificity and regulation, since a passive process, such as cell lysis for the provision of B_{12} or carbon (in which concentrations would be expected to be proportional to cell numbers) would result in the increase in growth of one organism leading to the increased growth of the other.

(6) Finally, a close relative of *L. rostrata*, *Chlamydomonas reinhardtii*, was unable to sustain the growth of *M. loti* over three subcultures, suggesting the carbon source provided to *M. loti* from *L. rostrata* could be unique to *L. rostrata*.

Kazamia (2012) concluded that the mutualism between *M. loti* and *L. rostrata* was specific and involved a direct exchange of nutrients with the potential involvement of molecular signalling or regulation such as quorum signalling. Furthermore, a mathematical model describing the interaction on the basis of the evidence described above was established by Grant et al. (2014).

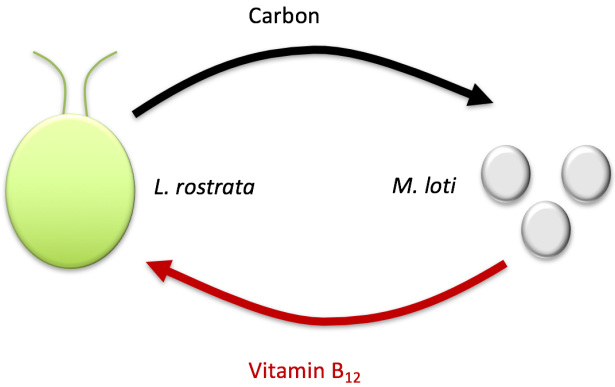


Figure 3.1 The *L. rostrata* + *M. loti* consortium

M. loti provides vitamin B_{12} (cobalamin) to *L. rostrata* and in return, *L. rostrata* provides a carbon source (Kazamia et al., 2012).

3.1.2 Expanding the *L. rostrata* + *M. loti* consortium

Ridley (2016) studied the consortium further and confirmed there was a growth penalty on *L. rostrata* when it received B_{12} from *M. loti* as opposed to when B_{12} was added directly to the culture. The growth penalty included a slower maximum growth rate and lower carrying capacity (lower final concentration of cells). The author made attempts to enhance the productivity of the consortium by introducing additional B_{12} producing bacteria, *Delftia acidovorans* and *Pseudomonas putida*. However, no consistent effect on growth was seen with either bacterium.

3.1.3 An industrially relevant consortium

Whilst the *L. rostrata* + *M. loti* consortium is an interesting model from a fundamental perspective, it is unlikely to be useful in industry in the near future due to *L. rostrata*'s relatively slow growth, and lack of a genome sequence and genome manipulation techniques (Kazamia, 2012; Ridley, 2016). It was initially thought *M. loti* could not enter symbiosis with *Chlamydomonas reinhardtii* (See section 3.1.1 point 6, Kazamia, 2012). However, Helliwell et al. (2015) developed a B_{12}-requiring strain of *C. reinhardtii* that could enter symbiosis with *M. loti*. The strain was developed using experimental evolution by exposing wild-type *C. reinhardtii* for many generations to concentrations of B_{12} far greater than its biological requirement. After approximately 600 generations, a mutant arose that required an exogenous source of B_{12} in order to survive. This B_{12} auxotrophy was found to be caused by the existence of a mutation in the gene for the B_{12}-independent form of methionine synthase (METE), leaving only the gene for the B_{12}-dependent enzyme (METH) functional. This mutation was determined by sequencing to have been caused by the insertion of a Gulliver-like transposable element, which left a 9 bp insertion, interfering with expression of the functional METE enzyme. For the purpose of this thesis, this strain is called *C. reinhardtii metE*$^{-}$. (The minus superscript is included in the nomenclature for consistency with usage elsewhere, and thust to avoid confusion.) It has since been found that *M. loti* could enter symbiosis with not only the *C. reinhardtii metE*$^{-}$ strain but also wild type *C. reinhardtii* (WT12) suggesting the METE mutation is not necessary for *M. loti* to survive in co-culture with *C. reinhardtii* (Freddy Bunbury, personal communication of unpublished data, January 2017).

Ridley (2016) compared the growth of axenic *C. reinhardtii* (WT12) to that of the *C. reinhardtii metE*$^{-}$ strain (supplemented with B_{12}) and found there was no fitness penalty for

the mutant. However, as in the *L. rostrata* + *M. loti* consortium, there was a slower growth rate as well as a lower carrying capacity of the *C. reinhardtii metE*$^{-}$ – *M. loti* consortium compared with axenic growth of *C. reinhardtii metE*$^{-}$ supplemented with B_{12}.

3.1.4 Aims

Little is known about the underlying genetic control of the *L. rostrata* + *M. loti* interaction, so the work I describe here set out to identify *M. loti* mutants affected in the symbiosis. I did this by screening mutants from a publicly-available collection, and generating and screening a library of transposon mutants.

There is also a need for additional consortia which may be industrially relevant and/or provide insights into the exchanges underlying B_{12}-dependent symbioses. During the screening of the mutants, I serendipitously identified a novel symbiosis, and studied it in more detail.

3.2 Results

3.2.1 Screening *M. loti* mutants for breakdown in symbiosis

3.2.1.1 Optimising the screen

To screen for mutants unable to carry out symbiosis with *L. rostrata,* I carried out a negative phenotype screen for *M. loti* mutants unable to provide B_{12} to *L. rostrata*. The principle behind this screen was to inoculate *M. loti* mutants into agar plates containing *L. rostrata.* The plates were then left to incubate under constant illumination at 20 µmol photons m^{-2} s^{-1} to allow the *L. rostrata* to grow around the *M. loti* inoculation points. If no growth was observed, the *M. loti* was isolated from the TY control plate and further testing carried out. Further details are outlined in Materials and Methods 2.8.1.

Initial optimisation experiments involved identifying the optimal concentration of *L. rostrata* throughout the agar to ensure algal growth when in proximity with the B_{12} released from *M. loti* without generating a large background population (Figure 3.2 A-D). Additionally, the most suitable agar concentration had to be identified that allowed for the passage of metabolites but with minimal movement of algae and bacterial cells. *L. rostrata* were inoculated into TP^+ agar at concentrations ranging from 0.5 to 2.0 µg/mL chlorophyll *a* as well as into TP^+ agar at a concentration between 0.6% to 1.5%. *M. loti* (at a concentration of approximately 1.0 OD_{600}) were then inoculated into the *L. rostrata*–inoculated agar plates using a hedgehog comb (Materials and Methods 2.8.2). I found that the algae could grow at any of the concentrations tested (Figure 3.2 A-D). Therefore, I determined the optimal concentration of *L. rostrata* in TP^+ agar to be 0.5 µg/mL chlorophyll *a* (Figure 3.2A) whereby the co-culture could establish whilst maintaining a low background concentration of *L. rostrata* to aid in identifying symbiosis mutants. The co-culture could grow in all agar concentrations tested (Figure 3.2 E-G). However, cracks began to form around the inoculation points between 1.0-1.5% agar (Figure 3.2F and G) which could interfere with identifying symbiosis mutants. Therefore, I determined the optimal concentration of agarose in the agar to be 0.6% (Figure 3.2E).

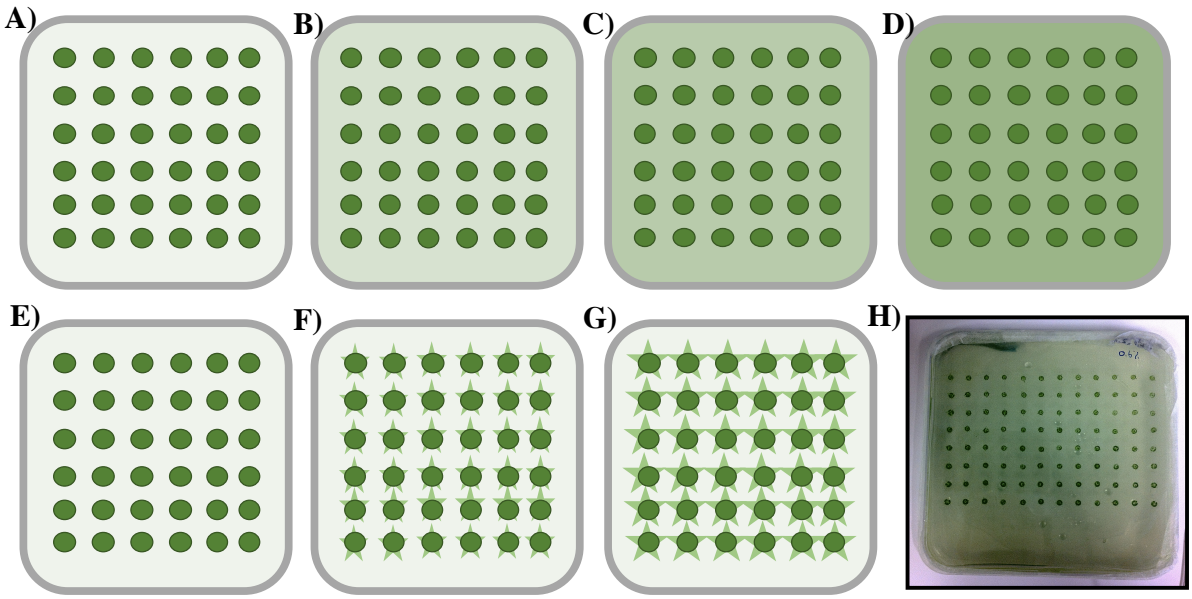


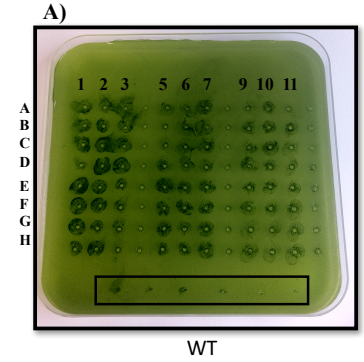
Figure 3.2 Illustration of the optimisation of TP$^+$ *L. rostrata* agar plates for *M. loti* screening

Illustration of the result of *M. loti* inoculated in TP$^+$ agar containing (A) 0.5 µg/mL chlorophyll *a*, (B) 1.0 µg/mL chlorophyll *a*, (C) 1.5 µg/mL chlorophyll *a* and (D) 2.0 µg/mL chlorophyll *a* of *L. rostrata*. Illustration of the result of *M. loti* inoculated in TP$^+$ agar with 1.5 µg/mL chlorophyll *a* of *L. rostrata* at the agarose concentrations (E) 0.6%, (F) 1.0% and (G) 1.5%. Image of optimal condition where *M. loti* was inoculaed in 0.6% TP$^+$ agar at 0.5 µg/mL chlorophyll *a* of *L. rostrata* (H). Note: Illustrations are used in this figure as it was difficult to represent the differences with images. All co-cultures were incubated for 7 days.

3.2.1.2 Screening selected *M. loti* mutants from an established library

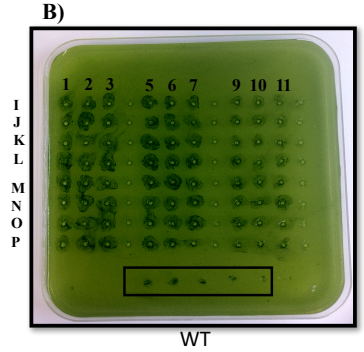
Shimoda et al. (2008) had previously generated a library of transposon mutants of *M. loti* to study its role in providing fixed nitrogen to leguminous plants. A total of 29,330 independent *M. loti* mutants were generated via a signature-tagged mutagenesis (STM) technique. Of these mutants, 3,680 had their transposon insertion site within protein coding genes determined via sequencing. Due to the cost of purchasing these mutants (£3 each) from Legume Base (https://goo.gl/jIpNh4) (see Materials and Methods 2.8.3) it was not financially feasible to test them all in the *L. rostrata* screen, so I chose 90 based on a view of the likelihood of the mutant gene being involved in the *L. rostrata* + *M. loti* mutualism. The transposon insertion sites included genes encoding ATP binding, substrate binding and permease proteins that together make up ABC transporters (in particular, sugar transporters). Additionally, mutants affected in genes involved in vitamin synthesis and uptake, other probable transport proteins and general secretory proteins were also chosen (Appendix 8.2). The selected mutants were obtained and screened as described above (Figure 3.3) to determine whether any of the genes disrupted in the mutants had an effect on symbiosis with *L. rostrata*. All the mutants showed the green halo around each inoculation point and therefore did not show any phenotypic effect on the growth of *L. rostrata*. I repeated the experiment two further times with no variation in this result. I concluded that none of the mutants had an effect on symbiosis with *L. rostrata.*

A)



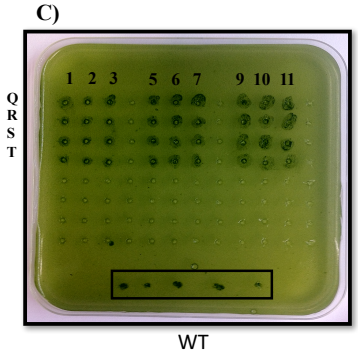
Position	Gene Product	Position	Gene Product	Position	Gene Product
A1-3	Thiamin phosphate pyrophosphorylase	A5-7	Sugar binding protein of sugar ABC transporter	A9-11	Probable secreted solute binding protein
B1-3	ATP Binding Protein	B5-7	ATP Binding Protein	B9-11	Probable sensor/response regulator hybrid
C1-3	ATP Binding Protein	C5-7	Response sensor protein	C9-11	ATP Binding Protein
D1-3	Probable sensor/response regulator hybrid	D5-7	ATP Binding Protein	D9-11	Sugar ABC transporter substrate-binding protein
E1-3	Sugar binding protein of sugar ABC transporter	E5-7	Alpha-galactoside ABC transporter substrate-binding protein	E9-11	Maltose ABC transporter substrate-binding protein
F1-3	Sugar ABC transporter (sugar-binding protein)	F5-7	ATP Binding Protein	F9-11	Sugar ABC transporter (sugar-binding protein)
G1-3	ATP Binding Protein	G5-7	ATP Binding Protein	G9-11	ATP Binding Protein
H1-3	Binding protein component of ABC sugar transporter	H5-7	Probable Transporter	H9-11	General Secretion protein G

B)



Position	Gene Product	Position	Gene Product	Position	Gene Product
I1-3	Sugar ABC transporter substrate-binding protein	I5-7	Probable sensory transduction regulatory protein	I9-11	C4 dicarboxylate transport protein
J1-3	ATP Binding Protein	J5-7	Thiamin/B1, ABC components	J9-11	WD Repeat, B Transducin-like
K1-3	Permease Protein	K5-7	Sugar ABC transporter substrate-binding protein	K9-11	ATP Binding Protein
L1-3	Sugar binding protein of ABC transporter	L5-7	General Secretion Protein D	L9-11	Permease Protein
M1-3	Permease Protein	M5-7	Permease Protein	M9-11	Probable Transporter
N1-3	ATP Binding Protein	N5-7	Thiamin biosynthesis	N9-11	ATP Binding Protein
O1-3	Metal Transporting ATPase	O5-7	Permease Protein	O9-11	Ribose-binding protein of ribose ABC transporter
P1-3	ATP Binding Protein	P5-7	ATP Binding Protein	P9-11	ATP Binding Protein

C)



Position	Gene Product	Position	Gene Product	Position	Gene Product
Q1-3	ATP Binding Protein	Q5-7	Permease Protein	Q9-11	Permease Protein
R1-3	Permease Protein	R5-7	Permease Protein	R9-11	ATP Binding Protein
S1-3	Permease Protein	S5-7	ATP Binding Protein	S9-11	Endopeptidase Clp ATP-binding chain A
T1-3	L Arabinose binding protein	T5-7	ATP Binding Protein	T9-11	Putative Transporter

Figure 3.3 Screening *M. loti* mutants selected from Legume Base STM *M. loti* mutant library

A) *M. loti* mutants screened in triplicate from A1-3 to H9-11, B) *M. loti* mutants screened in triplicate from I1-3 to P9-11 and C) *M. loti* mutants screened in triplicate from Q1-3 to T9-11. Two further screens were carried out for each Legume Base STM *M. loti* mutant inoculated in triplicate. Wild type *M. loti* inoculation points can be found at the bottom-centre of the *L. rostrata*-inoculated agar plates (black box). Each mutant was coded (A1-3, B1-3 etc.) based on its position in the screen and this code was translated into their associated Legume Base Code, Gene Annotation and Gene Product in Appendix 8.2.

3.2.1.3 Generating and screening *M. loti* transposon mutants

To screen a larger number of *M. loti* mutants for those unable to enter symbiosis with *L. rostrata*, I first had to generate the mutants. Mutagenesis of *M. loti* was carried out via conjugating *M. loti* with *E. coli* strain β2163 carrying a transposon for mutagenesis as outlined in Materials and Methods 2.8.1. Approximately 10,000 *M. loti* mutants, identified as such by growth on TY + kanamycin plates, were picked and transferred to 96-well plates containing TY + kanamycin medium. These plates were incubated overnight and then the mutants were inoculated into the *L. rostrata*-inoculated agar plates. The majority of these mutants had no obvious phenotypic effect on promoting *L. rostrata* growth. There were a few hundred mutants that appeared to have an effect, but repeated screening failed to reproduce the result indicating that they had been false positives. Figure 3.4A shows exit examples of two false positives (outlined by a red and orange square). There was an apparent increase in *L. rostrata* growth in the presence of the mutant shown in the red square, and inhibition of growth of *L. rostrata* in the presence of the mutant shown in the orange square. Both *M. loti* mutants grew on the TY + kanamycin control plate (Figure 3.4D), although there appeared to be less growth of the *M. loti* mutant outlined by the orange square. The two *M. loti* mutants were re-screened as shown in Figure 3.4C, but the original phenotypes were not seen. Another strain showed an increased bacterial growth phenotype outlined by the blue square in Figure 3.4B. I screened the strain again, as highlighted by the blue rectangles in Figure 3.4C and the phenotype was seen again. The cells were viable in the presence of kanamycin, and colonies had a greater diameter than the average *M. loti* colony (blue rectangles, Figure 3.4D). I concluded this bacterial strain was worth studying in further detail.

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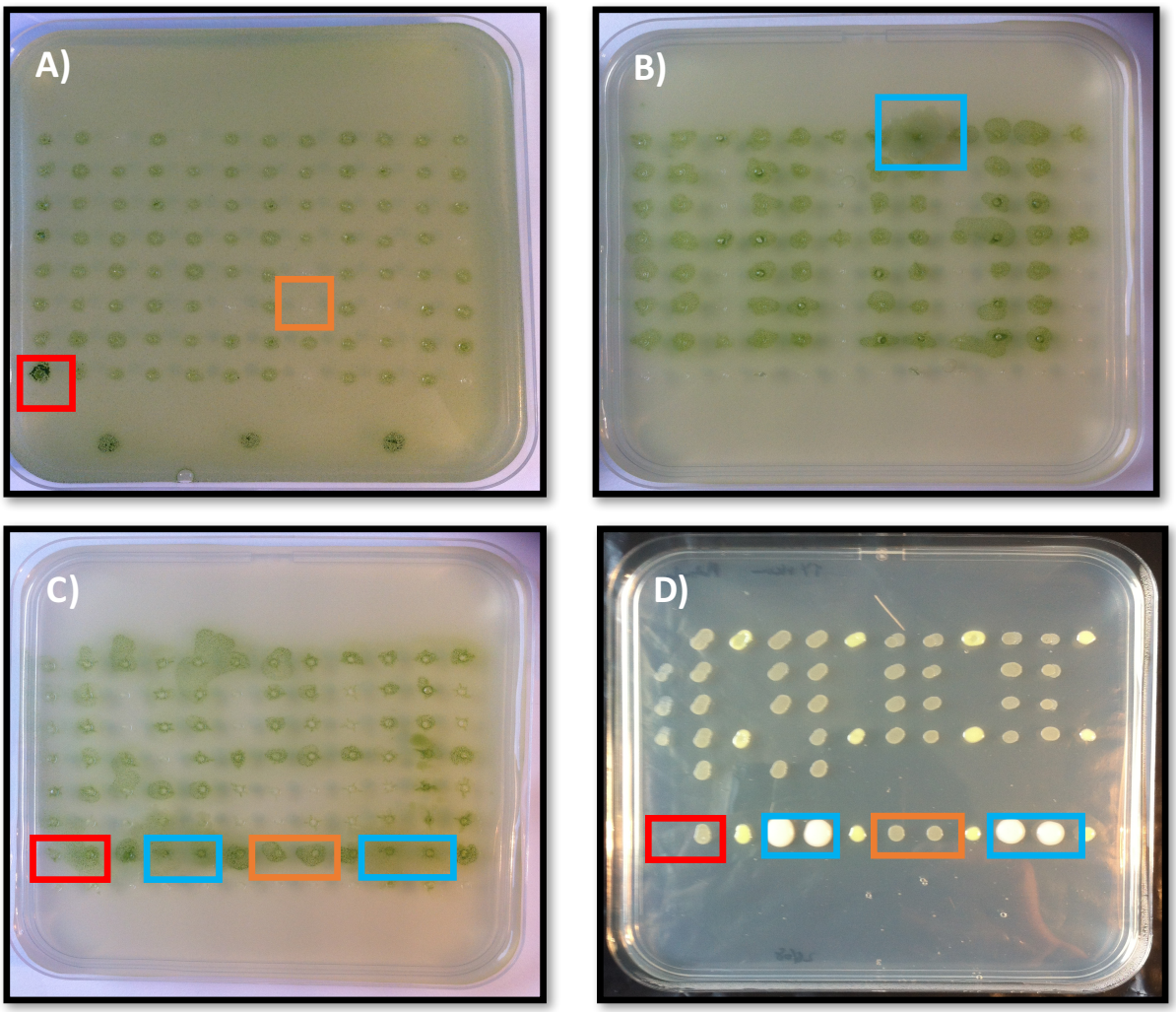

Figure 3.4 Confirmation of *M. loti* mutant false positives and the isolation of *R. erythropolis*

A) Plate containing *L. rostrata* and inoculated with individual *M. loti* strains highlighting two types of false positives. One shows improved growth of the alga (red square) and the other shows reduced growth of the alga (orange square). B) Plate containing *L. rostrata* highlighting the isolation of *R. erythropolis*. C) Plate containing *L. rostrata* showing a repeat plating of the two false positives (red and orange rectangles) and the strain subsequently identified as *R. erythropolis* (blue rectangles). D) TY+ kanamycin control plate with same bacteria inoculated as in panel C. As in C, the two false positives are highlighted by red and orange rectangles and *R. erythropolis* by blue rectangles.

3.2.1.4 16S rDNA sequencing of an isolated contaminant

Due to the obvious difference in colony size from a standard *M. loti* colony, the putative *M. loti* mutant (highlighted in blue, Figure 3.4) was isolated. 1000 bp segments of 16S rDNA were amplified by PCR (as described in Materials and Methods 2.4), and the sequence of the PCR products determined to identify the strain. I trimmed the sequences to include only high-quality reads. BLAST analysis showed 100% sequence identity to *Rhodococcus erythropolis* 16S rDNA (Figure 3.5). *R. erythropolis,* of the phylum Actinobacteria, is very distant in its taxonomy from *M. loti,* a member of the Alphaproteobacteria phylum (Figure 3.6) *R. erythropolis* had previously been identified as a putative B_{12} producer, given the presence of B_{12} synthesis genes according to the KEGG pathway database (https://goo.gl/G0wyjw) (Kanehisa et al., 2016). These results suggested that *R. erythropolis* might be able to supply vitamin B_{12} to *L. rostrata* and I subsequently investigated the growth of co-cultures.

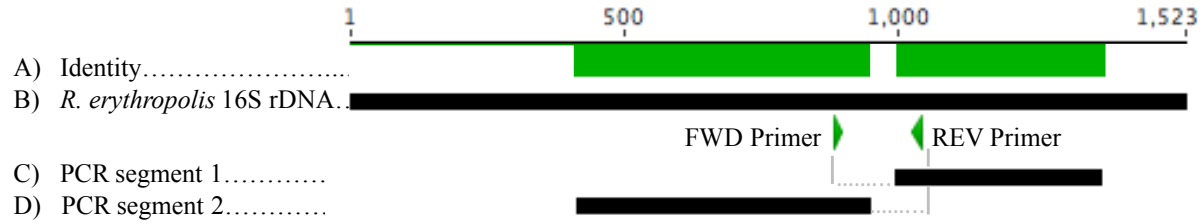

Figure 3.5 Sequence alignment of forward and reverse PCR products

A) Green bar above sequence represents identity. The bar is completely green where *R. erythropolis* 16S rDNA consensus sequence and forward and reverse PCR sequence overlap representing 100% similarity. B) Solid black lines represent the *R. erythropolis* 16S rDNA reference sequence. C) Solid black line represents PCR segment 1. D) Solid black line represents reverse PCR segment 2. Green arrow heads represent forward and reverse primer sites. Sequence alignment was carried out with NCBI Blast (NCBI Resource Coordinators 2016) and Geneious (Kearse et al., 2012).

Figure 3.6 Phylogenetic tree illustrating bacterial phyla.

M. loti is a member of the phylum Alphaproteobacteria (green square) whereas *R. erythropolis* is a member of the Actinobacteria phylum (red square) (NCBI Resource Coordinators, 2017). This figure was adapted from Hug et al. (2016) and is a maximum likelihood tree based on a concatenated dataset of 16 ribosomal proteins. The scale bar represents number of substitutions per site.

3.2.2 Investigating the *L. rostrata* + *R. erythropolis* symbiosis

3.2.2.1 Growth characteristics of the *L. rostrata* + *R. erythropolis* co-culture

Little is understood about bacterial interactions with *L. rostrata* outside the *L. rostrata* + *M. loti* symbiosis. The discovery of *R. erythropolis* as potentially being able to interact with *L. rostrata* provided an opportunity to identify any similarities or differences between *M. loti* and *R. erythropolis* in their interaction with *L. rostrata*. I carried out a growth experiment to determine any differences in growth rate between *M. loti* and *R. erythropolis* grown in TP^+ + Carbon (0.1% glycerol as per Kazamia, 2012) under standard heterotrophic conditions (Materials and Methods 2.3). I measured growth every 12 hours by optical density at 600 nm for 2.5 days and the starting OD_{600} for each of the cultures was 0.05. As expected, the *R. erythropolis* negative control did not grow without an added carbon source (Figure 3.7A). When I included 0.1% glycerol, *R. erythropolis* grew to OD_{600} 3.0 whereas *M. loti* grew to OD_{600} 2.3 after 60 hours. The maximum growth rates were 0.10 OD_{600} $hour^{-1}$ and 0.05 OD_{600} $hour^{-1}$ for *R. erythropolis* and *M. loti* grown in TP^+ + 0.1% glycerol respectively. I concluded that *R. erythropolis* was faster in its growth than *M. loti*.

I carried out a growth experiment to compare the growth of *L. rostrata* with *R. erythropolis* versus *M. loti*. The experiment was carried out under standard autotrophic conditions as outlined in Materials and Methods 2.3 and growth was measured by counting *L. rostrata* cells by Coulter counter every second day for 16 days. The growth rates and carrying capacity (maximum number of algal cells) appeared to be similar to that of the *M. loti* co-culture where the slow growth phase was reached after 14 days of growth (Figure 3.7B). I concluded there was no difference in growth rate of *L. rostrata* according to which bacterium was present.

To test if the *L. rostrata* + *R. erythropolis* symbiosis was stable, I sub-cultured the cells after 5 days of growth and repeated the sub culturing 6 times as outlined for *M. loti – L. rostrata* by Kazamia (2012). The experiment was carried out under standard autotrophic conditions as outlined in Materials and Methods 2.3.3 and growth was measured by counting *L. rostrata* cells by Coulter counter on the 5^{th} day of cultivation. Each of the sub-cultures started at an algal cell population of 100,000 cells per mL. The growth of *L. rostrata* + *R. erythropolis* was stable over 6 subcultures and average daily growth rates were estimated to be between 2.5-5.0 $x10^4$ algal cells per mL per day or an approximately 2-4-fold change in algal cell density over the 5 days of growth (Figure 3.7C). I concluded the growth of the co-culture was consistent over multiple subcultures and similar to that of the *L. rostrata* + *M. loti* co-culture as outlined by Kazamia (2012).

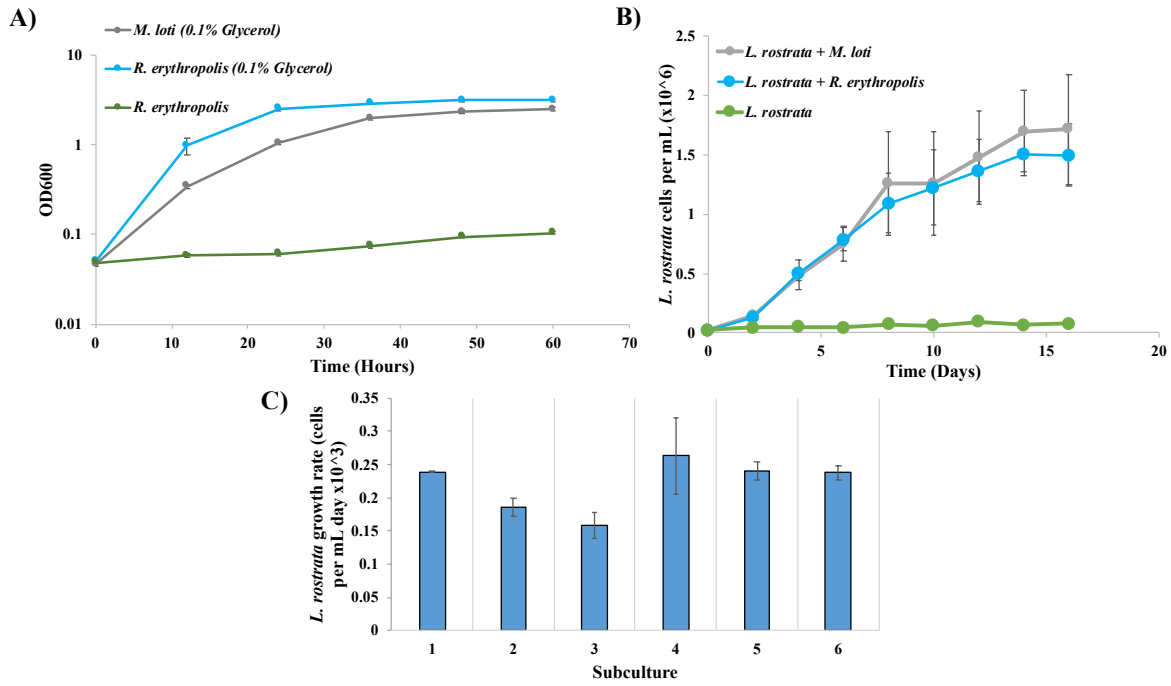

Figure 3.7 Growth characteristics of the *L. rostrata* + *R. erythropolis* co-culture

A) Growth of axenic *M. loti* and *R. erythropolis* in TP^+ + 0.1% Glycerol or TP^+ only (negative control) in logarithmic scale. B) Growth of *L. rostrata* with *M. loti* or *R. erythropolis* and axenic *L. rostrata* in TP^+. C) Growth rates of each *L. rostrata* + *R. erythropolis* sub-culture in TP^+. All subcultures started at an algal cell concentration of 1.0 x10^5 cells per mL. Error bars display standard deviation from three replicates.

To calculate the ratio of *R. erythropolis* cells to *L. rostrata* cells, I carried out two experiments on the *L. rostrata* + *R. erythropolis* mutualism. The experiments were carried out in duplicate under standard autotrophic conditions as outlined in Materials and Methods (2.3.3) and I measured the growth of *L. rostrata* by counting the cells by Coulter counter every second day for 14 days. The populations of *R. erythropolis* were determined by colony forming units (CFU's, Materials and Methods 2.3.1.3). *L. rostrata* (circles connected by solid blue and red lines) reached approximately 1.1 x10^6 cells per mL in one experiment and approximately 7.0 x10^5 cells per mL in the other (Figure 3.8A). *R. erythropolis* (triangles connected by broken blue and red lines) retained a constant cell count for the first 8 days of growth after which cell density increased to a final number of approximately 2.0 x10^9 CFUs per mL on day 14 (Figure 3.8A). Dividing the number of bacterial CFUs per mL by the number of algal cells per mL gives the ratio of bacteria to algae in a culture. The ratios were calculated and are shown in

Figure 3.8B. I concluded that the ratio of bacteria to algal cells in the *L. rostrata* + *R. erythropolis* co-culture between 1:1 and 1:7 was lower than that reported for *L. rostrata* + *M. loti,* between 1:10 to 1:30 (alga to bacteria) (Kazamia, 2012).

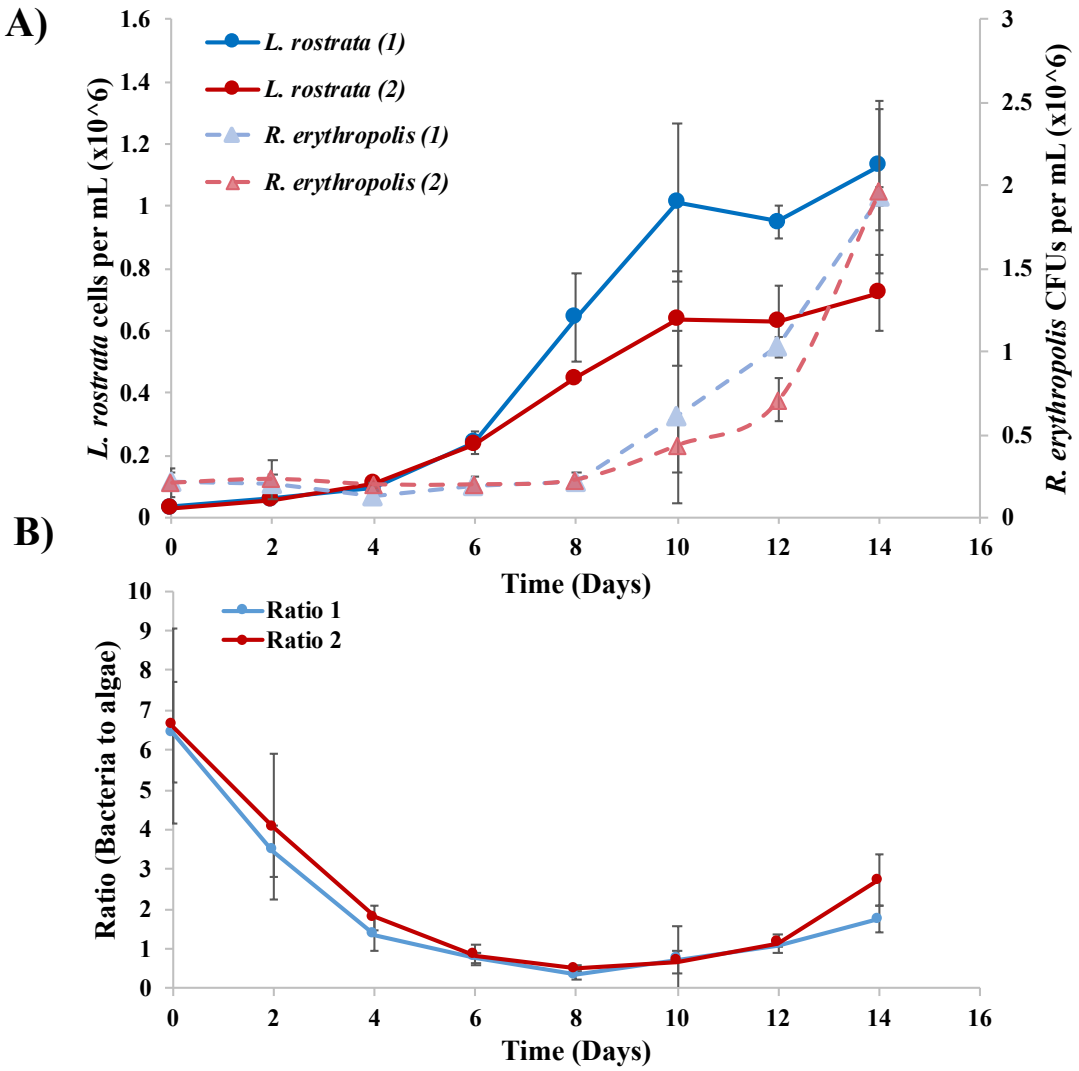

Figure 3.8 Population counts and ratio of the *L. rostrata* + *R. erythropolis* co-culture

A) Growth of *L. rostrata* + *R. erythropolis* co-culture with *L. rostrata* cell counts in TP^{+} and *R. erythropolis* CFU counts. B) Ratio of *L. rostrata* to *R. erythropolis*. Data replicated with two separate experiments. Experiment 1 is represented by blue lines and experiment 2 represented by red lines. Error bars display standard deviation from three replicates.

3.2.2.2 Measuring B_{12} concentration of *R. erythropolis*

To measure the B_{12} concentration of *R. erythropolis* cultured axenically versus in co-culture with *L. rostrata*, I carried out B_{12} assays using the B_{12} auxotroph *S. typhimurium metE cysG* (AR3612) (Materials and Methods 2.6). Briefly, the technique involves the growth of *S. typhimurium* AR3612 on boiled samples of *R. erythropolis* from axenic culture or in co-culture with *L. rostrata*. The growth of *S. typhimurium* AR3612 is dependent on the presence of available B_{12} in the sample. The B_{12} concentration in the sample is estimated by comparing the growth of *S. typhimurium* AR3612 on the sample to that of a standard curve showing growth at known concentrations of B_{12}. The population of *R. erythropolis* was recorded by measuring CFUs for axenic growth as well as in co-culture with *L. rostrata.* Measurements for B_{12} and CFUs were taken on day 0 (time point 0), day 1 (time point 1) and day 2 (time point 2) for axenic growth and day 0 (time point 0), day 8 (time point 1) and day 14 (time point 2) for growth in co-culture.

Figure 3.9A outlines the total B_{12} concentration in axenic cultures of *R. erythropolis* in TP^+ with added carbon (0.1% glycerol). Measured B_{12} in total samples (including bacterial cells and supernatant) and cell pellet samples of *R. erythropolis* increased to approximately 2400 pg/mL and 2600 pg/mL respectively after 2 days of growth. B_{12} measured in the supernatant of axenic cultures reached approximately 700 pg/mL. This is noticeably greater than the difference between cell pellet samples + total samples. It is possible the inconsistency could be explained by taking into account the standard deviation for each of the samples. For example, the total sample had a standard deviation of ± 280 pg/mL, ± 110 pg/mL for the cell pellet sample and ± 4400 pg/mL for the supernatant sample. The population of *R. erythropolis* was also recorded (triangles connected by broken lines) and by day 2 had reached 4.3 $x10^8$ CFUs per mL.

I also carried out B_{12} measurements for *R. erythropolis* in culture with *L. rostrata* in TP^+, to identify any difference in concentration compared to the axenic *R. erythropolis* culture. B_{12} concentration was measured with samples of supernatant, cell pellet and total (supernatant + cells) sample (Figure 3.9B). The concentrations measured in all three samples increased over the course of the experiment. Interestingly, B_{12} measured in supernatant sample reached approximately 18 pg/mL on day 8, which is markedly greater than the pellet concentration of 5 pg/mL. I also recorded the population of *R. erythropolis* (triangles connected by broken lines)

and by day 14 had reached 2.0 x10^6 CFUs per mL. I concluded that B_{12} concentration was higher in axenic conditions than in co-culture. However, it should be noted that *R. erythropolis* populations were higher in axenic conditions (to be expected with added carbon) than in co-culture, which could lead to the differences in B_{12} concentration.

I estimated the B_{12} concentration per cell by dividing the measured concentration of B_{12} by CFU count per ml. Figure 3.9C shows the calculated B_{12} concentration per cell in the total sample (cell + supernatant) for both axenic culture and co-culture over the three measured time points, day 0 (time point 0), day 1 for axenic or day 8 for the co-culture (time point 1) and day 2 for axenic or day 14 for the co-culture (time point 2). The only significant difference in B_{12} production between axenic and co-culture was during time point 1 (P= 0.025) where a concentration of 4.4 ag/cell of B_{12} was measured for *R. erythropolis* in axenic culture whereas in co-culture with *L. rostrata, R. erythropolis* generated a concentration of 49 ag/cell of B_{12}. Figure 3.9D outlines the calculated B_{12} concentration per cell in supernatant and cell pellet samples for both axenic and co-culture over the three measured time points, day 0 (time point 0), day 1 for axenic or day 8 for the co-culture (time point 1) and day 2 for axenic or day 14 for the co-culture (time point 2).The only significant difference in B_{12} concentration per cell between axenic and co-culture was in the supernatant samples during time point 1 (P= 0.0078) where a concentration of 1.0 ag/cell of B_{12} was generated by *R. erythropolis* in axenic culture whereas in co-culture with *L. rostrata, R. erythropolis* produced a concentration of 79 ag/cell of B_{12}. I concluded there is an increase in total B_{12} concentration per cell in co-culture compared to axenic *R. erythropolis* culture and that most of this extra B_{12} is found in the supernatant.

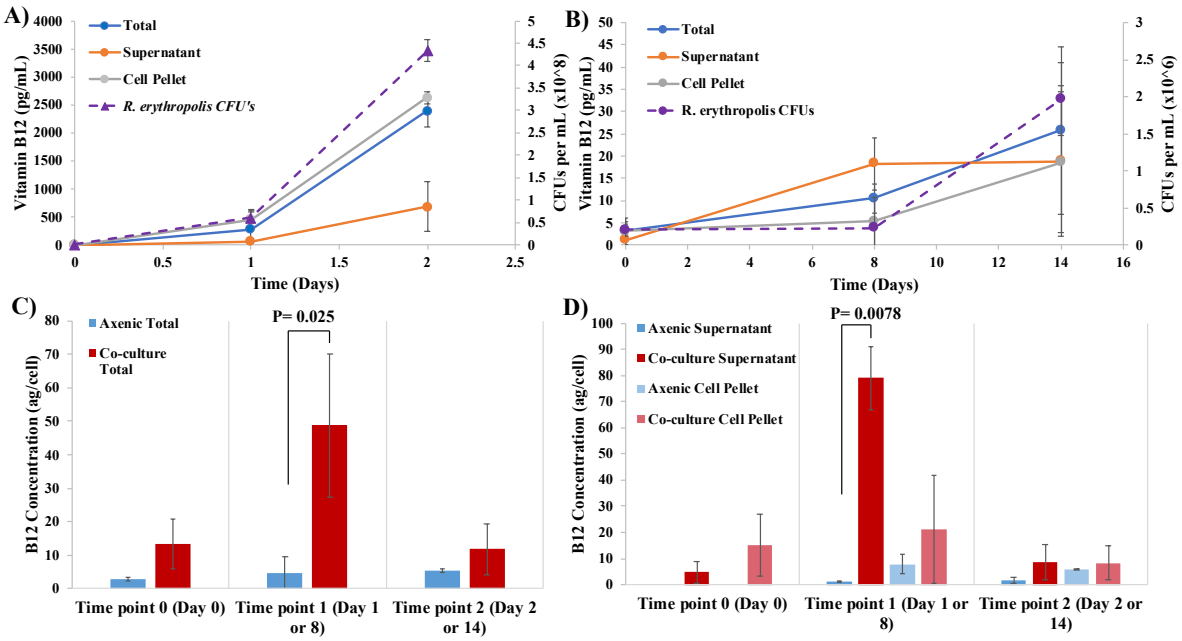

Figure 3.9 Vitamin B_{12} concentration of *R. erythropolis*

A) Vitamin B_{12} production of axenic *R. erythropolis* grown in TP^+ + 0.1% glycerol B) Vitamin B_{12} production of *R. erythropolis* in co-culture with *L. rostrata* in TP^+. C) Total (supernatant and cell pellet) B_{12} measured per cell of *R. erythropolis* grown axenically in TP^+ + 0.1% glycerol and in co-culture with *L. rostrata* in TP^+ over time point 0 (day 0 for both axenic and co-culture growth), time point 1 (day 1 for axenic growth or 8 for co-culture growth) and time point 2 (day 2 for axenic growth or 14 for co-culture growth) D) B_{12} measured in supernatant and cell pellet per cell of *R. erythropolis* grown axenically in TP^+ + 0.1% glycerol and in co-culture with *L. rostrata* in TP^+ over the time point 0 (day 0 for both axenic and co-culture growth), time point 1 (day 1 for axenic growth or 8 for co-culture growth) and time point 2 (day 2 for axenic growth or 14 for co-culture growth). Error bars display standard deviation from three replicates. Significance was determined by ANOVA and is illustrated by the connecting lines above the bars.

3.2.3 Measuring the effect of B_{12} producing bacteria on growth of an industrially relevant alga

I have previously shown that *R. erythropolis* co-cultured with *L. rostrata* was similar in growth rate and carrying capacity to the *L. rostrata* + *M. loti* co-culture (Figure 3.7B). However, a more industrially relevant algal strain to study is *Chlamydomas reinhardtii* due to its faster growth rate (compared to *L. rostrata*), well-characterised physiology, sequenced genome and well developed genetic tools (Kazamia, 2012; Ridley, 2016; Scott et al., 2010; Scaife et al., 2015). A B_{12}-dependent strain of *C. reinhardtii* (*C. reinhardtii metE*$^{-}$) was used instead of *L. rostrata*. I carried out an experiment to test if there were any differences in *C. reinhardtii metE*$^{-}$ growth if cultured with *R. erythropolis* rather than *M. loti*. The experiment was carried out under standard autotrophic conditions as outlined in Materials and Methods 2.3.3 and growth of *C. reinhardtii metE*$^{-}$ was measured by counting the cells by Coulter counter every second day for 14 days. The experimental data were plotted in a growth curve displayed in Figure 3.10A and the growth rates (Materials and Methods 2.3.5), calculated over 14 days of growth, are displayed in Figure 3.10B. The starting concentrations of the algal cells for each culture were approximately 50,000 algal cells per mL. After 14 days *C. reinhardtii metE*$^{-}$ - B_{12} (negative control) showed little growth at only 1.2 x10^5 algal cells per mL and *C. reinhardtii metE*$^{-}$ + B_{12} (positive control) had reached 5.7 x10^6 algal cell counts per mL (as expected. *C. reinhardtii metE*$^{-}$ + *M. loti* reached 7.8 x10^5 algal cell counts per mL whereas *C. reinhardtii metE*$^{-}$ + *R. erythropolis* reached 1.4 x10^6 algal cell counts per mL. However, the observed difference between the co-cultures (excluding the controls) was calculated as non-significant via ANOVA. I concluded that growth of *C. reinhardtii metE*$^{-}$ + *M. loti* and *C. reinhardtii metE*$^{-}$ + *R. erythropolis* co-cultures were similar over the 14 days tested.

The experiment was extended to include the co-culturing of multiple B_{12} producing bacteria to test whether they could improve the growth rate of *C. reinhardtii metE*$^{-}$ through the potential supply of additional B_{12}. Ridley (2016) found that co-culturing a *L. rostrata* + *M. loti* consortium with *Delftia acidovorans* or *Pseudomonas putida* gave a significant increase in growth over *L. rostrata* cultures with *M. loti* only. However, when this study was replicated the significance was lost (Ridley, 2016). Importantly, the experiments carried out by Ridley (2016) used co-cultures with a maximum of two B_{12} producing bacteria. Therefore, an experiment was carried out to identify whether additional B_{12} producing bacteria (two to four species) could improve the growth rate of the industrially relevant *C. reinhardtii metE*$^{-}$. The experiment was carried out under standard autotrophic conditions as outlined in Materials and

Methods 2.3.2 and growth of *C. reinhardtii metE*$^{-}$ was measured by counting the cells by Coulter counter every second day for 14 days. The experimental data were plotted in a growth curve displayed in Figure 3.10A and the growth rates of the cultures, averaged over 14 days, are displayed in Figure 3.10B. The starting concentrations of the algal cells for each culture were approximately 50,000 algal cells per mL. The results showed that *C. reinhardtii metE*$^{-}$ co-cultured with *M. loti* and *R. erythropolis* had a final algal cell count per mL of 2.6 x10^6. *C. reinhardtii metE*$^{-}$ was also grown with three B_{12} producing species simultaneously (*M. loti, D. acidovorans and P. putida*) over 14 days. The final density of *C. reinhardtii metE*$^{-}$ cells were 1.6 x10^6 algal cells per mL. When *C. reinhardtii metE*$^{-}$ was co-cultured with four B_{12} producing species (*M. loti*, *R. erythropolis*, *P. putida* and *D. acidovorans*), the alga had a final algal cell count of 1.2 x10^6 per mL. Overall, I observed no significant difference between the results of culturing multiple B_{12} producing species with *C. reinhardtii metE*$^{-}$ and the results of the *C. reinhardtii metE*$^{-}$ + *M. loti* co-culture.

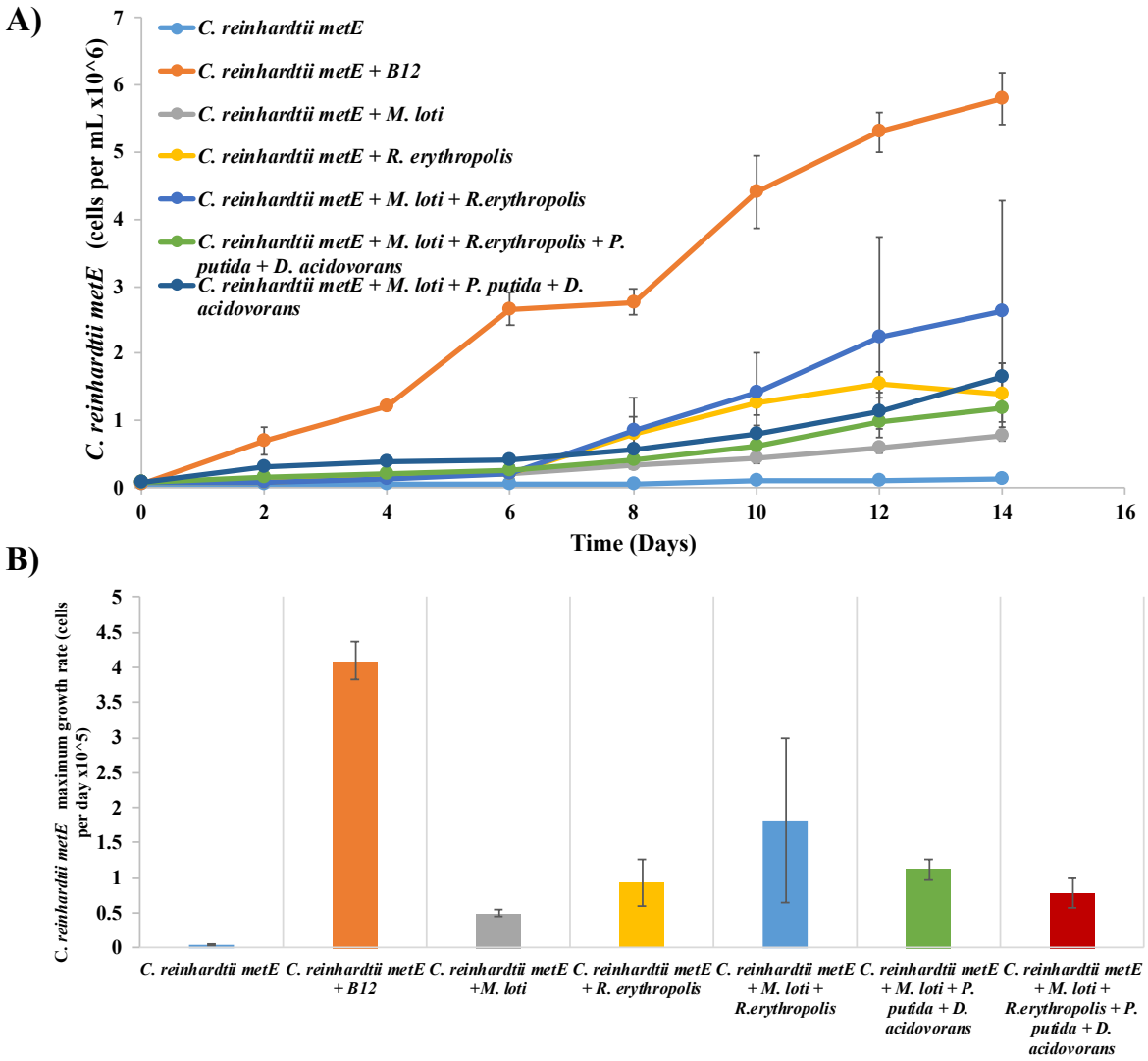

Figure 3.10 Investigating the growth effect of additional B_{12} producing bacteria on *C. reinhardtii metE*$^{-}$

A) Growth curve of *C. reinhardtii metE*$^{-}$ with additional B_{12} producing bacteria grown in TP^{+}. B) Calculated growth rates of the experimental data plotted in A. Error bars display standard deviation from three replicates. Significance was determined by ANOVA and is illustrated by the connecting lines above the bars.

3.3 Discussion

In this chapter, I attempted to identify *M. loti* transposon mutants unable to enter symbiosis with *L. rostrata*. In spite of screening 10,000 *M. loti* mutants and a number of commercially available ones, no mutants genuinely impaired in their ability to enter symbiosis were obtained. However, during the course of the screen a contaminating bacterium, identified as *R. erythropolis* through 16S rDNA sequencing, was isolated that appeared to feed off the carbon exudate from the alga (due to the large bacterial colony formation at the inoculation point) as well as provide $B_{12,}$ as evident from the algal growth around the inoculation point (Figure 3.4B, blue square; Figure 3.5). *R. erythropolis* is evolutionarily very distant from *M. loti* (Figure 3.6) The KEGG database predicted this species was a B_{12} producer as it contained a full set of the genes in the B_{12} synthesis pathway (outlined here: https://goo.gl/G0wyjw) (Kanehisa et al., 2016). As this was the first non-rhizobial bacterium to be identified that could potentially form a symbiosis with *L. rostrata*, further research was carried out to prove if the strain was capable of sustained symbiosis and then to characterize the interaction. I carried out growth experiments on axenic *R. erythropolis* (Figure 3.7A) as well as in co-culture with *L. rostrata* (Figure 3.7B). I carried out a long-term experiment involving six sub-cultures to test if the co-culture was stable (Figure 3.7C). I also measured the ratio of *R. erythropolis* to *L. rostrata* (Figure 3.8). Furthermore, I carried out quantitative B_{12} analysis on axenic *R. erythropolis* as well as in co-culture with *L. rostrata* to measure B_{12} production in the species (Figure 3.9). I cultured *R. erythropolis* with the more industrially relevant algal strain *C. reinhardtii metE*$^{-}$ to identify any growth difference from that with *M. loti* (Figure 3.10). Finally, to extend the analysis carried out by Ridley, (2016), I conducted experiments on the more industrially-relevant *C. reinhardtii metE*$^{-}$ to determine if additional B_{12} producing bacteria (up to 4 species) could increase the growth rate of the alga (Figure 3.10).

3.3.1 Understanding the shortfall of isolating symbiosis mutants

The fact that approximately 10,000 *M. loti* transposon mutants were generated and screened with no mutant isolated that was unable to enter symbiosis was an interesting result. There are a total of approximately 7.6 million base pairs and 7,300 protein coding genes in *M. loti*, which includes the chromosome and two plasmids (Kaneko et al., 2000). Shimoda et al. (2008) generated 29,330 transposon mutants and of these only 3680 (12.5%) were identified to contain a transposon in non-redundant protein coding genes. The mutagenesis techniques in

this study (derived from Monson et al., 2015) were similar in utilising a Tn5 transposon to Shimoda et al. (2008). Therefore, translating this efficiency to the 10,000 lines screened in this study one would expect at least 1250 mutants with transposons inserted into non-redundant protein coding genes.

Assuming approximately 1250 mutants with transposon insertions into protein coding genes were generated in this study, it must be asked why no mutant was isolated that was unable to enter symbiosis with *L. rostrata*. The transposon generating technique seemed to produce *M. loti* strains reliably that were kanamycin resistant and tetracycline sensitive, which suggests the transposon is indeed present but the plasmid which carried the transposon into the cell is not (the plasmid contains a tetracycline resistance cassette). Therefore, it could be the case that: 1) there are few genes required to enter symbiosis with *L. rostrata*; 2) these genes could be redundant, whereby the function of one gene could be replaced by another, such as the case for ABC transporter systems (Jensen et al., 2002; O'Connor et al., 2012); or 3) a gene required to enter symbiosis is also lethal to *M. loti* when absent.

3.3.2 Comparison of the growth dynamics of *L. rostrata* + *R. erythropolis* consortium with the *L. rostrata* + *M. loti* consortium

When *R. erythropolis* was initially isolated in this study, I noted that the species grew faster than *M. loti*. When the growth of the two species was compared through optical density measurements, I saw a difference in growth rate averaged over the first 48 hours of growth with *R. erythropolis* being greater than *M. loti* (Figure 3.7A). Additionally, after 60 hours there was a higher OD_{600} for *R. erythropolis* (OD_{600} 3.0) than for *M. loti* (OD_{600} 2.5) which could be due to a greater number of cells but also due to a difference in cell size causing greater light scattering.

I carried out an experiment to confirm that *R. erythropolis* could enter symbiosis with *L. rostrata* in the short term (14 days) and to compare the growth rate of *L. rostrata* in co-culture with *R. erythropolis* versus the growth rate in co-culture with *M. loti*. The experiment showed there was no significant growth difference of *L. rostrata* between the two co-cultures (Figure 3.7B). This is similar to the results observed by Kazamia (2012) where *L. rostrata* was co-cultured with B_{12} producers *Rm. leguminosarum* and *S. meliloti* as well as *M. loti*. Co-

culturing *L. rostrata* with each bacterium led to a similar level of *L. rostrata* growth over approximately the first 14 days.

Kazamia (2012) carried out a long-term growth experiment in which *Rm. leguminosarum*, *S. meliloti* and *M. loti* were each co-cultured with *L. rostrata* over multiple generations. The three co-cultures were sub-cultured 6 times and by the end of the 6 subcultures, only the *L. rostrata* + *M. loti* co-culture was still viable. The author hypothesised this stability could be due to a specific interaction between *L. rostrata* and *M. loti,* either because *M. loti* is better adapted to taking up the specific carbon source released from the alga (which is still unknown) or from the use of chemical signaling in *M. loti* such as quorum sensing. To test whether *R. erythropolis* was capable of long-term symbiosis, I subcultured the co-culture 6 times as carried out by Kazamia (2012). The continued viability of the co-culture showed that, unlike *Rm. leguminosarum* or *S. meliloti*, *R. erythropolis* was stable over the course of the experiment (Figure 3.7C). This suggests the *L. rostrata* + *M. loti* interaction is not as specific as originally thought and that in fact non-rhizobial species of bacteria are capable of long-term symbiosis with *L. rostrata*. The results illustrate the wide range of potential bacteria that could symbiose with *L. rostrata,* as *R. erythropolis* and *M. loti* are from very distant phyla (Figure 3.6).

Kazamia (2012) studied the ratio of algae to bacteria for the *L. rostrata* + *M. loti* co-culture and found it was approximately between 1:10 and 1:30. Ridley (2016) also repeated this experiment and found the ratio stabilized between 1:5 and 1:32 (*L. rostrata* to *M. loti*). In this study, I measured the ratio of the *L. rostrata* + *R. erythropolis* (Figure 3.8). It should be noted that I grew the co-culture in batch in this study, which contrasts with the studies of Kazamia (2012) and Ridley (2016) where semi-continuous culturing was carried out, allowing for an extended phase of rapid growth. Nonetheless, it appeared that the carbon exudates from *L. rostrata* supported a smaller ratio of *R. erythropolis* cells to algal cells than for *M. loti*. Even though the algae: bacteria ratio in a co-culture differs between the B_{12} producing bacteria *M. loti* and *R. erythropolis*, overall algal growth was similar between the two co-cultures when counting *L. rostrata* cells (Figure 3.7B). This suggests B_{12} availability for *L. rostrata* between the two co-cultures (*L. rostrata* in co-culture with *M. loti* versus *R. erythropolis*) must be similar as it has been observed that B_{12} is the limiting nutrient for growth of *L. rostrata* when cultured with B_{12} producing bacteria in laboratory scale (Figure 3.10A; Kazamia, 2012; Ridley, 2016). Therefore, because *L. rostrata* can support fewer individual cells of *R. erythropolis*

(algae: bacteria ratio between 1:7 to 1:0.5) than *M. loti* (algae: bacteria ratio between 1:5 to 1:32), each individual cell of *R. erythropolis* must provide more B_{12} per cell than *M. loti* to explain the similarity of *L. rostrata* growth between the co-cultures (Figure 3.8B).

3.3.3 Measuring the B_{12} concentration from *R. erythropolis*

Understanding the concentration of B_{12} in the *L. rostrata* + *R. erythropolis* consortium is important for understanding the fundamentals of the interaction. It is important to note that B_{12} concentration does not necessarily inform us on the level of production of B_{12} for the B_{12} producer as it does not consider anabolism and catabolism of B_{12} separately. For example, a high concentration of B_{12} could indicate high production or low breakdown of B_{12}. I conducted experiments to measure the B_{12} concentration of *R. erythropolis* both axenically and in co-culture with *L. rostrata*. Two main observations came out of this investigation: 1) the total B_{12} concentration observed in axenic conditions was approximately 95-fold greater than when *R. erythropolis* was co-cultured with *L. rostrata* (Figure 3.9A and B) and; 2) a higher overall concentration of B_{12} per cell was observed in the co-culture than in axenic conditions during the rapid growth phase and much of this extra B_{12} per cell was found in the supernatant (Figure 3.9C and D).

The higher concentration in axenic cultures can be partly explained by the difference in cell numbers (CFUs) at the time points at which B_{12} concentrations were measured; 4.33 $x10^8$ CFUs per mL of *R. erythropolis* in axenic conditions versus 2.0 $x10^6$ CFUs per mL *R. erythropolis* in co-culture with *L. rostrata*. In other words, higher bacterial cell densities are associated with higher B_{12} concentration (Figure 3.9A and B). The observation of an increase in total B_{12} concentration per cell in the co-culture compared to the axenic culture during rapid growth phase, with the majority of the B_{12} found in the supernatant, could reflect population growth (Figure 3.9C and D). As mentioned previously, there was a significant difference in cell densities between the axenic and co-cultures (approximately a 200-300-fold difference) (Figure 3.9A and B). This difference could be due to a limitation in carbon in the co-culture, as no carbon source was added (Figure 3.9B). This carbon limitation could induce stress and lead to more cell death and consequent release of B_{12} into the supernatant. However, there was also a recorded increase in overall B_{12} concentration per cell. Given this, it is also possible that the carbon stress led to a metabolic response involving the upregulation of B_{12} production and a consequential release of B_{12} either passively or actively (Figure 3.11). The B_{12} release could

be passive in that B_{12} release is a consequence of increased B_{12} production i.e. the B_{12} leaks from the cell. Alternatively, the B_{12} release could be active in that the B_{12} is 'intentionally' released into the supernatant to encourage the growth of carbon providing organisms such as algae. To test the hypothesis that carbon stress leads to an increase in B_{12} production and release, I carried out an experiment that measured the B_{12} in total (cell pellet and supernatant), cell pellet and supernatant samples of *R. erythropolis* in a series of decreasing carbon concentrations (0.1%, 0.01%, 0.001% and 0.0001% glycerol) in semi-continuous culture. However, incomplete results were obtained and therefore no conclusions could be drawn as the concentration levels of B_{12} produced in the lower carbon-supplemented cultures (0.01% - 0.0001%) were too small to be measured accurately in the B_{12} assays (data not shown).

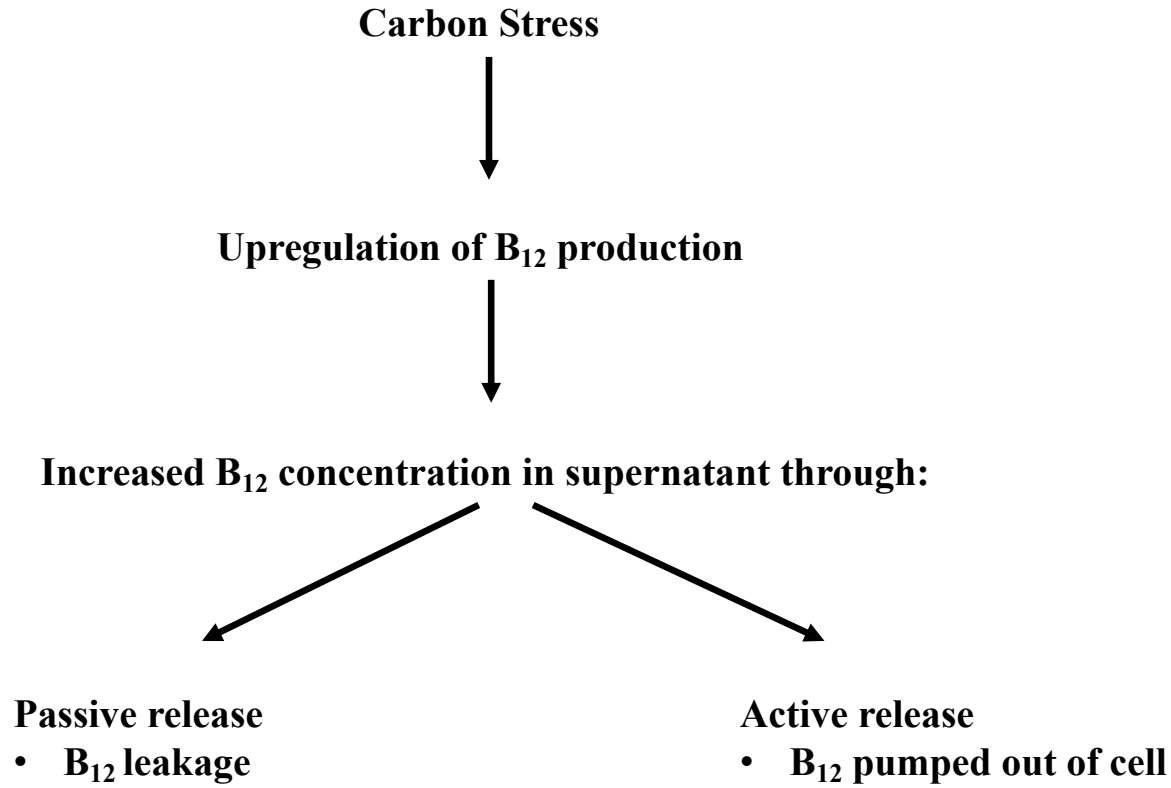

Figure 3.11 Flow diagram of potential *M. loti* B_{12} response to carbon stress.

As previously mentioned, Kazamia (2012) found an increase in total concentration of B_{12} per cell in co-culture compared to that in axenic conditions, although it was a 300-fold increase in contrast with the 12-fold increase in this study (Figure 3.9C). Nonetheless, there appears to be a common trend of an increase in B_{12} production in co-culture with algae.

One should recognize the limitations of the B_{12} assay used on this study. It is limited to measuring the B_{12} concentration at a particular time point, so it does not necessarily tell one about the rate of production. It is also limited to measuring B_{12} levels above the concentration required to promote growth of *S. typhimurium* AR3612 (greater than approximately 2 pg/mL) which is problematic when attempting to determine the low B_{12} concentrations produced during low bacterial growth. To provide further evidence as to whether B_{12} production per cell is upregulated under low growth conditions one could attempt to concentrate B_{12} samples prior to measurement by the *S. typhimurium* AR3612 assay. Alternatively, High-Performance Liquid Chromatography (HPLC) would provide more accurate means of quantifying B_{12} (Van Wyk and Britz, 2010; Chamlagain et al., 2015).

3.3.4 No improvement of growth rate with addition of B_{12} producing bacteria to B_{12}-dependent algae

Helliwell et al. (2015) and Ridley (2016) co-cultured *M. loti* with the more industrially relevant *C. reinhardtii metE*$^{-}$ and found that symbiosis could be established. This study focused on extending *C. reinhardtii metE*$^{-}$ bacterial symbiotic partners to *R. erythropolis* and found the two could enter symbiosis, at least in the short term (Figure 3.10). Furthermore, I observed no significant difference in algal growth between the co-culturing of *M. loti* or *R. erythropolis* with *C. reinhardtii metE*$^{-}$.

Ridley (2016) and Kazamia (2012) found that growth of B_{12}-dependent algae with B_{12}-producing bacteria did not result in as high a growth rate or carrying capacity as when the algae were grown with B_{12} supplemented directly. Ridley (2016) assessed whether adding additional B_{12} producing strains to the *L. rostrata* + *M. loti* consortium would lead to improved algal growth. Ridley's study looked at a maximum of two B_{12} producers but, after repeated attempts, found no increase in algal growth. My study focused on the more industrially relevant *C. reinhardtii metE*$^{-}$ strain and extended the number of bacteria to three and four species. The results of my study also found no significant benefit of adding more than one species of B_{12} producer to the consortium (Figure 3.10). On the other hand, there was no significant disadvantage to algal growth when more species were added. This opens the potential of carrying out further research on the probiotic principle of crop protection whereby commensal bacteria are cultured with algae to prevent the invasion of detrimental bacteria, which could improve productivity (Kazamia et al., 2014; Section 1.7.2).

3.3.5 Conclusions

The work described in this chapter includes an experiment in which 10,000 *M. loti* transposon mutants were generated and screened for a perturbation in symbiosis. The screen led to the discovery of a novel algae – bacteria consortium involving a non-rhizobial bacterium that appeared to be stable over multiple sub-cultures. I found the *L. rostrata* + *R. erythropolis* ratio to have fewer bacteria to algae than the previously characterized, *L. rostrata* + *M. loti* consortium. Furthermore, I saw a significant increase in B_{12} concentration per cell when *R. erythropolis* was co-cultured with *L. rostrata* in contrast with axenic cultivation, and most of this extra B_{12} was found in the supernatant. I found *R. erythropolis* was capable of entering symbiosis with the more industrially relevant *C. reinhardtii metE*$^{-}$ and, finally, the addition of multiple (up to four) B_{12} producing bacteria did not improve the growth rate or carrying capacity of the more industrially relevant *C. reinhardtii metE*$^{-}$ when cultured with *M. loti*.

4 ENGINEERING ALGAE-BACTERIA COMMUNITIES THROUGH NITROGEN PROVISION

4.1 Introduction

4.1.1 *Anabaena* sp. PCC 7120

Anabaena sp. PCC 7120 (*Anabaena* hereafter) is an isolate of *Anabaena azollae* (Kaneko et al., 2001), and its genome has been sequenced. *Anabaena* is a filamentous cyanobacterium that can fix its own nitrogen and has been used as a model organism to understand the complex metabolism of nitrogen fixation and cell differentiation (Buikema and Haselkorn, 1991; Liu and Chen, 2009). *Anabaena* cells come in two forms, vegetative cells and heterocysts (Figure 4.1). Vegetative cells are the photosynthesizing, carbon fixing cells, and the heterocysts are the non-photosynthetic, low-oxygen requiring, nitrogen fixing cells. In the presence of combined nitrogen (such as ammonium or nitrate), *Anabaena* grows as long filaments of vegetative cells. Under diazotrophic conditions (media lacking combined nitrogen), approximately 10% of the vegetative cells differentiate irreversibly into heterocysts forming a multicellular pattern of a single heterocyst followed by approximately 9 vegetative cells along filaments (Kumar and Mella-Herrera, 2010). The vegetative cells provide the heterocysts with fixed carbon and in return the heterocysts provide fixed nitrogen to the vegetative cells (Figure 4.1). For example, the vegetative cells provide glutamate, sucrose and alanine to the heterocysts, whereas the heterocysts provide glutamine and β-aspartyl-arginine (Muro-Pastor et al., 2005). Heterocysts and vegetative cells are therefore mutually interdependent and could be argued as being a simple “ecosystem” (Malatinszky et al., 2017).

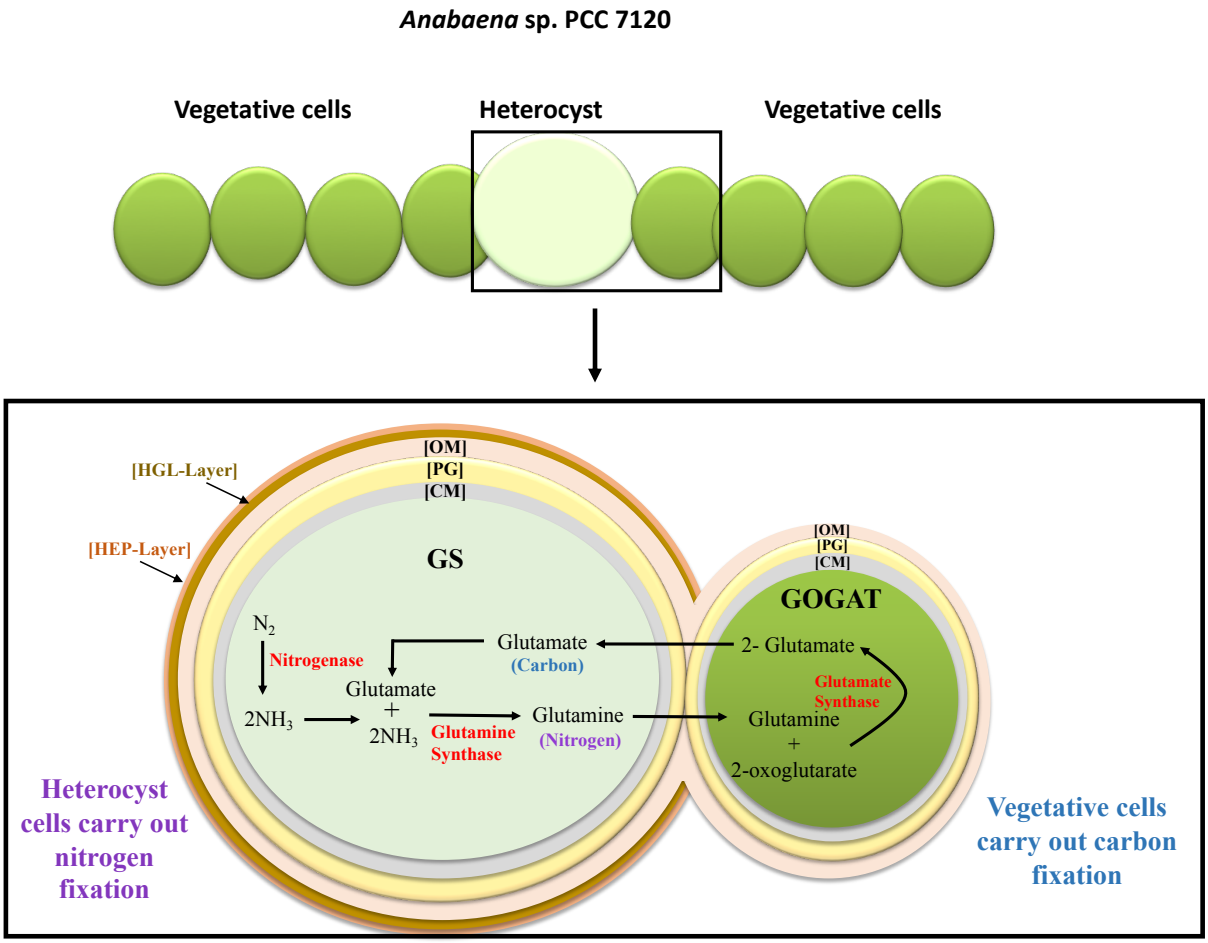

Figure 4.1 Illustration of *Anabaena* sp. PCC 7120 under diazotrophic conditions

Anabaena sp. PCC 7120 differentiates into two cell types under diazotrophic conditions – vegetative cells and heterocysts. Vegetative cells carry out photosynthesis and carbon fixation whereas heterocysts carry out nitrogen fixation. The figure outlines the GS-GOGAT cycle where GS is located in the heterocysts and GOGAT is located in the vegetative cells. GS (glutamine synthase) converts glutamate and ammonia to glutamine, whereas GOGAT (glutamine-oxoglutarate aminotransferase, or glutamate synthase) converts glutamine to glutamate. CM is the cytoplasmic membrane, PG the peptidoglycan layer, OM the outer membrane, HGL-Layer is the heterocyst specific glycolipid layer and the HEP-Layer is the heterocyst envelope polysaccharide layer.

Anabaena is a Gram-negative bacterium containing a cytoplasmic membrane (CM), a peptidoglycan layer and an outer membrane (OM). The CM and peptidoglycan layer surround each cell (vegetative and heterocysts) whereas the OM is continuous along the filament, defining a continuous periplasm. Furthermore, the heterocysts contain two additional envelope layers, the heterocyst specific glycolipid layer (HGL-layer) and the heterocyst envelope polysaccharide layer (HEP-layer) located on the outside of the OM, to minimise the diffusion

of oxygen (Nicolaison et al., 2009; Herrero et al., 2016) (Figure 4.1). Nitrogen fixation is carried out by the nitrogenase enzyme inside the heterocyst which converts atmospheric molecular nitrogen into ammonia in a highly expensive reaction, consuming chemical energy stored in 16 molecules of ATP per molecule of nitrogen fixed and 8 electrons (Reaction 4.1) (Bothe et al., 2010) (Figure 4.1).

Reaction 4.1

$$8H^+ + 8e^- + N_2 + 16MgATP \Rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

This ammonia is used to create glutamine from glutamate using the enzyme glutamine synthetase (GS). The glutamine is then transferred to the vegetative cells where the amide nitrogen is transferred to 2-oxoglutarate via glutamate synthase (GOGAT) yielding two molecules of glutamate (Reaction 4.2). This cycle is commonly known as the GS-GOGAT cycle. A major purpose of this cycle is to shuttle fixed carbon and nitrogen between the two cell types to ensure sufficient amino acid synthesis for protein synthesis (Muro-Pastor et al., 2005) (Figure 4.1).

Reaction 4.2

Glutamine Synthetase

$$Glutamate + ATP + NH_4^+ \Rightarrow Glutamine + ADP + Pi$$

(GS reaction carried out in the heterocyst cell)

Glutamate Synthase

$$2\text{-oxoglutarate} + Glutamine + NADPH + H^+ \Rightarrow 2\text{-}Glutamate + NADP^+$$

(GOGAT reaction carried out in the vegetative cell)

The GS-GOGAT cycle maintains a central position in diazotrophic metabolism connecting carbon and nitrogen metabolism. It is highly regulated and susceptible to modulation by external environmental factors such as carbon and nitrogen availability. In the presence of abundant carbon and absence of nitrogen, GS activity is upregulated (Figure 4.2). When abundant fixed nitrogen is available (e.g. ammonium), GS activity is downregulated (Figure 4.2). The regulation of GS is thought to maintain a balance between carbon use efficiency and a steady supply of glutamine. In particular, GS activity is downregulated in

Anabaena by a reversible protein-protein interaction involving GifA, which is a GS inactivation factor 7 (IF7) (Galmozzi et al., 2010). NtcA, a transcriptional regulator of the cAMP receptor protein (CAP) family is responsible for regulating the expression of *gifA* gene. Under diazotrophic conditions, NtcA is expressed and binds to a consensus binding site with the sequence $GTAN_8TAC$ found on the *gifA* promoter leading to the downregulation of *gifA* (therefore IF7) and a consequent upregulation of GS. When fixed-nitrogen is abundant, NtcA is downregulated leading to the activation of the *gifA* promoter (therefore expression of IF7) and a consequent downregulation of GS (Muro-Pastor et al., 2005; Galmozzi et al., 2010).

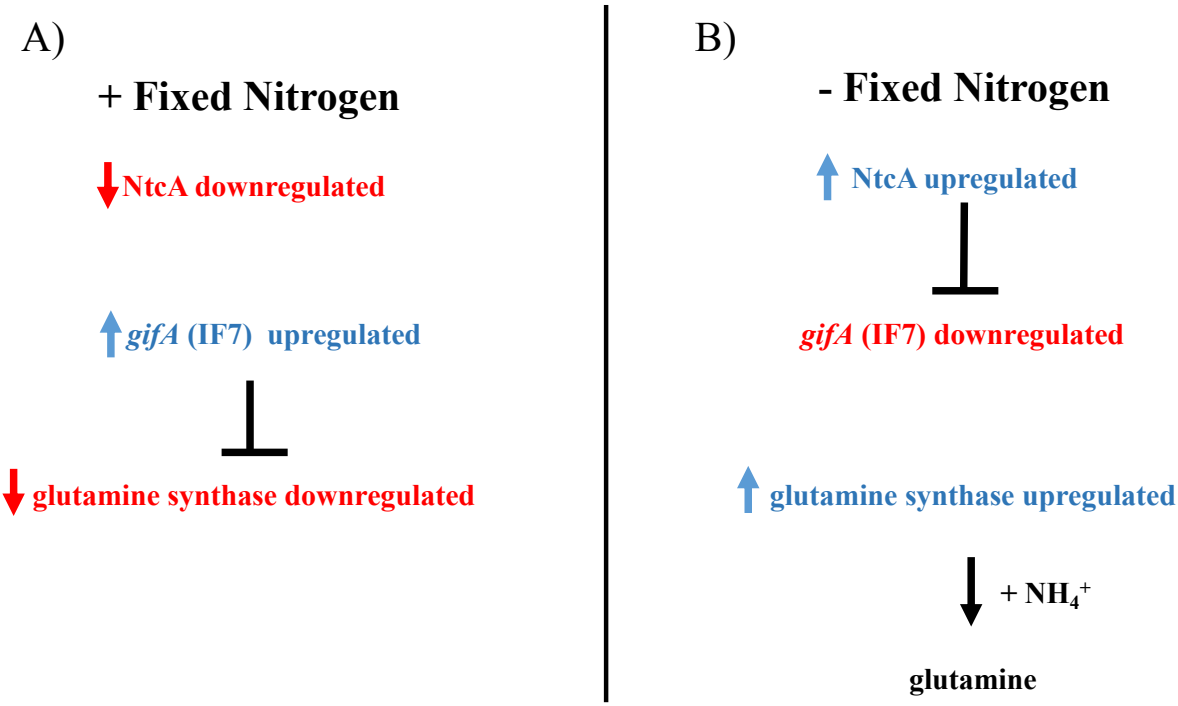

Figure 4.2 Flow diagram of glutamine synthase regulation in the presence (A) or absence (B) of fixed nitrogen.

Nitrogen is shuttled between the vegetative cells and heterocysts primarily as amino acids. This exchange of metabolites is thought to be carried out by diffusion along the continuous periplasm, with amino acids taken up by transporters in recipient vegetative cells along the filament (Flores and Herrero, 2010). The transporters identified to take up amino acids in *Anabaena* include four ABC-type amino acid uptake transporters known as the N-I, N-II, Bgt and N-III systems. The N-I system mainly transports proline and hydrophobic amino acids, but can also transport some other amino acids including glutamine and glutamate; the N-II system transports acidic and neutral polar amino acids; the Bgt system transports basic amino acids and, with low affinity, glutamine; and the N-III system mainly transports

hydrophobic amino acids, but has a relatively low specificity for other amino acids such as glutamate and glutamine. Whereas the Bgt and N-II systems are present in both vegetative cells and heterocysts, N-I is exclusively found in vegetative cells. However, it is still unknown where the N-III is located (Pernil et al., 2015). The transported amino acids, gene and predicted gene product of each transporter system are outlined in Table 4.1

Table 4.1 Identified amino acid transporters of *Anabaena* sp. PCC 7120

The order presented for the transported amino acids reflects the importance of the corresponding transporter in the uptake of the indicated amino acids. Amino acids in bold represent more than 95% of the total uptake through the indicated transporter (Pernil et al., 2008; Pernil et al., 2015).

Transporter	Transported amino acids	Gene (ORF)	Predicted Gene Product
N-I	**Pro**, **Phe**, **Leu**, Gly, Thr, Ala, Ser, Met, Asn, His, Orn, Gln, Glu	*natA* (*all1046*)	ATPase subunit
		natB (*alr1834*)	Periplasmic substrate-binding protein
		natC (*all1047*)	Transmembrane protein
		natD (*all1248*)	Transmembrane protein
		natE (*all2912*)	ATPase subunit
N-II	**Asp**, Glu, Asn, Gln, Met, Thr, Ala, Ser, Gly, His	*natF* (*alr4164*)	Periplasmic substrate-binding protein
		natG (*alr4165*)	Transmembrane protein
		natH (*alr4166*)	Transmembrane protein
		bgtA (*alr4167*)	ATPase subunit
Bgt	**Lys**, **Arg**, Orn, His, Gln	*bgtA* (*alr4167*)	ATPase subunit
		bgtB (*alr3187*)	Periplasmic substrate-binding and transmembrane domains
N-III	**Gly**, Pro, Glu, Phe, Leu, Ala, Gln	*natI* (*alr2535*)	Periplasmic substrate-binding protein
		natJ (*alr2536*)	Transmembrane protein
		natK (*alr2538*)	Transmembrane protein
		natL (*alr2539)*	ATPase subunit
		natM (*alr2541)*	ATPase subunit

4.1.2 Provision of fixed nitrogen for algal growth

Nitrogen is a major limiting nutrient for agriculture (including industrial scale algal growth). The cheap and efficient production of nitrogen fertilisers via the Haber Bosch process (conversion of atmospheric di-nitrogen to ammonia) revolutionized agriculture, helping to feed and sustain a growing population of up to 7.5 billion people alive today. However, the Haber Bosch process is energy intensive, requiring high temperature, 400–600 C, pressure, 15–25 MPa, and energy, about 32 GJ per ton ammonia (Patzek, 2004; Stal, 2003).

Possible options to mitigate the carbon intensive process of providing nitrogen to large scale algal production include 1) utilising domestic wastewater and 2) recycling spent medium and/or algal by-products from the extraction process (e.g. non-lipid portion of algal cells). Whilst the latter is sensible in any process, option 1 has several limitations. First, the wastewater must be decontaminated from pathogens, chemicals or heavy metals prior to use, to ensure reliable and consistent algal growth but most importantly to ensure purity in the case of algae grown for food or animal feed. Second, nitrogen concentrations in wastewater have been argued to be unable to support the growth of algae to produce sufficient concentrations for large-scale production systems, for example in making biofuels to contribute significantly to the world consumption of energy (Peccia et al., 2013).

A third option to introduce fixed nitrogen into growth media, bypassing the energy/carbon intensive Haber-Bosch process and domestic wastewater process, is to utilise nitrogen fixing bacteria capable of secreting fixed nitrogen into the medium. These are known as biofertilisers (Bhardwaj et al., 2014). Chaurasia and Apte (2011) demonstrated this concept with the engineering of the filamentous cyanobacterium *Anabaena* sp. PCC 7120 to increase its nitrogen fixing capabilities by overexpression of the *hetR* gene (a master regulator of hetercyst differentiation) which induced ammonium release, resulting in promotion of the growth of rice (*Oryza sativa*) seedlings. Furthermore, Ortiz-Marquez et al. (2014) and Barney et al. (2015) engineered the diazotrophic bacterium *Azotobacter vinelandii* to secrete ammonia for utilisation by the microalga *Chlorella sorokiniana*.

Alternatively, another approach could be to provide nitrogen in the form of amino acids from *Anabaena* sp. PCC 7120 as outlined by Picossi et al. (2005). Several mutants have been made in attempts to study amino acid transport in *Anabaena* that led unexpectedly to the release

of nitrogen into the culture medium as amino acids. These include mutants such as CSX60 and CSX60-R10. Strain CSX60 contains a knockout of *natA* which affects the N-I transport system. This knockout led to the accumulation of approximately 80 μM alanine and isoleucine, leucine and phenylalanine totaling approximately 30 μM in the medium after 48 hours of growth (cell concentration data were not published). Strain CSX60-R10 involves the knockout of *natA* and *bgtA* which affects the N-I, N-II and Bgt transport systems (Figure 4.3). This knockout led to the accumulation of approximately 100 μM alanine and approximately 18 μM each of isoleucine, leucine and phenylalanine in the medium after 48 hours of growth (cell concentration data were not published). The growth rate constants μ of CSX60 and CSX60-R10 were affected, being 0.22 ± 0.01 and 0.1 ± 0.01 day^{-1} respectively compared with 0.49 ± 0.01 day^{-1} for the wild type control.

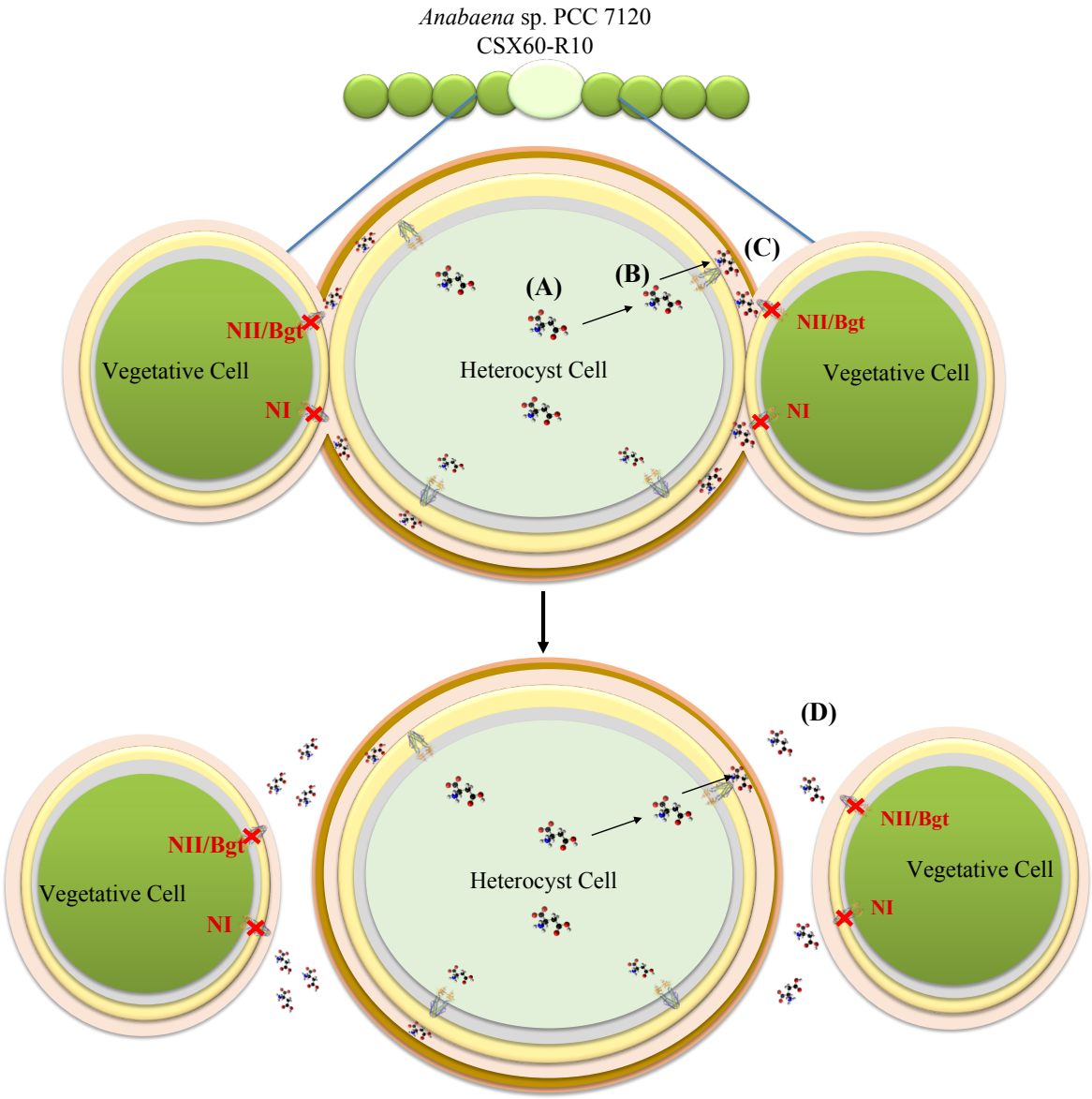

Figure 4.3 Illustration of the *Anabaena* sp. PCC 7120 CSX60-R10.

NII/Bgt and NI transporters are located in the cell membranes of the vegetative cells. Their purpose is to take up the amino acids which are synthesized and released from the heterocyst cell. *Anabaena* sp. PCC 7120 CSX60-R10 contains knockouts of these transporters. Therefore the following sequence of events is hypothesised: A) amino acids are synthesized in heterocysts; B) amino acids are transported across the heterocyst membranes; C) amino acids are 'trapped' inside the continuous periplasmic space leading to D) breaking apart of the filaments and consequential release of amino acids due to the vegetative cells being 'starved' of a nitrogen source (Picossi et al., 2005).

4.1.3 The *C. reinhardtii metE⁻* + *M. loti* consortium

C. reinhardtii metE⁻ is a vitamin B_{12}-dependent, freshwater eukaryotic green alga developed by Helliwell et al. (2015) and *M. loti* is a Gram-negative B_{12}-producing soil bacterium. The two organisms form a mutually dependent consortium when cultured together in which the alga provides carbon for the bacterium and in return the bacterium provides B_{12} to the alga. The original co-culture was based on the *L. rostrata-M. loti* consortium (Kazamia et al., 2012). However, *C. reinhardtii metE⁻* is a more useful model for B_{12}-dependent algal species as it has a sequenced genome and a well understood physiology, is a relatively fast grower, and has an established set of genetic manipulation tools (Kazamia, 2012; Ridley, 2016).

4.1.4 *Chlorella vulgaris*

Ca. vulgaris is a unicellular freshwater green alga that is widely studied and grown for its biotechnological applications. In the early 20th century, the high protein content (>55% of dry weight) of *Ca. vulgaris* initially attracted attention as a food source. It was first grown at large scale in the 1950s for CO_2 abatement. More recently, *Ca. vulgaris* is consumed for its claimed health-related benefits such as the stimulation of haematopoiesis and prevention of cardiovascular diseases, hypertension, cataract and atherosclerosis. Furthermore, *Ca. vulgaris* can accumulate large amounts of lipids when nitrogen starved producing a fatty acid profile suitable for use as a biofuel (Safi et al., 2014).

4.1.5 Aims

Large scale growth of microalgae requires the addition of fixed nitrogen. The Haber Bosch process is the primary means of providing this nitrogen. However, it is energy and carbon intensive. Biofertilisation via nitrogen fixing bacteria would be an attractive alternative to the Haber Bosch process. Biofertilisation for algae production has mainly focused on the heterotrophic bacterium *A. vinelandii* and not a lot is understood about the potential use of *Anabaena.* The aim of this study was to test whether an engineered strain of *Anabaena* could be grown as a consortium with eukaryotic algae and provide enough fixed nitrogen to the medium to provide enhanced growth for microalgae. The organisms chosen to test the potential for nitrogen provision from *Anabaena* were *C. reinhardtii metE⁻* grown in conjunction with *M. loti,* and *Ca. vulgaris.* Firstly, the study tests the possibility of co-cultivating strains

providing nitrogen (in the form of amino-acids and ammonium) of *Anabaena* with the *C. reinhardtii metE*$^{-}$ + *M. loti* consortium to determine if a stable tripartite consortium can be generated (Figure 4.4A). Second, nitrogen-providing (in the form of amino-acids and ammonium) strains of *Anabaena* will be co-cultured with *Ca. vulgaris* to test if they are able to enhance its growth (Figure 4.4B).

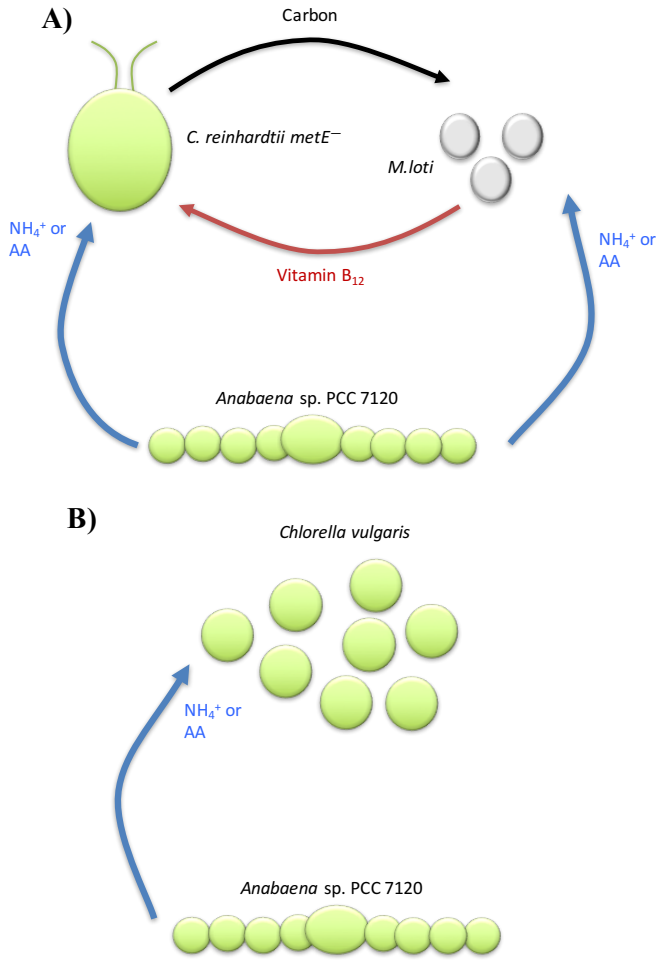

Figure 4.4 The proposed engineered consortia involving nitrogen-releasing strains of *Anabaena* sp. PCC 7120

A) Proposed nutrient exchanges when fixed-nitrogen releasing strains of *Anabaena* sp. PCC 7120 are added the *C. reinhardtii metE*$^{-}$ + *M. loti* co-culture. B) Proposed nutrient exchange when fixed-nitrogen releasing strains of *Anabaena* sp. PCC 7120 are co-cultured with to *Ca. vulgaris*.

4.2 Results

4.2.1 Identifying optimal media for growth of co-cultures

Engineering synthetic consortia requires the use of a medium that allows optimal growth rate for all member species with minimal compromise. Standard *Anabaena* medium is BG11 whereas the *C. reinhardtii metE*$^{-}$ + *M. loti* consortium typically grows well in TP^{+} medium (Pernil et al., 2008; Kazamia et al., 2012). *Ca. vulgaris* has been grown in a range of media including TAP and BG11 (Chia et al., 2013; Bono et al., 2015). I conducted initial growth experiments to identify which medium provided the highest growth rate for culturing *C. reinhardtii metE*$^{-}$ + *M. loti* with *Anabaena* and *Ca. vulgaris* with *Anabaena.* The media tested were TP^{+}, TP^{+}/BG11 and BG11 (Appendix 8.1). Growth experiments were carried out in non-bubbling, batch cultures in 50 mL conical flasks, shaking with constant illumination at 40 µmol photons m^{-2} s^{-1}. I measured growth daily using OD_{600} for *M. loti* and OD_{750} for *Ca. vulgaris*, *C. reinhardtii metE*$^{-}$, and *C. reinhardtii metE*$^{-}$ + *M. loti.* I calculated the maximum growth rate from the data collected in the growth experiments. The maximum growth rate was determined by calculating the difference in biomass (measured by OD_{600} for *M. loti* or OD_{750} for microalgae or cyanobacteria) from inoculation point up until slow growth phase divided by the time it took to reach slow growth phase. This time ranged from 2 days for *M. loti*, 8 days for *Ca. vulgaris*, and 14 days for *C. reinhardtii metE*$^{-}$, *C. reinhardtii metE*$^{-}$ + *M. loti* and *Anabaena*. With the maximum growth rates determined, I calculated the relative growth rates of the organisms cultured in the various media. The medium that allowed for the fastest growth rate of an organism was normalized as 100%. The growth rates in the alternative media were then divided by the fastest growth rate to yield a percentage value of the fastest growth rate. I recored this percentage as the relative growth rate.

Growth rates of *M. loti, C. reinhardtii metE*$^{-}$ + B_{12}, *C. reinhardtii metE*$^{-}$ + *M. loti* and *Ca. vulgaris* were highest in TP^{+} medium compared to TP^{+}/BG11 and BG11, hence were recorded as 100% efficient in TP^{+} (Figure 4.5A). Experiments with *M. loti*, *C. reinhardtii metE*$^{-}$ + $B_{12,}$ *C. reinhardtii metE*$^{-}$ + *M. loti* and *Ca. vulgaris* all showed reduced growth in TP^{+}/BG11 and BG11 media. Growth of *Anabaena* was optimal in BG11 medium compared with TP^{+} and TP^{+}/BG11, hence was recorded as 100% efficient in BG11. *Anabaena's* relative growth rate was 76% and 18% in TP^{+} and TP^{+}/BG11 respectively compared with that in BG11. I concluded that growth of *Anabaena* with *C. reinhardtii metE*$^{-}$ + *M. loti* in TP^{+} provided the

optimal growth rate with the least compromise. However, for growth of *Anabaena* with *Ca. vulgaris* either TP^+ or BG11 medium could be used.

The nitrogen source in BG11 is sodium nitrate ($NaNO_3$), whereas TP^+ is supplemented with ammonium chloride (NH_4Cl). To determine whether the differing nitrogen sources could be a cause for the decline in the growth rate of *Anabaena* in TP^+ compared with BG11 (as outlined in Figure 4.5A), I grew *Anabaena* in both BG11 and TP^+ lacking a nitrogen source (-N). I carried out the growth experiment in non-bubbling, batch cultures in 50 mL conical flasks, shaking with constant illumination at 40 µmol photons m^{-2} s^{-1}. Consistent with the previous experiment, the growth rate of *Anabaena* was higher in BG11 medium, hence the relative growth rate was normalized to 100% in BG11. Similar to the previous experiment, the relative growth rate of *Anabaena* in TP^+(-N) was found to be less than the growth measured in BG11(-N) (Figure 4.5B). Furthermore, the growth rate of *Anabaena* in TP^+(-N) was found to be less than the previous experiment (outlined in Figure 4.5A) at approximately 50% of the growth rate in BG11(-N) (Figure 4.5B). I concluded that the growth deficiency of *Anabaena* in TP^+ could not be explained simply by the source of nitrogen.

To attempt to improve the growth of *Anabaena* in TP^+, I grew *Anabaena* in TP^+ supplemented with BG11 ingredients at standard concentrations including BG11 trace elements, iron (ferric ammonium citrate), 100x BG11, $NaHCO_3$ and Na_2CO_3. Further details of the supplemented BG11 ingredients can be found in Table 4.3 (Section 4.3.1) and Appendix 8.1.4. I carried out the growth experiment in non-bubbling, batch cultures in 50 mL conical flasks, shaking with constant illumination at 40 µmol photons m^{-2} s^{-1}. Consistent with the previous experiment, the growth rate of *Anabaena* was highest in BG11 medium, hence the relative growth rate was normalized to 100% in BG11. The relative growth rate of *Anabaena* in TP^+ in this experiment was found to be consistent with the previous experiment at approximately 72% as fast as the growth rate in BG11 (Figure 4.5C). Supplementation with BG11 trace elements and iron resulted in slightly lower growth rates than in TP^+, which were 57% and 69% respectively compared to the rate in BG11. However, supplementation with 100x BG11 and carbonate ($NaHCO_3$, Na_2CO_3) resulted in a decline in the growth of *Anabaena* to 4% and 2% respectively of growth in BG11. I concluded that growth of *Anabaena* in TP^+ could not be rescued by supplementation of BG11 additives and so the co-culture of *Anabaena* with *C. reinhardtii metE*$^-$ + *M. loti* should be carried out in TP^+.

Figure 4.5D shows an image of the *Anabaena* cultures at the end of the experiment outlined in Figure 4.5C. When *Anabaena* was grown in BG11 (i), the cultures appeared the standard blue-green colour. However, when *Anabaena* was grown in TP^+ supplemented with BG11 ingredients, the colours of the cultures were unusual. *Anabaena* cultures grown in TP^+ (ii), TP^+ with BG11 trace elements (iii) and TP^+ with BG11 iron (iv) were dark blue in colour. An *Anabaena* culture grown in TP^+ with 100x BG11 (v) was a lighter shade of blue and an *Anabaena* culture grown in TP^+ with BG11 carbonate ($NaHCO_3$, Na_2CO_3) was green in colour (vi). Unfortunately, I did not conduct spectroscopic analysis on the cultures at the time. I concluded that *Anabaena* had undergone significant stress that led to an alteration in the amounts of proteins or pigments involved in the light harvesting machinery.

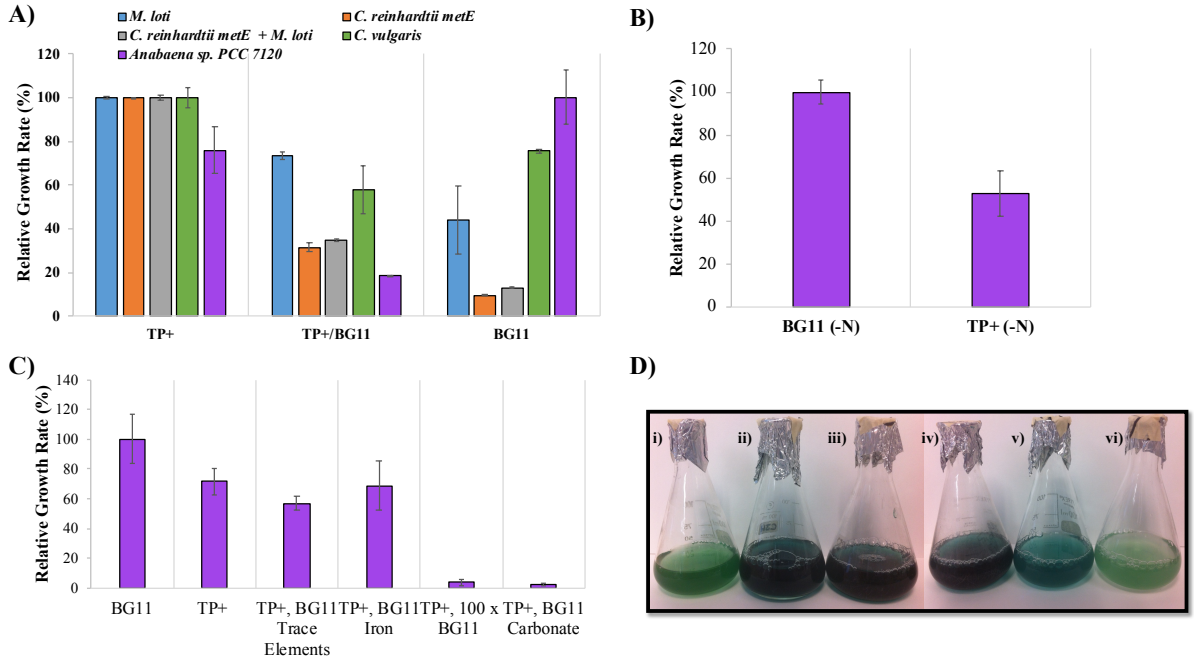

Figure 4.5 Relative growth rates of algae and bacteria in various media

A) Relative growth rate of *M. loti*, *C. reinhardtii metE*—, *C. reinhardtii metE*— + *M. loti*, *Ca. vulgaris* and *Anabaena* in TP^+, TP^+/BG11 and BG11. B) Relative growth rate of *Anabaena* in BG11 (lacking nitrogen) and TP^+ (lacking nitrogen). C) Relative growth rate of *Anabaena* in TP^+ supplemented with BG11 ingredients. D) Cultures at the end of the experiment outlined in C. *Anabaena* grown in i) BG11, ii) TP^+, iii) TP^+ with BG11 trace elements, iv) TP^+ with BG11 iron, v) TP^+ with 100x BG11, vi) TP^+ with BG11 carbonate ($NaHCO_3$, Na_2CO_3). Error bars display standard deviation from three replicates.

4.2.2 Removing *Anabaena* sp. PCC 7120 filaments for counting single cells

Accurate knowledge of population dynamics requires the ability to determine the populations of all species present in co-cultures. One technique investigated included the use of flow cytometry. Flow cytometry can discriminate populations of bacteria and algae based on both size and fluorescence (Veldhuis and Kraay, 2000). However, to use flow cytometry, *Anabaena* filaments (linear chains of connected cells) cannot be present otherwise obstruction will occur, preventing accurate readings and causing possible equipment failure. Possible techniques to remove *Anabaena* filaments include sonication to break up the filaments into individual cells or filtration of the culture through filters to purify the single cells from the filaments. However, sonication can be utilized only if it does not affect the population numbers of other species in the culture. I therefore conducted an experiment to determine the number of individual cells and filaments after sonication. *Anabaena* cultures of 1 mL at OD_{750} 0.1, 0.2 and 0.3 were subjected to 45 minutes of high strength sonication in a Bioruptor Sonicator Bath (Materials and Methods 2.9.2). I counted the single cells and filaments (between 2-10 cells in length and greater than 10 cells in length) in a 10 µL sample using a haemocytometer. I stopped counting single cells when I had counted 1000 single cells. The number of filaments counted was the lowest with cultures at OD 0.1, where approximately 10 filaments between 2-10 cells long were counted (Figure 4.6A). It was important to determine the effect of the same treatment on *C. reinhardtii metE*$^{-}$ cells, so I sonicated cultures under the exact same conditions as in Figure 4.6A and were counted by a Coulter counter. Under these settings, a decline in the number of *C. reinhardtii metE*$^{-}$ cells counted was detected from approximately 3.7 $x10^5$ pre-sonication to 1.9 $x10^5$ cells per mL post-sonication (Figure 4.6B). I concluded that the use of bath sonication was again not ideal, due to the decline in *C. reinhardtii metE*$^{-}$ cells at the sonication conditions that were tested for *Anabaena*.

I then decided to test the effect on *Anabaena* cells of sonicating at OD_{750} 0.1 with a probe (Materials and Methods 2.9.2) at a continuous amplitude of 16 microns for a range of time periods, namely 30, 45 and 60 seconds. The results showed that sonication for a time of 60 seconds was enough to break up the filaments into single cells completely (Figure 4.6C). *C. reinhardtii metE*$^{-}$ cell numbers were found to decline from 3.0-3.2 $x10^5$ cells per mL to 5.0-6.0 $x10^4$ cells per mL when probe sonicated for 30, 45 and 60 seconds at a strength of 16 microns (Figure 4.6D). I concluded that the use of probe sonication was not ideal, due to the decline in *C. reinhardtii metE*$^{-}$ cells at the conditions that were tested for *Anabaena*.

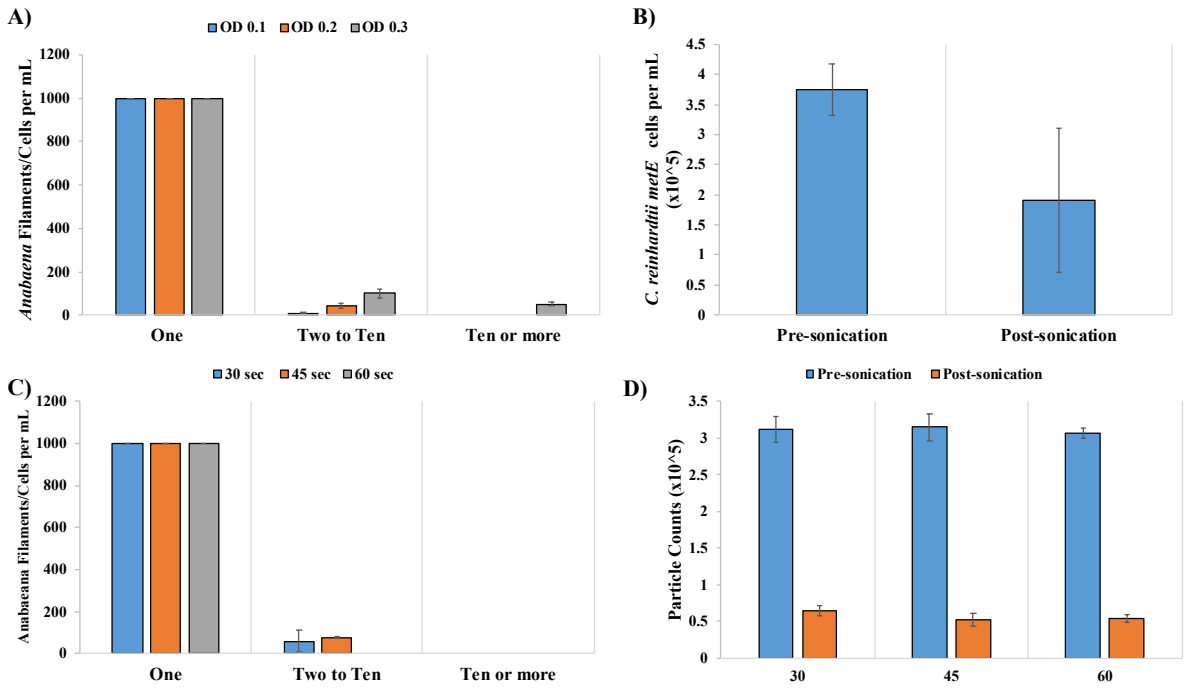

Figure 4.6 Investigating the effect of sonication on *Anabaena* and *C. reinhardtii metE*⁻

A) Population of *Anabaena* filaments of different lengths in 10 µL after bath sonication in a 1 mL volume for 45 minutes at 'high strength' (Bioruptor Sonicator Bath). B) Population of *C. reinhardtii metE*⁻ cells after bath sonication in a 1 mL volume for 45 minutes at 'high strength' (Bioruptor Sonicator Bath). C) Population of *Anabaena* filaments in 10 µL after probe sonication in a 1 mL volume at OD_{750} 0.1 with a probe at a continuous strength of 16 microns for 30, 45 and 60 seconds. D) Population of *C. reinhardtii metE*⁻ after probe sonication in a 1 mL volume at OD_{750} 0.1 with a probe at a continuous strength of 16 microns for 30, 45 and 60 seconds. Error bars display standard deviation from three replicates.

If sonication could not be used to break up the *Anabaena* filaments into single cells for quantification, it might still be advantageous to remove the *Anabaena* filaments from the cultures to count the algal or bacterial cells accurately by flow cytometry or Coulter counter. However, the disadvantage would be that *Anabaena* could no longer be accurately quantified. Therefore, I investigated an alternative option to separate the single cells from the filaments using filtration. I passed *Anabaena* cultures at OD_{750} 0.1 through one or two layers of mesh up to ten times. Total numbers of filaments (>20 cells in length) were counted from a volume of 10 µl. Figure 4.7A shows the results obtained using 25 µm diameter stainless steel mesh (Materials and Methods 2.9.3). The number of filaments in a pre-filtration sample was approximately 140. After passing the culture through the layers of mesh up to ten times, the

numbers of filaments 20 cells or more in length reached between 42-45. Figure 4.7B shows the results obtained using 25 µm diameter polypropylene filters (Materials and methods 2.9.3). The number of filaments in the pre-filtration sample was again approximately 140. After passing the culture through the layers of mesh up to ten times, the numbers of filaments reached between 16-17. I concluded that neither of the filters tested could remove all the filaments in the culture and so filtration was disregarded as an option to separate the filaments from the single cells. Filters with smaller diameter holes could have been tested. However, due to time constraints, previously established methods such as the measurement of CFUs or use of a haemocytometer to determine cell populations were utilized at the expense of accurately quantifying the population of *Anabaena.*

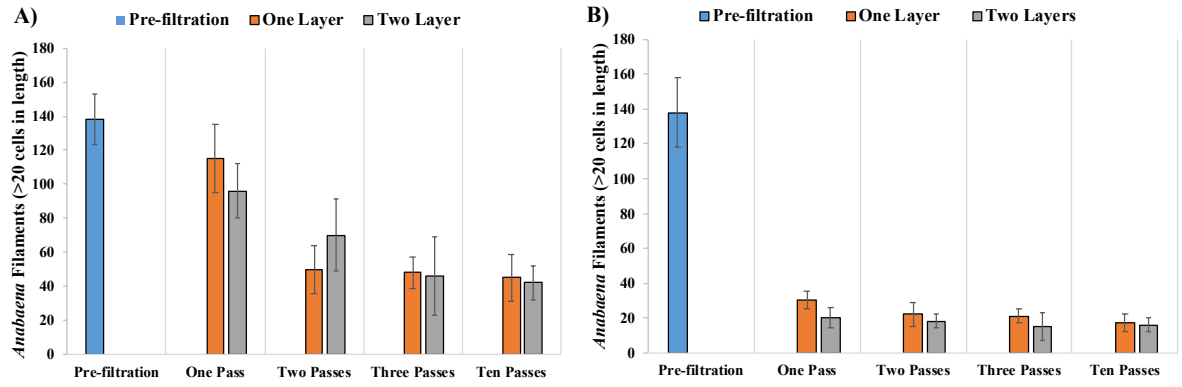

Figure 4.7 Investigating the use of filtration to remove filaments from culture

A) Number of *Anabaena* filaments (>20 cells in length) in 10 µL after passing through 25 µm diameter stainless steel mesh. B) Number of *Anabaena* filaments (>20 cells in length) in 10 µL after passing through 25 µm diameter polypropylene filters. Error bars display standard deviation from three replicates.

4.2.3 Growth of *C. reinhardtii metE*$^{-}$ and *M. loti* on amino acids

To engineer a consortium of organisms trading metabolites, it must be established whether the metabolites traded can indeed be taken up. The aim was to introduce a nitrogen-providing strain of *Anabaena* into the *C. reinhardtii metE*$^{-}$ + *M. loti* consortium previously established (Helliwell et al., 2014). The nitrogenous compounds I chose to be supplied by the engineered strains of *Anabaena* were ammonium (NH_4^+) and amino acids. I chose the CSX60-R10 strain as a candidate for nitrogen provision due to its release of amino acids at relatively high concentrations (155 µM). Importantly however, it was the only amino acid releasing strain still available from Professor Enrique Flores' laboratory (University of Sevilla, Spain) (Pernil

et al., 2008). I carried out a growth experiment under standard conditions with TP^+ medium (Materials and Methods 2.3) to investigate whether *C. reinhardtii metE⁻* and *M. loti* could take up the amino acids released by *Anabaena* strain CSX60-R10, namely alanine, isoleucine and phenylalanine (ammonium is a standard nitrogen ingredient of TP^+ medium, so it was used as a positive control).

The TP^+ positive control contained approximately 1.9 mM nitrogen initially (in the form of NH_4Cl). In contrast, the amino acid supplemented cultures (TP^+-N+AA) initially contained approximately 155 µM of total amino acids where 100 µM was in the form of alanine and 55 µM in the form of isoleucine, leucine and phenylalanine (approximately 18 µM each) which represents the total concentration of amino acids detected after 48 hours of growth (unfortunately the cell concentration was not published) of CSX60-R10 (Pernil et al., 2008). The TP^+ negative control (TP^+-N) contained no additional nitrogen (Appendix 8.1.3). Growth was measured for axenic *C. reinhardtii metE⁻*, *C. reinhardtii metE⁻* + *M. loti* co-culture and axenic *M. loti* (Figure 4.8A, B and C respectively). Minimal growth was observed for the negative control (TP^+-N) for all three growth experiments. In standard TP^+ medium (positive control) axenic *C. reinhardtii metE⁻, C. reinhardtii metE⁻* + *M. loti* and *M. loti* grew as expected. In TP^+(-N) + AA medium, I found that axenic *C. reinhardtii metE⁻* grew to a maximum of approximately 8.50 $x10^5$ algal cells per mL, *C. reinhardtii metE⁻* + *M. loti* grew to a maximum of approximately 4.7 $x10^5$ algal cells per mL and axenic *M. loti* grew to approximately 1.4 $x10^9$ CFUs per mL after five days of growth. I concluded that *C. reinhardtii metE⁻* and *M. loti* could metabolise the amino acids supplied both in axenic growth and in co-culture.

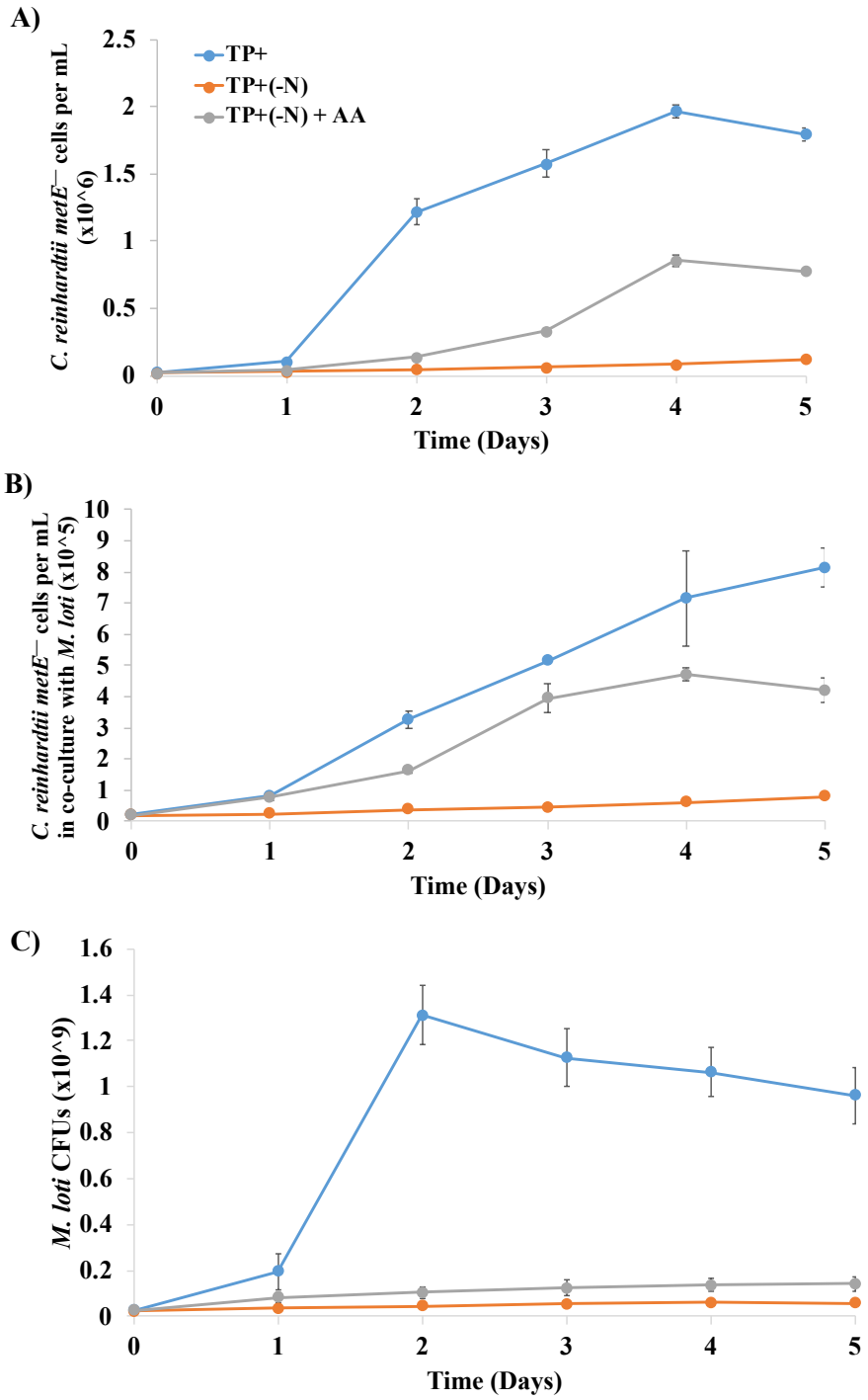

Figure 4.8 Growth of *C. reinhardtii metE⁻* and *M. loti* on amino acids

A) Growth of axenic *C. reinhardtii metE⁻*. B) Growth of *C. reinhardtii metE⁻* in co-culture with *M. loti* and C) Growth of axenic *M. loti*. TP^+ positive control medium contains the standard 1.9 mM nitrogen (in the form of NH_4Cl). TP+ (-N) +AA medium contains 100 µM alanine and approximately 18 µM each of isoleucine, leucine and phenylalanine equaling a total of approximately 155 µM nitrogen. TP^+ (-N) negative control medium contains no supplemented nitrogen. Error bars display standard deviation from three replicates.

4.2.4 Growth characteristics of amino acid-producing *Anabaena* strain CSX60-R10

Anabaena sp. PCC 7120 CSX60-R10 has previously been shown to release 100 μM alanine and approximately 18 μM each isoleucine, leucine and phenylalanine (Pernil et al., 2008). I carried out a growth experiment to determine the growth of this strain in different media under axenic, diazotrophic conditions. Figure 4.9A shows the growth over four days of CSX60 in BG11, BG11(-N) and TP^+ (-N) bubbling with air, and starting at OD_{750} 0.2. I originally carried out a growth experiment without bubbling, but growth was very poor for CSX60-R10 under diazotrophic conditions (data not shown). The results showed that the culture in BG11 reached OD_{750} 3.5 after four days whereas the cultures in BG11 (-N) and TP^+ (-N) reached OD_{750} 0.7 after four days. Figure 4.9B shows an image of the cultures after the growth experiment. It is apparent that CSX60-R10 grown in BG11 was the expected blue-green colour. However, CSX60-R10 cultures in BG11 (-N) or TP^+ (-N) were yellow in appearance. Figure 4.9C shows an absorbance spectrum of the cultures after the four days of growth. The BG11 culture showed the typical absorption peaks at 630 nm and 680 nm for phycocyanin and chlorophyll *a* respectively. However, the cultures of CSX60-R10 in BG11 (-N) and TP+ (-N) showed no observable peak at 630 nm and small peaks at 680 nm. Figure 4.9D shows the ratio of single cells to filaments (greater than 10 cell in length) after 4 days of growth in standard conditions (Materials and Methods 2.3). Prior to measuring the ratio, each of the samples was standardized to an OD_{750} of 0.8. The ratio for wild type dropped from approximately 500 to 105 single cells to filaments when grown in BG11 versus BG11(-N) respectively. In contrast, the CSX60-R10 strain climbed from approximately 3300 to 5200 single cells to filaments when grown in BG11 and BG11 (-N) respectively. When the CSX60-R10 strain was bubbled, the difference in ratio between BG11 and BG11(-N) climbed further from approximately 3000 to 41200 single cells to filaments. Overall, I concluded that the growth of *Anabaena* strain CSX60-R10 in nitrogen free media was impaired compared to that of the growth in media containing fixed nitrogen. Furthermore, bubbling with air was necessary to ensure maximum growth of the under nitrogen diazotrophic conditions.

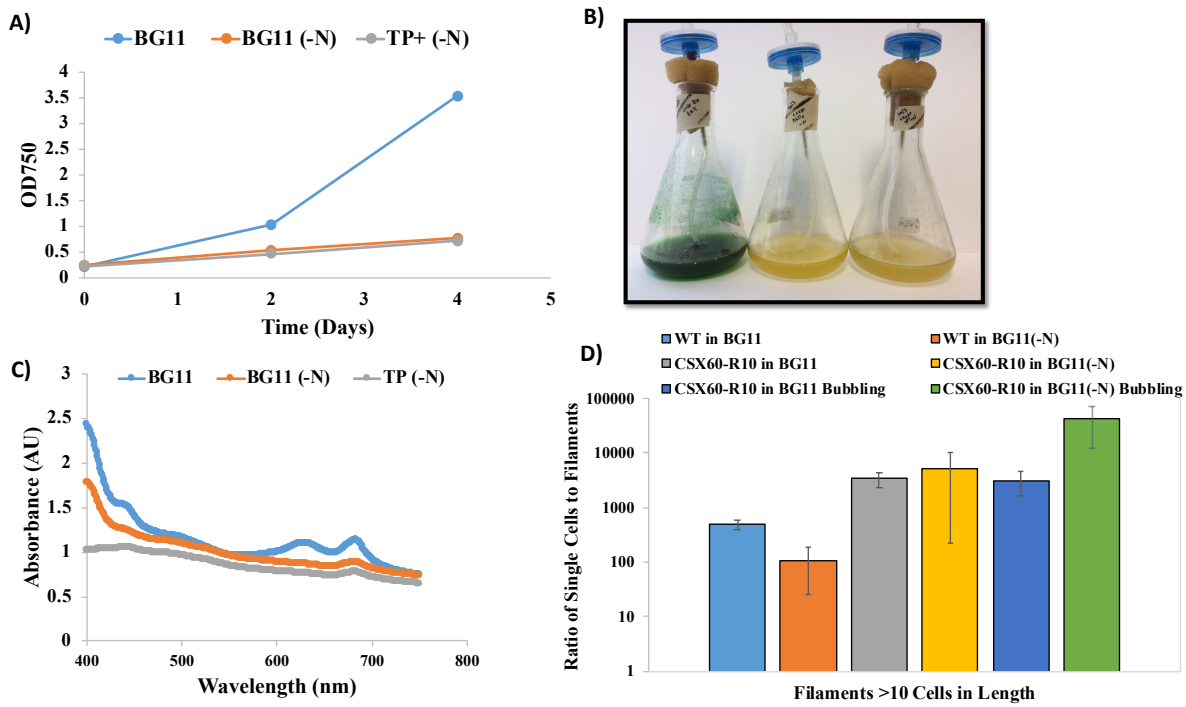

Figure 4.9 Investigating the growth of CSX60-R10 under diazotrophic conditions.

A) Growth curve of CSX60-R10 grown in BG11, BG11 (-N) and TP^{+}(-N) and bubbled with air. B) Photograph of cultures after 4 days of growth. From left to right: CSX60-R10 grown in BG11, BG11 (-N) and TP^{+}(-N). C) Spectrum of CSX60-R10 cultures after four days of growth in BG11, BG11 (-N) and TP^{+}(-N). D) Ratio of single cells to filaments (greater than 10 cells in length) on a logarithmic Y-axis with samples standardized to an OD_{750} of 0.8. Growth was carried out under standard conditions. Error bars display standard deviation from three replicates.

4.2.5 Growth characteristics of ammonium producing strains of *Anabaena*

Attempts were made to engineer *Anabaena* to release fixed nitrogen in the form of ammonium. Design and engineering of ammonium-releasing strains were mostly carried out (in consultation with the author of this dissertation and Dr. Dennis Nuernberg of Imperial College London, UK) by David Malatinszky in Patrik Jones' laboratory, Imperial College London, UK. A detailed discussion of how the strains were engineered will be documented in David Malatinszky's dissertation but an overview is outlined below. The aim of the work described here was to test the strains for ammonium production and ability to provide nitrogen to *C. reinhardtii metE*$^{-}$ + *M. loti* and *Ca. vulgaris* under diazotrophic conditions.

There are two features about these engineered strains that could lead to the release of ammonium into the supernatant. The first is the expression of GS inactivation factor (GifA) a protein that binds to and inhibits GS – the enzyme responsible for the conversion of glutamate and ammonium to glutamine (Reaction 4.2). If the GS is successfully inhibited and if ammonium is consistently being synthesized by the nitrogenase enzyme there should theoretically be an accumulation of ammonium within the cell. This accumulation could then result in the diffusion of ammonium (however, the exact mechanism of ammonium release is unclear) as demonstrated in previous studies (Chaurasia and Apte, 2011; Ortiz-Marquez et al., 2012; Ortiz-Marquez et al., 2014). Furthermore, half of the strains have their *amt* cluster deleted, which is an operon known to be involved in translocating ammonium across biological membranes, so *amt* deletion would theoretically prevent the ability to take up any released ammonium (Javelle et al., 2007; Paz-Yepes, 2008).

Table 4.2 outlines the genotype of each of the putative ammonium-producing *Anabaena* strains and Figure 4.10 illustrates the plasmids of the strains and their insertion into either *Anabaena* WT or *Anabaena* Δ*amt*. Each of the strains contains a plasmid based on the RSF1010 self-replicating plasmid conferring streptomycin and spectinomycin resistance (SmR). Furthermore, all of the plasmids contain the gene for GS inactivation factor (*gifA*) and the native terminator from *gifA* (T*gifA*). The strains differ in the type of promoter used and the background genotype (base strain). The promoters used include the P*nifHDK* (nitrogenase gene promoter conferring expression in the heterocyst cells only); P*gifA* (native *gifA* gene promoter); P*rbcLS* (promoter for the large and small subunits of the ribulose-1,5-*bis*phosphate carboxylase/oxygenase (RuBisCO) operon whereby expression is in the vegetative cells only); and P*petE* (copper inducible plastocyanin gene promoter). Promoters P*nifHDK* and P*petE* are expected to express *gifA* in the heterocyst cells where GS is expressed, thus leading to inhibition of GS and consequential nitrogen release. By contrast, promoters P*gifA* and P*rbcLS* are not expected to lead to nitrogen release, and are instead acting as negative controls, as P*gifA* is only active in environments with abundant fixed nitrogen and P*rbcLS* should only be active in the vegetative cells where GS is not expressed. The resulting four plasmids (containing four different promoters) were introduced via conjugation into two different base strains of *Anabaena*, wild type or Δ*amt*, creating a total of eight strains. The Δ*amt* strains contain a deletion in the *amt* gene cluster which includes three genes *amt4* (alr0990), *amt1* (alr0991) and *amtB* (alr0992).

Table 4.2 Engineered *Anabaena* sp. PCC 7120 strains used in this study

Strain No.	Description	Base Strain	Notes	Comments
160	P*nifHDK-gifA*-T*gifA*::RSF1010-SmR	wild type	Nitrogenase gene promoter (P*nifHDK*); Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*). Heterocyst-specific expression*	Predicted to release ammonium.
161	P*gifA-gifA*-T*gifA*::RSF1010-SmR	Δ*amt***	Native *gifA* gene promoter; Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*).	Control
162	P*rbcLS-gifA*-T*gifA*::RSF1010-SmR	Δ*amt***	Promoter for the large and small subunits of the Ribulose -1,5 –bisphosphate carboxylase/oxygenase (RuBisCO) operon (P*rbcLS*); Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*). Vegetative cell-specific expression.	Control
163	P*nifHDK-gifA*-T*gifA*::RSF1010-SmR	Δ*amt***	Nitrogenase gene promoter (P*nifHDK*); Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*). Heterocyst-specific expression*	Predicted to release ammonium.
164	P*gifA-gifA*-T*gifA*::RSF1010-SmR	wild type	Native *gifA* gene promoter; Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*).	Control
165	P*petE-gifA*-T*gifA*::RSF1010-SmR***	wild type	Copper inducible phycocyanin gene promoter (P*petE*): Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*).	Predicted to release ammonium.
166	P*rbcLS-gifA*-T*gifA*::RSF1010-SmR	wild type	Promoter for the large and small subunits of the Ribulose -1,5 –bisphosphate carboxylase/oxygenase (RuBisCO) operon (P*rbcLS*); Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*). Vegetative cell-specific expression.	Control
167	P*petE-gifA*-T*gifA*::RSF1010-SmR***	Δ*amt***	Copper inducible phycocyanin gene promoter (P*petE*): Glutamine Synthetase Inactivation Factor (*gifA*); Native Terminator from *gifA* (T*gifA*).	Predicted to release ammonium.

*expression under diazotrophic conditions only.
** strain contains the deletion of the *amt* cluster (alr0990, alr0991 and alr0992) involved in ammonium uptake.
*** expression only in the presence of copper (e.g. 3 μM Cu^{2+}).

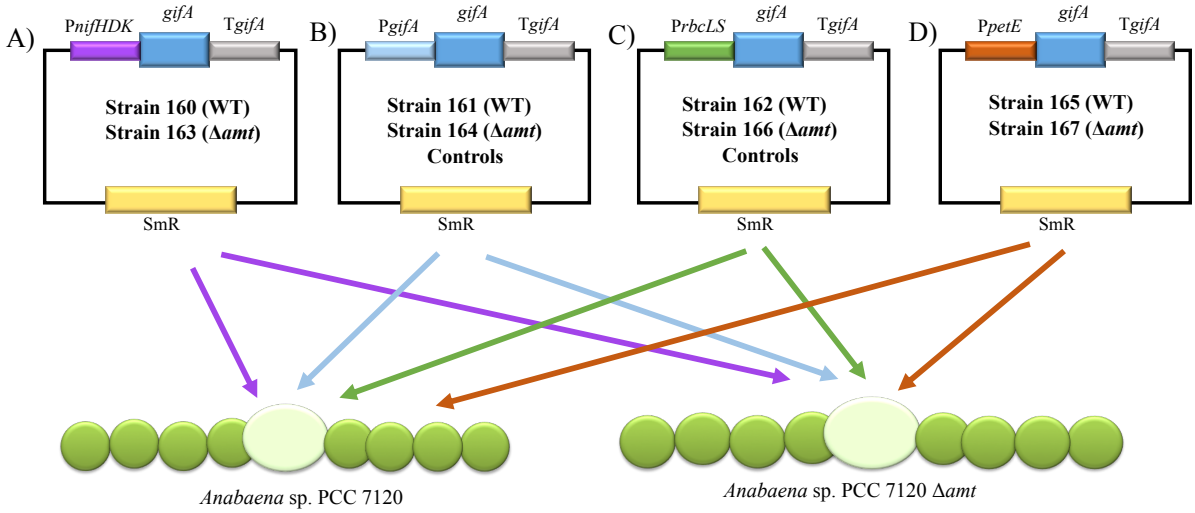

Figure 4.10 Illustration of plasmids inserted into *Anabaena* sp. PCC 7120.

A) RSF1010 – SmR plasmid containing a Nitrogenase gene promoter (PnifHDK), Glutamine Synthetase Inactivation Factor (*gifA*) and Native Terminator from *gifA* (T*gifA*). Strain 160 and Strain 163 contain this plasmid in the Anabaena WT and Δ*amt* respectively. B) RSF1010 – SmR plasmid containing a native *gifA* gene promoter (P*gifA*), Glutamine Synthetase Inactivation Factor gene (*gifA*) and Native Terminator from *gifA* (T*gifA*). Strain 161 and Strain 164 contain this plasmid in the Anabaena WT and Δ*amt* respectively. C) RSF1010 – SmR plasmid containing a promoter for the large and small subunits of the Ribulose -1,5 – bisphosphate carboxylase/oxygenase (RuBisCO) operon (P*rbcLS*), Glutamine Synthetase Inactivation Factor gene (*gifA*) and Native Terminator from *gifA* (T*gifA*). Strain 162 and Strain 166 contain this plasmid in the Anabaena WT and Δ*amt* respectively. D) RSF1010 – SmR plasmid containing a copper inducible phycocyanin gene promoter (P*petE*), Glutamine Synthetase Inactivation Factor gene (*gifA*) and Native Terminator from *gifA* (T*gifA*). Strain 165 and Strain 167 contain this plasmid in the Anabaena WT and Δ*amt* respectively.

It was important to determine the growth of the putative ammonium-producing strains under diazotrophic conditions. I carried out a growth experiment where the cultures were grown in 50 mL of BG11(-N) medium, shaking for 5 days with constant illumination at 40 µmol $m^{-2}s^{-1}$ and bubbled with air. I supplemented the cultures with 3 µM copper (II) sulphate ($CuSO_4$) because strains 165 and 167 require approximately 3 µM Cu^{2+} for expression from the copper-inducible P*petE* promoter (Buikema and Haselkorn 2001). All cultures were supplemented with 3 µM $CuSO_4$ to standardise the experiment and maintain direct comparisons between the cultures. The cultures (apart from the WT) were also supplemented with 2.5 µg/mL each of spectinomycin and streptomycin. Figure 4.11A shows the OD_{750} of the

strains after 5 days' growth starting at an initial OD_{750} of 0.05. Additionally, the figure also shows the concentration of chlorophyll *a* measured in each strain. The WT strain grew to OD_{750} 0.96 with a chlorophyll *a* concentration of 8.4 nmol chl *a* per mL. Strain 165 reached the highest OD with an OD_{750} 0.99 and 8.8 nmol chl *a* per mL. Strain 160 showed least growth with an OD_{750} 0.25 and 0.9 nmol chl *a* per mL. The ability of the strains to secrete ammonia into the medium was unverified, so the supernatant of the cultures after the 5-day growth experiment was tested for the presence and concentration of ammonium via an ammonium measuring kit (Materials and Methods 2.9.5) with the results shown in Figure 4.11B. Approximately 1.8 μM ammonium was measured in the supernatant of strains 163, 165 and 167 whereas approximately 1.5 μM ammonium was measured for strain 160. However, these were calculated as not significant compared to wild type by ANOVA. The remaining engineered strains showed a similar concentration to WT, at approximately 1 μM ammonium. To give an estimate of the ammonium release between the strains on a per cell basis, I measured the ratio of ammonium concentration in the supernatant to the cell density (OD_{750}) (Figure 4.11C). From these data, strains 160 and 163 were calculated to have the highest concentration of ammonium per OD_{750} and were also calculated as significantly different from wild type. Strain 167 was calculated as the next highest concentration of ammonium per OD_{750}. However, it was not found to be significantly different from wild type. Strain 161, 162, 164, 165 and 166 were calculated as not significantly different from wild type. Overall, I concluded that the strains with the highest chance of success for nitrogen provision to the algal species are strains 160, 163, 165 and 167.

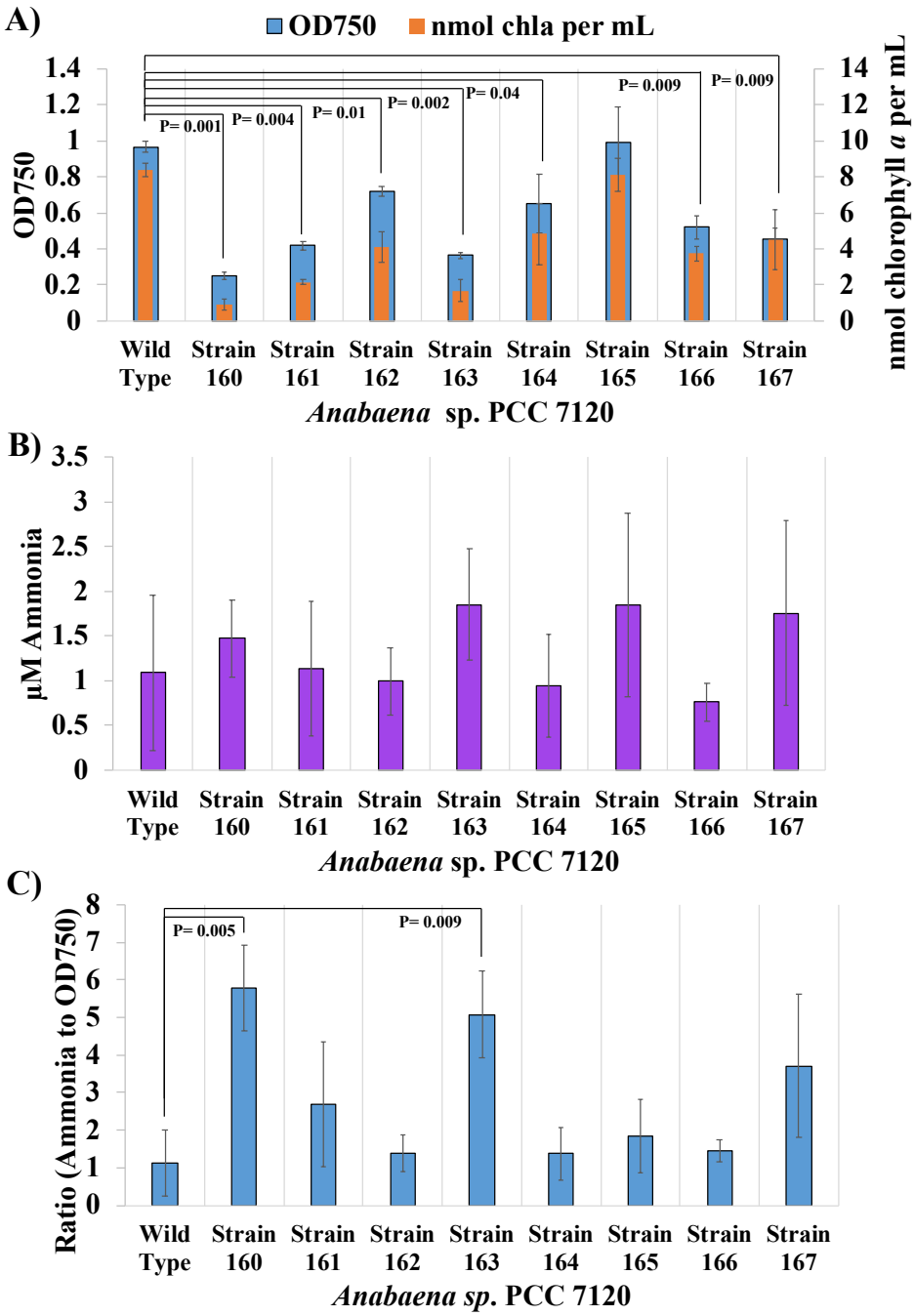

Figure 4.11 Investigating the growth and ammonium production of ammonium-producing strains of *Anabaena* under diazotrophic conditions

A) Measurement of OD_{750} and chlorophyll *a* for *Anabaena* strains. B) Measurement of extracellular ammonium concentration for *Anabaena* strains. C) Ratio of extracellular ammonium concentration per unit of biomass (as measured by OD_{750}). Growth was carried out under standard conditions in BG11(-N) medium with the exception that cultures were bubbled with air (Materials and Methods 2.3) and supplemented with 3 µM $CuSO_4$ and 2.5 µg/mL each of spectinomycin and streptomycin (apart from WT). Error bars display standard deviation from three replicates. Significance from wild type was determined by ANOVA and is illustrated by the connecting lines above the bars.

4.2.6 Tri-partite co-culture of nitrogen providing *Anabaena* strains with *C. reinhardti metE*$^{-}$ and *M. loti*

I cultured nitrogen-providing strains of *Anabaena* (CSX60-R10, and Malatinszky's strains 160, 163, 165, 167) and wild-type *Anabaena* with *C. reinhardtii metE*$^{-}$ + *M. loti* to engineer the first three-way trading mutualism involving the exchange of B_{12}, carbon and nitrogen. Negative controls of *C. reinhardtii metE*$^{-}$ grown on its own and with *M. loti* were also included. Cultures were grown for 8 days, bubbling with air at 25^{o}C, and shaking, after which OD_{750} was measured (Figure 4.12A). I supplemented the cultures with 3 µM $CuSO_4$ but no antibiotics were added. As expected, the *C. reinhardtii metE*$^{-}$ negative control and *C. reinhardtii metE*$^{-}$ + *M. loti* negative co-culture control did not grow well as there was no nitrogen available in the medium (TP^{+}-N). The *Anabaena* wild type co-cultured with *C. reinhardtii metE*$^{-}$ + *M. loti* had the highest OD_{750} and concentration of chlorophyll *a*. *Anabaena* strain 165 co-cultured with *C. reinhardtii metE*$^{-}$ + *M. loti* contained the next highest OD_{750} reading and chlorophyll *a* concentration. *Anabaena* strains 160, 163 and 167 co-cultured with *C. reinhardtii metE*$^{-}$ + *M. loti* showed low growth, with OD_{750} 0.22, 0.23 and 0.38 respectively. Finally, the amino acid-providing *Anabaena* strain CSX60-R10 co-cultured with *C. reinhardtii metE*$^{-}$ and *M. loti* also showed very little growth. I concluded that, of the tri-cultures with nitrogen-providing strains, the tri-culture of *C. reinhardtii metE*$^{-}$ and *M. loti* with ammonium-providing *Anabaena* strain 165 had the highest growth as measured by OD_{750}.

Figure 4.12B shows populations of *M. loti* in the cultures that were counted via colony forming units after 8 days of growth. The negative control containing only *C. reinhardtii metE*$^{-}$ showed no *M. loti* CFUs, which was expected. The negative co-culture control (*C. reinhardtii metE*$^{-}$ + *M. loti*) identified approximately 2.0 x10^{7} CFUs per mL. When *Anabaena* wild type and nitrogen-providing *Anabaena* strains were cultured with *C. reinhardtii metE*$^{-}$ and *M. loti*, *M. loti* populations were fairly similar between approximately 7.0-8.8 x10^{7} CFUs per mL. I concluded that *M. loti* CFUs per mL were not significantly different for *C. reinhardtii metE*$^{-}$ and *M. loti* cultured with wild type *Anabaena* versus the nitrogen providing strains of *Anabaena.*

Figure 4.12C shows the population of *C. reinhardtii metE*$^{-}$ counted by haemocytometer. As expected the *C. reinhardtii metE*$^{-}$ negative control had the lowest cell population. The *C. reinhardtii metE*$^{-}$ and *M. loti* negative co-culture control had the highest population of *C. reinhardtii metE*$^{-}$ cells. Finally, the population of *C. reinhardtii metE*$^{-}$ was

similar when any of the *Anabaena* species was inoculated with the co-culture, giving approximately 6.8-8.3 x10^4 cells per mL. I concluded that *C. reinhardtii metE*$^-$ cell counts per mL were not significantly different for *C. reinhardtii metE*$^-$ + *M. loti* cultured with wild type *Anabaena* versus the nitrogen providing strains of *Anabaena,* and unexpectedly the co-culture of *C. reinhardtii metE*$^-$ + *M. loti* (i.e. without additional *Anabaena* cells) had the highest counts of *C. reinhardtii metE*$^-$ cells per mL. However, I hypothesised this to be due to the death of *M. loti* and consequent release of nitrogenous compounds into the medium for *C. reinhardtii metE*$^-$ to utilize and therefore, would not be sustainable in the long term.

To estimate the proportion of *C. reinhardtii metE*$^-$ in these tri-cultures as a fraction of the total biomass, I divided the total *C. reinhardtii* cells per mL by the OD_{750}. Figure 4.12D shows the ratio of *C. reinhardtii metE*$^-$ cells per mL per OD_{750}. The negative co-culture control (*C. reinhardtii metE*$^-$ and *M. loti* only) showed the highest value at approximately 1.5 x10^6 cells per mL per OD_{750}. The value for the negative control (*C. reinhardtii metE*$^-$ only) was similar to when *C. reinhardtii metE*$^-$ and *M. loti* were co-cultured with *Anabaena* strain CSX60-R10, 160, 163 or 167 at approximately 3.0-3.7 x10^5 cells per mL per OD_{750} and the latter four values were calculated as significantly higher than the wild type control via ANOVA. The value was lowest when the *Anabaena* wild type or strain 165 was cultured with *C. reinhardtii metE*$^-$ and *M. loti*, between approximately 5.0-6.6 x10^4 cells per mL per OD_{750}. I concluded that *C. reinhardtii metE*$^-$ and *M. loti* co-cultured with either *Anabaena* strain CSX60-R10, 160, 163 or 167 had the highest ratio of *C. reinhardtii metE*$^-$ cells per mL to OD_{750}. Overall, I concluded that culturing nitrogen-producing strains of *Anabaena* proved no more beneficial to the growth of *C. reinhardtii metE*$^-$ and *M. loti* than culturing with the wild type *Anabaena.*

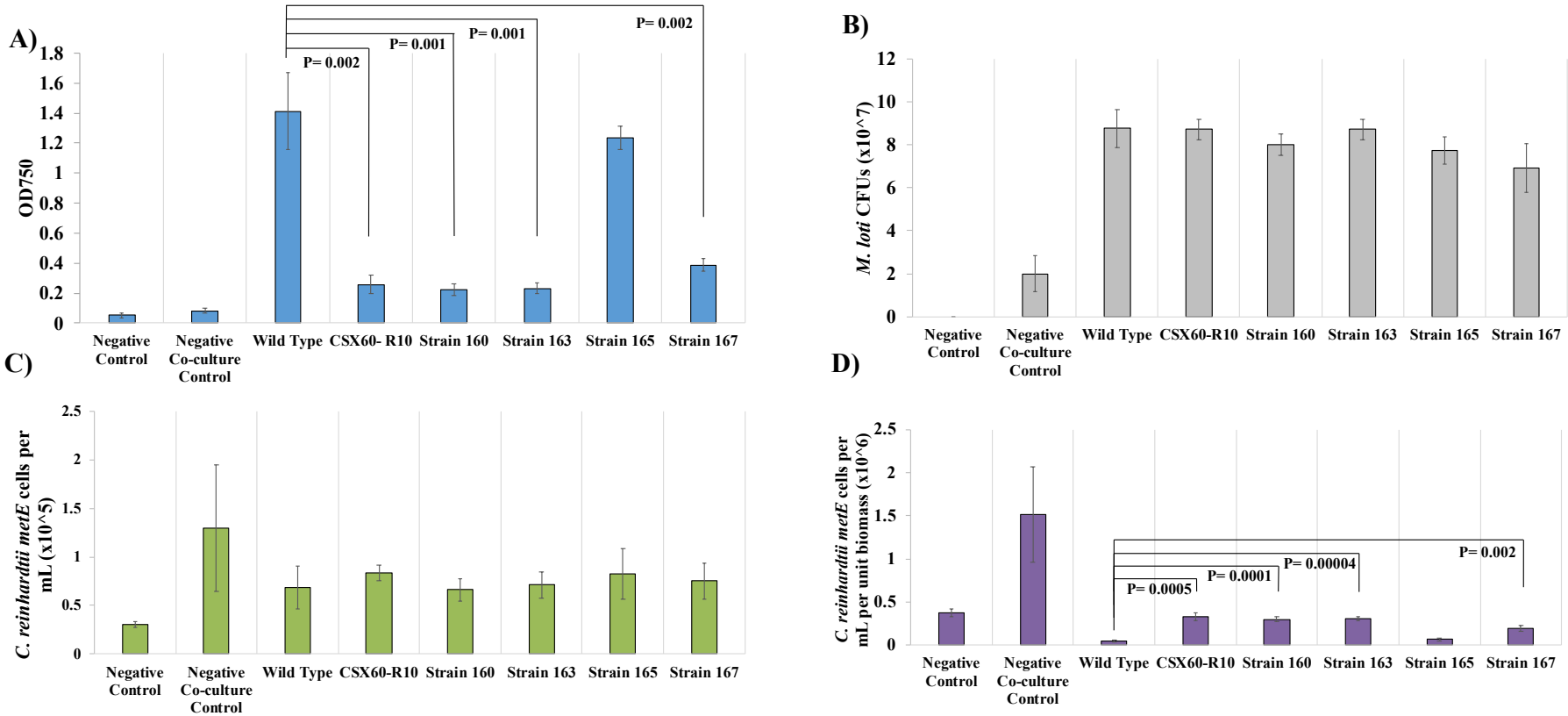

Figure 4.12 Investigating the potential of engineered strains of *Anabaena* to provide nitrogen to *C. reinhardtii metE⁻* and *M. loti*

A) Measurement of OD_{750} and chlorophyll *a*. B) Measurement of *M. loti* CFUs per mL. C) Measurement of *C. reinhardtii metE⁻* cells per mL. D) Ratio of *C. reinhardtii metE⁻* cells per mL per OD_{750}. Cultures were bubbled with air in TP^{+}(-N) for 8 days and supplemented with 3 µM $CuSO_4$. No antibiotics were added in this experiment. Negative control contained *C. reinhardtii metE⁻* only. Negative co-culture control contained *C. reinhardtii metE⁻* and *M. loti*. Error bars display standard deviation from three replicates. Significance from wild type was determined by ANOVA and is illustrated by the connecting lines above the bars.

4.2.7 Co-culture of nitrogen providing *Anabaena* strains with *Ca. vulgaris*

In the previous experiment, *Anabaena* was cultured with *C. reinhardtii metE*$^{-}$ and *M. loti* in TP^{+}-N. However, I identified in Figure 4.5 that TP^{+} is not the ideal medium in which to culture *Anabaena* and that it could lead to poor rates of nitrogen provision. Therefore, I carried out an investigation to test whether the nitrogen-providing *Anabaena* strains could promote the growth of algal strains in BG11 medium. I chose *Ca. vulgaris* for these experiments as it had previously been shown to grow well in BG11 (Feng et al., 2011; Belotti et al., 2014). *Ca. vulgaris* was grown in standard BG11 containing $NaNO_3$ as a nitrogen source for a positive control, and in BG11-N as a negative control. The experimental cultures had *Ca. vulgaris* co-cultured with wild type *Anabaena* or one of strains CSX60-R10, 160, 163, 165 and 167. The cultures were grown under standard conditions for 8 days in BG11-N, bubbled with air and supplemented with 3 µM $CuSO_4$. I supplemented CSX60-R10 with 5 µg/mL each of spectinomycin, streptomycin and 40 µg/mL of neomycin. Ammonium releasing *Anabaena* strains were supplemented with 2.5 µg/mL each of spectinomycin and streptomycin. WT was not supplemented with antibiotics. Figure 4.13A shows the growth of the cultures as measured by OD_{750} and Figure 4.13B shows the growth of the cultures as measured by *Ca. vulgaris* CFUs per mL. The negative control had poor growth at OD_{750} and low numbers of *Ca. vulgaris* CFUs per mL, which was expected. The positive control had high growth at OD_{750} and high numbers of *Ca. vulgaris* CFUs per mL, which was also expected. *Ca. vulgaris* co-cultured with wild type *Anabaena* gave OD_{750} 2.1 but low numbers of *Ca. vulgaris* CFUs per mL. When *Ca. vulgaris* was co-cultured with *Anabaena* strain CSX60-R10 there was low growth, reaching OD_{750} 0.16 and low numbers of *Ca. vulgaris* CFUs per mL. However, the ammonium-providing *Anabaena* strains 160, 163, 165 and 167 cultured with *Ca. vulgaris* gave OD_{750} measurements of approximately 1.2, 2.6, 2.8 and 1.6 respectively. Furthermore, the ammonium-providing *Anabaena* strains 160, 163, 165 and 167 promoted the growth of *Ca. vulgaris* to approximately 4.4 x10^7, 7.7 x10^7, 6.2 x10^6 and 3.2 x10^7 CFUs per mL. I concluded that the ammonium-producing *Anabaena* strain 163 promoted the highest growth of *Ca. vulgaris* under the conditions tested.

To understand the proportion of biomass due to *Ca. vulgaris* in these co-cultures, I divided the *Ca. vulgaris* CFUs per mL by the OD_{750}. Figure 4.13C shows the values of *Ca. vulgaris* CFUs per mL per OD_{750}. The negative control (*Ca. vulgaris* grown in BG11-N) had a

low value of 2.0 x10^6 CFUs per mL per OD_{750}. It is important to note that *Ca. vulgaris* populations were measured by CFUs and so only those cells that were viable were counted. Therefore, I hypothesised that the low value of the negative control was due to low algal cell viability as a consequence of growth in nitrogen-deficient medium. The positive control (*Ca. vulgaris* grown in standard BG11 containing $NaNO_3$ as a nitrogen source) showed a high value of 4.2 x10^7 CFUs per mL per OD_{750} which was expected. *Ca. vulgaris* co-cultured with wild type *Anabaena* had the lowest value of 2.0 x10^5 CFUs per mL per OD_{750}. *Ca. vulgaris* co-cultured with the amino acid–producing strain CSX60-R10 had a value of 7.0 x10^6 CFUs per mL per OD_{750} which was significantly different from wild type. Finally, cultivation with the ammonium-producing strains 160, 163, 165 and 167 gave values of 3.5 x10^7, 3.0 x10^7, 2.0 x10^6 and 1.9 x10^7 CFUs per mL per OD_{750} which were also calculated to be significantly different from the wild type. I concluded that *Ca. vulgaris* co-cultured with *Anabaena* strain 160 had the highest ratio of *Ca. vulgaris* CFUs per mL to OD_{750}.

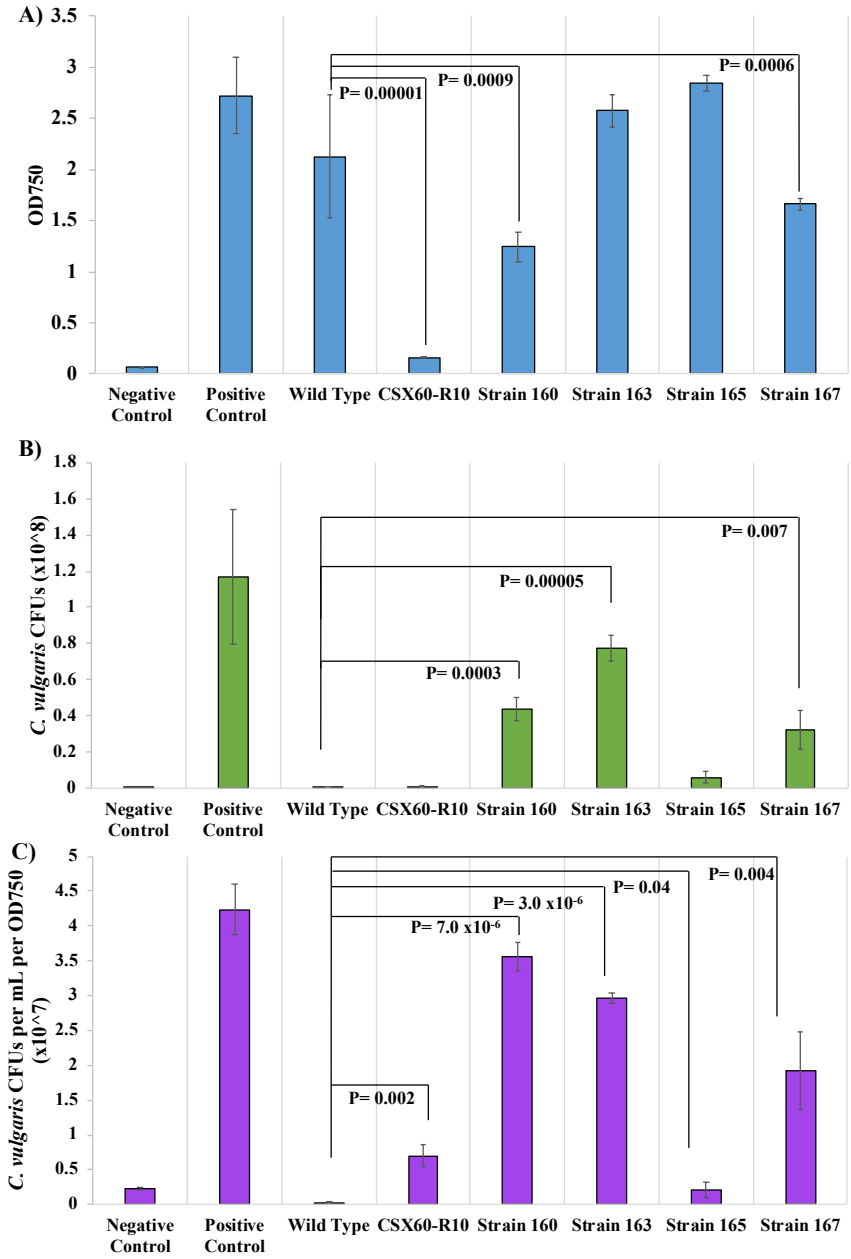

Figure 4.13 Investigating the potential of engineered strains of *Anabaena* to provide nitrogen to *Ca. vulgaris.*

A) Measurement of OD_{750}. B) Measurement of *Ca. vulgaris* CFUs per mL. C) Ratio of *Ca. vulgaris* CFUs per mL per OD_{750}. Cultures were bubbled with air in BG11(-N) for 8 days and supplemented with 3 µM $CuSO_4$. CSX60-R10 were supplemented with 5 µg/mL each of spectinomycin, streptomycin and 40 µg/mL of neomycin. Ammonium releasing *Anabaena* strains were supplemented with 2.5 µg/mL each of spectinomycin and streptomycin. WT was not supplemented with antibiotics. Negative control contained *Ca. vulgaris* only. Positive control contained *Ca. vulgaris* grown in standard BG11 (containing $NaNO_3$). Error bars display standard deviation from three replicates. Significance from wild type was determined by ANOVA and is illustrated by the connecting lines above the bars.

4.3 Discussion

In this chapter, I identified the appropriate conditions and techniques to study a co-culture of nitrogen-providing strains of *Anabaena* with eukaryotic algal species. The first step was to identify the ideal medium in which to culture the different species to ensure the highest growth rate (Figure 4.5). I made attempts to identify a technique to quantify the populations of all the species in the cultures including *Anabaena*, including the use of sonication and filtration (Figure 4.6 and 4.7). *C. reinhardtii metE⁻*, *C. reinhardtii metE⁻* + *M. loti* and *M. loti* were grown with the amino acids released by *Anabaena* strain CSX60-R10 to confirm the organisms were capable of metabolising them as a nitrogen source (Figure 4.8). The growth of *Anabaena* strain CSX60-R10 was measured under diazotrophic conditions (Figure 4.9). The growth of putative ammonium-releasing *Anabaena* strains was measured under diazotrophic conditions and the concentrations of ammonium ions in the supernatant were measured. Furthermore, I calculated the ratios of ammonium concentration (μM) per unit biomass (OD_{750}) (Figure 4.11). A tripartite culture involving the nitrogen releasing strains and *C. reinhardtii metE⁻* and *M. loti* was established in nitrogen free TP^+ medium (Figure 4.12). Finally, I established a co-culture of the nitrogen-producing *Anabaena* strains and *Ca. vulgaris* in nitrogen-free BG11 medium (Figure 4.13).

4.3.1 Identifying the optimal medium for growth

TP^+ medium has previously been used to grow *C. reinhardtii metE⁻* and the co-culture involving *C. reinhardtii metE⁻* + *M. loti* (Helliwell et al., 2014). In contrast, *Anabaena* is typically grown in BG11, which is quite different from TP^+ in its ingredients (Rippka et al., 1979) (Table 4.3) (Appendix 8.1). An investigation was carried out to determine the growth rates of each of the organisms in the different media options. Included in this investigation was a medium with a 50/50 mix of TP^+ and BG11 (labelled TP^+/BG11) (Figure 4.5A). I identified the optimal medium for a co-culture of *Anabaena*, *C. reinhardtii metE⁻* and *M. loti* as TP^+.

As outlined in section 4.2.1, a reduction in the growth rate of *Anabaena* was measured in TP^+ medium as well as TP^+/BG11 medium (Figure 4.5A). I hypothesised the different nitrogen sources could be the cause for the decline in growth rate as BG11 contains 1.8 mM of $NaNO_3$ whereas TP^+ contains 7.5 mM of NH_4Cl (Table 4.5). Therefore, *Anabaena* was grown in BG11 and TP^+ lacking a source of nitrogen (-N) (Figure 4.5B). However, there was still a

decline in growth rate in TP^+ (-N) relative to growth in BG11 (-N). The pH should not be a factor as the pH values of the two media are similar at approximately pH 7.5 and both are buffered with HEPES. I concluded that NH_4Cl was not a major contributor to the reduced growth rate.

In Figure 4.5A and 4.5B, I found that the growth of *Anabaena* was hindered in TP^+ (in the presence or absence of fixed nitrogen respectively). Furthermore, when *Anabaena* was grown in TP^+/BG11 there was a greater decline in the growth of *Anabaena* (Figure 4.5A), indicating the potential of a reaction between the ingredients of TP^+ and BG11 leading to a further detrimental growth effect on *Anabaena*. I hypothesised there was a missing BG11 ingredient in TP^+ required for optimal growth of *Anabaena*, and that, in addition, some of the ingredients, when combined, reacted in a way which led to a further decline in the growth of *Anabaena*. Therefore, I carried out an experiment in which TP^+ medium was supplemented with ingredients of BG11 separately (Figure 4.5C) (Table 4.3 and Table 4.4). However, no additive proved to rescue the growth rate of *Anabaena* to levels seen with BG11 alone. Furthermore, when supplemented with 100x BG11 stock the growth rate slowed to approximately 4% of that in BG11. The only ingredients in 100x BG11 stock that are not found in TP^+, are $NaNO_3$ and citric acid. The addition of one or both ingredients could react with an ingredient of TP^+ in a way that negatively affects the growth of *Anabaena*. When supplementing TP^+ with carbonate stocks, $NaHCO_3$ and Na_2CO_3, the growth rate decreased to approximately 2.5% of the BG11 growth rate, potentially highlighting further BG11 ingredients affecting the growth of *Anabaena*. Growth of *Anabaena* in TP^+ medium supplemented with BG11 phosphate was not carried out because the concentration of phosphate in BG11 is low in comparison with the phosphate concentrations in TP^+ (Table 4.3) (BG11 contains K_2HPO_4 at a concentration of 0.018 mM whereas TP^+ contains K_2HPO_4 and KH_2HPO_4 at concentrations of 4.1 mM and 2.7 mM respectively). Nonetheless, it cannot be discounted that high concentrations of phosphate could be a reason for the poor growth of *Anabaena* seen in TP^+. Overall, no BG11 ingredients could rescue the growth of *Anabaena* in TP^+ medium. In contrast, the addition of $NaNO_3$, citric acid, $NaHCO_3$ and Na_2CO_3 led to a major decline in growth rate suggesting some or all of these additives could be reacting with the ingredients of TP^+ leading to negative consequences for growth of *Anabaena*. I concluded, the original TP^+ medium remained the optimal growth medium for the co-culture of *Anabaena*, *C. reinhardtii metE*$^-$ and *M. loti.*

Table 4.3 BG11 and TP$^+$ ingredients and their concentrations

Nutrient	BG11 (concentration)	BG11 stock	TP$^+$ (concentration)	TP$^+$ Stock
Nitrogen	$NaNO_3$ (1.8 mM)	100x BG11	NH_4Cl (7.5 mM)	TP$^+$ Salts
Magnesium	$MgSO_4$ (0.06 mM)	100x BG11	$MgSO_4$ (0.8 mM)	TP$^+$ Salts
Calcium	$CaCl_2$ (0.2 mM)	100x BG11	$CaCl_2$ (0.3 mM)	TP$^+$ Salts
Citric Acid	Citric Acid (0.03 mM)	100x BG11	Nil	N/A
EDTA	Na_2 EDTA (0.03 mM)	100x BG11	Na_2 EDTA (0.1 mM)	TP$^+$ Trace Elements (Hutners)
Boric Acid	H_3BO_3 (0.005 mM)	BG11 Trace elements	H_3BO_4 (0.1 mM)	TP$^+$ Trace Elements (Hutners)
Manganese	MnCl (0.0009 mM)	BG11 Trace elements	$MnCl_2$ (0.03 mM)	TP$^+$ Trace Elements (Hutners)
Zinc	$ZnSO_4$ (0.0001 mM)	BG11 Trace elements	Nil	N/A
Molybdate	Na_2MoO_4 (9 x10^{-5} mM)	BG11 Trace elements	$(NH_4)MoO_4$ (0.005 mM)	TP$^+$ Trace Elements (Hutners)
Copper	$CuSO_4$ (5 x10^{-5} mM)	BG11 Trace elements	$CuSO_4$ (0.009 mM)	TP$^+$ Trace Elements (Hutners)
Cobalt	$CoNO_3$ (2 x10^{-5} mM)	BG11 Trace elements	$CoCl_2$ (0.012 mM)	TP$^+$ Trace Elements (Hutners)
Iron	$(NH_4)_5[Fe(C_6H_4O_7)_2]$ (0.004 mM)	BG11 Iron	$FeSO_4$ (0.03 mM)	TP$^+$ Trace Elements (Hutners)
Phosphate	K_2HPO_4 (0.02 mM)	BG11 Phosphate	K_2HPO_4 (4.1mM) KH_2HPO_4 (2.7 mM)	TP$^+$ Phosphate
Carbonate	Na_2CO_3 (0.02 mM) $NaHCO_3$ (1 mM)	BG11 Carbonate	Nil	N/A

Red font indicates the absence of a nutrient in TP$^+$. Blue font indicates nutrients that have substantial differences in their concentrations between TP$^+$ and BG11.

When *Anabaena* was grown in TP$^+$ supplemented with BG11 ingredients, I observed the cultures had changed their colour (Figure 4.5D) (Table 4.4). *Anabaena* grown in TP$^+$ (ii), TP$^+$ and BG11 trace elements (iii) and TP$^+$ and BG11 iron (iv) were blue in colour. This could be due to an increase in phycobiliprotein or alternatively, a decrease in chlorophyll *a* concentration. *Anabaena* grown in TP$^+$ and 100x BG11 (v) were a lighter shade of blue which

could be due to there being lower concentrations of biomass. *Anabaena* grown in TP^+ and BG11 carbonate ($NaHCO_3$, Na_2CO_3) were green in colour (vi), more so than the wild type, which could be due to reduction in the amounts of phycobiliprotein. This suggests TP^+ medium is inducing some element of stress in the *Anabaena* cultures affecting their photosynthetic machinery. I noted that although culturing *Anabaena* in TP^+ was optimal (apart from in BG11) in terms of growth rates, it could lead to less desirable results in terms of nitrogen provision than expected due to the stress indicated by the observed colour change of the culture.

Table 4.4 Summary of the experiment outlined in Figure 4.5C and D

Culture number	Media	Supplement	Relative growth rate (%)	Colour
i)	BG11	N/A	100%	Blue-Green
ii)	TP^+	N/A	70%	Blue
iii)	TP^+	BG11 Trace elements	60%	Blue
iv)	TP^+	BG11 Iron	69%	Blue
v)	TP^+	100x BG11	3.8%	Light Blue
vi)	TP^+	BG11 Carbonate	2.4%	Green

To maximise the productivity and potential industrial success of the engineered nitrogen providing *Anabaena,* I carried out a literature survey to identify a commercially interesting eukaryotic algal species capable of growing in BG11. I found in several publications that *Ca. vulgaris*, an established industrially-grown algal strain, was capable of growth in BG11 (Feng et al., 2011; Belotti et al., 2014). I compared the growth rates of *Ca. vulgaris* in TP^+, TP^+/BG11 and BG11 and I found the ideal medium was TP^+ with the growth rate in BG11 dropping to 75% to that of TP^+. This is similar to the decline in growth rate of *Anabaena* in TP^+ (also approximately 75%). This left a choice between growing the *Anabaena* - *Ca. vulgaris* co-culture in TP^+ with a decline in efficiency of *Anabaena* or growth in BG11 with a decline in efficiency of *Ca. vulgaris*. I chose BG11 for two reasons: 1) nitrogen is likely to be the limiting nutrient for growth of *Ca. vulgaris*, so maximising the health and productivity

of *Anabaena* and therefore nitrogen release should be of primary importance and 2) BG11 is an established medium used for the growth of *Ca. vulgaris* in several research papers.

4.3.2 Identifying techniques to measure the engineered consortia

When quantifying populations of species grown in co-culture it is important to understand the population dynamics of the consortium. Potential options to quantify the populations included the use of flow cytometry or a cell quantifier such as a Coulter counter. Flow cytometry is particularly useful as it can separate cells based on size, granularity and fluorescence (Davey et al., 1996). A Coulter counter can also quantify cells and differentiate single organisms in a consortium by cell size (Krediet et al., 2015). However, because *Anabaena* sp. PCC 7120 grows in filaments they cannot be directly measured in the flow cytometer as the filaments could obstruct the equipment leading to inaccurate readings and possible equipment failure. This is also problematic for cell quantification carried out in a Coulter counter as the filaments block the aperture and hence prevent cell counting. To circumvent this problem, I attempted to make use of sonication to break apart the filaments. Optimal settings were found for both bath and probe sonication (Figure 4.5A and B). However, I found that sonication also affected the cell counts of the eukaryotic algae, in this case *C. reinhardtii metE⁻,* and might have also led to the loss of some *Anabaena* cells. Therefore, sonication was not an ideal method to measure cell populations accurately. An alternative option I tested to remove the filaments was filtration. Two 25 μm filters were tested, stainless metal mesh and polypropylene. However, these were unable to separate all the filaments from the culture.

Alternative, more simple options to determine cell populations of *Anabaena*, *C. reinhardtii*, and *M. loti* in a tripartite culture included the use of CFUs, haemocytometer and OD_{750}. *M. loti* could be quantified through CFUs plated on TY agar (Materials and Methods 2.3.1.3) and *C. reinhardtii* could be quantified using a haemocytometer as the cells were large enough to differentiate from *Anabaena* cells (single or in filaments). In the *Anabaena – Ca. vulgaris* co-culture, the use of haemocytometer was not possible as *Anabaena* cells were similar in size to *Ca. vulgaris*. Instead *Ca. vulgaris* CFUs were counted from TAP agar plates inoculated with ampicillin (100 μg/mL). Total biomass for the engineered consortia was estimated through OD_{750}.

4.3.3 Nitrogen provision by amino acids

Fundamental studies on amino acids transporters in *Anabaena* led to the discovery of a strain that secretes into the supernatant alanine to a concentration of 100 μM and isoleucine, leucine and phenylalanine at approximately 18 μM each after 48 hours of growth (cell concentrations were not published) (Pernil et al., 2008). This strain was named CSX60-R10 and contains two gene knockouts, *bgtA* and *natA*, that lead to the loss of function of the N-I and N-II/Bgt transporters (Table 4.1). The strain was a starting point to test the potential of nitrogen provision by amino acid secretion.

I carried out an investigation to determine if the *C. reinhardtii metE*$^{-}$ and *M. loti* could utilise the secreted amino acids as a nitrogen source (Figure 4.8A). I found the *C. reinhardtii metE*$^{-}$ could metabolise the amino acids. However, the *C. reinhardtii metE – M. loti* co-culture was less efficient at utilizing the amino acids based on OD_{750} readings (Figure 4.8B). *M. loti* could also utilize the amino acids as shown by the doubling of growth compared with the negative control (Figure 4.8C). Compared with the positive control, the cultures grown with the amino acids reached slow growth phase at a lower population (i.e. lower carrying capacity). This is likely to be due to the differences in total nitrogen availability, as 1.9 mM nitrogen is present in standard TP^{+} medium (positive control) in contrast to the amino acid supplemented culture where only 155 μM nitrogen is available (this represents the total molar concentration of nitrogen present in alanine, isoleucine, leucine and phenylalanine) measured after 48 hours of growth by the CSX60-R10 strain (Pernil et al., 2008). Therefore, these experiments provided insight into the potential growth of *C. reinhardtii metE*$^{-}$ and *M. loti* after 48 hours of co-cultivation with CSX60-R10.

I carried out an experiment to compare the growth of CSX60-R10 in BG11 vs TP^{+} under diazotrophic conditions. I found the strain grew poorly in both BG11 and TP^{+} lacking nitrogen (Figure 4.9A). The strains grown in diazotrophic conditions were yellow after 4 days (Figure 4.9B). Spectroscopic analysis of the cultures showed a loss in the phycocyanin peak at OD_{630} nm and a decline in the chlorophyll *a* peak at OD_{680} compared to the positive control (Figure 4.9C). This decline in the absorption peaks is likely to be due to turnover of the phycobilisomes and also chlorophyll (and probably the associated proteins. Turnover of these proteins is a well-known consequence of nitrogen starvation (Richaud et al., 2001). It was noticed that there were fewer filaments in the CSX60-R10 strain than the wild type and the

number of filaments decreased further when bubbled. This was consistent with the observation that the ratio of single cells to filaments increased in the CSX60-R10 strain compared to that of WT when grown in diazotrophic conditions (note: all measurements were taken when cell densities were standardized to OD_{750} 0.8) (Figure 4.9D). This could be due to 1) a result of the stress caused by nutrient deprivation, or 2) bubbling leading to an increase in mechanical stress. Nonetheless co-cultivation of the CSX60-R10 strain with *C. reinhardtii metE⁻* + *M. loti*, was performed. As expected, the growth of the triculture was poor, reaching only OD_{750} 0.257 after 8 days of growth (Figure 4.12A). This could be due to the poor growth of CSX60-R10 measured in Figure 4.9A. The *M. loti* CFUs per mL reached 8.7 $x10^7$, which was not significantly different from the control of growth with wild type *Anabaena* (Figure 4.12B). The *C. reinhardtii metE⁻* cell numbers were not significantly different from the wild type control (Figure 4.12C). CSX60-R10 was also co-cultured with *Ca. vulgaris*, and the results also showed that the growth of *Ca. vulgaris* with this strain was no different from the wild type control (Figure 4.13B).

Given the low efficiency of growth and inability to provide adequate nitrogen to consortium members, the practical use for the CSX60-R10 strain in industry would be limited. More moderate mutants shown to lead to accumulation of a lower concentration of amino acids that were described by the same group (Enrique Flores, University of Seville, Spain) such as CSX60 (which has a knockout of *natA*) were unavailable as the mutant stocks were no longer viable (Enrique Flores, personal communication). I made attempts to re-engineer the more moderate amino acid releasing strains but were unsuccessful in the time available.

4.3.4 Nitrogen provision via ammonium

An alternative to utilizing the provision of amino acids as a nitrogen source is the provision of ammonium. Ammonium is beneficial in that it does not contain carbon, unlike amino acids, which might promote contamination when released into an open culture. David Malatinszky of Dr. Patrik Jones' lab at Imperial College London designed and engineered strains of *Anabaena* that expressed the gene encoding GS Inactivation Factor (*gifA*) fused to four separate promoters (P*nifHDK*, P*gifA*, P*rbcLS* and P*petE*) on a self-replicating plasmid. The plasmids were transformed into either a wild type strain or a Δ*amt* strain containing the deletion of the *amt* gene cluster which includes three genes *amt4* (alr0990), *amt1* (alr0991) and *amtB* (alr0992) (Table 4.2) (Figure 4.10).

In this chapter, these strains were tested for their biomass yield and concentration of chlorophyll *a*. In addition, the total ammonium concentrations in the supernatant were also measured. Strains 163, 165 and 167 showed the greatest total ammonium concentration at approximately 1.9 μM (although was calculated as non-significant compared with WT by ANOVA) (Figure 4.11B). However, when considering the ratio of ammonium to OD_{750}, strain 160 became a high performer along with strain 163 (Figure 4.11C). The next highest ammonium per OD_{750} was with strain 167. However, this was not calculated to be significantly different from the wild type strain. Even though strain 165 released a relatively high concentration of ammonium into the supernatant, its ratio of ammonium per OD_{750} was similar to that of the wild type. This could be due to the relatively high level of cell growth measured of strain 165 compared to strains 160 and 163. The ammonium concentrations measured in the supernatant by these strains are consistent with the fact they contain promoters that are active in the heterocyst. Strain 160 and 163 have *gifA* fused to a nitrogenase promoter which is active in the heterocysts under diazotrophic conditions, and strains 165 and 167 contain a promoter from the plastocyanin gene (*petE*) that is induced by the presence of copper. Therefore, these active promoters would be expected to lead to a down regulation of glutamine synthetase and consequent accumulation of ammonium by the nitrogenase enzyme. There was a slight difference in the accumulation of ammonium in the supernatant between the strains with background wild type or Δ*amt.* For example, strains 160 (wild type) and 163 (Δ*amt*) released ammonium to a concentration of approximately 1.5 and 1.9 μM respectively, which is consistent with the expectation that strain 163 should be less efficient at taking up external ammonium. However, for strains 165 (wild type) and 167 (Δ*amt*), the difference between the wild type and Δ*amt* strains was only observed when ammonium per unit OD_{750} was calculated as strain 167 had a higher ratio compared to strain 165. The low ammonium production for control strains 161, 164, 162 and 166 was expected. Strains 161 and 164 contain the native *gifA* promoter which is active only when abundant fixed nitrogen is present. The *rbcLS* promoter in strains 162 and 166 is expressed only in the vegetative cells and so the GS inactivation factor 7 (GifA) would have no effect on glutamine synthetase, which is found in the heterocysts. Nonetheless, these strains performed their function as controls, highlighting the efficacy of the underlying theory of constitutively expressing *gifA* to encourage the release of fixed nitrogen.

Due to time and incubator space constraints with bubbled cultures, only the highest performing ammonium-releasing strains 160, 163, 165 and 167 were taken forward to co-culture with *C. reinhardtii metE⁻* and *M. loti.* When the strains were cultured with *C. reinhardtii metE⁻* and *M. loti* in TP^+ (-N), there was no difference in growth of either *M. loti* or *C. reinhardtii metE⁻* compared to the *Anabaena* WT tri-culture control in terms of total *M. loti* CFU counts or *C. reinhardtii metE⁻* cell counts (Figure 4.12B and C). Based on the total *C. reinhardtii metE⁻* cells per OD_{750}, strains 160, 163 and 167 promoted higher growth than the wild type (Figure 4.12D). However, because total *C. reinhardtii metE⁻* growth was found to be similar when cultured with the ammonium providing *Anabaena* strains as well as the wild type, I concluded that co-culturing these *Anabaena* strains did not provide any additional growth benefit for the *C. reinhardtii* element of the *C. reinhardtii metE⁻* + *M. loti* consortium. It should be noted that it was not possible to select for the presence of the RSF1010-based plasmid in these experiments using antibiotics, as these would have detrimental effects on the other organisms. However, the plasmid would be expected to be retained over the time period of the experiment (D. Malatinszky, personal communication). In addition, a similar growth trend (as measured by OD_{750}) was observed in Figure 4.11 (containing antibiotics for putative nitrogen-releasing strains) and Figure 4.12 (antibiotic-free). This observation would not be expected if the plasmids had been lost.

The *Anabaena* strains were co-cultured with *Ca. vulgaris* in BG11 (-N) medium (Figure 4.13). All four ammonium-producing strains promoted the growth of *Ca. vulgaris* when compared to the WT co-culture. However, the most notable was strain 163, producing 7.7 $x10^7$ *Ca. culgaris* CFUs per mL (P=0.00005 compared to wild type). This equates to approximately 114 times the growth of the wild type control and 66% of the positive control in which a nitrogen source ($NaNO_3$) was provided. Based on the ratio of *Ca. vulgaris* CFUs per mL to OD_{750}, strain 160 produced the highest co-culture ratio with 3.5 $x10^7$ CFUs per mL to OD_{750}. This suggests *Ca. vulgaris* takes up a higher proportion of the biomass in the co-culture with strain 160.

4.3.5 Understanding the difference between culturing nitrogen-providing organisms with *C. reinhardtii metE⁻* + *M. loti* versus *Ca. vulgaris*

There is a clear difference in the provision of nitrogen to *C. reinhardtii metE⁻* + *M. loti* versus *Ca. vulgaris.* None of the nitrogen-providing strains of *Anabaena* promoted the growth

of *C. reinhardtii metE⁻* + *M. loti* any more than when *C. reinhardtii metE⁻* + *M. loti* was cultured with wild type *Anabaena* (Figure 4.12). Nonetheless, a greater overall OD_{750} was measured when *C. reinhardtii metE⁻* + *M. loti* was cultured with any strain of *Anabaena* than without (Figure 4.12A). This OD_{750} reading was likely to be due to the individual growth characteristics of the *Anabaena* strains and not due to *C. reinhardtii metE⁻* or *M. loti* as their populations were approximately equal whether grown with wild type or engineered *Anabaena* (Figure 4.12B and C). It is interesting that none of the engineered *Anabaena* strains promoted the growth of *C. reinhardtii metE⁻* over wild type *Anabaena*. I hypothesise this is unlikely to be due to loss of the plasmid because the same relative OD_{750} measures (which were largely due to the *Anabaena* – see above) for wild type, strain 160 and strain 165 (both of which have their plasmids in a wild type background) were seen whether the plasmids were selected directly (Figure 4.11A) or not (Figure 4.12A). Similarly, the OD_{750} value for strain 163 compared with strain 167 (which differ only in which plasmid they have, and not the delta *amt* background) was similar whether the plasmids were selected (Figure 4.11A) or not (Figure 4.12A). Further discussion is made below as to why the engineered *Anabaena* strains failed to provide nitrogen to the *C. reinhardtii metE⁻* + *M. loti* co-culture.

For the co-cultures containing *Ca. vulgaris*, clear increases from the negative control were again seen in overall OD_{750} with most of the *Anabaena* strains (with the possible exception of CSX60-R10) (Figure 4.13A). However, in this case, there were clear differences between the number of *Ca. vulgaris* cells per ml when grown in co-culture with several of the strains, compared with the number of *Ca.vulgaris* cells per ml when grown in co-culture with wild type *Anabaena*. For example, strains 160, 163 and 167 seemed to give a significant increase in the yield of *Ca.vulgaris*. In addition, it should be noted that the growth of *Ca.vulgaris* in the co-cultures would be likely to have been partially inhibited by one or more of the antibiotics used. Although neomycin (for CSX60-R10) at the concentration used probably has little effect on *Ca.vulgaris* growth (Galling & Wolff, 1971) streptomycin might have caused a significant reduction (Qiang et al., 2010; Perales-Vela et al., 2016). Data are not available for the effects of spectinomycin.

The difference between co-culturing ammonium releasing *Anabaena* strains with *C. reinhardtii metE⁻* + *M. loti* versus *Ca. vulgaris* could be due to the media used to grow the cultures. The cultures of *C. reinhardtii metE⁻*, *M. loti* and *Anabaena* were grown in TP^+, whereas the cultures of *Ca. vulgaris* and *Anabaena* were grown in BG11. When *Anabaena* was

grown in TP^+ as well as TP^+ supplemented with BG11 ingredients an interesting observation was made in that the cultures had a reduced growth rate as well as a change of colour and thus appears to be inducing stress in *Anabaena* (as outlined in section 4.2.1 and 4.3.1). It is also possible this stress of growing in TP^+ medium could lead to the rapid loss of the RSF1010 plasmid from the ammonium providing strains of *Anabaena* especially with the absence of antibiotic selection of the plasmids. Overall, the culturing of *Anabaena* in TP^+ seemed to be inducing a detrimental response affecting, at the very least, the photosynthetic machinery, which could then result in lower nitrogen fixation rates.

4.3.6 Other examples of nitrogen provision by bacteria

To the best of my knowledge, the only previous use of an engineered strain of *Anabaena* providing nitrogen to another organism was reported by Chaurasia and Apte (2011). These authors engineered *Anabaena* to secrete ammonium by overexpressing *hetR*, the master regulator of heterocysts. They then co-cultivated the strain with rice (*Oryza sativa*) and found it increased the growth of the root fresh weight and shoot fresh weight by 27% and 48% respectively compared to that of the control with wild-type *Anabaena*.

Several other studies on biological nitrogen provision to microalgae have been carried out with the use of *Azotobacter vinelandii*. Bali et al. (1992) first engineered a strain of *A. vinelandii* lacking a functional NifL, a nitrogen fixation regulatory protein. This led to ammonium being secreted into the medium to a concentration of approximately 6.5 mM. *A. vinelandii* is a heterotrophic organism, and in this case was grown in the presence of 2% sucrose. Based on the accumulation of ammonium in the medium by this mutant, a number of research groups have focused on optimising nitrogen release by *A. vinelandii* to make it a suitable biofertiliser for the growth of both microalgae and higher plants. Table 4.5 summarises reports in the literature of various nitrogen-releasing strains of *A. vinelandii* that have been co-cultured with various microalgae or cyanobacterial species. Co-cultures were tested in both autotrophic and mixotrophic conditions, bubbled or non-bubbled. The data for each study were presented differently (e.g. different starting cell concentrations or measured via OD_{750} versus cell counts) which is a problem for directly comparing the growth rates of the various nitrogen receiving strains. Therefore, to be able to compare the growth rates, the published growth data for the recipient strains were standardised to the maximum doubling time measured in hours

(Materials and Methods 2.3.6 - although it is not clear that exponential growth was occurring in all cases, this is assumed for the purpose of calculating doubling time).

When comparing the growth rates of the nitrogen receiving strains from the literature, the strain that provided the lowest doubling time was *Anabaena* strain 163 tested in this study. The doubling time of *Ca. vulgaris* when co-cultured with strain 163 was 18 hours and 51 hours when *Ca. vulgaris* was co-cultured with wild type *Anabaena*. The next highest growth rate recorded was wild type *A. vinelandii* co-cultured with *Scenedesmus sp. BA032* with a doubling time of 20 hours (Villa et al., 2012). It should be noted that the differences in doubling times could be due to the differences in ammonium uptake and metabolism among the recipient algae species. Nonetheless, *Anabaena* has proved to be an effective provider of fixed nitrogen, which offers the advantage of autotrophic growth rather than heterotrophic growth. The ability of *Anabaena* to fix its own carbon minimises the requirement for carbon supplementation, thus saving costs and minimising the risk of contamination. Indeed, there were examples outlined in Table 4.5 where *A. vinelandii* co-cultures were grown in autotrophic conditions, but *A. vinelandii* cell counts were not measured or published in most of these publications. Consequently, sustainable growth of the co-cultures cannot be assumed. One publication did measure *A. vinelandii* CFUs (Ortiz-Marquez et al., 2012) and it was found that the population of *A. vinelandii* declined over the course of the experiment. It therefore seems likely that *Anabaena* may be preferred as a nitrogen source over *A. vinelandii*.

Table 4.5 Published examples of nitrogen providing strains of bacteria to microalgae or cyanobacteria

Numbers in red indicate the maximum growth rate of the nitrogen receiving strain when co-cultured with a wild type control (unless otherwise stated) in the relevant study. Category in bold represents the work carried out in this study.

Nitrogen providing strain	Characteristic of the nitrogen providing strain	Nitrogen receiving strain	Maximum doubling time (T_d) per hour (hr) of the nitrogen receiving strain Wild type control T_d (hr)	Growth conditions	Reference
A. vinelandii ΔnifL	Ammonium release through the deletion of a nitrogen fixation regulatory protein	*Pseudokirchneriella* sp. strain C1D	53 79	Autotrophic (non-bubbling)	Ortiz-Marquez et al., 2012
A. vinelandii ΔnifL	Ammonium release through the deletion of a nitrogen fixation regulatory protein	*Ca. sorokiniana* strain RP	88 and 43 623 and 130	Autotrophic (non-bubbling and bubbled with air respectively)	Ortiz-Marquez et al., 2012
A.vinelandii wild type	Naturally released siderophores	*Scenedesmus* sp. BA032	20 N/A**	Mixotrophic (non-bubbling)	Villa et al., 2014
A. vinelandii AV6	Ammonium release through a mutation in the ammonium binding site of glutamine synthetase.	*Ca. sorokiniana* strain RP	60 No growth *	Autotrophic (bubbled with air)	Ortiz-Marquez et al., 2014
A. vinelandii AZBB103	Ammonium release through the deletion in an ammonium transporter (*ΔamtB)*	*Ca. sorokiniana* UTEX 1602	26 No growth	Mixotrophic (non-bubbling)	Barney et al., 2015
A. vinelandii ΔnifL	Ammonium release through the deletion of a nitrogen fixation regulatory protein	*Synechococcus elongatus cscB*	T_d not quantifiable 0.075 (OD_{750} mL^{-1} day^{-1})*** T_d not quantifiable No growth (OD_{750} mL^{-1} day^{-1})***	Autotrophic (non-bubbling)	Smith and Francis 2016
A. vinelandii AV11	Ammonium release through an IPTG inducible glutamine synthetase gene .	*S. obliquus* C1S	65 No growth *	Autotrophic (bubbled with air	Ambrosia et al., 2017
***Anabaena* strain 163**	**Ammonium release via the overexpression of *gifA* and deletion of the *amt* cluster (Table 4.2)**	***Ca. vulgaris***	**18** **51**	**Autotrophic (bubbled with air)**	**This study**

*expression under diazotrophic conditions only.

** strain contains the deletion of the *amt* cluster (alr0990, alr0991 and alr0992) involved in ammonium uptake.

*** expression only in the presence of copper (e.g. 3 μM Cu^{2+}).

4.3.7 Conclusions

In this chapter, I have shown the optimal growth medium for the *Anabaena*, *C. reinhardtii metE⁻* and *M. loti* tripartite co-culture to be TP^+, whereas BG11 was identified as optimal for the *Anabaena*, *Ca. vulgaris* co-culture (Figure 4.5). The use of flow cytometry or cell counting (via a Coulter counter) for determining cell populations involving *Anabaena* was not possible. Instead I concluded that measuring populations through OD_{750}, haemocytometer and colony forming units was the best option (Figure 4.6 and 4.7). *C. reinhardtii metE⁻* and *M. loti* were shown to be able to metabolise the amino acids released from the CSX60-R10 strain (Figure 4.8). The growth of the amino acid releasing strain CSX60-R10 was impaired under diazotrophic conditions and the cells appear to have lost their phycocyanin and chlorophyll *a*. Additionally, I found the ratio of single cells to filaments was higher than wild type, a difference which was exaggerated under bubbling conditions (Figure 4.9). Amino acid releasing strain CSX60-R10 did not provide significant ammonium to either the *C. reinhardtii metE⁻ – M. loti* co-culture or *Ca. vulgaris* for the species to grow beyond the wild type control (Figure 4.12 and 4.13). The ammonium releasing strains 160, 163, 165 and 167 were identified as producing the highest extracellular ammonium concentration (Figure 4.11). These strains were not successful in maintaining adequate growth rates for the *C. reinhardtii metE⁻* - *M. loti* co-culture (Figure 4.12). However, they were successful in allowing improved growth of *Ca. vulgaris* in nitrogen-free BG11 medium when compared with growth of *Ca. vulgaris* in the presence of the WT control. Strain 163 was shown to provide the greatest enhancement of *Ca. vulgaris* growth (Figure 4.13). This observation has important implications for the future biotechnological exploitation of *Chlorella*.

5 INVESTIGATING THE PRODUCTIVITY OF SCALING AN ALGAE-BACTERIA CONSORTIUM

5.1 Introduction

5.1.1 An introduction to algae biotechnology

Research on algae (including cyanobacteria) at the small scale has been beneficial for our understanding of algal and plant biology in areas ranging from the evolution, genetics and biochemistry of photosynthesis and metabolism (e.g. Stiller et al., 2014; Lea Smith et al., 2016), to ecological processes such as carbon, nitrogen and phosphorus cycling (e.g. Sayre et al., 2010; Zehr, 2011; Cottingham et al., 2015; Lea-Smith et al., 2015). Algae are also grown at large scale to produce low value goods such as food and animal feed (Becker, 2007; Parvin et al., 2008) or high value compounds such as antioxidants, pigments, fatty acids, cosmetics and nutraceuticals (Borowitzka, 2013).

Historically, algal species such as *Spirulina* have been harvested from natural lakes for human consumption. For example, the Kanembu people harvested the alga (known as Dihé) from Lake Chad and consumed it as a sauce (Kiple and Ornelas, 2000) (Figure 5.1A). Similarly, the Aztec people of modern day Mexico harvested the alga (known as 'Techuitlatl') from Lake Texcoco and consumed it as cakes (Parvin et al., 2008) (Figure 5.1B). More recent algal production has come in the form of photobioreactors (PBRs) and open raceway ponds. These methods allow for an element of control over parameters such as nutrients and additives in the media and the use of selective environments determined by pH and salinity (Craggs et al., 2012; Singh and Sharma, 2012; Juneja et al., 2013). PBRs are enclosed systems, designs for which vary. Typically, arrays of transparent tubes are used to grow the algae or, alternatively, glass walls have also been used (Zedler et al., 2016). Additionally, the light can be provided by either

natural light or artificial light (e.g. LEDs). Open raceway ponds are typically a shallow, closed-loop oval channel, exposed to the air and sunlight, and the culture is mixed with a paddle wheel. PBRs are more efficient for algal growth in the capture of light energy, use of cultivation area and overall productivity. In contrast, open raceway ponds are less expensive with regard to cost of materials and construction, although there is poor gas transfer and a lack of temperature control (Jorquera et al., 2010; Slade and Bauen, 2013). Nevertheless, a limited number of algal species can be grown in raceways.

Figure 5.1 Multiple cultures have harvested algae for consumption.

A) Modern day Kanembu people of Chad drying their algae harvest in the sun. B) Ancient Aztecs harvesting algae from local lakes.

5.1.2 The challenges of algal biotechnology

The growth of algae to produce high value compounds is well-established and the industry is capable of making profit (Borowitzka, 2013). However, to date there are no examples of commercialised algal biofuel production contributing significantly to the supply of liquid fuels, simply due to the costs of production outweighing the value of the fuel. The factors leading to the high costs of algal production for low value commodities include: maintenance of equipment, low algal yields because of poor light penetration, poor gas exchange and contamination, and high energy costs of harvesting and downstream processing of algae (Kazamia et al., 2014).

Commercial cultivation of algae is typically carried out as axenic monocultures (free of other organisms) where maximum sterility is required. This contrasts with nature where

algae typically live intimately with many other organisms (Jaggman and Philipp, 2014). However, maintaining sterility in large facilities is labour-intensive and expensive, especially in open systems such as raceway ponds where algae are exposed directly to the environment. Contaminating organisms can come in the form of bacteria, fungi, algae and even animals such as zooplankton (Kazamia et al., 2012). Whilst PBRs are closed systems they periodically suffer from fouling, and contaminants can be harder to eliminate as it is often necessary to flush out the reactors completely with large volumes of sterilised clean water (Lee, 2001). Options exist to minimise contamination by employing extreme environments such as high pH or high salinity (Wang et al., 2013), but this is not always an option with some species of algae.

5.1.3 The potential application of communities in algal biotechnology

The contrast between growth of algal monocultures in industry and growth in communities in nature has been highlighted previously, and the potential benefits of replicating consortia in algal biotechnology have been discussed (Kazamia et al., 2012). The main advantages proposed include an increase in algal productivity (accrued biomass) of mixed cultures compared with axenic cultures, enhanced crop protection from pathogens and pests due to the competitive exclusion principle, reduction of energy inputs, increased stability of the crop to include population control and resilience to environment perturbations, and, finally, the division of labour between consortium members leading to increased ability to engineer complex and broader metabolic pathways (see section 1.7) (Ortiz-Marquez et al., 2013; Jagmann and Philipp, 2014; Kazamia et al., 2014; Nalley et al., 2014; Pandhal and Noirel, 2014; Smith and Crews, 2014).

Several studies exist that identify an advantage in using communities in large scale algae production. Hena et al. (2015) isolated algal communities from a dairy wastewater plant. They then screened through the species to identify those that were high in lipid production capacity and nutrient removal capacity when grown on the dairy wastewater. Ten of these species were then selected and combined to create a consortium. They showed that this consortium of 10 native algal strains produced the maximum amount of biomass and algal oil in wastewater compared to when the species were grown axenically. Bell et al. (2016) grew *Chlorella vulgaris* in a 200 L open raceway pond and then transferred to a 2000 L raceway pond. DNA was extracted from samples from the culture and used to determine the community

composition. A positive correlation of growth between *Ca. vulgaris* and *Pseudomonas sp.* was seen, leading the authors to suggest a symbiotic relationship between the two organisms. This putative relationship and the use of high pH were suggested as major contributors to the successful growth of the culture. Beyter et al. (2016) measured algal and bacterial composition in a 400,000 L open pond over 350 days and found that high algal diversity was correlated with high and stable algal productivity. Finally, Narwani et al. (2016) grew algae in 10 L tanks and found, in a variable temperature environment, increased diversity had a stabilising effect on biomass production over time. However, increased algal diversity also resulted in a lower mean biomass compared to the growth of the monoculture although the latter fluctuated from higher peaks to lower troughs of biomass.

5.1.4 The *C. reinhardtii metE*$^{-}$ + *M. loti* model consortium

Bacteria produce B_{12} for a variety of enzymes such as B_{12}-dependent reductive dehalogenases, isomerases and methyltransferases. B_{12}-dependent reductive dehalogenases detoxify aromatic and aliphatic chlorinated organics such as chlorinated phenol and chlorinated ethene. B_{12}-dependent isomerases play important roles in fermentation, and B_{12}-dependent methyltransferases play important roles in amino acid metabolism (Banerjee and Ragsdale, 2003). In particular, the amino acid methionine in both bacteria and algae is synthesised with the help of one of two isoforms of the enzyme methionine synthase, METE and METH. METE is a vitamin B_{12} independent isoform whereas METH requires B_{12} as a cofactor in order to function (Croft et al., 2005). *De novo* B_{12} synthesis can occur via two distinct pathways, 1) 'early cobalt insertion' (or anaerobic) pathway and 2) 'late cobalt insertion' (or aerobic) pathway. The pathways are complex involving around 30 genes (Warren et al., 2002) (Figure 5.2). *M. loti* synthesizes B_{12} via the 'late cobalt insertion' (aerobic) pathway. Furthermore, despite the ability to synthesize B_{12}, *M. loti* retains both METE and METH isoforms of methionine synthase (Kaneko et al., 2000).

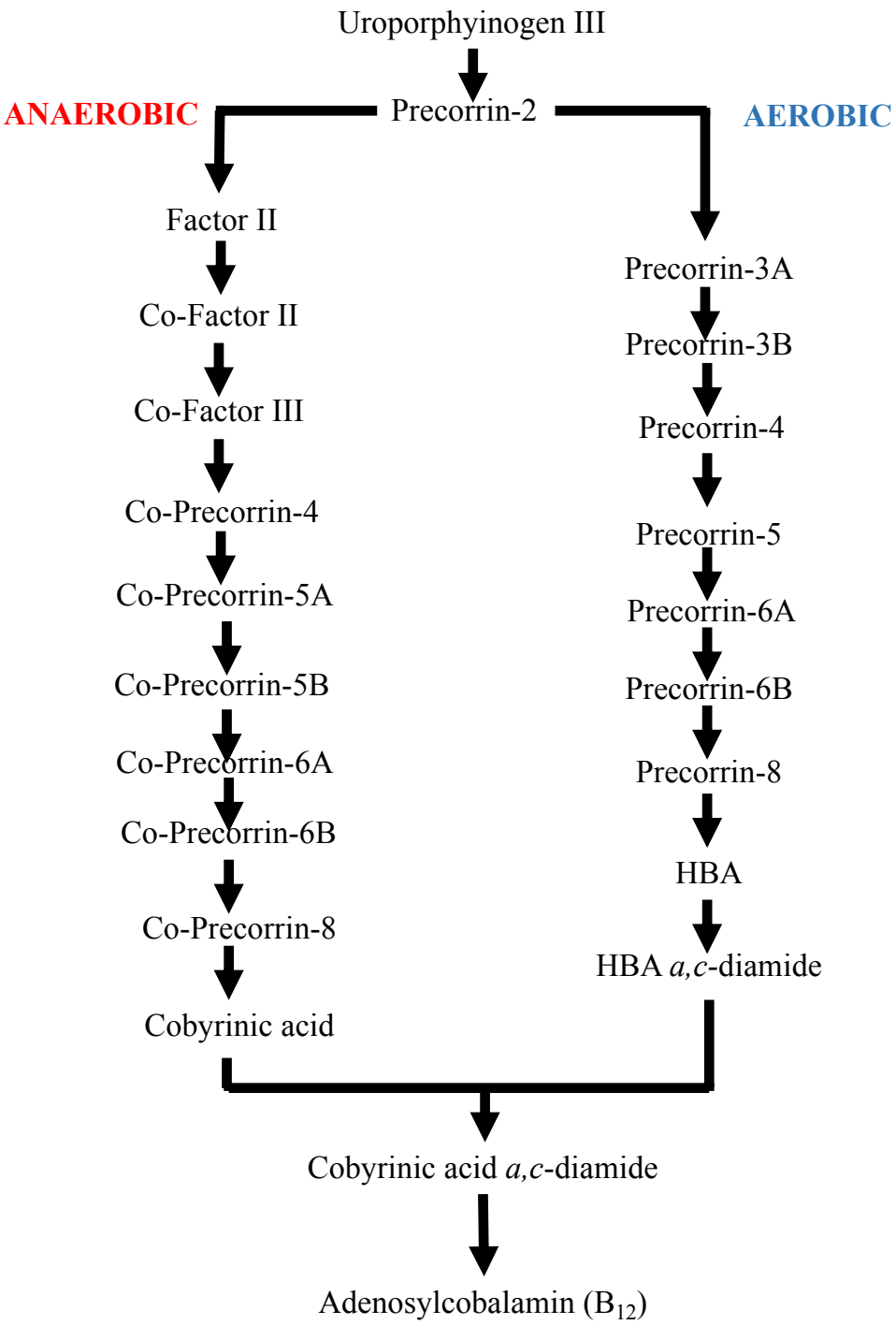

Figure 5.2 Vitamin B_{12} synthesis pathways in bacteria.

Vitamin B_{12} producing bacteria synthesise B_{12} either via the anaerobic or aerobic pathway (Moore et al., 2013; Kanehisa et al., 2016).

The work described here uses a *C. reinhardtii metE⁻* strain that is dependent on vitamin B_{12} due to the insertion of a type-II Gulliver-related transposable element into the B_{12}-independent methionine synthase gene (*METE*) (Helliwell et al., 2015). When co-cultured with *M. loti*, *C. reinhardtii metE⁻* can grow by utilizing the B_{12} released by the bacterium (Helliwell et al., 2015). It was shown that there is no growth penalty of *C. reinhardtii metE⁻* when supplemented with B_{12} as it had a similar growth rate to the ancestral B_{12}-independent wild-type *C. reinhardtii* (WT12) (Ridley, 2016).

The *C. reinhardtii metE⁻* + *M. loti* co-culture was based on the *L. rostrata* + *M. loti* consortium (Kazamia et al., 2012). *C. reinhardtii metE⁻* was used in preference to *L. rostrata* as it is a more favourable alga to use as a model for B_{12}-dependent algal species in studying interactions that depend on B_{12}. *C. reinhardtii* has a sequenced genome and a well understood physiology, is a relatively fast grower, and has an established set of genetic manipulation tools (Kazamia, 2012; Ridley, 2016).

There have been several studies on large scale growth of *C. reinhardtii* in monoculture. For example, Gianelli and Torzillo (2012) grew *C. reinhardtii* in 110 L photobioreactors to maximise hydrogen production and collection. Gimpel et al. (2014) investigated the production of recombinant proteins from engineered *C. reinhardtii* in 100 L bags, and finally Zedler et al. (2016) grew transgenic, cell-wall deficient *C. reinhardtii* strains expressing a cytochrome P450 (CYP79A1) or bifunctional diterpene synthase (cis-abienol synthase, TPS4) in 100 L green glass wall photobioreactors (GGW PBR).

5.1.5 Aims

Little is known about the growth of defined algal-bacterial co-cultures at pilot scale, or the differences in growth efficiencies when algal cultures are scaled from the lab to pilot scale. In this study, I aimed to test the consequences of progressing *C. reinhardtii metE⁻* + B_{12} and *C. reinhardtii metE⁻* + *M. loti* co-culture from 40 mL laboratory scale, to 10 L pre-pilot scale and then 60 L pilot scale. I aimed to test whether the co-culturing of the B_{12}-dependent alga with B_{12} producing bacteria proved to be as efficient in terms of growth as supplementing the culture directly with B_{12} at 60 L pilot scale. I also wished to determine whether co-culturing with *M. loti* could provide protection from contaminant/invading bacteria.

5.2 Results

5.2.1 Growth characteristics observed at lab scale (50 mL)

I conducted an experiment to compare the growth of *C. reinhardtii metE⁻* supplemented with B_{12} with growth when co-cultured with *M. loti* at laboratory scale (50 mL). *C. reinhardtii metE⁻* + B_{12}, *C. reinhardtii metE⁻* + *M. loti* and *C. reinhardtii metE⁻* – B_{12} (used as a negative control) were grown in 50 mL of TP^+ medium, with shaking for 14 days under constant illumination at 40 µmol photons m^{-2} s^{-1}. Samples were taken every second day and *C. reinhardtii* cell numbers were measured by a Coulter counter. The results showed that *C. reinhardtii metE⁻* + B_{12} reached a maximum cell number of approximately 3.1 x10^9 per mL and *C. reinhardtii metE⁻* + *M. loti* reached approximately 1.0 x10^9 algal cells per mL over the same time period. *C. reinhardtii metE⁻* – B_{12} (negative control) did not grow, which was expected (Figure 5.3A, C). This result indicated the culture supplemented with B_{12} grew faster than the culture supplemented with *M. loti.*

Previous research has found that wild-type *C. reinhardtii* (WT12) has a similar growth rate to that of *C. reinhardtii metE⁻* when supplemented with B_{12} hence I concluded the *metE⁻* mutation rendered no other growth penalty in axenic conditions (Ridley, 2016). Therefore, the two strains could be used interchangeably as positive controls.

I carried out an experiment to allow more accurate comparisons to larger scales (10 L and 60 L) in which the growth would be tested under natural conditions where light and dark cycles occur. Therefore, the 50 mL cultures were grown under light:dark cycles (16:8 respectively) in TP^+ medium. This experiment was carried out in conjunction with Freddy Bunbury (Smith lab, University of Cambridge, UK) and wild-type *C. reinhardtii* (WT12) was used as the positive control. Similar to the experiment conducted under constant illumination, the cultures were grown in 50 mL of TP^+ medium, shaking for 20 days although with illumination at 100 µmol $m^{-2}s^{-1}$ because this was the light intensity used in other experiments conducted by Freddy Bunbury. Samples were taken approximately every second day and *C. reinhardtii* cell numbers were measured by a Coulter counter. *C. reinhardtii* (WT12) grew to approximately 2.9 x10^6 algal cells per mL after 20 days whereas *C. reinhardtii metE⁻* + *M. loti* grew to approximately 1.3 x10^6 algal cells per mL over the same time (Figure 5.3B). I concluded that co-culturing *C. reinhardtii metE⁻* with *M. loti* for a source of B_{12} imposed a

growth penalty (compared to when *C. reinhardtii metE*$^{-}$ was supplemented with B_{12}) whether the cultures were grown in either constant or diurnal light conditions. Overall, these results at laboratory scale are similar to those seen in previously published data (Kazamia et al., 2012; Ridley, 2016).

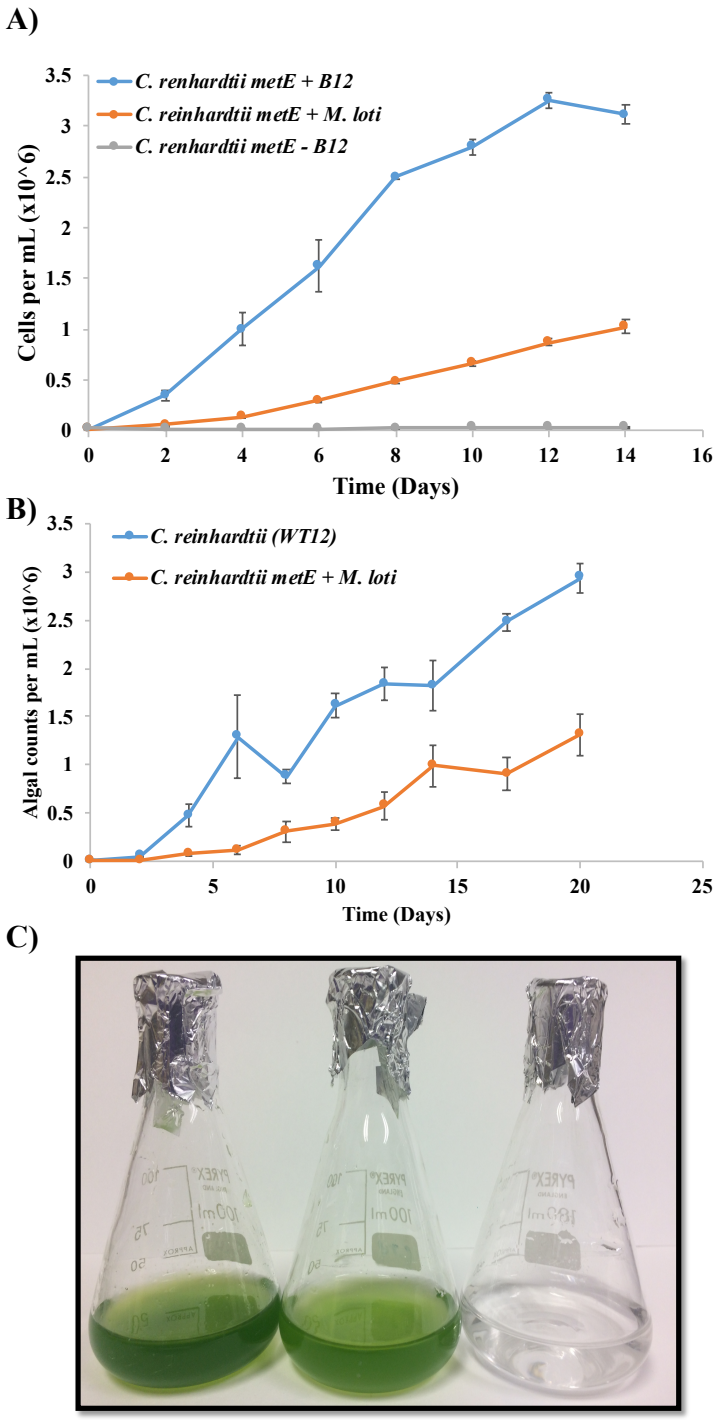

Figure 5.3 Laboratory scale growth of axenic *C. reinhardtii* (WT12 and *metE*$^{-}$ + B_{12}) and *C. reinhardtii metE*$^{-}$ + *M. loti*

A) Growth curve of *C. reinhardtii metE*$^{-}$, *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* in TP^{+} under constant light conditions. B) Growth curve of *C. reinhardtii* (WT12) and *C. reinhardtii metE*$^{-}$ + *M. loti* in TP^{+} under 16:8 hr light/dark cycles. C) Image of the 50 mL laboratory scale growth under constant light in 250 mL conical flasks. Flasks are (from left to right) *C. reinhardtii metE*$^{-}$ + B_{12}, *C. reinhardtii metE*$^{-}$ + *M. loti* and *C. reinhardtii metE*$^{-}$. Error bars display standard deviation from three replicates.

5.2.2 Growth characteristics observed at pre-pilot scale (10 L)

I next carried out a growth experiment at 10 L pre-pilot scale to compare to the growth of the algae-bacteria consortium at 50 mL laboratory scale and subsequently to the 60 L pilot scale. Pre-pilot scale cultivation (10 L) of *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* was carried out in 10 L tubular photobioreactors at the Algal Innovation Centre (AIC), University of Cambridge, UK. The cultures were bubbled with air under natural light conditions during the month of April, 2016 over 8 days (due to time constraints only 8 days of growth could be recorded). Samples were taken every second day and *C. reinhardtii* cell counts were determined by a Coulter counter and dry cell weight determined as outlined in Materials and Methods 2.3.1.1 and 2.10.1.3. The results showed that *C. reinhardtii metE*$^{-}$ + B_{12} grew to approximately 6.0 x10^5 algal cells per mL and *C. reinhardtii metE*$^{-}$ + *M. loti* grew to approximately 2.0 x10^5 algal cells per mL over the time period (Figure 5.4A). Total dry cell (TDC) weight for *C. reinhardtii metE*$^{-}$ + B_{12} was 0.21mg/mL and the TDC for *C. reinhardtii metE*$^{-}$ + *M. loti* was 0.17mg/mL, both after 8 days of growth (Figure 5.4B). A picture of the PBRs used at 10 L pre-pilot scale is shown in Figure 5.4C. I concluded that, similar to the results at laboratory scale, *C. reinhardtii metE*$^{-}$ grew faster with supplemented B_{12} as opposed to supplementation with *M. loti.* However, the cell density after 8 days of both *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* was lower at 10 L than at laboratory scale suggesting a limitation of growth such as light, temperature or contamination (the 10 L growth experiment was not carried out under sterile conditions).

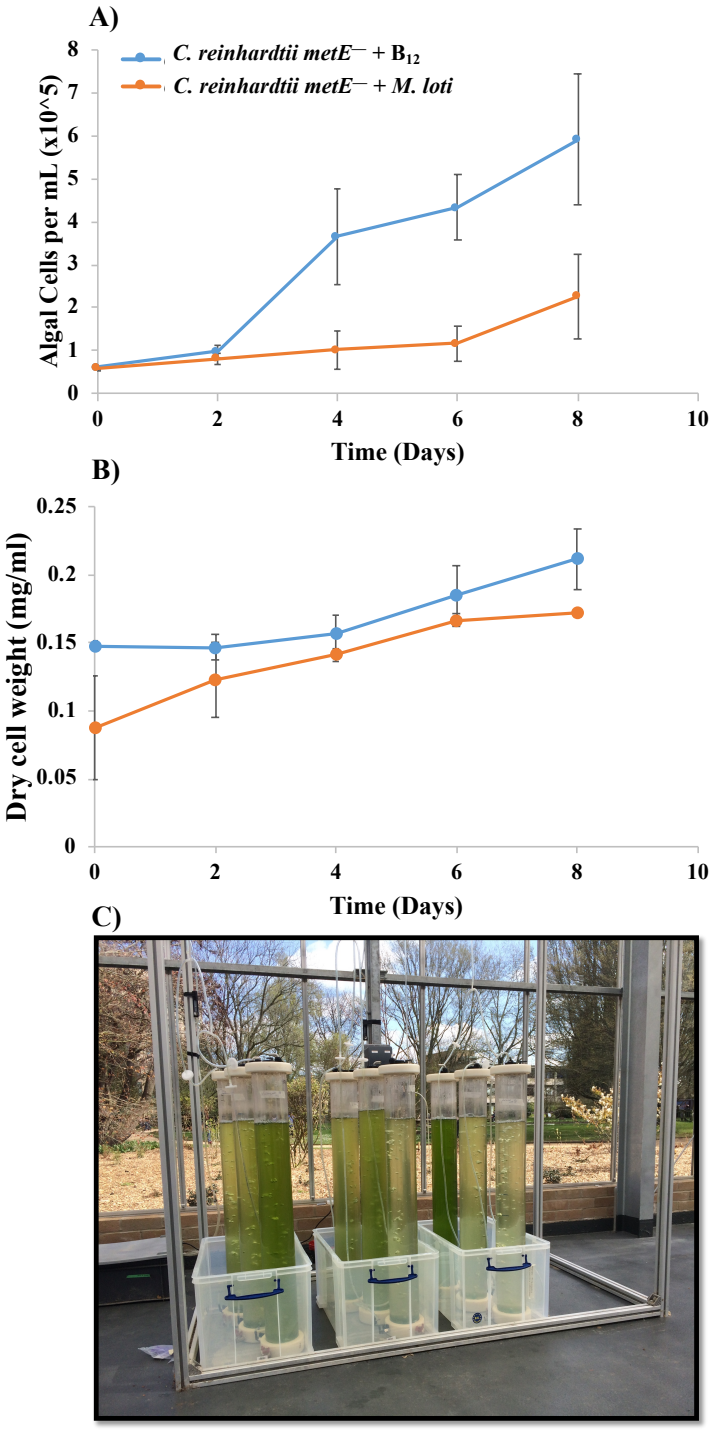

Figure 5.4 10L Pre-pilot scale growth of axenic *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii* + *M. loti*

A) Algal cell counts for *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii metE*⁻ + *M. loti*. B) Dry cell weight of *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii metE*⁻ + *M. loti*. Cultures were grown in TP^+ under natural light conditions. C) Image of the 10 L pre-pilot scale tubular bioreactors. Error bars display standard deviation from three replicates.

5.2.3 Growth characteristics observed at pilot scale (60 L)

The next stage was to compare growth with that at 60 L pilot scale. The pilot scale experiments with *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* were carried out in 60 L GGW PBRs inside a greenhouse of the Experimental Unit of A4F in Lisbon, Portugal. The cultures were grown in TP^{+} medium and bubbled with air under natural light conditions over the months of June and July 2016 for 13 days. Replicates of the cultures were not possible as there were not enough GGW PBRs. Samples of the cultures were taken every weekday. The viability of the algal cells in the cultures was measured using a Muse cell counter (Materials and Methods 2.10.2.3), and 64% of the algal cells in the *C. reinhardtii metE*$^{-}$ + B_{12} culture and 44% of the algal cells in the *C. reinhardtii metE*$^{-}$ + *M. loti* culture were found to be alive by the end of the experiment (Figure 5.5A). A decline in the percentage of live algal cells was apparent over the course of the experiment, with a consistent difference between the two cultures becoming apparent from day 10. Total cell counts for *C. reinhardtii* were measured with a Muse cell counter (Materials and Methods 2.10.2.3) and the results showed that both *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* grew to approximately 1.5 x10^{6} algal cells per mL after 13 days of growth (Figure 5.5B). However, typical algal culture growth characteristics such as rapid growth and slow growth phases were not observed, with only a slight increase in algal cells per mL of approximately 66% of starting algal cell counts for both cultures (Figure 5.5B). The final viable algal cell density for *C. reinhardtii metE*$^{-}$ + B_{12} was approximately 1.0x10^{6} algal cells per mL and for *C. reinhardtii metE*$^{-}$ + *M. loti* it was approximately 7.0 x10^{5} algal cells per mL (broken lines Figure 5.5B). Therefore, I concluded that the production rate of new algal cells did not exceed those dying off. The number of viable algal cells was approximately the same at the end of the experiment compared to the beginning of the experiment for *C. reinhardtii metE*$^{-}$+ B_{12} whereas the number of viable cells for *C. reinhardtii metE*$^{-}$ + *M. loti* declined.

The measurements of OD_{750} and dry cell weight (Figures 5.5C and D) for both cultures showed an increase in cell densities over the course of the experiment, with similar results between both cultures for most of the growth period. Chlorophyll *a* (see Materials and Methods 2.5) concentration increased for both cultures over the first 9 days with deviation beginning at day 10 when chlorophyll *a* concentration in the *C. reinhardtii metE*$^{-}$ + *M. loti* culture began to decline (Figure 5.5E). Chlorophyll *a* per algal cell (total chlorophyll *a* divided by algal cell counts) reached a final concentration of 4.9 fmol of chlorophyll *a* per cell for *C. reinhardtii*

metE^{-} + B_{12} and 3.2 fmol of chlorophyll *a* per cell for the co-culture (Figure 5.5F). The increase in chlorophyll *a* per algal cell was approximately the same for both cultures over much of the course of the experiment but with deviation occurring at day 10. By dividing the total chlorophyll *a* by the number of viable algal cells in the culture shown in Figure 5.5A, an increase was seen in the fmol of chlorophyll *a* per cell, reaching a maximum of approximately 7.3 fmol chlorophyll *a* per viable algal cell for both cultures (broken line Figure 5.5F). The increase in chlorophyll *a* per viable algal cell was approximately the same for both cultures over the course of the experiment.

Total vitamin B_{12} concentrations in the cultures were measured over the course of the experiment. They increased steadily over the course of the experiment with detection of 24 pg/mL B_{12} from *C. reinhardtii metE*$^{-}$ + B_{12} on day 0 and reaching a maximum of approximately 60 pg/mL on day 13, whereas a concentration of 13.0 pg/mL B_{12} was detected on day 3 for *C. reinhardtii metE*$^{-}$ + *M. loti* reaching a maximum of approximately 55 pg/mL on day 13 (Figure 5.5G). B_{12} was detected only from day 3 onwards for *C. reinhardtii metE*$^{-}$+ *M. loti* so it is presumed the B_{12} concentration was too low for detection in the B_{12} assay on days 0 to 2. I concluded there was a problem regarding the B_{12} concentration in the *C. reinhardtii metE*$^{-}$ + B_{12} culture as 100 pg/mL was expected from the beginning of the experiment (this was investigated in section 5.2.4 below).

I recorded average daily light exposure (averaged over the number of light hours i.e. 15 hours) and average daily temperature (averaged over 24 hours) and the results were found to vary between 450-650 µmol photons m^{-2} s^{-1} and 17-26 ^{o}C (Figure 5.5H). Images of the *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* cultures at 60 L pilot scale on day 13 are shown in Figure 5.5I and J. Culture samples from both PBRs were observed under the microscope daily to assess motility, cell division, autofluorescence and bacterial populations (data not shown). Motility of algal cells for both cultures appeared to decline over the course of the experiment. Approximately half the algal cells observed were undergoing cell division throughout the experiment for both cultures. Approximately 90% of algal cells for both cultures were observed to autofluoresce throughout the experiment indicating the existence of chlorophyll. Finally, bacterial populations in both cultures appeared to increase over the course of the experiment.

Overall, I concluded the growth in algal cells per mL of *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M.* loti cultures were low and the total populations were similar between the cultures at 60 L pilot scale, which differed from the results observed at 50 mL laboratory (Figure 5.3) and 10 L pre-pilot scale (Figure 5.4).

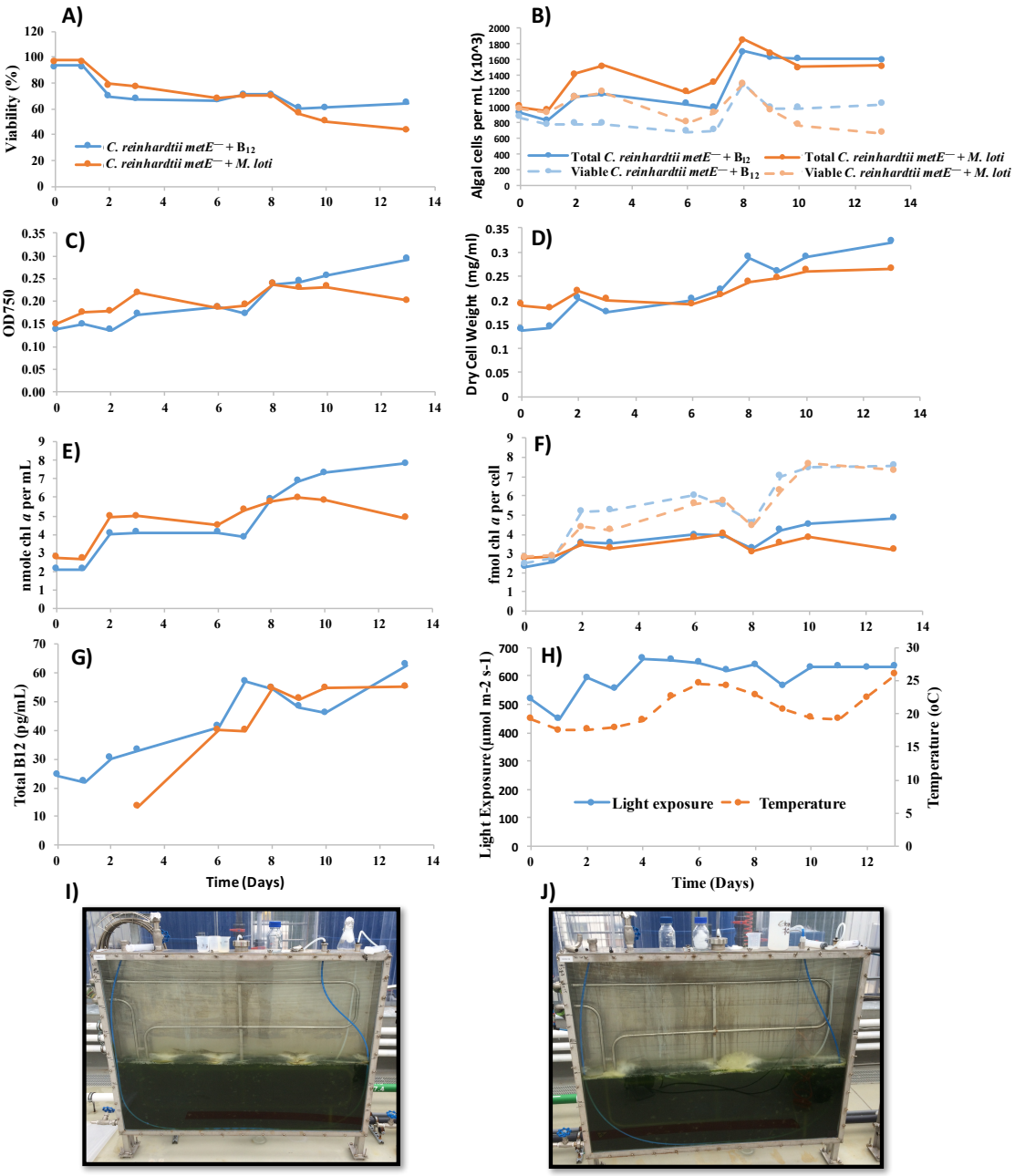

Figure 5.5 60L pilot scale growth of *C. reinhardtii metE⁻* + B_{12} and *C. reinhardtii metE⁻* + *M. loti*

A) Viability (percentage of live cells) of algal cells. B) Total algal cell counts (solid lines) and calculated live cells (broken lines). C) Optical Density at 750 nm. D) Dry cell weight in mg/mL. E) nmol chlorophyll *a* per mL. F) Concentration of chlorophyll *a* per cell. G) Total B_{12} concentration detected in medium. H) Average daily light exposure (µmol photons m^{-2} s^{-1}) and temperature (°C). I) Final day of growth of *C. reinhardtii metE⁻* + B_{12} culture J) Final day of growth of *C. reinhardtii metE⁻* + *M. loti culture.* Cultures were grown in TP^{+} under natural light conditions. No error bars are displayed as only one experiment for each culture was possible.

5.2.4 Investigating the B_{12} measurement at 60 L pilot scale

In section 5.2.3, I reported that the initial B_{12} concentration measured in the *C. reinhardtii metE*$^{-}$ + B_{12} culture was 24 pg/mL which was lower than the expected 100 pg/mL that it should have contained (Figure 5.6G). I carried out several experiments to identify what could have been the cause for this discrepancy. B_{12} concentrations were measured by a bioassay as outlined in Materials and Methods 2.6. Briefly, the technique involves the growth of *S. typhimurium* AR3612 on boiled samples whose B_{12} concentration is to be measured. The growth of *S. typhimurium* AR3612 is dependent on the presence of available B_{12} in the sample. The B_{12} concentration in the sample is estimated by comparing the growth of *S. typhimurium* AR3612 on the sample to growth measured in the presence of known concentrations of B_{12} using a standard curve.

Firstly, I carried out an experiment to confirm whether boiling the B_{12} samples influenced the activity of the B_{12}. Even though boiling B_{12} samples prior to the B_{12} assay is common practice in this technique to ensure sterility, and B_{12} is known to be stable at 100 ^{o}C (Harris, 1988), the experiment was carried out as a control. As per normal, B_{12} was added to TP^{+} medium at 8 different final concentrations ranging from 0 to 125 pg/mL for two separate B_{12} assays. One assay tested the B_{12}-inoculated TP^{+} medium after boiling (at 100 ^{o}C) for 15 minutes and in the other the medium was not boiled but instead filter sterilised. I found that both filter sterilised and boiled samples of B_{12} promoted the growth of *S. typhimurium* AR3612 similarly at all concentrations, approximately reaching OD_{595} 1.4 at the final concentration of 120 pg/mL of B_{12} (Figure 5.6A). I concluded that boiling had no effect on the biological activity of B_{12} in this assay.

The samples from the 60 L pilot scale cultures for both *C. reinhardtii metE*$^{-}$ + B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* were sent from the A4F laboratory in Portugal to the Howe laboratory at the University of Cambridge, UK where the B_{12} analysis was carried out. The time taken for the package to be delivered was approximately 4 weeks, so I considered that some degradation may have occurred in transit. Furthermore, it could have been possible the samples were subjected to freeze-thawing as the samples arrived partially thawed, even though the samples were packaged with dry ice in a polystyrene container. I carried out a B_{12} bioassay to determine if four weeks in a frozen state together with exposure to freeze-thaw cycles could have led to the degradation of B_{12} and hence a lower measurement in the B_{12} assay (Figure

5.6G). As in the previous experiment, B_{12} was added to TP^+ medium at 8 different concentrations ranging from 0 to 125 pg/mL for two separate B_{12} assays. One assay tested the B_{12}-inoculated TP^+ medium after 4 weeks in a frozen state combined with 4 freeze-thaw cycles. The control samples were made fresh prior to the assay and did not undergo 4 weeks in a frozen state nor did they undergo the freeze-thaw cycles. Both the control and the test assay underwent boiling as a sterilisation step (Materials and methods 2.6.2). I found that both the control and the test samples promoted the growth of *S. typhimurium* AR3612 to approximately the same extent at all concentrations measured, reaching OD_{595} 1.3 at the final concentration of 120 pg/mL of B_{12} (Figure 5.6B). I concluded that 4 weeks in a frozen state along with four freeze-thaw cycles had no degradation effect on B_{12} in this assay.

The B_{12} stock used in the 60 L pilot scale experiment carried out at A4F, Portugal, was different from the B_{12} stock used in the Howe laboratory, University of Cambridge. I hypothesised the actual concentration of B_{12} used in the 60 L pilot scale experiment may not have been as high as originally thought, possibly due to the age of the B_{12} stock which was unknown. A B_{12} bioassay was carried out to determine whether the B_{12} stock from A4F was less effective than the B_{12} stock used in the Howe laboratory. Two B_{12} assays were carried out, one using the B_{12} stock from the Howe laboratory (standard sample) as a control and the other using the B_{12} stock from A4F. Again, B_{12} was inoculated into TP^+ medium at 8 different final concentrations ranging from 0 to 125 pg/mL for the two separate B_{12} assays. I found the standard stock (Howe laboratory stock) promoted the growth of *S. typhimurium* AR3612 as expected, given the same stock was used for the calibration curve (Figure 5.6C). However, the A4F stock was not as effective at promoting the growth of *S. typhimurium* AR3612 as the standard stock. The growth of *S. typhimurium* AR3612 on 100 pg/mL of A4F stock reached a cell density of approximately OD_{595} 0.5 which equates to only 10 pg/mL of the standard stock. This measurement (10 pg/mL) is only approximately 2-fold different from the B_{12} concentration measured in the *C. reinhardtii metE*$^-$ + B_{12} culture in Figure 5.5G. I concluded that the actual concentration in the A4F stock was not as high as originally thought, which would have led to the lower B_{12} concentrations measured in the 60 L pilot scale experiment (Figure 5.5G).

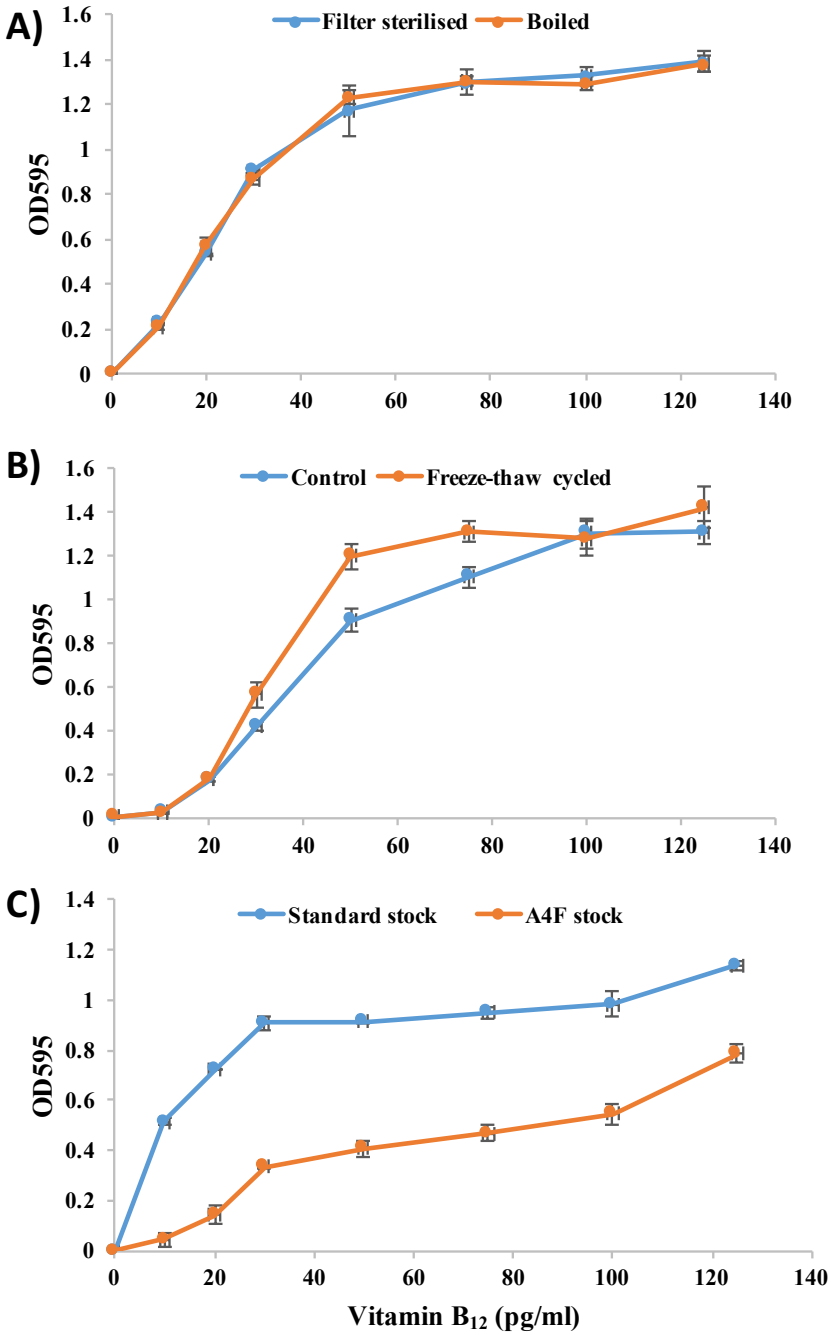

Figure 5.6 Investigating B_{12} activity through bioassays

A) B_{12} bioassay of B_{12} sterilised through filters or boiled (100 ^{o}C) for 15 minutes. B) B_{12} bioassay of B_{12} exposed to 4 weeks in a frozen state as well as 4 freeze-thaw cycles versus control B_{12} samples not exposed to these conditions. C) B_{12} bioassay of B_{12} obtained from the Howe laboratory stock (standard stock) versus B_{12} originating from the A4F stock. Error bars display standard deviation from three replicates.

5.2.5 Comparison of the doubling time of *C. reinhardtii* (*metE*$^{-}$ + B_{12} or WT12) when grown as a monoculture or in co-culture with *M. loti* at different scales

To compare accurately the growth of the algae between cultures (*C. reinhardtii* (*metE*$^{-}$ + B_{12} or WT12) or *C. reinhardtii metE*$^{-}$ + *M. loti*) at the different volumes, I calculated the cell doubling time ($T_{d,}$ calculated in hours) for all cultures (Materials and Methods 2.3.6, and making the assumption that growth over the period analysed was exponential). The T_d for all cultures at all scales was calculated based on total algal cell counts. The T_d of *C. reinhardtii metE*$^{-}$ + B_{12} was significantly lower than for *C. reinhardtii metE*$^{-}$ + *M. loti* when grown under constant light at 50 mL laboratory scale (Figure 5.7 A and B). The T_d of *C. reinhardtii metE*$^{-}$ + B_{12} was again significantly lower than for *C. reinhardtii metE*$^{-}$ + *M. loti* when grown under diurnal light at 50 mL laboratory scale. The T_d of *C. reinhardtii metE*$^{-}$ + B_{12} was also significantly lower than for *C. reinhardtii metE*$^{-}$ + *M. loti* when grown at 10 L pre-pilot scale. The T_d of *C. reinhardtii metE*$^{-}$ + B_{12} was also lower than *C. reinhardtii metE*$^{-}$ + *M. loti* when grown at 60 L pilot scale with a T_d of 389 hours and a T_d of 512 hours respectively (although significance could not be determined at this scale as biological replicates were not possible). I concluded the T_d of *C. reinhardtii metE*$^{-}$ + B_{12} was lower than *C. reinhardtii metE*$^{-}$ + *M. loti* at all scales measured.

I also compared the T_d of a monoculture of *C. reinhardtii* (*metE*$^{-}$ + B_{12} or WT12) at the various volumes (under constant or diurnal light at 50 mL). When comparing the T_d between *C. reinhardtii* (*metE*$^{-}$ + B_{12} or WT12) at different volumes, the T_d was lowest when *C. reinhardtii metE*$^{-}$ + B_{12} was grown in 50 mL laboratory scale under constant light conditions at a T_d of 47 hours (Figure 5.7 A and B). The next lowest T_d was *C. reinhardtii* WT12 when grown in 50 mL laboratory scale under diurnal light at a T_d of 53 hours which was significantly different from *C. reinhardtii metE*$^{-}$ + B_{12} when grown in 50 mL laboratory scale under constant light conditions. When *C. reinhardtii metE*$^{-}$ + B_{12} was grown in 10 L pre-pilot scale, it had a T_d of 62 hours which was significantly different from *C. reinhardtii metE*$^{-}$ + B_{12} when grown in 50 mL laboratory scale under constant light conditions. No significant difference was measured between *C. reinhardtii* WT12 when grown in 50 mL laboratory scale under diurnal light and *C. reinhardtii metE*$^{-}$ + B_{12} when grown in 10 L pre-pilot scale. *C. reinhardtii metE*$^{-}$ + B_{12} had the highest T_d when grown in 60 L pilot scale at 389 hours. I concluded, there was a trend of increasing T_d when culture volume increases for *C. reinhardtii metE*$^{-}$ + $B_{12.}$

When comparing the T_d of *C. reinhardtii metE⁻* + *M. loti* at different volumes, the T_d was lowest when *C. reinhardtii metE⁻* + *M. loti* was grown in 50 mL laboratory scale under diurnal light conditions at a T_d of 60 hours (Figure 5.7 A and B). This was significantly different from the next lowest T_d of 61 hours for *C. reinhardtii metE⁻* + *M. loti* when grown in 50 mL laboratory scale under constant light. *C. reinhardtii metE⁻* + *M. loti* grown in 10 L pre-pilot scale had a T_d of 124 hours and was significantly different from *C. reinhardtii metE⁻* + B_{12} when grown in 50 mL laboratory scale under constant light conditions. Significant difference was also measured between *C. reinhardtii metE⁻* + *M. loti* when grown in 50 mL laboratory scale under constant light and *C. reinhardtii metE⁻* + *M. loti* when grown in 10 L pre-pilot scale. *C. reinhardtii metE⁻* + *M. loti* had the highest T_d when grown in 60 L pilot scale at 512 hours. I concluded that there was a trend that T_d of *C. reinhardtii metE⁻* + *M. loti* increased when the volume increased.

A)

Culture	Condition	Doubling Time (T_d in hours)
C. reinhardtii metE^{-} + B_{12}	50 mL laboratory scale (constant light)	47 ± 0.07
C. reinhardtii WT12	50 mL laboratory scale (diurnal light)	53 ± 0.7
C. reinhardtii metE^{-} + B_{12}	10 L pre-pilot scale	62 ± 5.23
C. reinhardtii metE^{-} + B_{12}	60 L pilot scale	389
C. reinhardtii metE^{-} + *M. loti*	50 mL laboratory scale (constant light)	61 ± 0.07
C. reinhardtii metE^{-} + *M. loti*	50 mL laboratory scale (diurnal light)	60 ± 0.28
C. reinhardtii metE^{-} + *M. loti*	10 L pre-pilot scale	124 ± 3.5
C. reinhardtii metE^{-} + *M. loti*	60 L pilot scale	512

B)

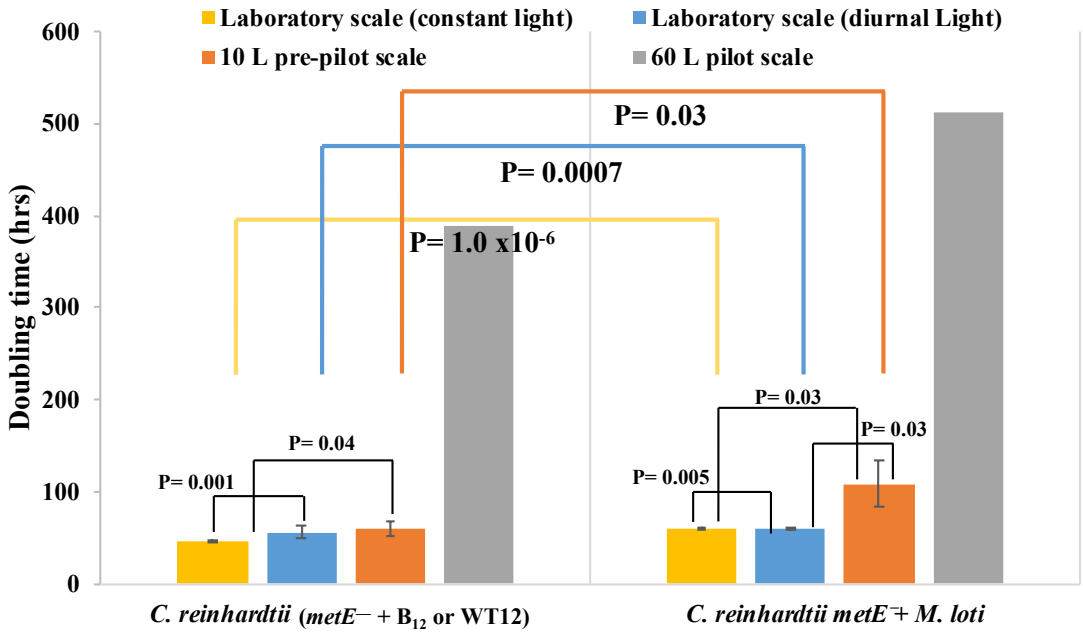

Figure 5.7 Doubling time of the algae at different scales of volume

A) Table and B) graph outlining the doubling time (T_d in hours) of *C. reinhardtii* (*metE*$^{-}$ + B_{12} or WT12) and *C. reinhardtii metE*$^{-}$+ *M. loti* at laboratory scale (50 mL) (*metE*$^{-}$ + B_{12} in constant light and WT12 in diurnal light), pre-pilot scale (10 L) and pilot scale (60 L). Error bars (not applicable to 60 L pilot scale growth rates) display standard deviation from three replicates. Significance was determined by ANOVA and is illustrated by the connecting lines above the bars.

5.2.6 Bacterial growth in the cultures at the various scales

Little is known about the bacterial populations when grown with algae at various scales of volume therefore, for each culture grown at the various scales, I measured the bacterial populations. It is important to note that the laboratory scale cultures were grown in sterile conditions, whereas the 10 L pre-pilot scale as well as the 60 L GGW PBRs were grown in non-sterile conditions, which will influence the species and numbers of bacteria in the cultures. Total bacterial numbers were estimated by counting CFUs on TY agar plates (Materials and Methods 2.3.1.3; note that this technique provides an estimate only, as not all bacterial species will grow on TY) approximately every second day for 50 mL laboratory and 10 L pre-pilot scale and every day (except for weekends) for 60 L pilot scale for 20, 8 and 13 days respectively. Total CFUs of *M. loti* in the 50 mL laboratory cultures grown under diurnal illumination increased over the course of the experiment (Figure 5.8A). No bacteria were observed throughout the experiment in the *C. reinhardtii* WT12 control (Figure 5.8A). The ratio of bacteria to algae peaked on day 1 and then declined and stabilised (Figure 5.8B). The bacterial cell density of both sets of cultures at the 10 L scale increased over the course of the experiment (Figure 5.8C). For *C. reinhardtii metE*$^{-}$ + B_{12} the ratio of bacteria to algae stabilised at approximately 5:1 whereas, for *C. reinhardtii metE*$^{-}$ + *M. loti*, the ratio of bacteria to algae peaked on day four and then began to decline (Figure 5.8D). Bacterial cell counts in both cultures also increased over the 13-day experiment at 60 L scale (Figure 5.8E). The bacteria to algae ratios for both cultures at 60L scale increased over the course of the experiment (Figure 5.8F). I concluded the bacterial populations followed a similar trend of increasing over time at all the volumes, although the ratio of bacteria to algae differed throughout the various scales, which could be attributed to differences in exposure to contaminating organisms.

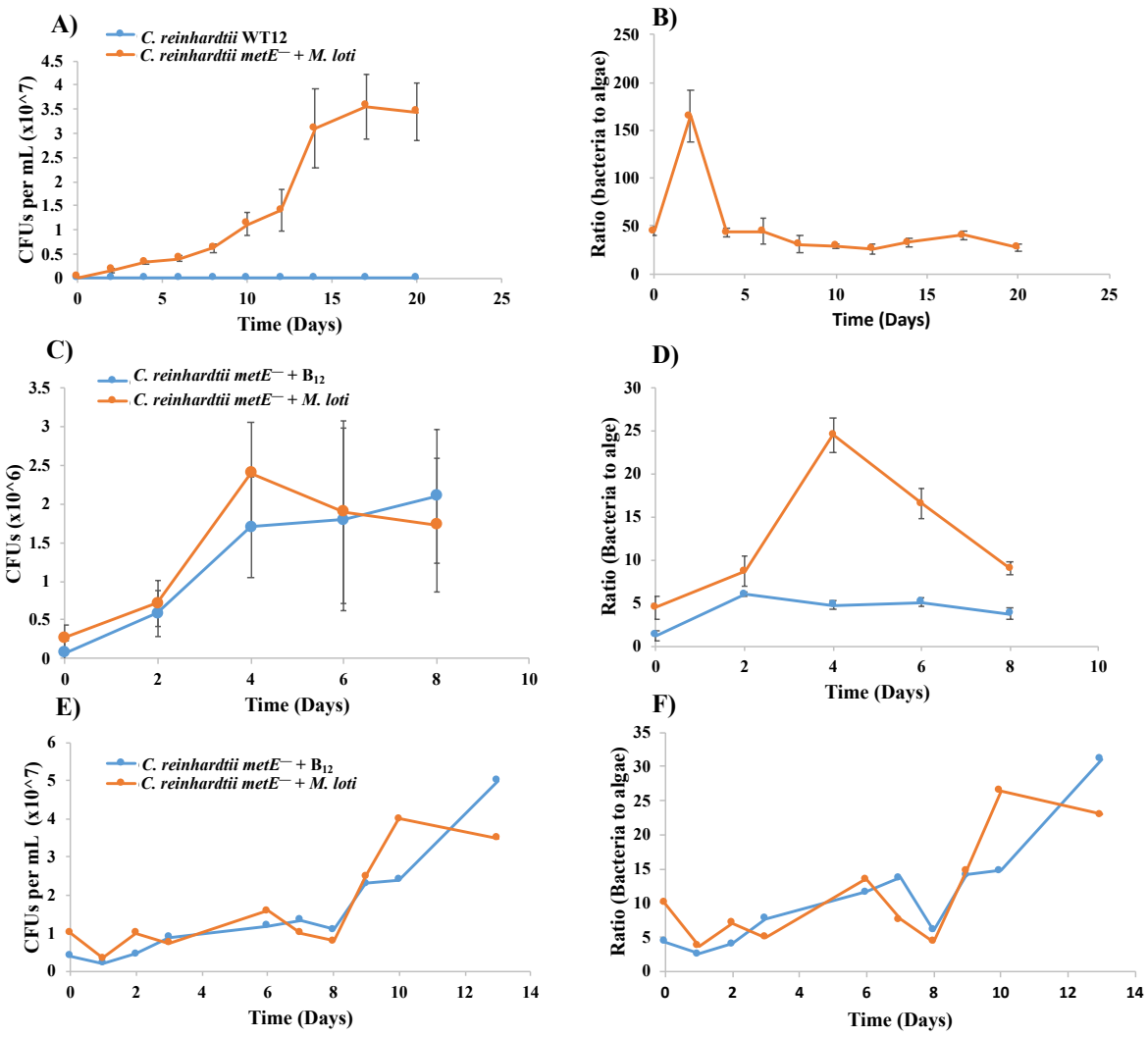

Figure 5.8 Total bacterial CFUs and bacteria to algae ratios at all scales

A) CFUs per mL of *M. loti* in *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii metE*⁻ in laboratory scale growth B) Ratio of bacteria to algae in laboratory scale growth. C) CFUs per mL of bacteria in *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii metE*⁻ + *M. loti* in 10 L pre-pilot scale growth D) Ratio of bacteria to algae *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii metE*⁻ + *M. loti* in 10 L pre-pilot scale growth E) CFUs per mL of bacteria in *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii metE*⁻ + *M. loti* in 60 L pilot scale growth. F) Ratio of bacteria to algae in *C. reinhardtii metE*⁻ + B_{12} and *C. reinhardtii metE*⁻ + *M. loti* in 60 L pilot scale growth. No error bars are displayed for 60 L pilot scale as only one experiment for each culture was possible. Error bars (laboratory and pre-pilot scale only) display standard deviation from three replicates.

5.2.7 Characterising the bacterial contaminants isolated in the 60 L GGW PBRs

Little is known about which bacterial species may contaminate large-scale cultures, such as the 60 L pilot scale cultures. Therefore, I carried out an experiment to identify the bacterial species in the cultures that could grow on TY plates. The bacterial species were initially characterised through their colony morphologies. Briefly, each culture was diluted 1.0 $x10^3$ and 5.0 $x10^3$ fold to ensure single colonies grew for easy identification. Samples containing 10 µL of the diluted cultures were then placed at the end of TY plates. I then tilted the plates to allow the drops to form vertical lanes across the plate. The plates were dried and incubated for 2 days at 25°C (Materials and Methods 2.10.2.6). Up to three different bacterial morphologies (different colony phenotypes) were seen on TY agar plates for each culture. These three morphologies appeared to re-occur during the experiment suggesting they were potentially stable inhabitants of the culture (red, purple and black dotted squares, Figure 5.9A). Experiments were then carried out to determine what these species of bacteria were and if they were found in both of the 60 L cultures. Colonies representing the three morphologies identified for each culture on day 13 were re-streaked on fresh TY plates. I amplified 1000 bp segments of 16S rDNA by PCR (as described in Materials and Methods 2.4), and their sequence was determined to confirm the identity of the strain. I trimmed the sequences to include only high-quality reads. BLAST analysis for bacteria from both cultures showed almost 100% sequence identity to the same three species; *Ochrobactrum anthropi* (red dotted square, Figure 5.9A), *Pseudomonas oryzihabitans* (black dotted square, Figure 5.9A) and *Stenotrophomonas maltophilia* (purple dotted square, Figure 5.9A) (Figure 5.9B, C and D respectively). To determine whether any of the identified contaminants were predicted to synthesise their own B_{12}, I searched for the presence of the B_{12} synthesis pathway in the KEGG database for each of the organisms. The search found *O. anthropi* and *P. oryzihabitans* encoded the necessary enzymes to produce B_{12}, but *S. maltophilia* did not (See *O. anthropi* https://goo.gl/4gwwLo; *P. oryzihabitans* https://goo.gl/Uu20Dj; *S. maltophilia* https://goo.gl/VwmTOC; Kanehisa et al., 2016). I concluded that *O. anthropi* and *P. oryzihabitans* were putative B_{12} producers and *S. maltophilia* was not.

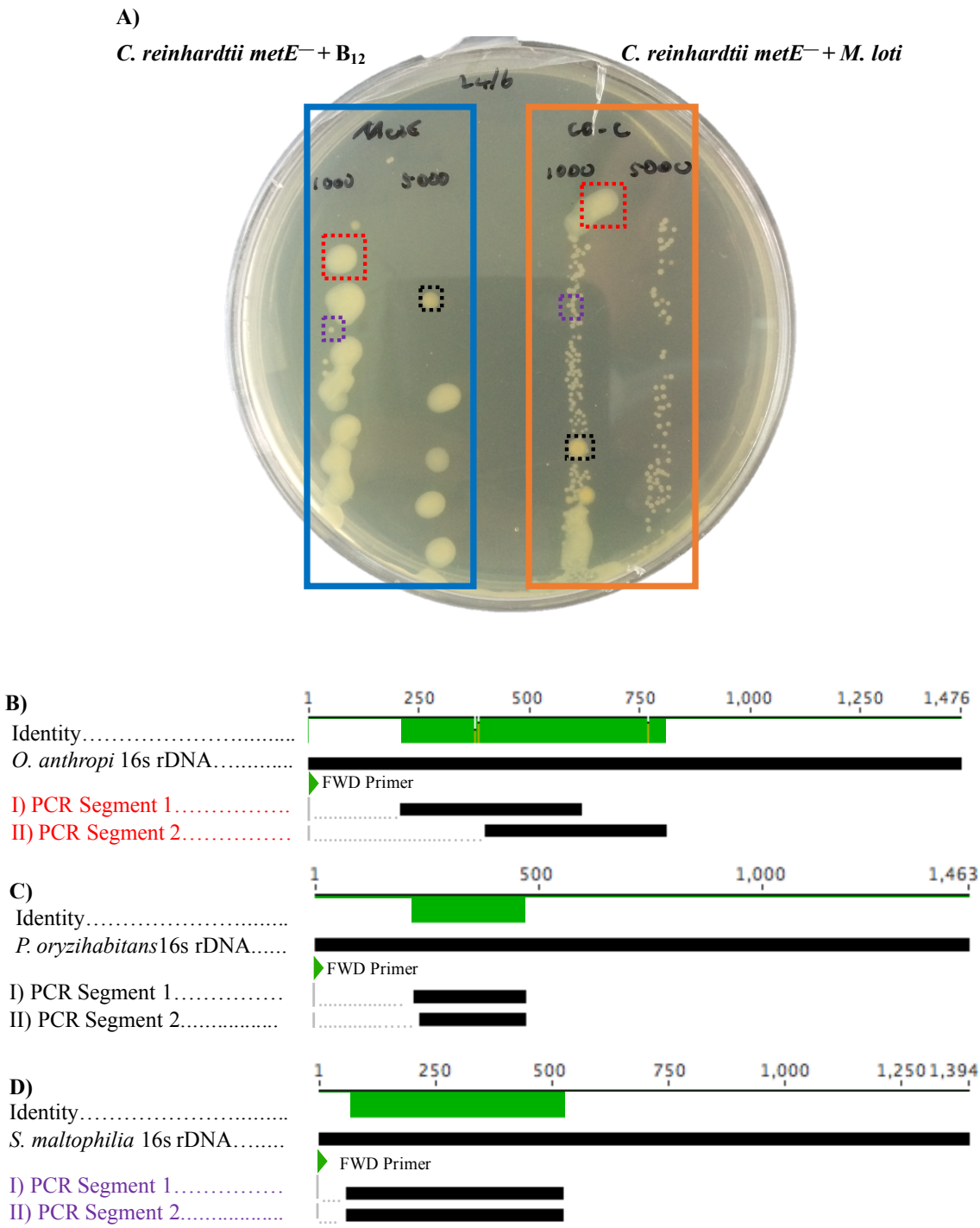

Figure 5.9 Sequence alignment of PCR products

A) Image of bacterial colonies on TY plates isolated from *C. reinhardtii metE*$^{-}$+ B_{12} and *C. reinhardtii metE*$^{-}$+ *M. loti* cultures. Dotted squares indicate the different colony morphologies repeatedly observed B) Sequenced PCR products of 16S rDNA from colonies isolated from 60 L pilot scale cultures of I) *C. reinhardtii metE*$^{-}$+ B_{12} and II) *C. reinhardtii metE*$^{-}$+ *M. loti* aligned to *O. anthropi* 16S rDNA sequence. Red text indicates the PCR was carried out on the bacterial colonies highlighted by the red dotted square in Figure 5.7A. C) Sequenced PCR products of 16S rDNA from colonies isolated from 60 L pilot scale cultures of I) *C. reinhardtii metE*$^{-}$+ B_{12} and II) *C. reinhardtii metE*$^{-}$+ *M. loti* aligned

to *P. oryzihabitans* 16S rDNA sequence. Black text indicates the PCR was carried out on the bacterial colonies highlighted by the black dotted square in Figure 5.7A. D) Sequenced PCR products of 16S rDNA from colonies isolated from 60 L pilot scale cultures of I) *C. reinhardtii metE⁻*+ B_{12} and II) *C. reinhardtii metE⁻*+ *M. loti* aligned to *S. maltophilia* 16S rDNA sequence. Purple text indicates the PCR was carried out on the bacterial colonies highlighted by the purple dotted square in Figure 5.7A. The green bar above sequence represents identity. The bar is completely green where bacterial 16S rDNA consensus sequence and PCR sequence overlap representing 100% similarity. A break in the green bar represents non-identical sequence. Solid black lines represent the sequences that were aligned. Green arrow heads represent forward primer sites. Sequence alignment carried out with NCBI Blast (NCBI Resource Coordinators 2016) and Geneious (Kearse et al., 2012).

To determine whether any of these contaminant bacterial species could enter symbiosis with *C. reinhardtii metE⁻,* I carried out a growth experiment which cultured each of the contaminant bacterial species or *M. loti* with *C. reinhardtii metE⁻*. The cultures were grown in 50 mL TP^+ medium, shaking with constant illumination at 40 µmol photons m^{-2} s^{-1} for 7 days. I measured *C. reinhardtii metE⁻* cell counts via a Coulter counter for each of the cultures on day 7. *C. reinhardtii metE⁻* + *M. loti* reached approximately 3.5 x10^7 cells per mL; however, *C. reinhardtii metE⁻* + *O. anthropi, C. reinhardtii metE⁻* + *P. oryzihabitans* and *C. reinhardtii metE⁻* + *S. maltophilia* did not grow (Figure 5.10A). I concluded that none of the bacterial contaminants could enter symbiosis with *C. reinhardtii metE⁻*.

The ability of the identified contaminant bacterial species to synthesis B_{12} was examined. The KEGG database had predicted that *O. anthropi* and *P. oryzihabitans* are B_{12} producers and *S. maltophilia* is not a B_{12} producer. To test this, I carried out a B_{12} assay (Materials and Methods 2.6) on the bacterial cells to see whether B_{12} synthesis was taking place. I grew the bacteria for 24 hours in TY medium and they were then washed with TP^+ medium three times. I grew the strains in TY medium because *S. maltophilia* could not grow on minimal medium (TP^+) supplemented with any of the following sugars (glycerol, glucose and sucrose) (data not shown). The results showed that 0.4, 0.05, and 0.2 ag of B_{12} per cell was present for *M. loti*, *O. anthropi*, *P. oryzihabitans* respectively after 24 hours of growth in TY medium. In contrast, B_{12} was not detected in the cells of *S. maltophilia* (Figure 5.10B). The CFUs per mL recorded for the cultures were 2.3 x10^8, 5.4 x10^9, 6.0 x10^8 and 2.7 x10^9 for *M. loti*, *O. anthropi, P. oryzihabitans* and *S. maltophilia* respectively after 24 hours of growth in

TY medium (Figure 5.10B). I concluded that *O. anthropi* and *P. oryzihabitans* could synthesise B_{12} and *S. maltophilia* did not.

One reason why the B_{12} producing bacterial contaminants do not enter symbiosis with *C. reinhardtii metE⁻* could be that B_{12} was synthesized but not released into the medium. I carried out an experiment to test whether B_{12} was being released into the supernatant by measuring the B_{12} in the medium supernatant. *M. loti*, *O. anthropi*, *P. oryzihabitans* were grown in 50 mL TP^+ + 0.1% glycerol for 24 hours as TP^+ does not contain B_{12}. I spun down the cultures and isolated the supernatant. The results found 0.08 ag of B_{12} per cell for *M. loti*. However, no B_{12} was measured for *O. anthropi* or *P. oryzihabitans* (Figure 5.10B). Furthermore, CFUs per mL were recorded as 6.3 $x10^8$, 9.0 $x10^7$, 6.7 $x10^8$ after 24 hours of growth in TP^+ + 0.1% glycerol for *M. loti*, *O. anthropi* and *P. oryzihabitans* respectively indicating bacterial growth had occurred (Figure 5.10B). I concluded that *O. anthropi* and *P. oryzihabitans* either did not release B_{12} or released it to very low concentrations so that it was not detected. This could be a possible reason for the inability to enter symbiosis with *C. reinhardtii metE⁻*.

Another reason why the bacterial contaminants did not enter symbiosis with *C. reinhardtii metE⁻* could be that the bacteria were unable to metabolise the carbon released from the alga. I carried out a growth experiment to test whether *M. loti*, *O. anthropi, P. oryzihabitans* and *S. maltophilia* could metabolise any potential carbon exudates from *C. reinhardtii metE⁻*. *C. reinhardtii metE⁻* was grown in TP^+ with B_{12} supplementation up to approximately 3.0 $x10^6$ cells per mL. I centrifuged the culture to separate the cells from the supernatant. The supernatant was then filter sterilised with a 0.45 µm filter. I added 1 mL of this supernatant to 1 mL of 2xTP^+ (two times the concentration of standard TP^+ to ensure adequate supply of nutrients). The bacteria were inoculated into this medium as well as TY and 2xTP^+ (+ 0.1% glycerol) for positive controls and incubated for 4 days at 30 ^oC. I measured the growth of the bacteria by OD_{600} at the end of the 4 days (Figure 5.10C). The results found all the bacteria grew on TY, which was expected. Furthermore, *M. loti*, *O. anthropi* and *P. oryzihabitans* grew on the 2xTP^+ (+ 0.1% glycerol) but *S. maltophilia* did not, which was also expected. Finally, none of the bacterial species grew with the *C. reinhardtii metE⁻* supernatant. The results from this experiment were inadequate to conclude whether the bacterial contaminants could feed off the carbon source as *M. loti*, a known symbiont of *C. reinhardtii metE⁻,* was not able to grow in the supernatant sample.

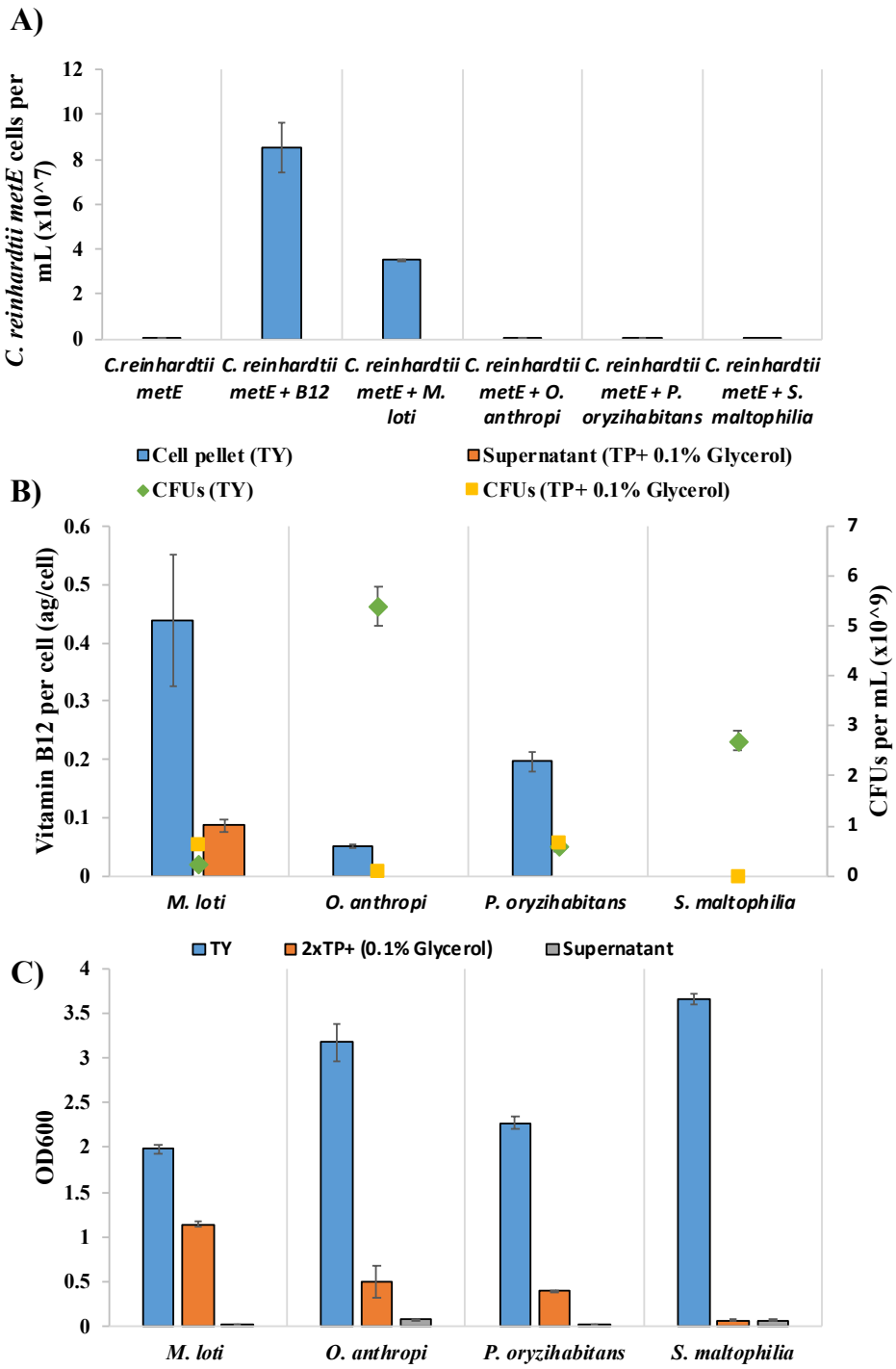

Figure 5.10 *C. reinhardtii metE*⁻ co-culture and B_{12} production of contaminant bacterial species

A) Algal cell counts of *C. reinhardtii metE*⁻ co-cultured with *M. loti*, *O. anthropi*, *P. oryzihabitans* and *S. maltophilia* after 7 days of cultivation. B) Vitamin B_{12} production and CFUs per mL of *M. loti*, *O. anthropi*, *P. oryzihabitans* and *S. maltophilia* after 24 hours of cultivation. C) Bacterial growth as measured by OD_{600} after 4 days of growth at 30 °C for *M. loti*, *O. anthropi*, *P. oryzihabitans* and *S. maltophilia* in TY, 2xTP⁺ (+ 0.1% glycerol) and *C. reinhardtii metE*⁻ supernatant. Error bars display standard deviation from three replicates

5.3 Discussion

In this chapter, I compared the growth of *C. reinhardtii metE⁻* supplemented with B_{12} as well as in co-culture with the B_{12} producing heterotrophic bacterium *M. loti* at laboratory scale (50 mL), pre-pilot scale (10 L) and pilot scale (60 L). The results showed that B_{12} supplementation at laboratory scale resulted in a lower T_d compared to co-culturing the B_{12}-requiring cells with B_{12} producing *M. loti* (Figure 5.3; Figure 5.7) as shown in previous research (Kazamia et al., 2012; Ridley, 2016). Growth at pre-pilot scale (10 L) and pilot scale (60 L) showed a similar trend (Figure 5.4; Figure 5.5). Furthermore, the T_d of the cultures (both monoculture and co-culture) appeared to increase as the volume increased suggesting laboratory-scale experiments are not indicative of the productivity one should expect at larger scales (Figure 5.7A and B). It should be noted that there were variations in bioreactor design between the three different volumes which could contribute to the differences in T_d measured (Figure 5.3C, 5.4C and 5.5C). The concentration of B_{12} measured in the *C. reinhardtii metE⁻* + B_{12} culture at 60 L pilot scale was less than the expected 100 pg/mL. I identified through B_{12} assays that the A4F B_{12} stock had apparently been degraded, which was probably due to the age of the stock (Figure 5.6). Nonetheless, laboratory studies indicate the concentration actually present should have been sufficient to support *C. reinhardtii metE⁻* growth (Bertrand et al., 2012; Kazamia et al., 2012). I found throughout the various scales of culture, that the bacterial populations followed a similar trend of increasing in cell numbers over time, although the ratio of bacteria to algae differed (Figure 5.8). Several bacterial species were isolated from the pilot scale culture and were identified as *O. anthropi, P. oryzihabitans* and *S. maltophilia* (Figure 5.9). None of these contaminants could support the growth of *C. reinhardtii metE⁻* (Figure 5.10A). *O. anthropi* and *P. oryzihabitans* were confirmed to synthesise B_{12}, but *S. maltophilia* was not, as predicted by the KEGG database (a bioinformatics resource, Figure 5.10B). Furthermore, B_{12} was not detected in the supernatant for any of the contaminants (Figure 5.10B). Finally, none of the bacterial species including *M. loti* could grow on the supernatant of *C. reinhardtii metE⁻* (Figure 5.10C).

5.3.1 Algal growth analysis

Laboratory scale data revealed that *C. reinhardtii* (WT12) and *C. reinhardtii metE*$^{-}$+ B_{12} grew faster than *C. reinhardtii metE*$^{-}$ co-cultured with B_{12} producing *M. loti* in constant and diurnal light conditions (Figure 5.3; Figure 5.7). Overall the T_d was lower under constant light conditions than for diurnal light conditions for axenic *C. reinhardtii*, even though the light intensity used for diurnal conditions was greater than for continuous light (but unlikely to be so great as to cause photodamage), both in terms of intensity during illumination and total photon flux over a 24 h period (Figure 5.7). As mentioned, the growth rates of *C. reinhardtii* (WT12) and *C. reinhardtii metE*$^{-}$ + B_{12} were reported to be similar (Ridley, 2016), so the difference in growth rate measured in this study could be due to the difference in light exposure. This hypothesis is consistent with previously published studies on the effect of constant versus diurnal light on algal growth. Jacob-Lopez et al. (2009) found an 250% increase in biomass production (as measured in mg/L) of the cyanobacterium *Aphanothece microscopica Nägeli* when grown in constant light conditions versus diurnal light conditions (16:8 hr light/dark cycles). Goncalves et al. (2014) found a consistent increase in specific growth rates and maximum biomass productivities for a range of algal species (including *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Synechocystis salina* and *Microcystis aeruginosa*) when grown under constant light conditions versus diurnal light conditions (10:14 and 14:10 hr light/dark cycles). However, the shorter T_d in constant light was not seen for the co-culture, and the reason for this is unclear. The growth differences between axenic *C. reinhardtii* (WT12 or *metE*$^{-}$) and the co-cultures also suggest that B_{12} is a limiting nutrient for growth of *C. reinhardtii metE*$^{-}$ + *M. loti* when grown in those conditions. This agrees with previously published data on co-culturing *M. loti* with the B_{12}-dependent alga *L. rostrata* (Kazamia et al., 2012) and *C. reinhardtii metE*$^{-}$ (Ridley, 2016). It is important to note that laboratory scale growth was carried out in 50 mL conical flasks where there was good light penetration, nutrient exposure (via shaking), and sterility (Figure 5.3C). The temperature was regulated at 25^{o}C but there was no bubbling.

Pre-pilot scale (10 L) data revealed a similar trend to that of the laboratory scale data whereby *C. reinhardtii metE*$^{-}$ supplemented with B_{12} grew slightly faster than *C. reinhardtii metE*$^{-}$ + *M. loti* (Figure 5.4) suggesting that B_{12} was a limiting nutrient for growth. However, the T_d for *C. reinhardtii metE*$^{-}$+ B_{12} and *C. reinhardtii metE*$^{-}$ + *M. loti* at 10 L pre-pilot scale was higher than the T_d measured at laboratory scale under constant illumination (Figure 5.7A

and B). Again, it is important to note the practical differences at this scale which could contribute to differences in T_d measured. The cultures were grown in tubular bioreactors in non-sterile conditions and bubbled with air (Figure 5.4C) and finally, the temperature of the medium was not directly regulated and the temperature in the greenhouse varied between 20 ^oC and 30 ^oC.

The T_d for the cultures at 60 L pilot-scale showed a similar trend to that of the lower volumes. The T_d for *C. reinhardtii metE*$^-$ + B_{12} was approximately 389 hours, whereas *C. reinhardtii metE*$^-$ + *M. loti* was approximately 512 hours (Figure 5.7A and B). Significance between the cultures could not be determined as replicates were not possible. There was deviation between the two cultures beginning after day 8 when higher concentrations of total chlorophyll *a* per mL were seen in the *C. reinhardtii metE*$^-$ + B_{12} culture (Figure 5.5E). This corresponded with a drop in viability of *C. reinhardtii metE*$^-$ + *M. loti* that was more pronounced than for *C. reinhardtii metE*$^-$ + B_{12} (Figure 5.5A). The chlorophyll *a* per viable algal cell was similar between cultures but the total number of viable algal cells was lower in the co-culture (Figure 5.5F). Due to the limited number of replicates, it is difficult to conclude whether (or not) this variation of total chlorophyll *a* per mL and percentage of viable cells is a standard feature between the cultures.

The initial B_{12} concentration in *C. reinhardtii metE*$^-$ + B_{12} culture was measured at approximately 25 pg/mL (Figure 5.5G) which was lower than the expected 100 pg/mL we calculated to supplement the culture. This discrepancy was shown to be attributable to the effective concentration in the A4F stock being lower than expected (Figure 5.6). Nonetheless, laboratory studies indicate that a minimum of 20–50 pg/mL exogenous B_{12} is sufficient to sustain algal growth axenically (Bertrand et al., 2012; Kazamia et al., 2012). According to this information, there should have been enough B_{12} in the culture to sustain algal growth. Indeed, this was supported by the lower T_d of *C. reinhardtii metE*$^-$ + B_{12} (389 hours) compared to *C. reinhardtii metE*$^-$ + *M. loti* (512 hours) – a difference that was also found in the lower volume cultures (Figure 5.7). Vitamin B_{12} concentration for both *C. reinhardtii metE*$^-$ + B_{12} and the *C. reinhardtii metE*$^-$ + *M. loti* culture was found to increase over the course of the experiment (Figure 5.5G). This is likely to be due to the production of B_{12} from increasing concentration of bacterial contaminants in the culture (Figure 5.8E).

Previous work on scaling up cultures of *C. reinhardtii* (cell wall deficient strains) has been carried out by Zedler et al. (2016). Two transgenic strains and a WT control were scaled up to 100 L in the same PBRs as outlined in this study. However, the cultures were grown in sterile mixotrophic conditions and were found to be far more productive than the cultures grown here, with cell density reaching 5-15 $x10^6$ cells per mL over 7 days compared with 5-6 $x10^5$ cell per mL over 13 days in this study. The difference between dry cell weight was also significant, with 0.22-0.34 g/L increase in dry weight over 7 days reported by Zedler et al. (2016), compared with 0.075-0.183 g/L over 13 days in this study. It is important to note that the dry cell weight measurement also considers the bacteria present in the culture which were numerous in the experiments described here (see Section 5.2.6). The two studies were also carried out at similar times of the year (June/July) with similar temperatures and light exposure, so this cannot account for the difference seen in the results. Gimple et al. (2005) also grew *C. reinhardtii* (with cell walls) at pilot scale (110 L) in photoautotrophic conditions. However, there were several differences including the growth of the strains with sterile HSM medium in hanging polybags, and bubbling with 5% CO_2 (in contrast with 0.5% CO_2 in this study). The results obtained by Gimple et al. (2005) also showed the cultures were more productive in terms of biomass, with 0.267 ± 0.011 g/L after 12 days compared to 0.075-0.183 g/L over 13 days in this study.

Overall, the data obtained here showed a significant reduction in growth rates as the volume of the experiments increased (Figure 5.7). This could be due to several reasons including variation in equipment design at each scale, including the conical flask at laboratory scale, tubular bioreactor at 10 L pre-pilot and 60 L rectangular GGW PBR for pilot scale. For example, the GGW PBRs contain a lot more 'traps' which accumulate algal biomass. These 'traps' include the corners of the rectangular GGW PBRs and the connections of the temperature regulating piping. The illlumination was very different between the systems. For example, the light intensity at laboratory scale was 40 or 100 µmol photons m^{-2} s^{-1} (under constant and diurnal light conditions respectively) and for pilot scale ranged from between 400 – 600 µmol photons m^{-2} s^{-1} (Figure 5.5H). Light penetration as a result of the GGW design may have been a limitation as the GGW PBRs allow light to enter only from one side, whereas the conical flasks and 10 L tubular bioreactors allow light to enter from all angles. The exposure to 400-600 µmol photons m^{-2} s^{-1} is not suspected to be of concern as *C. reinhardtii* had been grown in very similar conditions one year earlier by Zedler et al. (2016), with higher algal growth recorded than in this study (discussed below). Furthermore, other studies showed that

when *C. reinhardtii* was grown under similar light intensities there was minimal adverse effect on the algal cells (Falk et al., 1990; Bonente et al., 2012). Finally, the cells were inoculated at high density to encourage cell shading and reduce the intensity to individual cells, so actual light exposure per cell would typically be less than 400-600 μmol photons m^{-2} s^{-1}. As mentioned previously, Zedler et al. (2016) used the same GGW PBR to grow cell wall-less C. *reinhardtii* in 100 L of TAP medium (therefore growing the strains under mixotrophic conditions) and, importantly, found the growth of the algal strains to be similar at laboratory scale to the 100 L pilot scale. This suggests the carbon in the TAP medium (in the form of acetate) could be driving the difference in productivity of the cultures in the GGW PBR as the TP^{+} medium utilized in this study contained no fixed carbon. Indeed, previous studies indicate this is to be expected. For example, Moon et al. (2013) found an approximately 7 times greater increase in dry cell weight of *C. reinhardtii* when grown in mixotrophic conditions compared to phototrophic conditions after 5 days of cultivation at 100 mL laboratory scale. Zedler et al. (2016) reported an approximately 30-fold increase in dry cell weight for cells grown in mixotrophic conditions after 5 days, compared to the approximately 2-fold increase observed here over 13 days under phototrophic conditions. It therefore appears that the difference between phototrophic and mixotrophic growth at pilot scale is much higher than at laboratory scale. However in this study, *C. reinhardtii metE*$^{-}$ was grown in non-sterile (as well as phototrophic) conditions, which is in contrast to the sterile experiments conducted by Moon et al. (2013) and Zedler et al. (2016). This difference could be a contributing factor to the relatively slow phototrophic growth of *C. reinhardtii metE*$^{-}$ in this study, if contaminating bacterial species were negatively affecting algal growth (Ridley, 2016).

5.3.2 Bacterial analysis

The number of bacterial CFUs for the 50 mL laboratory scale, 10 L pre-pilot and 60 L pilot scale experiments showed a similar trend whereby the total number measured on TY plates increased over the time course of the experiment (Figure 5.8A, C, E). The relatively constant 25:1 to 45:1 (Figure 5.8B) long-term ratio of bacteria to algae for the 50 mL laboratory scale *C. reinhardtii metE*$^{-}$ + *M. loti* culture was similar to that of the *L. rostrata* + *M. loti* co-culture, which ranged from 10:1 to 30:1 (Kazamia et al., 2012). The bacteria to algae ratio for the 10 L pre-pilot scale growth of *C. reinhardtii metE*$^{-}$+ B_{12} was stable at approximately 5:1 whereas the ratio for *C. reinhardtii metE*$^{-}$ + *M. loti* peaked at 24:1 then declined to 9:1 at day 8 (Figure 5.8D), a pattern resembling the initial days of the 50 mL laboratory scale culture. The

ratio for both cultures in the 60 L pilots scale increased over time to approximately 20:1 to 30:1 (Figure 5.8F). It is likely that this ratio differed from that of the laboratory scale because of the numerous species of environmental bacteria present in the 60 L cultures (each with their own characteristics) which could be selected for by the concentration and types of nutrients being released from the dead algal cells present (Figure 5.5A).

Several contaminating species were isolated from the 60 L pilot scale cultures (Figure 5.9A). The 16S rDNA of the contaminating species present in the 60 L pilot scale culture were partially sequenced and allowed identification as *O. anthropi,* an alphaproteobacterium, as well as *P. oryzihabitans* and *S. maltophilia*, both gammaproteobacteria (Figure 5.9B). All three species are Gram negative, undertake aerobic metabolism and are common in aqueous environments (Freney et al., 1988; Berg et al., 1999; Chain et al., 2011). Importantly, *M. loti* was not observed on the TY plates (*M. loti* is known to grow on these plates) and was not detected in any of the colonies analysed by PCR and sequencing. It could be that *M. loti* was either no longer in existence in the culture or the population present was too low to be detectable. Either way, *M. loti* was unable to protect from invasive species, in contrast to what has been predicted from the competitive exclusion principle (see section 1.7).

When the three bacteria were co-cultured seperately with *C. reinhardtii metE*$^{-}$ none of the contaminants could effectively establish a symbiosis with the alga (Figure 5.10A). This could be due to the bacteria not releasing B_{12} into the medium or the inability to utilise the carbon exudate from the alga or both. Of the contaminants detected, only *O. anthropi* and *P. oryzihabitans* were predicted (via the KEGG database) to synthesis B_{12}, in both cases via the 'late cobalt insertion' (aerobic) pathway. I confirmed in this study through a B_{12} assay that the two organisms produce B_{12} (Figure 5.10B). According to the KEGG database, *O. anthropi* is predicted to contain only the B_{12}-dependent (METH) isoform of methionine synthase whereas *P. oryzihabitans*, as with *M. loti*, is predicted to retain both METE and METH isoforms. *S. maltophilia*, the non-B_{12} synthesising strain (Figure 5.10B), was also predicted to contain both the METE and METH isoforms of methionine synthase. According to this information there does not seem to be a direct connection between the ability to synthesise B_{12} or not and the isoform of methionine synthase present in a bacterium. This is in agreement with the literature as it has been found that many prokaryotes (*E. coli* being one such example) encode both types of enzymes and appear to switch between them when B_{12} is present in the environment (Hamilton, 1974; Helliwell et al., 2011). The concentrations of B_{12} released into the supernatant

were measured for each of the bacterial species and only *M. loti* was found to release B_{12} to a concentration high enough for detection (Figure 5.10B). When the bacterial species were inoculated into the supernatant of *C. reinhardtii metE*$^{-}$ to test their ability to grow on any carbon released, none grew, including the *M. loti* which is a known symbiont (Figure 5.10C). This suggests the carbon exudate was either removed through filtration (an unlikely possibility as small carbon-containing molecules such as amino acids are small enough to bypass the filters) or there could be an element of contact required between the bacterial and algal cells for the carbon to be utilised. The bacterium *Kordia algicida* is known to have a strong algicidal effect on algae such as *Skeletonema costatum* when in physical contact with the algae, although physical contact is not essential (Skerret et al., 2002; Sohn et al., 2004; Mayali and Azam, 2004; Paul and Pohnert, 2011). Furthermore, the bacterium *Pseudomonas fluorescens* has been shown to require physical contact with the diatom *Stephanodiscus hantzschii* in order to kill the algae (Jung et al., 2008). The observations here suggest a requirement for physical contact between *C. reinhardtii metE*$^{-}$ and a bacterial partner could be a real possibility. Not withstanding this, the contaminant species were persistently present over the course of the pilot scale experiment, which suggests they must have been feeding off a carbon source. Even if the bacteria were not able to obtain fixed carbon from live algae directly, the carbon could have come from algal cell lysate, which might have been present at high concentrations because the viability of algal cells was only at around 50% by the end of the pilot scale experiment (Figure 5.5A) (Ramanan et al., 2016). It is also possible these bacteria could have been feeding off carbon exudates or metabolites released from other bacterial inhabitants. In conclusion, it could be the case that the contaminant species cannot enter symbiosis with *C. reinhardtii metE*$^{-}$ due to the limited release of B_{12} into the medium. However, it is still not clear whether these species can metabolise the carbon released from the alga.

Other studies have analysed the bacteria present in algal populations. For example Ribalet et al. (2008) isolated *P. oryzihabitans* from a bloom of the marine diatom *Skeletonema marinoi*. However, it was found that growth of *P. oryzihabitans* was inhibited by the bioactive polyunsaturated aldehydes (PUAs) produced by the diatom (Ribalet et al., 2008). Beyter et al. (2016) studied the bacterial and eukaryotic composition of an algal pond over the course of a year. The study found that both alphaproteobacteria (of which *O. anthropi* is a member) and gammaproteobacteria (of which *P. oryzihabitans* and *S. maltophilia* are members) persisted over the course of the experiment. Unfortunately, only the classes to which the contaminants belonged, and not the genera, were directly reported by Beyter et al. (2016). Bell et al. (2016)

studied the bacterial diversity with growth of *Chlorella vulgaris* in outdoor raceway ponds. Interestingly, they identified that bacteria of the order Rhizobiales (of which *O. anthropi* is a member) and genus *Pseudomonas* (of which *P. oryzihabitans* is a member) declined over the course of the experiment and were largely undetectable by day 7. However, no similar strains to *S. maltophilia* were detected. Therefore, the identification of the contaminants in this study is broadly consistent with bacteria identified in previous studies, although the profile over time may be different.

5.3.3 Application to industry

I concluded from this research that B_{12}-independent *C. reinhardtii* should be used in preference to B_{12}-dependent *C. reinhardtii* (where B_{12} supplementation or co-culture with B_{12} producing bacteria is required) in large scale industrial cultures. This is because no benefit was identified, in terms of growth productivity or protection from contaminants, and there would be an increase in costs (B_{12} supplements and time-related costs) when growing B_{12}-dependent strains. However, if the growth of a B_{12}-dependent strain is necessary, the choice between supplementing the algae with B_{12} or with B_{12} producing bacteria depends on certain conditions. For example, if one has the required resources to make sterility possible, the B_{12}-dependent algae could be grown in mixotrophic medium (where B_{12} supplementation can be used and B_{12} producing bacteria cannot be used due to the carbon source). In this situation they grow approximately 25-fold faster (based on comparison of the first 7 days of growth from this study with that reported by Zedler et al., (2016)) than in autotrophic (TP^+) medium (where B_{12} producing bacteria can be utilised to provide B_{12} to the B_{12}-dependent algae due to the absence of fixed carbon). Additionally, the cost of supplemented B_{12} is low at approximately £0.05 per mg when bought in 25 mg quantities from Sigma Aldrich, UK (e.g. when 1mg/L is used, a saturating concentration, it would cost £5 per 100 L of algal culture). Therefore, B_{12} supplementation to the alga should be the option if the financial benefit of growing B_{12}-dependent algae 25 times faster (including the cost of sterilised, mixotrophic media and the B_{12} additive) is greater than the benefit of the long term/low cost option involving non-sterile, autotrophic growth with B_{12} producing bacteria. Conversely, if sterility is not possible, autotrophic growth with B_{12} producing bacteria could be utilised (although there was a lower doubling time when *C. reinhardtii metE*$^-$ was supplemented with B_{12} as opposed to *M. loti* at 60 L pilot scale as outlined in Figure 5.7B). Furthermore, economically viable growth of this algae-bacteria co-culture might be improved in open ponds (in contrast to the GGW PBRs used

in this study) as the ponds allow for greater light exposure and even mixing (Rawat et al., 2013). However, one must keep in mind the challenges this system faces such as low CO_2 transfer rates and exposure to contamination (Rawat et al., 2013).

5.3.4 Conclusions

In this chapter I aimed to test the feasibility of growing *C. reinhardtii metE*$^{-}$ supplemented with B_{12} as well as *C. reinhardtii metE*$^{-}$ in co-culture with the B_{12} producing bacterium *M. loti* at laboratory scale (50 mL) through to pre-pilot scale (10 L) and pilot scale (60 L) and whether growth in the presence of B_{12} producing bacterium *M. loti* provided adequate B_{12} for growth and offered any protection from contaminating organisms. I found that growth at increasing scale led to longer algal cell doubling times. I also found that although *M. loti* appeared to provide an adequate supply of B_{12} for growth, the number of cells were very low and did not provide protection against contaminating microorganisms.

6 GENERAL DISCUSSION

6.1 Overview

Microalgae are a remarkably diverse group of microorganisms not only in phylogeny but also in their metabolic capability. The biotechnological potential of these organisms has only recently been identified. As a result, vast resources have been directed for the use of microalgae in the production of high value compounds such as fatty acids and pigments to low value compounds such as biofuel (Gangl et al., 2015). Whilst there has been reasonable success in the production of high value compounds, it still proves difficult to produce algae efficiently enough to produce biofuel. Major contributors to the high cost of algae production arise from contamination and culture instability (Kazamia et al., 2012). A potential solution to these problems could be to replicate the natural habitat of algae in communities as opposed to cultivating algae in isolation. Algae are known to interact with all kinds of organisms, from higher plants to coral, fungi, and bacteria (Hawksworth, 1988; Baker, 2003; Pereira et al., 2015; Ramanan et al., 2016). Unfortunately, our understanding of algal community dynamics in nature and its implications for algal biotechnology is limited. The work in this dissertation was an exploration of natural and engineered algae-bacteria mutualisms for use in industry.

In the introduction to this dissertation, the stated aims of this work were to gain a further understanding of the fundamental characteristics of algae-bacteria mutualisms, the potential for engineering algae-bacteria communities through the provision of metabolites such as nitrogen and, finally, the potential of scaling an algae-bacteria mutualism for use in industry. Each of these aims has been explored and developed throughout this thesis and a summary of how each question has been addressed is provided in the following section.

The first aim was initially focused on the genetics of *M. loti*'s mutualism with *L. rostrata*. Chapter 3 outlines the screening of *M. loti* transposon mutants (both generated and purchased) with *L. rostrata* to isolate any genes whose mutation would perturb the mutualism. After approximately 10,000 mutants screened, no genuine mutants affecting the growth of *L. rostrata* were isolated. However, during the screening process, a contaminant bacterium identified as *R. erythropolis* was isolated that could enter symbiosis with *L. rostrata*. This was the first example of a non-Rhizobiales bacterium known to do this. Furthermore, *R. erythropolis* was also found to enter symbiosis with *C. reinhardtii metE*$^{-}$ and it was found that

co-culturing multiple (up to four) B_{12} producing bacterial species with *C. reinhardtii metE*$^{-}$ did not improve the growth of the algae any more than when co-culturing it with a single B_{12} producing bacterium such as *M. loti* or *R. erythropolis*. The second aim focused on engineering algae-bacteria communities through the provision of nitrogen from engineered strains of *Anabaena* sp. PCC 7120. Chapter 4 outlines the successful use of the ammonium-releasing *Anabaena* sp. PCC 7120 strain 163 to enhance growth of the green alga, *Ca. vulgaris*. *Anabaena* strain 163 was found to be the highest performing biofertiliser (as measured by doubling time of the nitrogen receiving strain of algae) recorded in the literature to date. The third aim focused on investigating the use of an algae-bacteria co-culture in industry by culturing the *C. reinhardtii metE*$^{-}$ + *M. loti* consortium at 60 L pilot scale as well as investigating the differences in growth efficiencies seen during the scaling of the co-culture from 50 mL laboratory scale, to 10 L pre-pilot scale and finally to 60 L pilot scale. Chapter 5 outlines the successful cultivation of *C. reinhardtii metE*$^{-}$ + *M. loti* and the *C. reinhardtii metE*$^{-}$ + B_{12} control at 50 mL, 10 L and 60 L. It was found there was a trend of longer doubling times as the volume increased for both the monoculture and co-culture. It was also found that although *M. loti* appeared to provide adequate supply of B_{12} for growth of *C. reinhardtii metE*$^{-}$ at 60L pilot scale, the number of cells were low and did not provide any obvious benefits such as excluding contamination as predicted by the competitive exclusion principle (see section 1.7.2). Overall, my dissertation contributes to the understanding of natural and engineered algae-bacteria communities and their potential for the use in industrial algal biotechnology. The specific findings have been discussed separately in their respective chapters, but overall conclusions and lessons will be outlined in the following sections.

6.2 The specificity of algae-bacteria communities in nature

There is a vast quantity of evidence suggesting microalgae interactions with bacteria are ubiquitous. For example, Park et al., (2007) reported that six out of the eight bacterial contaminants isolated from a *Chlorella elipsoidea* culture enhanced algae growth (by 50-300%) when co-inoculated with the species in a controlled co-culture. Furthermore, the bacterium *Dinoroseobacter shibae* was shown to deliver both vitamin B_{12} and vitamin B_1 (thiamine) to its algal host *Prorocentrum minimum* (Wagner-Dobler et al., 2010). This study focused on the *L. rostrata – M. loti* mutualism which was thought to be specific and regulated as outlined in Chapter 3 (Kazamia et al., 2012, Grant et al., 2014). However, recent evidence suggests the degree specificity of the interaction is unclear. For example, it was identified that

growth of *L. rostrata* and *C. reinhardtii metE⁻* could be sustained by the closely related, *Rm. leguminosarum* and *S. meliloti,* although the ability to provide B_{12} to the algae was not as effective as *M. loti* (Bhardvaj 2016; Helliwell et al., 2015). In chapter 3 of this study, I identified a contaminant, *R. erythropolis,* which was also found to support the growth of both *L. rostrata* and *C. reinhardtii metE⁻* with no obvious deviation in B_{12} provision from *M. loti.* In contrast, the B_{12} producing strains of *P. putida* and *D. acidovorans* isolated from the growth of *L. rostrata* in outdoor buckets were individually found to be unable to support *L. rostrata* (personal communication Ridley, 2016). Furthermore, neither *O. anthropi* nor *P. oryzihabitans* (both B_{12} producing bacterial contaminants isolated from the 60 L GGW PBR in Chapter 5) could enter symbiosis with *C. reinhardtii metE⁻*.

I hypothesised, that the ability of a bacterium to synthesise B_{12} is not enough to establish symbiosis with a B_{12}-dependent alga. An additional requirement may be a system of releasing B_{12} into the medium. It was found that algal (*L. rostrata* or *C. reinhardtii metE⁻*) symbionts *M. loti*, *R. erythropolis*, *S. meliloti* and *R. leguminosarum* all secrete B_{12} into the medium when grown axenically (Chapter 3, section 3.2.2.2; Bhardvaj, 2016) (Table 6.1). However, B_{12} producing bacteria unable to enter symbiosis with *L. rostrata* or *C. reinhardtii metE⁻* such as *P. putida*, *O. anthropi* and *P. oryzihabitans* were not measured to secrete B_{12} or secreted very low concentrations of B_{12} (Chapter 5, section 5.2.5; Bhardvaj, 2016) (Table 6.1). It has not been tested as to whether *D. acidovorans* releases B_{12}. Bhardvaj (2016) hypothesised that the difference in B_{12} release may be due to the presence of a BtuB protein which is an outer membrane B_{12} transport protein that binds to exogenous B_{12} and transports it into the bacterial cell (Chimento et al., 2003) (Figure 6.1). Bhardvaj (2016) found that *M. loti, S. meliloti* and *R. leguminosarum* do not possess the BtuB protein and that *P. putida* does possess the BtuB protein. If the hypothesis is correct, this would suggest that B_{12} is being released by all the B_{12} producing bacteria (outlined above), but those strains where B_{12} is detected in the medium (*M. loti, S. meliloti* and *R. leguminosarum*) would be unable to take up the released B_{12} due to the absence of the BtuB protein, thus leading to the ability to enter symbiosis with the algae. In contrast, *P. putida* could take up the released B_{12} due to the presence of BtuB, thus leading to the inability to enter symbiosis with the algae as no B_{12} would be made available to the algae. Expanding this hypothesis to the bacterium isolated in this study, the algal symbiont *R. erythropolis* was found not to contain BtuB whereas the non-symbionts *D. acidovorans*, *O. anthropi* and *P. oryzihabitans* do appear to contain the protein according to NCBI Blast (NCBI Resource Coordinators, 2016). This provides further evidence in support of the hypothesis that

the absence of the BtuB protein is required for a B_{12} producing bacterium to enter symbiosis with a B_{12}-dependent alga. Future work on the interactions could focus on engineering BtuB knockouts in those bacterial species that do contain BtuB and measuring any subsequent increase in B_{12} release and ability to enter symbiosis with a B_{12} dependent alga. In reverse, one could try to introduce a *btuB* gene into those lacking BtuB and measure whether they no longer release B_{12} or enter symbiosis with B_{12} dependent algae. Futhermore, one could determine whether the BtuB protein has been lost by the algal symbionts or the BtuB protein was gained by the non-symbionts by carrying out a phylogenetic and bioinformatic analysis. Finally, the bioinformatic analysis could focus on identifying common to symbionts that are not found in the non-symbiont B_{12} producing bacteria, which could also be required for symbiosis.

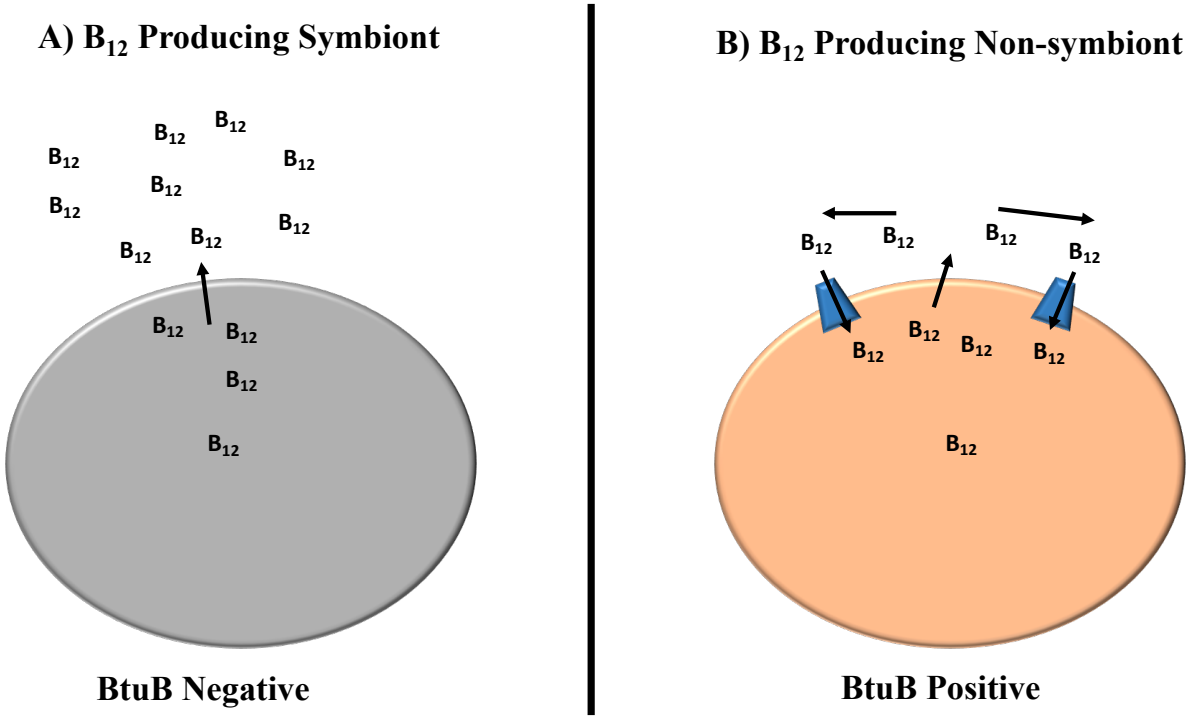

Figure 6.1 Hypothesis for the absence of BtuB as a requirement for a B_{12} producing bacterium to enter symbiosis with a B_{12} dependent alga.

A) B_{12} producing bacterial symbiont releasing B_{12} into the supernatant without subsequent B_{12} uptake due to the absence of BtuB. B) B_{12} producing bacterial non-symbiont releasing B_{12} into the supernatant with subsequent B_{12} uptake due to the presence of BtuB.

Table 6.1 B_{12} producing symbionts (BtuB negative) and B_{12} producing non-symbionts (BtuB positive)

B12 producing symbionts	B12 producing non-symbionts
M. loti	*P. putida*
R. leguminosarum	*D. acidovorans*
S. meliloti	*O. anthropi*
R. erythropolis	*P. oryzihabitans*
BtuB Negative	**BtuB Positive**

6.3 The potential of community engineering techniques in algal biotechnology

The potential benefits of growing algae in communities have been largely recognized not only for the biotechnological potential but also for the fundamental understanding of species interactions (Kazamia et al., 2012). We are not limited to the use of naturally existing algal communities for biotechnology but also engineered communities which have the potential to overcome inefficiencies, improve productivity and sustainability of algae biotechnology. There are many examples of engineered interactions which were created using various techniques such as 'screening for symbiosis', 'environmental selection', 'trait-based engineering', 'directed evolution' and 'genetic engineering'. Each of these techniques have their own advantages and disadvantages as I outlined in Gangl et al., (2015).

'Screening for symbiosis' involves identifying naturally occurring symbiosis through a high throughput screening technique. For example, Le Chevanton et al., (2013) screened algal-bacterial cultures with a high-throughput optical technique to identify interactions that gave the highest chlorophyll a fluorescence. From this technique, two bacterial strains were found to enhance biomass accumulation and nitrogen provision for *Dunaliella* sp. (Le Chevanton et al., 2013). 'Environmental selection' involves the manipulation of the environmental conditions to force organisms to form symbioses. For example, Hom and Murray, (2014) created an obligate

mutualism between the yeast *S. cerevisiae* and *C. reinhardtii* whereby *S. cerevisiae* provided carbon dioxide by metabolizing glucose and in return *C. reinhardtii* provided ammonia by metabolizing nitrite. The authors selected for the mutualism by screening a range of different conditions to identify those that allowed both organisms to grow (Hom and Murray, 2014). 'Trait-based engineering' is the creation of communities by organisms with unique but complementary growth requirements. This is based on the principle of resource-use complementarity, which allows in principle, individuals to cohabit and lead to an increase in productivity (see section 1.7.1). Stockenreiter et al., (2016), cultivated 12 different algal species in randomly selected communities of three, five and seven species in municipal wastewater as their only source of nutrients. The highest performing communities in terms of biomass production were no better than the highest performing monocultures. However, the highest lipid content was found in the multispecies communities. The authors concluded the need to hand pick communities based on known physiology and ecology for increased algal biomass production (Stockenreiter et al., 2016). 'Directed evolution' is the selection of organisms to interact in a community that collectively achieve a particular trait after many generations. Mooij et al., (2013) used this method to select for carbon storage molecules in the form of starch and lipids in algae. Algal inocula were taken from several different surface waters and cultured in a carbon dioxide-rich light period (nitrogen absent) and then a nitrogen-rich dark period. Cycling between these two conditions over many generations selected for organisms able to produce energy storage compounds (starch/lipids) to power nitrogen assimilation in the dark periods (Mooij et al., 2013). Finally, 'genetic engineering' involves the manipulation of organisms in a clearly defined manner. Chapter 4 in this study outlines the use of engineered strains of nitrogen fixing strains of *Anabaena* to provide ammonium to *Ca. vulgaris.* In particular, *Anabaena* strain 163 was identified to promote the growth of *Ca. vulgaris* the most efficiently out of all the strains tested. Furthermore, it was identified to promote the growth of an alga at the highest maximum growth rate recorded in the literature to date.

Each of the techniques has its own advantages and disadvantages and so the choice of which technique to use will depend on the purpose for engineering the communities. The 'screening for symbiosis' and 'environment selection' techniques have the advantage of screening or selecting from a large range of organisms. However, significant investment in time, materials, and manpower may be required for success. 'Trait-based engineering' and 'directed evolution' approaches offer the potential to create highly complex communities with improved productivity. However, these techniques are in their infancy and further evidence of

their efficacy is required. The 'genetic engineering' technique has the advantages of precisely engineering metabolite trading. However, it is labour intensive as a way of creating symbiotic interactions, it is limited to the few species that have sequenced genomes and established engineering methods, and obtaining regulatory approval for genetically modified organisms may prove a barrier. It is also possible that multiple techniques could be used to reach the same goal. For example, nitrogen provision from one organism to another need not be carried out by the 'genetic engineering' technique alone, as Le Chevanton et al. (2013) utilised the 'screening for symbiosis' technique to improve nitrogen provision to the alga *Dunaliella* sp., and Hom and Murray (2014) engineered nitrogen provision through 'environmental selection' as outlined above.

It is also possible that the most successful communities could be engineered through a combination of the techniques outlined above. For example, in the case of *Anabeana* strain 163 providing ammonium to *Ca. vulgaris* in Chapter 4 of this study, an improvement of ammonium provision from *Anabaena* strain 163 or possibly ammonium uptake from *Ca. vulgaris* could be selected for through repeated subculture of the community and/or screening for algal chlorophyll fluorescence via a high throughput optical screening technique, as outlined by Le Chevanton et al. (2013).

6.4 Future of microbial communities in algal biotechnology

Despite the large amount of work researching the potential of communities at laboratory scale volumes (50-500 mL) for algal biotechnology in the last decade, there are relatively few examples of their use at large scale volumes (Stockenreiter et al., 2012; Praveenkumar et al., 2014). A possible reason for this could be the relatively inconsistent methodology and results in the laboratory scale publications. Experiments in the literature range from combining a minimum of 2 algal species up to a maximum of 22 algal species in various growth media, carried out in the laboratory or in the field, in sterile or non-sterile conditions, in different volumes, bioreactors and light intensities (Liu, 2016). The results of these large groups of experiments have been summarized into several meta-analyses that have inconsistent conclusions. Most analyses found that increasing species richness tends to increase resource capture and biomass production (Schlapfer and Schmid, 1999; Balvanera et al., 2006; Cardinale et al., 2007), whereas another found that resource capture and biomass production saturate at low levels of species richness (Cardinale et al., 2006). However, when the resource

capture and biomass production of these polycultures are compared to monocultures (containing the single most productive species) only 12% of all experiments found that polycultures had greater biomass than any monoculture of the component species - a phenomenon called 'transgressive over-yielding' (TO) (see section 1.7.1) (Weis et al., 2008). Several hypotheses have been proposed to explain the limited examples of transgressive over-yielding in algal community studies including: 1) the experiments have not been run for long enough as it has been found the probability of achieving TO increases the longer the experiment runs (Cardinale et al., 2007); 2) the existence of statistical bias in biodiversity experiments which limits the ability to identify TO (Schmid et al., 2008); 3) species niche differentiation (species utilizing different environments/nutrients) may not be large enough among the community members to promote TO (Loreau et al., 2004); and 4) experiments have been intentionally simplified and homogenized to improve experimental control for example, the use of homogenous light, temperature and nutrients (Weis et al., 2008).

To the best of my knowledge, no examples exist in the literature that grow specific algae communities (including algae-bacteria communities) at large scale (i.e. greater than 10 L). However, one study has been carried out which investigated the community structure of the microorganisms co-existing with an algal species to provide an insight into the existence of naturally occurring mutualisms. Bell et al. (2016) grew *Ca. vulgaris* in 200 L outdoor raceway ponds for 10 days and then transferred the culture to a 2,000 L raceway pond for a further 6 days of growth. The microbial community was determined by sequencing small subunit rRNA sequences. *Ca. vulgaris* dominated the culture throughout the experiment as expected. However, although the bacterial diversity increased over the course of the experiment the archaeal community declined. Furthermore, a positive correlation between *Ca. vulgaris* and *Pseudomonas* sp. was observed throughout the course of the experiment leading the authors to suggest a potential symbiotic relationship could be involved, supported by the fact that such a relationship had been previously described by Guo and Tong (2013). In chapter 5 of this study, the B_{12}-dependent algal strain *C. reinhardtii metE*$^{-}$ was grown in 60 L GGW PBRs with supplemented B_{12} or co-cultured with *M. loti*. The doubling time of *C. reinhardtii metE*$^{-}$ at 60 L scale was longest at this scale compared with that of laboratory (50 mL) and pre-pilot (10 L) scale. Indeed, it was found that as the volume increased the doubling time for the algal cells also increased. This increase in doubling time could be due to differences in light penetration, nutrient availability or contamination as a result of growth in the various reactors for example, Erlenmeyer flasks, tubular bioreactors and GGW PBRs (Figure 5.3, 5.4 and 5.5).

As discussed earlier, research into the productivity of algal communities has produced limited examples of 'transgressive over-yielding'. Future research could focus on testing whether the hypotheses presented are correct explanations for the relatively poor performance of communities in the literature. For example, algal communities should be tested over the long term (with sub-culturing to select for the communities with highest growth performance), under the conditions likely to be experienced in the location of industrial scale production. As Stockenrieter et al. (2016) outlined, hand-picked selection of algal communities based on known ecological and physiological factors (also known as 'trait-based engineering') may also be beneficial to improve productivity as the algae (and other microorganisms) occupy complementary niches. Due to the expense of scaling a culture, the polycultures should be well characterised and replicable at low volumes. If this can be achieved, working with an established company (such as A4F who collaborated with this author in chapter 5 of this study) to test the polyculture at large scale is advised due to the technically demanding nature at such volumes.

6.5 Conclusion

The limited use of algal communities at industrial scale is likely to be due to the minimal data present in the literature supporting the potential benefits suggested in recent reviews (Liu, 2016). However, this does not mean the potential benefits of communities do not exist, rather that standardized methodologies that replicate the conditions faced in industry must be utilized and further knowledge of basic understanding of algal interactions in nature must be further understood. In Chapter 3 of this study, it was shown that *L. rostrata* could be supported by a non-Rhizobiales B_{12}-producing bacterium *R. erythropolis* providing evidence that the mutualism is possible with a wider range of bacteria. However, results in Chapter 5 showed that not all B_{12}-producing bacteria could support B_{12}-dependent algae and that the presence or absence of the protein BtuB in a bacterium correlated with the ability to enter symbiosis with the algae. In Chapter 4, *Anabaena* was engineered to successfully provide nitrogen in the form of ammonium to *Ca. vulgaris*, thus providing evidence to support the use of biofertilisation as an option for algal production and the use of 'genetic engineering' as a tool to precisely engineer consortia for a desired interaction. Finally, Chapter 5 outlined the first example in the literature of scaling an algal-bacterial co-culture from 50 mL laboratory scale through to 10 L pre-pilot scale and 60 L pilot scale. *M. loti* provided adequate B_{12} for growth of *C. reinhardtii metE*$^{-}$ throughout all scales tested, although cell populations at 60 L pilot scale were low and did not provide protection against contaminating organisms. Overall, there is much to be learnt about natural algal communities as well as understanding the most efficient techniques and methodologies to engineer communities to help us improve the efficiency of algae production for industry.

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8 APPENDICES

8.1 Growth Medium

For all growth media, DI water was used to make up to the final volume of solutions.

8.1.1 TAP medium

For 1 L of TAP medium:

- 16 g NH_4Cl
- 2 g $CaCl_2.H_2O$
- 4 g $MgSO_4.7H_2O$
- 7.26 g KH_2PO_4
- 14.34 g K_2HPO_4
- 1 mL glacial acidic acid
- 2.42 g Tris base
- 1ml Hutner's trace elements, as described in Merchant *et al.* (2006)
- 15 g Agar (for solid medium)

8.1.2 TP^+ medium

1 L of a modified high phosphate form of TAP medium, as described by Kazamia et al., (2012), comprising:

- 16 g NH_4Cl
- 2 g $CaCl_2.2H_2O$
- 4 g $MgSO_4.7H_2O$
- 7.26 g KH_2PO_4
- 14.34 g K_2HPO_4
- 1ml Hutner's trace elements, as described in Merchant *et al.* (2006)
- 15 g Agar (for solid medium)

8.1.3 BG11 medium

For 1 litre of BG11 medium:

- 10 mL 100 x BG11
- 1 mL trace elements
- 1 mL iron stock
- 1 mL phosphate stock (autoclaved separately and added after cooling to minimise precipitation)
- 1 mL Na_2CO_3 stock (autoclaved separately and added after cooling to minimise precipitation)

For 1 L of 100 x BG11:

- 149.6 g $NaNO_3$
- 7.49 g MgSO4. $7H_2O$
- 3.6 g $CaCl_2.2H_2O$
- 0.6 g Citric Acid
- 1.12 mL of 0.25 M solution (pH 8.0) Na_2EDTA

For 100 mL of Trace Elements:

- 0.286 g H_3BO_3
- 0.181g $MnCl_2.4H_2O$
- 0.022 g $ZnSO_4.7H_2O$
- 0.039 g $Na_2MoO_4.2H_2O$
- 0.008 g $CuSO_4.5H_2O$
- 0.005 g $Co(NO_3)_2.6H_2O$

For 100 mL of:
Iron stock

- 0.6 g Ferric citrate

Phosphate Stock

- 3.05 g K_2HPO_4

Na_2CO_3 stock

- 2 g Na_2CO_3

1M NaHCO3

- 8.4 g $NaHCO_3$ (filter sterilise)

For Buffering (*Anabaena* sp. PCC 7120 only)

- Add 20mL/L of 1 M HEPES pH 7.5 (Always keep in fridge)

For 1L of BG11 plates:

- Add 10 mL/L of TES Buffer

  For 100 mL of TES buffer:

  - 22.9 g, pH to 7.5 with NaOH
- 3 g Sodium thiosulphate
- 15 g Agar

8.1.4 BG11-N medium (nitrogen free)

For 1 litre of $BG11_0$ medium:

- 10 mL 100 x $BG11_0$
- 1 mL trace elements
- 1 mL iron stock
- 1 mL phosphate stock (autoclaved separately and added after cooling to minimise precipitation)
- 1 mL Na_2CO_3 stock (autoclaved separately and added after cooling to minimise precipitation)

For 1 L of 100 x BG11-N:

- 7.49g MgSO4. $7H_2O$
- 3.6g $CaCl_2.2H_2O$
- 0.6g Citric Acid
- 1.12 mL of 0.25 M solution (pH 8.0) Na_2EDTA

For 1L of BG11-N plates:

- Add 10 mL/L of TES Buffer
    - For 100 mL of TES buffer:
    - 22.9 g, pH to 7.5 with NaOH
- 3 g Sodium thiosulphate
- 15 g Agar

8.1.5 Tryptone-yeast (TY)

For 1 L of TY:

- 5g tryptone
- 3g yeast extract
- 0.875g $CaCL_2.2H_2O$
- 15g agar (for solid medium)

8.1.6 Lysogeny broth (LB)

For 1 L of LB:

- 12.5g tryptone
- 6.25g yeast extract
- 12.5g NaCl
- 15g agar (for solid medium)

8.1.7 TBE Buffer

For 5x stock solution in 1 L of H_20

- 54 g tris base
- 27.5 g of boric acid
- 20 mL of 0.5 M EDTA (pH 8.0)

8.1.8 Solutions for vitamin B_{12} assay

8.1.8.1 10x M9 salts

For 1 Litre of 10x M9 salts

- Na_2HPO_4 60 g
- KH_2PO_4 30 g
- NH_4Cl 10 g
- NaCl 5 g

This was made up to a litre with distilled water and then autoclaved

The following ingredients were then added:

- Calcium chloride solution (0.1 M) 50ml
- $MgSO_4$ (1M) 50ml
- Glucose (20%) 200ml
- Cysteine (5 mg/ml) (filtersterilised)* 15ml
- Methionine(5mg/ml) (filtersterilised) 15ml
- Agar (172 ml H_2O + 2.57 g bacto-agar)** 172ml

*Prepare fresh as it precipitates after a while.

**Do not autoclave M9 salts and agar together, the solution turns brown.

8.1.8.2 Minimum medium agar (10 plates)

The following reagents were autoclaved separately and subsequently mixed to constitute 200ml of minimum medium agar (10 * 9cm diameter petri dishes).

- 10 x M9 salts 20ml
- 20% glucose 4ml
- 1 M $MgSO_4$ 0.4ml
- 0.1 M $CaCl_2$ 0.2ml
- L-cysteine (5 mg/ml)* 2 ml
- L-methionine (5 mg/ml) 2 ml
- H_2O (+/- 15g bacto-agar) 171.4ml

*Prepare fresh as it precipitates after a while.

8.1.8.3 M9-based media for liquid assay (250ml=250 assays)

- 10x M9 media 50mL
- 20%glucose 10mL
- 1M $MgSO_4$ 1mL
- L-cysteine 5mL
- 0.1M $CaCl_2$ 0.5mL
- H_2O 183.5mL

8.2 Selected *M. loti* strains from Legume Base STM library

Plate code	Gene Product	Mutant code (Legume Base)	Gene Annotation
A1-3	Thiamin phosphate pyrophosphorylase	01T01h04	mll5789
A5-7	Sugar binding protein of sugar ABC transporter	01T02g01	mll1918
A9-11	Probable secreted solute binding protein	01T03b08	mll0854
B1-3	ATP Binding Protein	01T03c06	mll4176
B5-7	ATP Binding Protein	01T03c09	mll3403
B9-11	Probable sensor/response regulator hybrid	01T03e04	mll0976
C1-3	ATP Binding Protein	01T03h12	mll1680
C5-7	Response sensor protein	01T04b06	mll1798
C9-11	ATP Binding Protein	01T05c04	mll4992
D1-3	Probable sensor/response regulator hybrid	01T05f10	mll0997
D5-7	ATP Binding Protein	01T06e07	mll0708
D9-11	Sugar ABC transporter substrate-binding protein	01T06g10	mlr6435
E1-3	Sugar binding protein of sugar ABC transporter	02T03b10	mlr2326
E5-7	Alpha-galactoside ABC transporter substrate-binding protein	02T03h05	mlr6444
E9-11	Maltose ABC transporter substrate-binding protein	02T04e02	mlr3050
F1-3	Sugar ABC transporter (sugar-binding protein)	04T02h08	mll4149
F5-7	ATP Binding Protein	04T05c09	mlr3154
F9-11	Sugar binding protein of sugar ABC transporter	05T01d10	mlr2244
G1-3	ATP Binding Protein	05T04c03	mlr3054
G5-7	ATP Binding Protein	05T05d10	mlr4152
G9-11	ATP Binding Protein	06T01a08	mll1002
H1-3	Binding protein component of ABC sugar transporter	06T01d02	mll3319
H5-7	Probable Transporter	06T01e06	mll2542
H9-11	General Secretion protein G	06T01g02	mll6827

Plate code	Gene Product	Mutant code (Legume Base)	Gene Annotation
I1-3	Sugar ABC transporter substrate-binding protein	06T02a04	mlr7091
I5-7	Probable sensory transduction regulatory protein	06T02f02	mll2301
I9-11	C4 dicarboxylate transport protein	06T02g06	mll7237
J1-3	ATP Binding Protein	06T05h07	mll4203
J5-7	Thiamin/B1, ABC components	07T02a07	mll3867
J9-11	WD Repeat, B Transducin-like	07T02d04	mll2837
K1-3	Permease Protein	07T02g05	mll7374
K5-7	Sugar ABC transporter substrate-binding protein	07T03h12	mlr7582
K9-11	ATP Binding Protein	07T04f04	mll0504
L1-3	Sugar binding protein of ABC transporter	07T05a06	mlr2439
L5-7	General Secretion Protein D	09T01e12	mll6829
L9-11	Permease Protein	09T04g04	mll0705
M1-3	Permease Protein	09T04h09	mll1724
M5-7	Permease Protein	09T06g04	mlr1893
M9-11	Probable Transporter	10T05d10	mll1210
N1-3	ATP Binding Protein	11T01d01	mll3598
N5-7	Thiamin biosynthesis	14T01d03	mll5795/6
N9-11	ATP Binding Protein	14T03f06	mll7362
O1-3	Metal Transporting ATPase	14T05d05	mll2475
O5-7	Permease Protein	14T05d10	mll5112
O9-11	Ribose-binding protein of ribose ABC transporter	14T05g07	mll2148
P1-3	ATP Binding Protein	17T01d06	mll849
P5-7	ATP Binding Protein	18T02g09	mll5705
P9-11	ATP Binding Protein	18T03a03	mlr7066

Plate code	Gene Product	Mutant code (Legume Base)	Gene Annotation
Q1-3	ATP Binding Protein	20T01d08	mll7583
Q5-7	Permease Protein	22T01d03	mlr6436
Q9-11	Permease Protein	23T01f04	mll5110
R1-3	Permease Protein	23T02c09	mlr4772
R5-7	Permease Protein	23T04c02	mlr1694
R9-11	ATP Binding Protein	27T01h04	mll5657
S1-3	Permease Protein	27T02e08	mlr4912
S5-7	ATP Binding Protein	29T03c04	mlr3336
S9-11	Endopeptidase Clp ATP-binding chain A	38T06a09	mll0663
T1-3	L Arabinose binding protein	40T01g02	mll7082
T5-7	ATP Binding Protein	40T02c09	mll3378
T9-11	Putative Transporter	40T02d03	mll6284

8.3 Publications

8.3.1 An engineered community approach for industrial cultivation of microalgae

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An Engineered Community Approach for Industrial Cultivation of Microalgae

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Abstract

Although no species lives in isolation in nature, efforts to grow organisms for use in biotechnology have generally focused on a single-species approach, particularly where a product is required at high purity. In such scenarios, preventing the establishment of contaminants requires considerable effort that is economically justified. However, for some applications in biotechnology where the focus is on lower-margin biofuel production, axenic culture is not necessary, provided yields of the desired strain are unaffected by contaminants. In this article, we review what is known about interspecific interactions of natural algal communities, the dynamics of which are likely to parallel contamination in industrial systems. Furthermore, we discuss the opportunities to improve both yields and the stability of cultures by growing algae in multi-species consortia.

Introduction

Microalgae (eukaryotic photosynthetic microbes) and cyanobacteria (oxygenic photosynthetic bacteria) represent a highly diverse collection of microorganisms. They live in a range of environments including all aquatic ecosystems, both freshwater and marine, and are also found in terrestrial habitats including on hard surfaces and snow. Many taxa are capable of growing heterotrophically as well as phototrophically, and some obligate heterotrophs exist that, although ancestrally photosynthetic, have lost the ability to photosynthesize. These include the dinoflagellate *Crypthecodinium cohnii*, which is of commercial importance as a source of docosahexaenoic acid (DHA).[1] Algae are currently cultivated on a relatively small scale for high-value products such as the carotenoid astaxanthin from *Haematococcus pluvialis* and the phycobiliprotein phycocyanin from the cyanobacterium *Aphanizomenon flos-aquae*.[2] Certain strains are marketed as dietary supplements, including the cyanobacterium *Spirulina* sp. (*Arthrospira platensis*) and the green alga *Chlorella vulgaris*.

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Bulk growth of algae for products of lower value—aimed at displacing commodities traditionally made from fossil oil—has received considerable attention in the research community in recent years.[3] However, the scale-up required to achieve this poses a number of problems, ranging from the energy costs of maintaining the cultivation systems, low yields in large-scale cultures due to factors such as poor light penetration, mass transfer (where exogenous carbon dioxide is supplied, or oxygen needs to be removed), the energy costs of downstream processing and production, and biological contamination.[4–6] This work considers how understanding the ecology of the organisms under cultivation—their interaction with others in the environment—can be harnessed to enhance productivity and increase the financial and environmental benefits of cultivating algae.

Applying Community Ecology to Algal Cultivation

Most studies that target increasing yields in industrial cultures are aimed at an individual species level, which assumes that cultivation will be in monoculture. However, because contamination is inevitable without stringent sterile practice, which at industrial scale is neither cost-effective nor likely to be achievable, understanding the growth dynamics of an algal population cultivated in reactors is fundamentally an ecological problem.[7,8] Moreover, monocultures are by their nature unstable and prone to perturbation. According to a review by Smith and Crews, the genetic uniformity of monocultures encourages proliferation of pathogens and invaders, a problem common in traditional single-crop agriculture.[9] Monocultures are predicted to be unstable by the basic tenets of community ecology, which describe natural systems as increasing in complexity over time.[10] Given the opportunity, multiple species with diverse niche specificities can coexist alongside each other, maximizing the use of the available resources. Invasions by organisms from neighboring environments will continue until a “climax” stable state is reached; this state is predicted to be resilient to change provided abiotic conditions remain constant.[11]

Therefore, a strategy based on community approaches to cultivation is beginning to emerge. By starting with what would be an “end-point” consortium in a natural system, it may be possible to avoid the development of unwanted alternatives. In the following section we review the advantages—and disadvantages—of growing algae in consortia of species, rather than as monocultures. The principles that we draw on are from aquatic community ecology, the key concepts of which are summarized in Table 1.[12–32]

MAXIMIZING PRODUCTIVITY

One of the tenets of community ecology is that productivity is enhanced when diverse organisms are grown together. This has been illustrated for a range of habitats, and famously in a long-term experiment on grasslands. For a period of 7 years, it was demonstrated that grassland plots with a mixture of 16 species attained 2.7 times more biomass than the respective monocultures.[12] “Overyielding” is said to occur when the biomass production of a consortium of species is greater than that of the average monoculture of the species contained in the mixture.[21] While “transgressive overyielding” describes the state at which the mixture outperforms even the most productive of the monocultures of the constituent species.[22] Evidence has shown that when functionally diverse algae are grown together, the resulting communities are more productive than monocultures of individual species. An

aquatic experiment showed that diverse algal communities (grown in biofilms) increased the uptake and storage of nitrate from streams and significantly increased in biomass content compared to monocultures.[33] Behl et al. analyzed the rate of carbon uptake and productivity for 85 assembled communities composed of species from four phylogenetic groups: chlorophytes, diatoms, cyanobacteria and chrysophytes.[34] The researchers found that all algal communities consisting of species from two, three, or four different groups showed overyielding compared with cultures of individual species, with transgressive overyielding in more than half of the assemblages studied.

An important explanation for increased productivity in mixed cultures is through resource-use complementarity. When species that have different growth requirements are grown together, competition between members of the community is reduced compared with that experienced by individuals in dense monocultures. This allows more individuals to cohabit, increasing the net biomass of the culture. One trait that distinguishes different algal species is the portfolio of pigments they use to absorb light. Although oxygenic photosynthetic organisms generally use chlorophyll *a* as the major pigment in photosystems, the accessory light harvesting pigments differ (Fig. 1). In cyanobacteria grown under iron-replete conditions, phycobilisomes on the surface of the thylakoid membranes contain phycobilin proteins containing phycocyanin and phycoerythrin. Phycobilins are also found in red algae, whereas green algae (chlorophytes) contain chlorophyll *b*, as do all land plants. Chlorophyll *c* is the major accessory pigment in the Chromalveolata. Therefore, a possible explanation for the overyielding of biodiverse algal cultures observed by Behl et al. could be maximal use of the available light resource.[34]

CROP PROTECTION

Contaminating organisms that invade algal cultures can reduce yields in different ways: predators and pathogens are able to do so directly by killing the algae, while competing microalgae can take over as the dominant strain. For biomass production this may not be a problem, but when a specific algal strain is required, such as an oil producer or a strain with useful pigments, this will reduce productivity. In principle it could be possible to address all of these challenges by growing algae in culture with carefully selected cohabitating species.

The effect of predators can be decreased through biomanipulation of the food web, whereby an ecosystem is deliberately altered by adding or removing species. This is common practice in the freshwater management industry, where the goal is to minimize microalgal production.[35] In the context of algal cultivation, which is the reverse scenario, if production were to be hampered by invading zooplankton, the addition of zooplanktivores (such as small fish) to the reactors might increase yields.[8,36] This is unlikely to be feasible in closed photobioreactors, but may also not be practicable in open ponds. An alternative solution would be crop protection through “interference” (Table 1). By introducing multiple inedible algal species to grow alongside the desired strain, the foraging efficiency of invading zooplankton may be decreased due to the increased energetic costs of finding the desired prey.[37] This technique of pest control was recently investigated by Shurin et al. in a set of laboratory experiments, the results of which are summarized in Fig. 2.[27] Communities containing one, two, five, or ten species of algae in various combinations were subjected to

grazing by *Daphnia pulex*. Although the total biomass of algal food resources increased with diversity, survival of introduced *Daphnia* grazers declined markedly when five or ten species of algae were grown together.

However, there may be a cost to co-cultivation of a range of algal species when only a single species is of commercial interest. Since the growth of a desired strain may decrease in a dense polyculture due to increased shading by co-cultured strains. When stability of a monoculture against invasions is the primary concern, this may be enhanced by manipulating the abiotic environment to make the establishment of competitors less likely. This is why extremophiles have been preferred in commercial ventures, such as *Spirulina* sp., which is grown under alkaline conditions, or *Dunaliella salina*, which is cultured in highly saline medium.

A community solution to the problem of competitors may be engineered through co-culturing with partners that produce allelopathic chemicals. Chemical interactions are an important part of phytoplankton competition and are particularly important with dinoflagellates and cyanobacteria, which are able to produce chemicals that are toxic to most other algae in the environment.[38] This allows the organisms to bloom under the right conditions for growth, often causing what are known as Harmful Algal Blooms (HABs).[39] However, some species have evolved to withstand the toxins produced during HABs and are able to cohabit with the toxin-producing strains. If either HAB-forming or HAB-tolerant species were identified as interesting candidates for biofuel production, growth in consortia with toxin-producing strains could be a possible solution to competitive invasion. A similar approach has been taken in water treatment, where barley straw is often used to control populations of unwanted algae. Toxins produced from the straw liquor are known to inhibit the growth of some algae but not others.[32]

Bacterial contaminants often invade cultures of algae because they are able to scavenge algal exudates as a source of fixed carbon. If the bacteria compete with algae for other nutrients, they often overtake the growth of the microalgae, leading to the establishment of anoxic conditions.[40–42] Bacterial fouling (surface growth) can be very severe in closed bioreactors, requiring these systems to be shut down and fully flushed before operation can resume. This leads to yield losses and has an associated financial burden. We have previously suggested that bacterial contamination may be decreased through co-culturing algae with symbiotic (probiotic) bacteria that enhance algal growth, because invading bacteria would be less likely to establish as the bacterial niche is already occupied.[43] There is some empirical evidence from fish aquaculture that supports this theory. For example, Sharifah and Eguchi reported that *Roseobacter* clade bacteria that are symbiotic with *Nannochloropsis oculata* (grown commercially for fish food) successfully inhibited the growth of the fish pathogen *Vibrio anguillarum*.[44]

CAPITALIZING ON MUTUALISMS

Mutualisms can be of benefit in industrial biotechnology of microalgae in a range of ways. Mutualistic exchange of metabolites can replace external inputs of scarce or expensive resources. For example, half of all algae are known to require vitamin B_{12} (cobalamin) for growth, but this is only synthesized by prokaryotes.[45] Model laboratory consortia have been

described in which vitamin B_{12}-dependent algae can obtain cobalamin from vitamin B_{12}-synthesizing bacteria, in exchange for a source of fixed carbon, and, indeed, in the case of the *Dinoroseobacter shibae* partnership with its dinoflagellate host, vitamin B_1 is also exchanged (Fig. 3A).[36,43] If this system were to be employed industrially, the bacteria could replace exogenous addition of vitamins into the medium, reducing material and energy inputs into the culture. Other described mutualisms include the provision of iron via siderophores from bacteria to algae in exchange for fixed carbon.[18]

It is possible to envisage a system in which the mutualism between algae and bacteria depends on provision of nitrogen by the bacteria, a macronutrient that is acknowledged as one of the key drivers of microalgal productivity in natural systems.[48,49] Modelling the potential for algal biodiesel production in the United States indicated that the availability of nitrogen and phosphorus fertilizers were the major limiting factors for large-scale cultivation.[50] In a recent study, *Azotobacter vinelandii*, a nitrogen-fixing bacterium, was genetically engineered to secrete ammonium into the surrounding medium.[47] Co-culture of the strain in medium that did not contain exogenous carbon or nitrogen with oil-producing microalgae including *Chlorella sorokiniana, Pseudokirchineriella* sp. and *Scenedesmus obliquus*, allowed for growth of the algae (Fig. 3B).[47] This shows the potential to grow algae industrially in the absence of nitrogenous fertilizer input by co-culturing with appropriate bacteria. As nitrogenous fertilizer is made through the energy-intensive Haber-Bosch process that has been estimated to contribute up to 40% of all energy inputs into microalgae biofuel systems, provision of nitrogen via a symbiont could significantly reduce the lifecycle energy and carbon footprint of the resulting fuel.[51] Another possible sustainable approach would be to grow algae on wastewater that is rich in nitrogen and phosphorus, thereby recycling nutrients from domestic and agricultural effluent.[52]

It is likely that the range of options for co-culturing algae with bacteria will increase as our understanding of interspecific interactions between these organisms improves. Evidence suggests that microalgal interactions with bacteria are ubiquitous, although the physiological basis is often not known. For example, Park et al. reported that six out of the eight bacterial contaminants isolated from a *Chlorella elipsoidea* culture enhanced algal growth when co-inoculated with the species in a controlled co-culture.[53] Similarly, Do Nascimento et al. found that the inoculation of *Rhizobium* strain 10II into cultures of the oleaginous microalga *Ankistrodesmus* sp. strain SP2-15 resulted in up to a 30% increase in accumulation of chlorophyll, biomass, and lipids compared with axenic monocultures of the alga.[54]

IMPROVING THE STABILITY OF DESIRED STRAINS

Regulation has been observed in the specific mutualism between the vitamin B_{12}-dependent green alga *Lobomonas rostrata* and the soil bacterium *Mesorhizobium loti*, for which the ratio of algal-to-bacterial numbers equilibrated to around 1:30 in semi-continuous co-culture.[43] Mathematical modelling of the dynamics of the two species in co-culture revealed that the population growth of one organism could be predicted entirely based on the expected carrying capacity of the co-cultured symbionts.[55] Although the mechanism remains unknown, the biological implication is that the symbionts are controlling the the growth of one another when in co-culture. Understanding regulatory processes in symbioses

may benefit biotechnology by providing a means to maintain the long-term stability of a co-culture and its fidelity.

It is well understood that bacterial genomes are highly dynamic and can adapt to selection pressures easily. Therefore, when proposing the engineering of an organism for a desired output, long-term stability of the genetic alteration should be an important consideration. An example of the highly dynamic nature of bacterial genomes is highlighted in a recent large-scale effort to re-sequence strains of wild type *Synechocystis* sp. PCC6803 (originally sequenced at University of California at Berkeley in 1971) that are being maintained in various culture collections around the world.[56] The study revealed that strains presumed to be identical had in fact accumulated mutations likely to have effects on glucose tolerance, metabolism, motility, phage resistance, and stress responses.[57,58] Likewise, genetically modified organisms are no less dynamic, and engineering the production and/or secretion of metabolites useful for biotechnology may create a significant energy burden. This burden can cause selection pressure to lose the introduced genes if there are no benefits in providing the service (i.e., only one organism is providing something useful). However, mutual exchange of essential metabolites may provide an environment whereby those genes are required to function in order to survive, thus mitigating against any selection pressure in favor of reversion. For example, Hosoda et al. engineered a syntrophic (cross-feeding) community of *Escherichia coli* in which two strains cohabited: one auxotrophic for isoleucine and the other for leucine.[59] Neither strain was able to survive on its own, but growth was possible in synergistic co-culture. Kerner et al. engineered a similar system, in which *E. coli* were either tyrosine or tryptophan auxotrophs, but improved on the previous attempts by introducing an element of control to the system.[60] By tuning the metabolic exchange via gene expression or chemical inducer they were able to regulate the growth rates and strain ratios. This was taken further to demonstrate successful interspecific associations, when an *E. coli* strain auxotrophic for glutamine was engineered to provide lipoic acid to *Dictyostelium discoideum*, an amoeba, in exchange for the amino acid.[61]

Towards Designing Algal Communities

We have identified four main advantages for using community approaches to cultivate microalgae. It is possible to increase productivity of microalgal cultures by cultivating consortia of species that have complementary functional traits and therefore overyield, or to decrease loss of productivity by cultivating microalgae with species from other life domains (such as non-photosynthetic bacteria or zooplanktivores), which can increase resistance to predators and contaminants. We have highlighted the importance of engineering co-dependence among introduced members of the consortium via mutualisms with the benefit of reducing energy and material inputs. Finally, in agreement with Brenner et al. we believe that for a stable and robust co-culture, whenever a new organism is introduced into a consortium it should contribute something useful to the culture “economy” in addition to receiving something in return, for example through the division of labor or specialization.[62] In that way, interacting organisms rely on each other through trading to establish a stable and long-lasting culture.

Of course the use of consortia of microbes in biotechnology is not novel; multispecies systems are often employed to increase yields in microbial-based processes such as anaerobic digestion, fermentation, and bioremediation.[63] In these traditional systems microbial communities are allowed to develop naturally; the most efficient assemblages are chosen for application and subsequently carefully maintained. Although this approach is not common in algal biotechnology, recently Mooij et al., demonstrated that by providing a selection pressure for algae to accumulate storage compounds linked directly to fitness, communities rich in starch and/or lipid assembled stochastically.[64]

These directed selection approaches will prove very useful to understanding the complex and advantageous interactions of microorganisms. In parallel to these efforts, we proposed a synthetic ecology approach to assembly of consortia aimed to be more productive and/or more resistant to contamination.[36] Synthetic ecology differs from selection approaches by introducing an element of design and use of transferrable building blocks (such as specific species, engineered symbioses, or growth conditions) to assemble a desired community of microorganisms. However, all community approaches to cultivation remain unproven at scale. Indeed, the stability of an engineered consortium may face the same challenges as monocultures. A range of unanswered questions remains, the most fundamental of which is how much complexity within a consortium is required before challenges faced by monocultures (instability, invisibility, etc.) are overcome? The use of high-throughput sequencing in microbial ecology is revealing the highly complex and dynamic nature of communities, but these natural systems may well harbor considerable functional redundancy, which could be simplified for the benefit of biotechnological purposes.[65]

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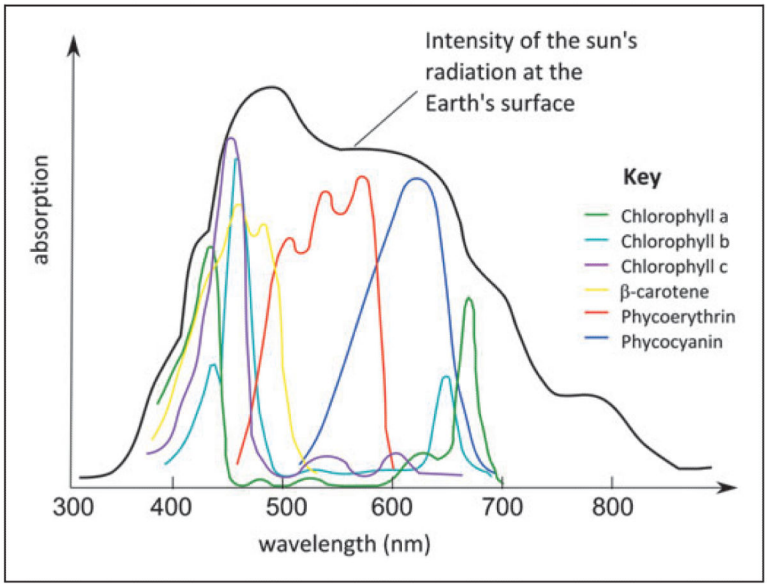

Fig. 1.

Absorption spectra of pigments present in algae shown in comparison to the intensity of the sun's radiation at the Earth's surface. In vivo absorption spectra are shown for chlorophyll *a* (λ_{max} = 435, 665 nm), chlorophyll *b* (λ_{max} = 480, 650 nm), chlorophyll *c* (λ_{max} = 645 nm), *β*-carotene (λ_{max} = 450 nm), phycoerythrin (λ_{max} = 490, 546, 576 nm), and phycocyanin (λ_{max} = 618 nm).

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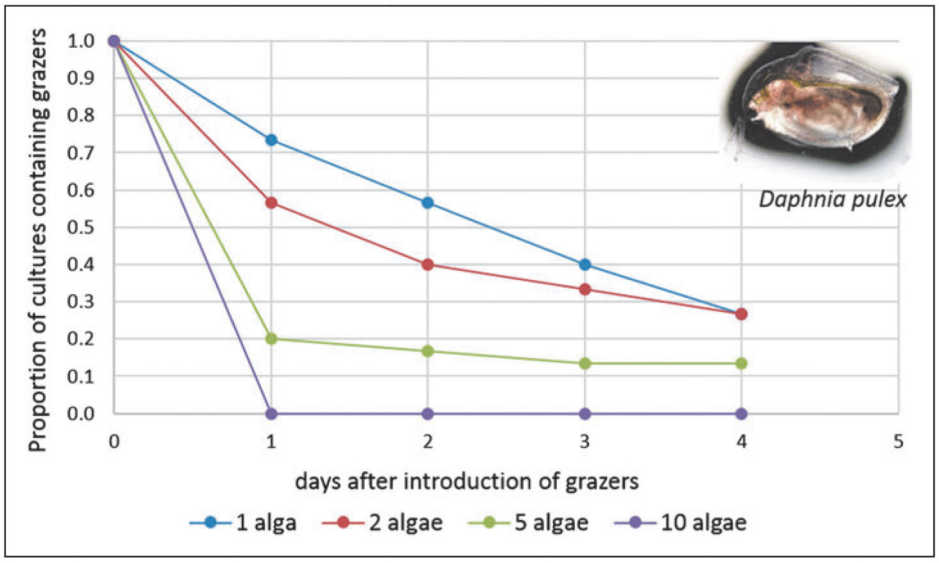

Fig. 2.

Survival of *Daphnia pulex* (water flea) in cultures of algae with different species composition, taken from a study by Shurin et al.[27] In this experiment, animals were added to 25-day-old cultures of algae composed of either one, two, five, or ten algal species (belonging to the groups Chlorophyta, Cyanophyta, Bacillariophyta and Heterokontophyta) and the number of cultures in which the fleas survived was assessed daily. For each combination, three biological repeats were assayed. The photograph is an image of *Daphnia* under the light microscope at 10× magnification.

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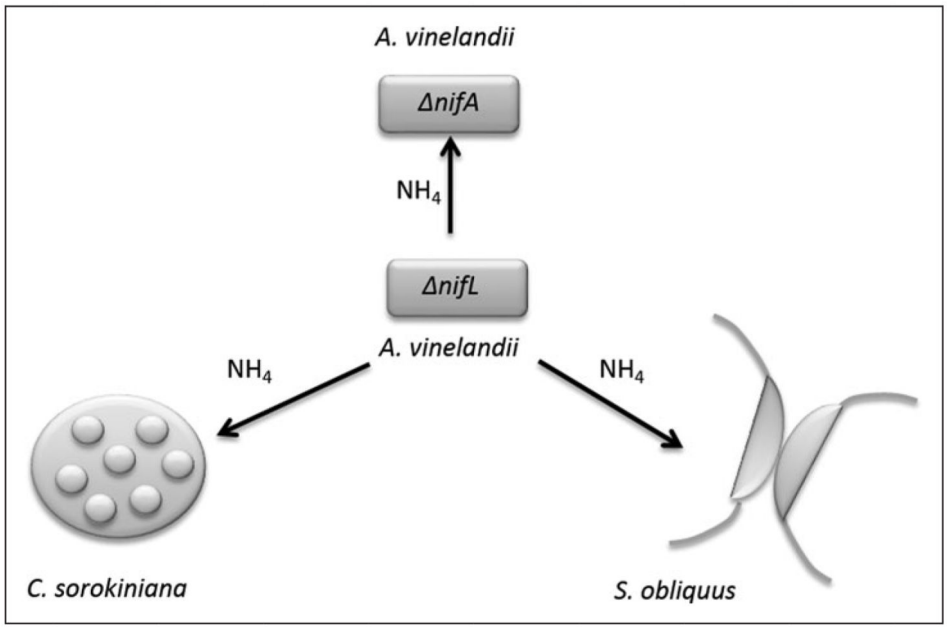

Fig. 3.

An engineered symbiotic interaction described by Ortiz-Marquez et al., in which a nitrogen-fixing bacterium, *Azotobacter vinelandii*, was modified to express constitutively a nitrogenase (Δ*nifL*) providing ammonium for non-diazotrophic organisms (unable to fix nitrogen), including the green algae *Chlorella sorokiniana* and *Scenedesmus obliquus*.[47]

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Table 1
Key Concepts from Aquatic Community Ecology and Freshwater Management

CONCEPT[a]	DESCRIPTION
Productivity- resource use complementarity	The productivity of a community is maximized when cohabitants have complementary resource requirements. Direct competition is decreased compared with a population of equal magnitude when members require the same resources.[12–14]
Reciprocal exchange of nutrients	As part of a complex range of possible symbioses, many species have evolved to exchange metabolites that limit their growth in the environment. Such mutualisms strengthen the stability of the populations.[15–18]
Regulation	Partners to a mutualism often regulate the growth of their symbionts in order to establish a fair (cost-efficient) exchange of nutrients.[19,20]
Overyielding	Overyielding occurs when the net productivity of a community is greater than the average of the individual monocultures grown in the same environment. This is likely to be due to resource use complementarity, mutualisms, and enhanced stability against environmental perturbations.[21,22]

PRACTICES FROM FRESHWATER MANAGEMENT/ INDUSTRY[b]	DESCRIPTION
Biomanipulation	Biomanipulation is an ecosystem approach to freshwater management commonly aimed at reducing algal loads in lotic systems (such as lakes). Food web dynamics are used to shift community composition. For example, top predators may be introduced to reduce the density of zooplanktivorous fish, allowing the zooplankton grazers to decrease the algal load.[23–25]
Interference grazing	Grazing zooplankton are able to consume certain algal species, dependent on their mouth size (known as 'gape-limitation'). If a range of algal species of various sizes are grown together, grazers can be forced to spend more energy looking for prey that they can eat than they gain from feeding, which leads to their elimination from culture.[26,27]
Probiotic control of unwanted bacteria	Bacteria are capable of eliminating other species from culture either directly or indirectly. Direct control is through the production of toxins that kill off competitors, while indirect control is through competition for resources. Once bacteria are present in the system, unwanted contaminants have a decreased likelihood of establishing.[28,29]
Allelopathic control of contaminants	Allelopathy is a biological phenomenon that has been extensively documented in plant communities. Secondary metabolites that affect the survival, reproduction, and growth of neighboring species are released into the environment. Both positive and negative interactions have been recorded.[30–32]

[a] Core ecological principles.

[b] Main practices in freshwater management that have been used to direct communities towards a desired composition.

8.3.2 Biotechnological exploitation of microalgae

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REVIEW PAPER

Biotechnological exploitation of microalgae

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Abstract

Microalgae are a diverse group of single-cell photosynthetic organisms that include cyanobacteria and a wide range of eukaryotic algae. A number of microalgae contain high-value compounds such as oils, colorants, and polysaccharides, which are used by the food additive, oil, and cosmetic industries, among others. They offer the potential for rapid growth under photoautotrophic conditions, and they can grow in a wide range of habitats. More recently, the development of genetic tools means that a number of species can be transformed and hence used as cell factories for the production of high-value chemicals or recombinant proteins. In this article, we review exploitation use of microalgae with a special emphasis on genetic engineering approaches to develop cell factories, and the use of synthetic ecology approaches to maximize productivity. We discuss the success stories in these areas, the hurdles that need to be overcome, and the potential for expanding the industry in general.

Key words: Chlamydomonas; downstream processing; metabolic engineering; microalgae; recombinant proteins; transformation.

Introduction

Microalgae are a large and diverse group of photosynthetic organisms ranging from prokaryotic cyanobacteria to eukaryotic algae spread across many phyla (Guiry, 2012). This diversity offers great potential that has yet to be exploited to any great extent. Microalgae are found in freshwater and marine habitats and produce half of the atmospheric oxygen on earth. The ability to grow autotrophically makes their cultivation potentially simple and cost-effective, and microalgae have attracted increasing interest as sources of natural food additives, cosmetics, animal feed additives, pigments, polysaccharides, fatty acids, and biomass (Hallmann, 2007; Borowitzka, 2013; Leu and Boussiba, 2014). An important aspect of using microalgae for industrial purposes is the GRAS (generally regarded as safe) status of numerous algae. This is essential for products intended for animal or human consumption and could significantly reduce downstream processing costs. Recently, major advances in developing microalgae as biotech platforms have been made; this is especially

the case from a genetic engineering perspective. Here we give an overview of state-of-the-art engineering tools, previous successes with recombinant protein expression, and advances made in engineering cyanobacteria for high-value compound production. Furthermore, the largely untapped potential of algae grown in synthetic communities and the challenges associated with downstream processing of microalgae are discussed.

High-value natural products in microalgae

Microalgae are the source of several forms of high-value compounds such as carotenoids, polyunsaturated fatty acids (PUFAs), proteins, antioxidants, and pigments. Characterized by high protein and nutrient contents, some species such as *Arthrospira platensis* (a cyanobacterium, also known as *Spirulina platensis*) and *Chlorella vulgaris* (a green alga) are used as feed, food additives, and diet supplements (Yaakob *et al.*, 2014). In other cases, specific high-value compounds are isolated from appropriate strains.

Relatively few proteins have been purified from microalgae for commercial use, but *Spirulina* is a rich source of phycocyanin, a protein that constitutes 14% of the dry weight of this cyanobacterium (McCarty, 2007). The US Food and Drug Administration (FDA) has approved phycocyanin from *Spirulina* as a blue food colorant. Moreover, phycocyanobilin, the tetrapyrrole chromophore of phycocyanin, manifests fluorescent properties that have been exploited for labelling of antibodies in immunofluorescence and flow cytometry. In mammalian tissues, it can be enzymatically reduced to phycocyanorubin (a close homologue of bilirubin) and inhibits the activity of NADPH oxidase, thus reducing the generation of reactive oxygen species. It has been suggested that a regular intake of phycocyanobilin may provide protection against cancer and other diseases (Sørensen *et al.*, 2013). Moreover, recent studies have proven the beneficial health effect of microalgae (*Chlorella*, *Spirulina)* by increasing natural killer cell levels and stimulating immune and anti-inflammatory system response in humans (Nielsen *et al.*, 2010; Kwak *et al.*, 2012).

Carotenoids are important products that are extracted from microalgae, and indeed the first commercialized product derived from algae was β-carotene. It is produced in very high amounts by *Dunaliella salina*, a halophilic alga growing in saline habitats, which makes the cultures less susceptible to contamination. What differentiates *Dunaliella* β-carotene from the synthetic product (present only in the form of all-*trans* isomer) is that it is rich in the 9-*cis* isomer, and a negative effect of the use of all-*trans* isomer, such an effect on plasma cholesterol levels and atherogenesis, has been reported from mice studies (Harari *et al.*, 2008; Borowitzka, 2013). Another example of a carotenoid with a well-established and growing market in the nutraceutical area is astaxanthin from the freshwater green alga *Haematococcus pluvialis*. Astaxanthin is mainly used as a feed supplement and pigmentation source for salmon and shrimp farming, but due to its high antioxidant properties (10-fold greater than other carotenoids) and protective activities, it has also many applications in the pharmaceutical and cosmetic industries. Astaxanthin has also been shown to prevent bacterial infection, vascular failure and cancer (Ambati *et al.*, 2014).

Fatty acids are the other natural components produced commercially from microalgae. Several marine algal species are rich in omega-3 long chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid (DHA; e.g. from *Isochrysis* strain T-iso and *Pavlova lutheri*), eicosapentaenoic acid (EPA; e.g. *Nannochloropsis gaditana*, *Nannochloropsis oculata*), and alpha-linolenic acid (e.g. *Rhodomonas salina*, *Tetraselmis uecica*). Oils from *Nannochloropsis*, *Rhodomonas*, and *Tetraselmis* have higher antioxidant properties than fish oils, partly because of a high content of valuable carotenoids (fucoxanthin, lutein, neoxanthin, alloxantin) and polyphenols. Given the fact that intensive fishing endangers many fish species, algal oils may provide an alternative to fish oils in diets in the future (Ryckebosch *et al.*, 2014).

Other algal species such as *D. salina* and *Botryococcus braunii* can accumulate up to 60% of storage lipids as triacylglycerides (TAGs), potentially making them a valuable source of oil for biodiesel production (Scott *et al.*, 2010). Compared with plants, algae exhibit higher productivities and theoretically could give 10- to 100-fold higher yields of oil per acre, although such capacities have not yet been achieved on a commercial scale (Greenwell *et al.*, 2010). Nevertheless, algae appear to be a potential solution to the controversial food vs fuel problem that is associated with the use of fertile land to produce plant-derived biofuels. TAGs are not the only way in which microalgae could be exploited for biofuels. Under anaerobic conditions and sulphur depletion, some microalgae produce hydrogen gas, which could be used as an alternative to fossil fuels in the future (Melis *et al.*, 2000; Zhang *et al.*, 2002). Recently, Scoma *et al.* (2012) have attempted to produce hydrogen from *Chlamydomonas reinhardtii* using natural sunlight. Although successful, the overall yields did not exceed those obtained in lab-scale settings (Scoma *et al.*, 2012) and so this technology is at an early stage.

While the above compounds are well-established microalgal products with known potential, other microalgae are being studied for new compounds with useful properties. Some cyanobacteria have a poor reputation in the popular press for causing toxic blooms, because they are able to produce hepatotoxins and neurotoxins (e.g. anatoxin, jamaicadine, L-beta-*N*-methylamino-L-alanine) (Aráoz *et al.*, 2010). However, research on cyanobacteria is undergoing a renaissance, because some identified metabolites and their derivatives have been shown to have potential as next generation antiviral (Huheihel *et al.*, 2002), anticancer (Leão *et al.*, 2013), and antibacterial drugs. Several of these drugs have even successfully reached phase II and III clinical trials (Dixit and Suseela, 2013). A large number of promising natural compounds are derived from filamentous marine genera such as *Lyngbya*, *Symploca*, and *Oscillatoria*. Usually they are short peptides built from non-canonical amino acids by the hybrid polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS) (Tan, 2013).

Some of the most promising candidate anticancer agents are derivatives of dolastatin 10, a peptide originally isolated

from sea hare *Dolabella auricularia*. The first one, TZT-1027 (soblidotin) is a microtubule polymerization inhibitor that exhibits antitumour activity in preclinical models, manifesting stronger activity than paclitaxel (Watanabe *et al.*, 2006; Akashi *et al.*, 2007). Another analogue of dolastatin 10 (monomethyl auristatin E) linked to an antibody is already approved by the FDA and used in therapies for patients with Hodgkin lymphoma (Deng *et al.*, 2013). In summary, algae and cyanobacteria are an apparently under-explored source of natural high-value compounds and there is a rising interest in their exploitation. Just as numerous plant secondary metabolites have been used for biotechnological and biomedical purposes, there is huge scope for the identification of correspondingly valuable compounds within the vast microalgal population. New discoveries are made regularly, and new compounds and applications for industrial purposes are to be expected in the near future.

Genetic tools in microalgae, and the development of microalgal cell factories

As well as containing a range of high-value compounds, microalgae offer real potential as cell factories for the production of other compounds and proteins. With the advancement and availability of algal genome data, transformation protocols have been developed for a number of microalgae and this means that they can now be used to enhance the levels of natural high-value products, or for the expression of genes in order to produce novel products, or recombinant proteins including antibodies, hormones, vaccines, and insecticidal protein at economically viable levels (Hallmann, 2007; Gong *et al.*, 2011). To date, there have been reports of successful genetic manipulation of over 40 different microalgae species including the green algae *C. reinhardtii*, *D. salina*, *Chlorella vulgaris*, and *H. pluvialis*, and the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Walker *et al.*, 2005). However, the genetic toolkits developed for *C. reinhardtii* and *P. tricornutum* are the most advanced, and both nuclear and chloroplast transformation has been achieved, so these species will be reviewed as examples.

In recent years, there have been rapid developments of genetic tools for microalgae in an attempt to generate more effective microalgal cell factories. Microalgae have several distinct advantages compared with plant-based production and some other bioreactor systems. First, many microalgae can double their biomass in less than 24 hours, with some species such as *Chlorella sorokiniana* having a doubling time under optimum conditions of less than three hours (Sorokin, 1967). Therefore, only a short period of time is required for large-scale production compared with plants. Furthermore, the time from creation of a new transgenic line to industrial scale-up can be as short as two months (Schmidt, 2004; Mayfield *et al.*, 2007). Second, genes for recombinant proteins can be expressed from the nuclear or chloroplast genomes (León-Bañares *et al.*, 2004), and eukaryotic microalgae possess post-translational modification pathways which allow glycosylated proteins to be produced and secreted out of the cell (Hempel and Maier, 2012; Lauersen *et al.*, 2013*a*, 2013*b*). Third, many microalgae can be grown either phototrophically or heterotrophically in enclosed photobioreactors, preventing transgenes from escaping into the environment. This is a particular concern for transgenic plants where transgenic DNA might spread to soil bacteria or to related plant species by means of pollen transfer (Gong *et al.*, 2011). Prior to introducing the desired gene into an algal genome, a few fundamental factors such as DNA delivery method, selection method, and control of gene expression have to be taken into account.

DNA delivery method

A variety of different methods have been employed for delivering transgenes into algal cells (Table 1), and the method of choice is very much determined by the cell size and nature of the cell wall of the chosen algal species (Stevens and Purton, 1997). The first demonstrations of stable transformation of an alga involved the bombardment of *C. reinhardtii* cells with DNA-coated microprojectiles (so-called biolistics) and proved successful for the delivery of DNA into both the chloroplast (Boynton *et al.*, 1988) and nucleus (Debuchy *et al.*, 1989). Biolistics was also used to transform the mitochondrial genome of *C. reinhardtii* (Randolph-Anderson *et al.*, 1993).

Subsequently, the nuclear genome of *C. reinhardtii* has been genetically transformed using other delivery methods including agitation in the presence of glass beads or silicon carbide whiskers, electroporation, *Agrobacterium*-mediated transformation, and, more recently, positively charged aminoclay nanoparticles (Kindle, 1990; Brown *et al.*, 1991; Dunahay, 1993; Kumar *et al.*, 2004; Kim *et al.*, 2014). Unlike biolistics or

Table 1. *DNA delivery methods for C. reinhardtii and P. tricornutum*

Organelle	Species	Delivery methods	References
Nucleus	*C. reinhardtii*	Microparticle bombardment, electroporation, *Agrobacterium tumefaciens*, glass beads, silicon carbide whiskers, and aminoclay nanoparticles	Boynton *et al.*, 1988; Brown *et al.*, 1991; Dunahay, 1993; Kim *et al.*, 2014; Kindle, 1990; Kumar *et al.*, 2004
Nucleus	*P. tricornutum*	Microparticle bombardment, electroporation	Apt *et al.*, 1996; Falciatore *et al.*, 1999; Miyahara *et al.*, 2013; Zhang and Hu, 2014
Chloroplast	*C. reinhardtii*	Microparticle bombardment, glass beads	Boynton *et al.*, 1988; Kindle *et al.*, 1991
Chloroplast	*P. tricornutum*	Electroporation	Xie *et al.*, 2014
Mitochondria	*C. reinhardtii*	Microparticle bombardment	Randolph-Anderson *et al.*, 1993

electroporation, methods using glass beads, silicon carbide whiskers, or *Agrobacterium* do not involve any specialized equipment and can be done in any laboratory at low cost, although silicon carbide whiskers can be difficult to obtain and may potentially be a health hazard (León-Bañares *et al.*, 2004). The glass bead method remains popular, and has also been employed for chloroplast transformation as a simple alternative to biolistics (Kindle *et al.*, 1991; Economou *et al.*, 2014). For nuclear transformation by electroporation, cell wall-less strains (either mutants or cells treated with autolysin) are normally used (Brown *et al.*, 1991; Shimogawara *et al.*, 1998). However, *C. reinhardtii* with an intact cell wall was recently transformed using a series of multi-electroporation pulses (Yamano *et al.*, 2013). The cheaper *Agrobacterium*-mediated gene transfer technique can also be used with walled strains and has been reported to give high transformation frequency compared with glass bead transformation (Kumar *et al.*, 2004), and this can be increased further by inducing the *Agrobacterium* prior to infection using acetosyringone and glycine betaine (Pratheesh *et al.*, 2014).

Nuclear transformation using aminoclay nanoparticles (positively charged nanoparticles based on 3-aminopropyl-functionalized magnesium phyllosilicate) is still new, but it has also been reported to give high transformation rates, and again this simple method can be used with wild-type strains possessing a cell wall (Kim *et al.*, 2014). This method might have general applicability to other algal species that have traditionally been transformed using particle bombardment (Walker *et al.*, 2005) since the aminoclay nanoparticles (45 nm) are several orders of magnitude smaller than the gold particles (1 μm to 3 μm) used in the bombardment. The size of particles is an important factor, because smaller particles increase the chance of cell wall penetration and genome integration (Kim *et al.*, 2014).

The nuclear genome of the diatom *P. tricornutum* is most commonly transformed via particle bombardment (Apt *et al.*, 1996; Falciatore *et al.*, 1999). Diatoms are generally quite difficult to transform due to challenges such as controlling and manipulating their life cycles, as well as penetrating their rigid cells walls (Falciatore *et al.*, 1999). Nonetheless, recently the *P. tricornutum* nuclear genome was successfully transformed by electroporation (Miyahara *et al.*, 2013; Zhang and Hu, 2014). More remarkable, given the four membranes that surround a diatom chloroplast, is a recent demonstration that electroporation can be used to deliver DNA into the chloroplast genome (Xie *et al.*, 2014).

Chloroplast genome engineering is particularly attractive given the ability to target transgenes into specific, predetermined loci via homologous recombination, and the high levels of expression that can be achieved. To date, chloroplast transformation by biolistics has been reported for five other algal species: *Dunaliella tertiolecta*, *Euglena gracilis*, *H. pluvialis*, *Porphyridium* sp., and *Tetraselmis cordiformis* (Purton *et al.*, 2013; Cui *et al.*, 2014).

Selectable markers, reporter genes, and promoters

Different types of selectable marker and reporter genes can be used in identifying putative transformants from the population of untransformed cells (Table 2). Endogenous selectable markers for nuclear transformation of the haploid *C. reinhardtii* allow the rescue of auxotrophic or non-photosynthetic mutants with examples for nuclear transformation being *ARG7*, *NIT1*, and *OEE1* that allow rescue of recessive mutants defective in arginine biosynthesis, nitrate metabolism, and photosynthesis, respectively (Stevens and Purton, 1997). A wide range of dominant markers are commonly used in *C. reinhardtii* and confer resistance to various antibiotics or herbicides, as detailed in Table 2. For chloroplast transformation, several dominant markers (e.g. *aadA* and *aphA6*) have been developed (Table 2). In addition, endogenous chloroplast genes have been used as selectable markers for phototrophic rescue of the corresponding chloroplast mutant: for example, the *atpB* gene rescues an *atpB* mutant defective in the ATP synthase. The use of phototrophic markers is not possible for nuclear or chloroplast transformation of wild-type *P. tricornutum* since this diatom is an obligate phototroph. However, an engineered strain expressing a transgene encoding a glucose transporter has been described that is capable of heterotrophic growth on the sugar (Zaslavskaia *et al.* 2001), and this potentially could be used to develop non-phototrophic recipient strains. Reporter genes based on fluorescent proteins, luciferases, or colorimetric enzymes have been developed for both the nucleus and chloroplast (Table 2), with the key to good expression of such transgenes being the use of synthetic genes that are codon-optimized for expression in the algal host (Fuhrmann *et al.*, 1999; Zaslavskaia *et al.*, 2000; Purton *et al.* 2013).

For transgene expression in the nucleus or the chloroplast, promoters and untranslated regions (UTRs) from highly expressed endogenous genes are typically used. For nuclear expression these include genes for subunits of the photosynthetic complexes, ribulose bisphosphate carboxylase, or components of the light-harvesting apparatus (Walker *et al.*, 2005), and for chloroplast expression, genes for core photosynthetic subunits such as *atpA*, *psbA*, *psaA*, and *psbD* (Purton, 2007). However, such chloroplast genes are typically under feedback control via *trans*-acting factors that bind to the 5′ untranslated region (UTR). Recently, (Specht and Mayfield, 2013) have shown that re-engineering these 5′UTRs can overcome such control and result in elevated levels of transgene expression.

With the advancement of microalgal biotechnology, many tools and techniques for the genetic manipulation of microalgae have been developed. However, nuclear transformation still faces problems such as poor transgene integration, codon bias, positional effects, and gene silencing which can lead to poor or unstable transgene expression. Moreover, there are still limited molecular genetic tools for many of the microalgal species that have high commercial value. Chloroplast transformation is a promising strategy for precise engineering and high-level expression of transgenes, but its application is still largely limited to *C. reinhardtii*, and again there is a pressing need to develop reliable methods for commercial species. Hence, more work has to be carried out so that microalgae can be exploited as light-driven bioreactors in the future.

Table 2. *Common marker and reporter genes used in C. reinhardtii and P. Tricornutum*

Species	Marker/reporter genes	Description	Reference
C. reinhardtii	***Marker genes***		
	Chloroplast		
	atpB	ATP synthase subunit	Boynton *et al.*, 1988
	psbH	Thylakoid membrane protein (PSII subunit)	Cullen *et al.*, 2007
	aadA	Adenylyl transferase (resistance to spectinomycin)	Goldschmidt-Clermont, 1991
	Nucleus		
	ARG7	Argininosuccinate lyase	Debuchy *et al.*, 1989
	NIT1	Nitrate reductase	Kindle *et al.*, 1989
	ble	Protein conferring resistance to bleomycin, phleomycin and zeomycin	Stevens *et al.*, 1996
	aph7"	*Streptomyces hygroscopicus* aminoglycoside phosphotransferase	Berthold *et al.*, 2002
	bptII	Neomycin phosphotransferase III	Hall *et al.*, 1993
	aphVIII	Aminoglycoside 3′phosphotransferase (resistance to paromomycin, kanamycin and neomycin)	Sizova *et al.*, 1996
	als	Acetolactate synthase (resistance to sulphonylurea herbicides)	Kovar *et al.*, 2002
	cry1-1	Ribosomal protein S14	Nelson and Lefebvre, 1995
	oee-1	Oxygen evolving enhance protein	Chang *et al.*, 2003
	cat	Chloramphenicol acetyltransferase (resistance to chloramphenicol)	Tang *et al.*, 1995
	Reporter genes		
	gus	β-Glucuronidase	Kumar *et al.*, 2004
	gfp	GFP	Kumar *et al.*, 2004
	luc	Luciferase	Fuhrmann *et al.*, 2004
	chgfp	Modified GFP	Fuhrmann *et al.*, 1999
	ars	Arylsulphatase	Davies *et al.*, 1992
P. tricornutum	***Marker genes***		
	Chloroplast		
	cat	Chloramphenicol acetyltransferase	Xie *et al.* 2014
	Nuclear		
	ble	Resistance to bleomycin, phleomycin and zeomycin	Apt *et al.*, 1996; Zaslavskaia *et al.*, 2001
	nptII	Neomycin phosphotransferase III	
	hpt	Hygromycin B phosphotransferase	
	nat/sat1	Nourseothricin resistance	
	cat	Chloroamphenicol acetyltransferase (resistance to chloroamphenicol)	
	Reporter genes		
	egfp	Modified GFP	Zaslavskaia *et al.*, 2001, 2000
	glut1	Glucose transporter	
	hup1	Hexose transporter	

Production of recombinant proteins in microalgae

Several previous studies have shown that microalgae are promising production platforms for recombinant proteins. Major successes and recent advances in recombinant protein production are summarized in the following.

Monoclonal antibodies are complex molecules used to treat several human diseases. To date most antibodies are produced in mammalian cells due to their ability to carry out post-translational modifications, particularly glycosylation and disulphide bond formation. Recently, efforts have also been directed towards producing them in bacteria and yeast (Spadiut *et al.*, 2014). The first expression of a functional antibody in microalgae was achieved in the chloroplast of *C. reinhardtii*. A large single chain antibody directed against the herpes simplex virus (HSV) glycoprotein D was successfully expressed in this green alga. The genetic construct was integrated into the chloroplast genome via homologous recombination and expression was driven by *atpA* or *rbcL* promoters and 5′ UTRs. The antibodies were shown to accumulate as soluble proteins in the chloroplast and could bind to HSV proteins in ELISA experiments. Heavy and light chains assembled by forming disulphide bonds, however, no other post-translational modifications were detected (Mayfield *et al.*, 2003). In another study, a fully functional monoclonal antibody against anthrax protective antigen 83 (PA83) was expressed in the *C. reinhardtii* chloroplast. Separate constructs for the heavy and light chain were introduced and the antibody was shown to assemble spontaneously by forming the necessary 16 disulphide bonds (Tran *et al.*, 2009).

The production of antibodies in microalgae is, however, not restricted to *Chlamydomonas*. A monoclonal antibody was also successfully expressed in the endoplasmic reticulum (ER) of the diatom *P. tricornutum*. It was directed against the Hepatitis B virus surface protein and accumulated to 8.7 % of total soluble protein. Heavy and light chains, retained in

the ER by DDEL retention peptides, were shown to assemble into complete antibodies and were glycosylated (Hempel *et al.*, 2011). When the same group omitted the ER retention signal from their constructs, functional antibodies were efficiently secreted and accumulated in the culture medium in an active form (Hempel and Maier, 2012).

Another group of valuable therapeutics are immunotoxins, which are most commonly used in the treatment of cancer. Immunotoxins are antibodies coupled to eukaryotic toxins and are difficult to produce in both prokaryotic and eukaryotic hosts. Tran *et al.* (2012) were able to show that fully functional immunotoxins can be expressed in algal chloroplasts. An antibody directed against the B-cell surface epitope CD22 was genetically linked to Exotoxin A from the human pathogenic bacterium *Pseudomonas aeruginosa* and expressed in the chloroplast of *C. reinhardtii*. It was shown that the immunotoxins were enzymatically active, bound specifically to CD22 on B-cells, and inhibited cell proliferation *in vitro*. Tests in mouse xenograft models showed reduced tumour progression (Tran *et al.*, 2012). In another study the successful expression of an immunotoxin comprising an antibody recognizing B-cell CD22 coupled to gelonine—a ribosome inhibiting protein—in the *C. reinhardtii* chloroplast was demonstrated. The immunotoxin was shown to inhibit the survival of two B-cell lymphoma lines (Ramos and CA-46) while leaving Jurkat T-cells intact, thereby proving their specificity (Tran *et al.*, 2013).

Several vaccine antigens have been produced in the chloroplast of *C. reinhardtii* (Sun *et al.*, 2003; He *et al.*, 2007; Demurtas *et al.*, 2013). The GRAS status of the alga makes the production of antigens without costly purification from residual toxins or impurities attractive and could even be exploited for oral delivery of vaccines. An algal-produced *Staphylococcus aureus* vaccine was shown to protect orally vaccinated mice against sub-lethal and lethal doses of *S. aureus* infection (Dreesen *et al.*, 2010). Vaccines blocking malaria transmission were also successfully expressed in the *C. reinhardtii* chloroplast (Gregory *et al.*, 2012; Jones *et al.*, 2013). In addition, Bayne *et al.* (2013) showed that it is possible to produce influenza haemagglutinin (HA) in the heterotrophic alga *Schizochytrium sp.* thereby presenting an alternative to current egg-based vaccine production platforms (Bayne *et al.*, 2013).

Other proteins expressed in microalgae include mammary-associated serum amyloid (M-SAA), which could be used as viral and bacterial prophylaxis in newborn mammals (Manuell *et al.*, 2007); domains 10 and 14 of human fibronectin, which have potential as antibody mimics; proinsulin and vascular endothelial growth factor, as well as the high mobility group protein B1 involved in wound healing (Rasala *et al.*, 2010). Furthermore, a human selenoprotein, Sep15 (Hou *et al.*, 2013) and antitoxins against botulinum neurotoxin have been successfully produced (Barrera *et al.*, 2014).

Further efforts to develop microalgae as a production platform have been made. Other than the therapeutic proteins mentioned, several enzymes have been successfully produced in *C. reinhardtii*. The industrially relevant Xylanase 1 was expressed in the nucleus of *C. reinhardtii* both in a soluble and secreted form (Rasala *et al.*, 2012). The first membrane-bound enzyme expressed in the *C. reinhardtii* chloroplast was CYP79A1 from *Sorghum bicolor*. It was shown to be active and produced *p*-hydroxyphenylacetaldoxime, the precursor for a plant natural product, from endogenous tyrosine (Gangl *et al.*, 2015). In another study, a bifunctional diterpene synthase was expressed in the chloroplast of *C. reinhardtii*, and is the largest recombinant protein expressed to date. This synthase is of interest for exploring the potential of terpenoid production in microalgae (Zedler *et al.*, 2014; see next section for details).

Despite the huge variety of recombinants successfully produced in algae to date, only one report exists of transferring recombinant protein production to a larger scale. A milk amyloid A-producing strain of *C. reinhardtii* was grown in three 100 l bags in a greenhouse setting and the maximum rate of MAA production achieved was 0.051 ± 0.016 g/l/d (Gimpel *et al.*, 2014). The lack of other examples shows that the knowledge transfer from the lab scale to industrially relevant growth conditions for recombinant protein production in microalgae is still not established.

Metabolic engineering of microalgae to produce other high-value compounds

Microalgae offer additional potential as light-driven cell factories for the production of novel metabolites. Plant secondary products are one of the most important forms of target compound. Plants are a source of a broad spectrum of diverse bioactive compounds, also known as secondary or specialized metabolites. Often their biosynthesis is restricted to specific developmental stages, tissues or even cells and they can be involved in many processes important for plant growth and survival, such as protection from pathogens or herbivores, attraction of pollinators, and adaptation to environmental stress. A large number of specialized metabolites are now used by the pharmaceutical, chemical, and food industries, and approximately 50% of all approved medicines including anticancer drugs are of natural origin (Dai *et al.*, 2014; Lassen *et al.*, 2014*b*).

Many of these plant-derived compounds can be produced by chemical synthesis, but in some cases due to complexity of their structure they require difficult multistep regio- and stereospecific reactions, therefore overall yields can be very low. This applies particularly to the synthesis of terpenoids—a particularly large, complex, and important family of plant secondary metabolites. One solution to this problem can be extraction from the plant source, but if a product is accumulated in scarce amounts, the result may not be economically viable. An example is paclitaxel, a cytostatic drug known under the commercial name Taxol. Its accumulation in *Taxus baccata* depends on the maturity of the tree and ranges from 0.064 g (27-year-old tree) to 8.038 g (136-year-old tree) per tree (Nadeem *et al.*, 2002), whereas chemical synthesis is complicated and requires 40 different steps (Nicolaou *et al.*, 1994).

Towards engineering metabolic pathways

An alternative method for production of high-value chemicals is to use synthetic biology or metabolic engineering tools.

Both terms are often used interchangeably, however, there are significant differences between the two fields. Synthetic biology is more about designing and using defined synthetic DNA parts (biobricks), constructing genetic circuits and molecular switches to control the expression and metabolism, whereas metabolic engineering is a broader term involving protein engineering and pathway optimization in order to improve production yields of the desired product (Stephanopoulos, 2012). Nonetheless, both are understood as transferring biosynthetic pathways from one organism to another (or combining pathways from different organisms).

In order to produce some specialized high-value compounds such as terpenoids, it is often necessary to perform highly specific and complex enzymatic reactions, which can be catalysed by cytochrome P450s (P450s). For example, biosynthesis of both artemisinin and paclitaxel requires the involvement of P450s. Unfortunately the expression of plant P450s in *Escherichia coli* and *Saccharamoyces cerevisiae* is not always simple due to special requirements for post-translational modification and protein localization, and in consequence plant P450s are often inactive upon expression in these hosts (Chemler and Koffas, 2008).

P450s are monooxygenases performing stereospecific hydroxylations and they are anchored in the plant ER. In order to function they need to be powered by single electron transfers from NADPH-dependent cytochrome P450 reductase (CPR). The expression level of these proteins is relatively low and the activity is often limited by NADPH and the substrate pool (Jensen *et al.*, 2011). It has been demonstrated that these energy-consuming reactions can be bypassed by relocating P450-dependent metabolic pathways to the thylakoid membranes of tobacco chloroplasts, where the reducing power generated by photosystem I (PSI) in the form of reduced ferredoxin (Fd) is a cheap and essentially unlimited source of electrons for the P450s (Nielsen *et al.*, 2013) (Fig. 1). More recently, it has been shown that a P450 can be expressed in the chloroplasts of transgenic *C. reinhardtii* and it was furthermore shown that the enzyme was targeted into the chloroplast membranes and was highly active (Gangl *et al.*, 2015). This strongly suggests that it will be possible to produce pharmacologically important terpenoids not only in plant chloroplasts (Bock and Warzecha, 2010), but also in microalgal chloroplasts.

Cyanobacteria as a tool for synthetic biology

During the last decade, cyanobacteria have been gaining renewed interest as a chassis for metabolic engineering and synthetic biology approaches. Currently many efforts are being undertaken to use genetically modified cyanobacteria as a production platform for biofuels such as isoprene (Zhou and Li, 2010), bioplastics like polyhydroxybutyrate (Wang *et al.*, 2013) and polylactic acid (Angermayr *et al.*, 2014), fatty acids (Ruffing, 2014), ethylene (Guerrero *et al.*, 2012; Ungerer *et al*, 2012), and sugars (Jacobsen and Frigaard, 2014). In many cases reported yields are not yet satisfactory, however, the ability to perform photosynthesis and the idea of converting sunlight and carbon dioxide into high-value chemicals are undoubtedly attractive.

As indicated above, expression of foreign pathways involving P450s in *E. coli* often causes problems and is ineffective, due to difficulties with proper folding, post-translational modifications, and targeting to the membranes. Unlike most native bacterial P450 enzymes that are soluble, cyanobacterial P450s are membrane bound, exactly like plant P450s (Robert *et al.*, 2010). All the above considerations indicate that cyanobacteria and eukaryotic microalgae can be suitable candidates for expression of foreign P450s.

Expression of *p*-coumarate-3-hydroxylase from *Arabidopsis thaliana* in the cyanobacterium *Synechococcus* sp. PCC 6803 for the production of caffeic acid was the first reported successful expression of a plant cytochrome P450 in cyanobacteria (Xue *et al.*, 2014). Further genetic strain modifications resulted in a 25-fold increase of production rate of *p*-coumaric acid (a precursor for caffeic acid) giving yields of 82.6 mg/l secreted to the media (Xue *et al.*, 2014). Another

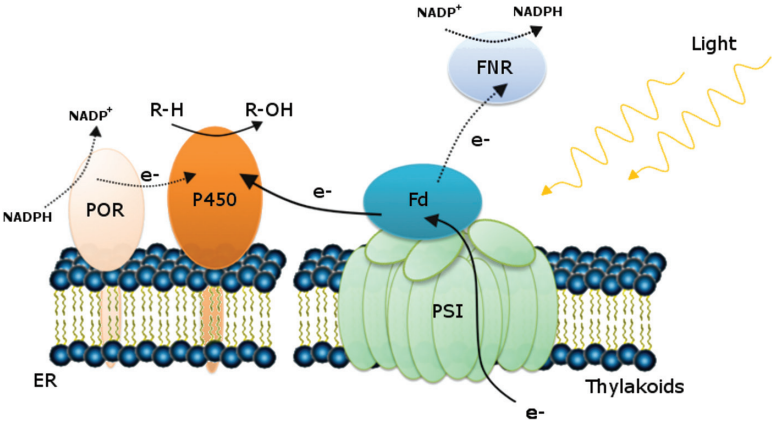

Fig. 1. Relocation of cytochrome P450s from the ER into the thylakoid membranes for coupling with photosynthetic electron flow. Most cytochromes P450 function in the ER where they interact with specific enzymes (cytochrome P450 reductases) that provide the reducing power to drive complex substrate modifications. It has now been shown that these key enzymes can be expressed in the chloroplasts of plants, algae, and cyanobacteria, where the P450s are active and able to use reducing power from ferredoxin. This provides a novel route for the formation of complex metabolites involving P450 enzymes.

example of stable expression of a P450 enzyme (CYP79A1 from the dhurrin pathway) in *Synechococcus* sp. PCC 7002, and direction of the protein to the thylakoid membranes by fusing the protein with the PsaM subunit of PSI, has recently been reported (Lassen *et al.*, 2014*a*). Successful heterologous expression of the mevalonic acid (MVA) pathway in *Synechocystis* sp. PCC 6803 (Bentley *et al.*, 2014) and production of β-phellandrene (Bentley *et al.*, 2013) also opens up the opportunity to express terpenoid biosynthetic pathways in cyanobacteria. Moreover, excretion of produced metabolites into the growth media (Lassen *et al.*, 2014*a*; Xue *et al.*, 2014) suggests that cyanobacteria have an unexplored potential as good platforms for metabolic engineering. Overall, it is clear that these organisms have potential as production hosts but it is fair to say that improvements are required before they can attain mainstream status as production chassis.

Techniques to engineer algal communities

In their natural environment, algae live intimately with many other organisms. These communities exhibit complex interactions including metabolite exchange, cell aggregation, and biofilm formation (Jagmann and Philipp, 2014). For example, at least half of all algal species require an exogenous supply of vitamin B_{12}, and bacteria can be a source for this co-factor in return for a carbon source, thus forming a tightly regulated symbiosis (Croft *et al.*, 2005; Kazamia *et al.*, 2012). Further examples include the symbiosis between a unicellular prymnesiophyte alga and a cyanobacterium (UCYN-A) with the former providing a carbon source in return for fixed nitrogen (Thompson *et al.*, 2012), and the dynamic symbiosis between the haptophyte alga *Emiliania huxleyi* and the bacterium *Phaeobacter gallaeciensis* BS107, which can be mutualistic or parasitic according to environmental conditions (Seyedsayamdost *et al.*, 2011).

In contrast, algal biotechnology has traditionally involved large-scale cultivation of axenic cultures (Scott *et al.*, 2010; Day *et al.*, 2012). This difference has been widely recognized and consequently there are many reviews outlining the potential benefits of using consortia in algal biotechnology. The main advantages proposed include an increase in algal productivity (accrued biomass) of mixed cultures compared with axenic cultures, enhanced crop protection from pathogens and pests due to the competitive exclusion principle, reduction of energy inputs, increased stability of the crop to include population control and resilience to environment perturbations, and finally, the division of labour between consortium members leading to increased ability to engineer complex and broader metabolic pathways (Table 3) (Ortiz-Marquez *et al.*, 2013; Jagmann and Philipp, 2014; Kazamia *et al.*, 2014; Nalley *et al.*, 2014; Pandhal and Noirel, 2014; Smith and Crews, 2014).

Although the proposed benefits of creating algal communities are great, engineering the communities in practice is likely to be complex. We therefore focus on examples where engineering of efficient algal communities has been shown to be effective.

Table 3. *Key benefits of engineering algal communities for biotechnological exploitation*

Benefit	Reasoning	Reference
Improved algal productivity	Resource-use complementarity is when algal species with different growth requirements are grown together; the competition for resources between the organisms is lowered allowing the individual species to cohabit, thus increasing net biomass of the culture.	Kazamia *et al.*, 2014; Nalley *et al.*, 2014; Smith and Crews, 2014
Enhanced crop protection	The competitive exclusion principle is when a culture contains a diverse array of organisms. Resources or niches are therefore occupied leading to an increased ability to competitively exclude invaders.	Kazamia *et al.*, 2012; Nalley *et al.*, 2014; Smith and Crews, 2014
Reduction of energy inputs	The use of organisms to provide scarce or expensive resources such as vitamin B_{12} or fixed nitrogen into the media.	Kazamia *et al.*, 2014; Ortiz-Marquez *et al.*, 2013
Increased stability and resilience	Stability of communities can be defined as the minimal population fluctuation despite a disturbance, and resilience can be defined as the ability for a community to revive after a disturbance. Increasing algal diversity and richness has been shown to improve the stability and resilience of algal cultures.	Nalley *et al.*, 2014
Increasingly broad and complex engineering of metabolic pathways	Microbial communities engineered to produce valuable products have many advantages due to the division of labour. The ability to compartmentalize complex pathways into a number of strains could lead to optimized functionality and lowering metabolic burden on any one cell.	Jagmann and Phillip, 2014

Genetic engineering for symbiosis

Genetic manipulation can be used to engineer organisms to interact in a clearly defined way by the trading of metabolites. Examples of this include engineering the nitrogen-fixing bacterium *Azotobacter vinelandii* to secrete fixed nitrogen into the growth media, replacing the need for addition of synthetic nitrogen inputs for algal growth (Ortiz-Marquez *et al.*, 2012; Ortiz-Marquez *et al.*, 2014), and the engineering of the cyanobacterium *Synechocystis sp.* PCC 6803 to secrete a carbon source for *E. coli* which it can utilize to produce low- to high-value compounds (Niederholtmeyer *et al.*, 2010). The benefits of using one organism to provide nutrients for another include the potential to lower production costs (nutrient inputs and energy consumption) and reduce the carbon footprint of the process. However, creating these communities may require considerable investment of time and resources, limiting the size to a small number of interactions therefore limiting the benefit of the competitive exclusion

principle (Table 3). Given the limited ability to exclude contaminants, this strategy, if applied on its own, would be best suited for contained cultures (with low risk of contamination) for production of high-value products in contrast to cultures exposed to the environment (for low-value product) where contaminants will easily occupy niches and even take advantage of the engineered community.

Screening symbioses

Screening of naturally developing populations can offer a simpler method of identifying mutually beneficial symbioses from a large number of organisms. For example, Do Nascimento *et al.* (2013) showed an increase of up to 30% in chlorophyll, biomass, and lipid accumulation in the oleaginous microalga *Ankistrodesmus* sp. strain SP2-15 when co-cultured with the bacterium *Rhizobium* strain 10II (Do Nascimento *et al.*, 2013). The positive interaction was identified by subculturing non-axenic algal cultures (without added organic carbon) for three years, which was followed by the isolation of bacterial strains able to utilize carbon exudates from the algal strains. The bacteria were then screened for induction of positive growth effects on various algal strains via growth curves. Le Chevanton *et al.* (2013) used a similar approach where bacteria were isolated from algal cultures (Le Chevanton *et al.*, 2013). However, the cultures were initially screened with a high-throughput optical technique to identify interactions that gave the highest chlorophyll *a* fluorescence. Le Chevanton *et al.* thus identified two bacterial strains enhancing biomass accumulation and nitrogen provision for *Dunaliella* sp. The final example given here highlights the importance of screening a diverse range of organisms for symbiosis. Lorincz *et al.* (2010) serendipitously created an artificial tripartite consortium based on the known bipartite symbiosis involving *C. reinhardtii* (alga) trading carbon to *A. vinelandii* (bacterium) in exchange for nitrogen. *Alternaria infectoria* (fungus) had spontaneously contaminated the co-culture, which led to a positive growth effect by providing amino acids (in particular cystathionine) to the consortium (Lőrincz *et al.*, 2010). Analysis of naturally developing populations is therefore advantageous in its potential for screening of a large range of interactions. However, significant investment in time, materials, and manpower may be required for success.

Environmental selection for symbiosis

A novel approach to form communities is through manipulation of the environmental conditions to force organisms to form symbioses. For example, Hom and Murray (2014) created an obligate mutualism between the yeast *S. cerevisiae* and *C. reinhardtii* by testing a range of different conditions to identify those that allowed both organisms to grow. Under the successful conditions, *S. cerevisiae* provided carbon dioxide by metabolizing glucose and in return *C. reinhardtii* provided ammonia by metabolizing nitrite. The authors reproduced this mutualism with many additional algal and yeast species, which gives a sense of optimism about applying this technique to a range of other organisms (Hom and Murray, 2014). Similarly Ortiz-Marquez and colleagues created an artificial symbiosis between a strain of *A. vinelandii* engineered to secrete fixed nitrogen (ammonium) and the alga *Chlorella sorokiniana* by co-culturing the species in nitrogen- and carbon-deficient media (Ortiz-Marquez *et al.*, 2012). An important similarity between these two examples was the requirement of a solid support for the symbiosis to occur (i.e. a lack of agitation or shaking), outlining the importance of cell-cell proximity and spatial structure. It should be noted that this approach is new and only a few positive examples have been reported, so it remains to be seen how widely the approach can be applied. It also remains to be seen how robust symbioses generated in this way will be.

Trait-based engineering

Trait-based engineering is the creation of communities by organisms with unique but complementary growth requirements. This is based on the principle of resource-use complementarity, which allows, in principle, individuals to cohabit and lead to an increase in productivity. Examples of trait-based complementarity include the co-culturing of algal species with varying accessory light-harvesting pigments maximizing the utility of the visible light spectrum (Kazamia *et al.*, 2014; Nalley *et al.*, 2014). Trait-based engineering could be applied to make use of the varying temperatures that occur during a 24-h cycle as well as seasonal fluctuations, by co-culturing organisms that optimally grow at a range of temperatures, such as *Detonula confervacea* with a temperature optimum of 11 °C to *Chlorella pyrenoidosa*, with a temperature optimum of 40 °C (Eppley, 1985; Myers, 1984). Another feature that could be considered in this context is the introduction of species that control organisms such as zooplankton, which are known for their predation on algal species. Kazamia *et al.* (2014) and Nalley *et al.* (2014) outlined the use of fish as a means to control zooplankton (Kazamia *et al.*, 2014; Nalley *et al.*, 2014). Trait-based engineering has particular benefits for open-raceway production, where algal species are exposed to fluctuating environmental conditions. However, this approach will require a thorough understanding of the surrounding environment of the raceway pond to be able to handpick organisms for optimal community growth.

Directed evolution

Another approach is to take advantage of artificial selection as a means of creating efficient co-cultures. Selecting for the ability of organisms to work together over many generations may be an efficient and productive means of achieving certain traits from mixed communities. This approach has been applied to fermentation, anaerobic digestion, bioremediation, and the production of polyhydroxyalkanoate and polyphosphates (Zeng *et al.*, 2003; Serafim *et al.*, 2008; Johnson *et al.*, 2009; Sabra *et al.*, 2010). Mooij *et al.* (2013) used this method to select for carbon storage molecules in the form of starch and lipids in algae. Algal inocula were taken from several

different surface waters and cultured in a carbon dioxide-rich light period (nitrogen absent) and then a nitrogen-rich dark period. Cycling between these two conditions over many generations selected for organisms able to produce energy storage compounds (starch/lipids) to power nitrogen assimilation in the dark periods (Mooij *et al.*, 2013). This particular example, due to the controlled environmental conditions (nitrogen cycling), may be best utilized with photobioreactors. However, directed evolution could be applied for production of algae in open-raceway ponds for the selection of resistance to extreme conditions such as high pH or salt concentrations as applied to the production of *Arthrospira* sp. and *D. salina*, respectively.

In summary, metabolic engineering has the advantages of precisely engineering metabolite trading as well as the ability to fine tune population dynamics (You *et al.*, 2004; Kerner *et al.*, 2012). However, it is labour intensive as a way of creating symbiotic interactions and obtaining regulatory approval for genetically modified organisms may prove a barrier. The metabolic engineering approach is further limited to organisms whose genomes are readily manipulated. Screening and environmental selection for symbiosis has the advantage of using a potentially large range of organisms to form symbioses, and trait-based engineering and directed evolution approaches offer the potential to create highly complex communities with improved productivity. However, these techniques are in their infancy and further evidence of their efficacy is required.

Algal community research has made great progress in recent years and a number of additional technologies will be utilized alongside the engineering approaches discussed above. Some of these emerging technologies include the monitoring of algal populations through the use of quantitative PCR; crop protection using traditional chemicals such as pesticides and herbicides (McBride *et al.*, 2014); the highly parallel screening of beneficial and novel microbial symbiotic interactions through the use of microdroplet technology (Park *et al.*, 2011); development of online databanks or libraries detailing functional traits of bacteria and algae so communities can be designed for optimum trait functionality (Guiry *et al.*, 2014); and accurate modelling of communities to help predict environments that induce and stabilize microbial interactions (Kim *et al.*, 2008; Klitgord and Segre, 2010; Grant *et al.*, 2014).

Downstream processing of microalgal products

As described above, microalgae are light-driven cell factories that can produce a wide spectrum of natural products, or which can be engineered to produce diverse high-value compounds. Extraction and purification of these products are critical processes that can contribute to up to 60% of the total production cost (Molina Grima *et al.*, 2003). Research to date has focused on developing methods for downstream processing of algal biomass and for oil extraction and biofuel production. Many have reviewed downstream processing for biofuel production from different algae including *Chlorella*, *Dunaliella*, *Nannochloris*, *Nannochloropsis*, *Porphyridium*, *Schizochytrium*, and *Tetraselmis* (Mata *et al.*, 2010; Chen *et al.*, 2011; Halim *et al.*, 2012; Lee *et al.*, 2012; Kim *et al.*, 2013; Pragya *et al.*, 2013; Rawat *et al.*, 2013; Rios *et al.*, 2013; Ahmad *et al.*, 2014). In this section, we review the common steps for processing microalgae in order to prepare biofuels, recombinant proteins and other high-value products.

Downstream processing methods for biofuels production

Typically, downstream processing of microalgae used for the production of biodiesel consists of harvesting in a two-step operation in order to separate the biomass from the culture media using solid–liquid separation technologies. The first step is bulk harvesting where the biomass is concentrated by flocculation, flotation, or gravity sedimentation reaching up to 7% total solids; the second step is thickening where the biomass slurry is concentrated into a paste by more energy-intensive processes like centrifugation or filtration (Brennan and Owende, 2010). Such technologies are chosen according to the characteristics of the microalga species and the nature and quality of the final product (Amaro *et al.*, 2011), however, they are limited in their abilities to separate biomass from the media and the operating cost that can fluctuate between 20% and 30% of the total production cost (Gudin and Therpenier, 1986).

From this point, the harvested biomass paste can undergo two different pre-treatments depending on the final product requirements, the so-called dry and wet route. The dry route, which involves technologies such as spray drying, drum drying, freeze-drying, or sun drying, is the preferred method to obtain dry biomass as it offers high extraction yields, albeit with high costs and energy use [e.g. harvesting and drying combined with extraction cost is 50% of the total production cost (Pragya *et al.*, 2013)].

An alternative to the dry route is the wet route, in which wet biomass needs to be disrupted first to release the intracellular products. Cell wall composition plays an important role in this route; cell walls from microalgae are typically a thick and rigid matrix of polysaccharides and glycoproteins that require costly downstream processing steps during the production of bio-products (Kim *et al.*, 2013). For example, *H. pluvialis* possesses a thick cell wall that makes this alga highly resistant to chemical and physical disruption, thus significantly increasing astaxanthin production costs (Hagen *et al.*, 2002). The unit operations for the wet route can be: mechanical (ultra-sonication, high pressure homogenization, microwave, bead beating, and electroporation); chemical (acids, alkalis, and organic solvents); biological (enzymes); or osmotic shock. Selection of methods depends again on the biomass characteristics. Assessment of the dry and wet routes has demonstrated that both have a positive energy balance for production of biofuels. This evidence also shows that the wet route has more potential for high valuable biofuels whereas the dry route seems more attractive for short-term biofuel productions (Xu *et al.*, 2011). Whether the biomass goes into

the dry or wet route, the physical state of the output is a dried biomass or disrupted concentrate; this stream is processed for lipid extraction. Different methods are available including: (i). Organic solvents: algal oil can be recovered by using solvent such as chloroform, methanol, benzene, diethyl ether, and *n*-hexane. Other metabolites such as β-carotene and astaxanthin are also extracted by solvents (Molina Grima *et al.*, 2003). However, solvent extraction can result in high toxicity if the product is used for animal or human consumption. (ii). Supercritical fluid extraction: this is a green technology based on CO_2 at supercritical conditions used as a non-toxic extracting solvent in order to separate the lipids from matrix (dried biomass or disrupted concentrate). It has been applied to extract lipids and other high valuable compounds from algae, however, the operational cost is high (Mendes *et al.*, 1995; Thana *et al.*, 2008).

Strategies to facilitate downstream processing of recombinant protein produced in microalgae

The potential of *C. reinhardtii* to produce recombinant proteins for industrial, nutritional, and medical uses has been discussed above, but it is notable that hardly any reports have described large-scale production or recovery of target proteins. Moreover, downstream processing of engineered microalgae is largely based on laboratory scale techniques. More than 30 proteins with biotechnological applications have been expressed in *Chlamydomonas* (Rasala and Mayfield, 2014) including a number that have been expressed in the chloroplast. Although the chloroplast has been shown to be a robust platform for commercial production of proteins, it has some limitations as chloroplast-expressed proteins cannot be secreted, thus, cells must be processed after cultivation to recover and purify the product. Regardless of the nature of the bio-product produced in the microalgae, downstream processing follows the same route as in biofuels; therefore the operations to recover high-value products that require high purity (recombinant proteins, pigments, carotenoids, PUFAs, terpenoids, etc.) include harvesting, cell disruption (if the compound is intracellular), and purification. Figure 2 illustrates the recovery and purification process of recombinant proteins produced in microalgae.

Since recombinant protein production in *Chlamydomonas* is largely limited to small-scale cultivation, the current harvesting is mainly done by conventional centrifugation. Harvested biomass can be processed using the approach of dry and wet routes. For the dry route, the drying biomass has potential in the production of edible vaccines and enzymes; research suggests that dry algal extract is an ideal vehicle for oral delivery of vaccines and enzymes as the cell wall protects the antigens and enzymes (Yoon *et al.*, 2011). Production of vaccines that can be administrated orally is benefited by the effectiveness of freeze-drying to preserve antigen-expressing *Chlamydomonas*; some vaccine-producing strains have been reported to be stable for up to 20 months; therefore, the cost is reduced by eliminating extraction and purification steps (Specht and Mayfield, 2014). Algal-based recombinant vaccines administered orally as dry biomass have demonstrated effectiveness in two studies, namely protection against *S. aureus* infection (Dreesen *et al.*, 2010) and a potential malaria vaccine (Gregory *et al.*, 2013).

The wet route for extraction offers the possibility of recovery of recombinant proteins with high purity. However, cell disruption techniques can be limited since the proteins of interest can be compromised during harsh conditions. Cell lysis can be accomplished by combining mechanical, physical, and chemical methods. The most common methods are freezing, sonication, and lysis with a buffer, and any combination of them will result in a disrupted concentrate that must go through more purification steps. Cell disruption methods adopted by the community vary considerably, some common approaches are: freezing followed by lysis buffer (Gregory *et al.*, 2013), and lysis buffer coupled with sonication (Rasala *et al.*, 2012; Demurtas *et al.*, 2013).

There is an alternative third route in the process, termed the 'supernatant route'. This route is only possible when nuclear-expressed proteins are coupled with a signal peptide in order to be secreted into the culture media and thereby facilitate product recovery (Table 4). After cultivation, biomass is discarded as the recombinant proteins are present in

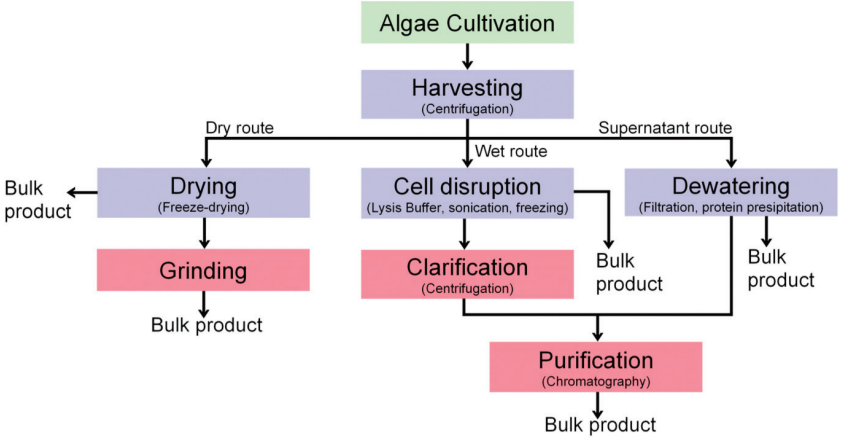

Fig. 2. Downstream processing of recombinant proteins extracted from transgenic algae systems. A flow-chart for the downstream processing of algal extracts by the 'dry route' involving freeze-drying or the 'wet route' involving cell disruption and fractionation.

Table 4. *Secretion of recombinant proteins from C. reinhardtii*

Signal peptide	Protein secreted	Reference
Arylsulphatase (ARS2)	Luciferase and erythropoietin	Eichler-Stahlberg *et al.*, 2009
POXA1b (from *Pleurotus ostreatus*)	Laccase	Chiaiese *et al.*, 2011
Arylsulphatase (ARS1)	Xylanase	Rasala *et al.*, 2012
Carbonic anhydrase 1 (CAH1)	Gaussia luciferase	Lauersen *et al.*, 2013*a*
Luciferase	Luciferase	Ruecker *et al.*, 2008
Carbonic anhydrase 1 (CAH1)	Ice-binding protein	Lauersen *et al.*, 2013*b*

the media. The proteins are then concentrated by filtration, lyophilization, or precipitation and further purified. Lauersen *et al.* (2015) optimized culture conditions and parameters of an engineered *Chlamydomonas* strain able to secrete an ice-binding protein with potential use in the food industry; they found that photomixotrophic cultivations led to accumulation of ~10 mg l^{-1} of this protein in a small scale. Larger scale experiments (10 L) conducted in plastic bags under the same conditions resulted in ~12 mg l^{-1} of recombinant protein accumulated in the medium. Compared with other microbial systems where secretion of recombinants proteins into the culture media is well established, microalgae still needs further development and optimization in order to have comparable secretion yields.

Thus, although microalgae appear to be an attractive platform for biomanufacturing of high-value proteins for industrial, nutritional, and medical uses, downstream operations have technical challenges and such processes are still limited to small-scale proof of concept studies. On the other hand, the high cost of downstream processing of high-value products from transgenic algae can be avoided in applications where no product purification is required, thus resulting in less expensive processing. The next step is to explore higher scale production levels in order to make the platforms competitive with other expression platforms. The first (and so far only) example of large-scale production of an algal-expressed therapeutic protein was MAA in a volume of 100 l in a greenhouse (Gimpel *et al.*, 2014).

Conclusions

In this review, we have highlighted the biotechnological potential of microalgae from several different angles. Microalgae are a valuable source of natural products, including carotenoids, antioxidants, and pigments, in addition to being used as feed stock or for the production of biodiesel. Advances in genetic engineering also mean that some species can be transformed and used as cell factories for other high-value products, including recombinant proteins. Several vaccine antigens, antibodies, and some enzymes have now been produced in the model alga *C. reinhardtii*. In addition, efforts have been directed towards metabolic engineering in algae and cyanobacteria. On a different note, we have also summarized the current research into engineering algal communities and the potential benefit of co-culturing bacteria and algae to increase productivity, reduce energy inputs, and protect cultures from pathogens. Considering these aspects, the great potential of microalgae in biotechnology becomes evident. However, large-scale production remains a challenge and microalgae still struggle to compete with existing platforms. It remains to be seen whether microalgae will be used for a wide range of industrial applications or only for more specialized applications, where there are severe shortcomings in competing platforms. The niche for microalgae still needs to be developed. The GRAS status of many microalgae, their inexpensive culturing, and potential for large-scale growth in bioreactors are definitely distinctive advantages of these photosynthetic microorganisms and upcoming years will reveal where the industry is headed.

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8.3.3 A young algaeneers' perspective: Communication and networking are key to successful multidisciplinary research.
- A letter to the editor of Algal Research (opinion piece i.e. not peer reviewed).

8.3.4 Electrochemical Characterisation of Bio-Bottle-Voltaic (BBV) Systems Operated with Algae and Built with Recycled Materials

biology

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Article

Electrochemical Characterisation of Bio-Bottle-Voltaic (BBV) Systems Operated with Algae and Built with Recycled Materials

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Abstract: Photobioelectrochemical systems are an emerging possibility for renewable energy. By exploiting photosynthesis, they transform the energy of light into electricity. This study evaluates a simple, scalable bioelectrochemical system built from recycled plastic bottles, equipped with an anode made from recycled aluminum, and operated with the green alga *Chlorella sorokiniana*. We tested whether such a system, referred to as a bio-bottle-voltaic (BBV) device, could operate outdoors for a prolonged time period of 35 days. Electrochemical characterisation was conducted by measuring the drop in potential between the anode and the cathode, and this value was used to calculate the rate of charge accumulation. The BBV systems were initially able to deliver ~500 mC·bottle^{-1}·day^{-1}, which increased throughout the experimental run to a maximum of ~2000 mC·bottle^{-1}·day^{-1}. The electrical output was consistently and significantly higher than that of the abiotic BBV system operated without algal cells (~100 mC·bottle^{-1}·day^{-1}). The analysis of the rate of algal biomass accumulation supported the hypothesis that harvesting a proportion of electrons from the algal cells does not significantly perturb the rate of algal growth. Our finding demonstrates that bioelectrochemical systems can be built using recycled components. Prototypes of these systems have been displayed in public events; they could serve as educational toolkits in schools and could also offer a solution for powering low-energy devices off-grid.

Keywords: algae; bioelectrochemistry; renewable energy; recycled materials

1. Introduction

The world's increasing population and energy demand and the recognition of the environmental consequences and limited availability of fossil fuels have driven extensive research into the development of renewable energy sources, including biologically based ones [1]. These technologies include Microbial Fuel Cells (MFCs), which are bioelectrochemical systems that exploit the electron-producing respiration processes of heterotrophic microbes [2,3]. Biophotovoltaics (BPVs), by contrast, function as biological solar cells, using the photosynthetic activity of microalgae or

cyanobacteria to harvest solar energy and generate an electrical current [4]. The simple nutrient requirements of photosynthetic microorganisms also mean that they are relatively inexpensive to culture, a key advantage for bioenergy applications [5].

In biophotovoltaic systems, the primary electron source is provided by a natural process known as water photolysis, performed during photosynthesis [6]. Photolysis results in the splitting of water into protons, oxygen, and electrons. A portion of those electrons can be exported to the extracellular space [7] to be donated to an electrode called the anode. Following this, those electrons travel through an external circuit to reach a second electrode called the cathode. The cathode has a catalytic surface on which the electrons combine with protons and oxygen to regenerate water [4].

The factors determining the electrical output of biophotovoltaic systems, including intracellular metabolic pathways and the ability to export electrons outside the cells, are not completely understood yet. To date, the current output of these systems remains relatively low, with maximal current density output reported to date being 1–2 $A \cdot m^{-2}$ when microfluidic approaches are used [8,9].

A number of photosynthetic organisms have been the subject of bioelectrochemical studies. Prokaryotic photosynthetic microorganisms such as *Synechocystis sp.* PCC6803 [10] and *Oscillatoria limnetica* [11] are widely used. Their tendency to form biofilms on conductive materials is thought to optimise electron transport to the anode [12]. Eukaryotic photosynthetic microorganisms, such as *Chlorella vulgaris*, *Dunaliella tertiolecta*, *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, and *Thalassiosira pseudonana*, have also been studied [13–15]. Plant Microbial Fuel Cells (PMFCs) are systems where photosynthetic macro-organisms, generally vascular plants, operate in conjunction with MFC systems. In those devices, the organic compounds generated from the plants are metabolised by microorganisms in the rhizosphere to generate electricity [16]. Bombelli et al. have also tested bryophytes and moss as non-vascular photosynthetic organisms in bryoMFCs [17].

At present, because of the high energy demands of western society, bioelectrochemical systems (e.g., BPV, MFCs, and plant-MFCs) are not perceived as a viable alternative to conventional electricity supplies. However, in particular conditions, the limited electrical output delivered by bioelectrochemical systems could constitute a valuable solution to specific problems. For example, sensors with a low current requirement located in remote areas such as rainforests, where the environmental concerns related to the use of batteries and their impracticable replacement are relevant, could offer a testbed for demonstrating the effectiveness of these technologies.

It would be particularly attractive to be able to construct biophotovoltaic systems from recycled materials. The purpose of this study was to construct a prototype device of this kind and measure its current output in an outdoor location in London (UK) over an extended time period of ~35 days (August–September 2017). The design is shown in Figure 1A. The prototype was built inside a two litre PET plastic (polyethylene terephthalate) bottle. Given the use of plastic bottles, the prototype was named 'Bio-Bottle-Voltaic' (BBV). The cathode and the electrical connectors were embedded into a de novo constructed plastic lid, the internal structure of which is described in Figure 1B,C. The anode was made from shredded aluminum and placed inside the bottle. Further details of the BBV construction are given in the Materials and Methods, and the blueprints to recreate those systems are available online [18]. All the BBV systems described in this investigation were operated with the eukaryotic green alga *C. sorokiniana*, forming a biofilm layer over the anodic surface (Figure S1).

The results displayed in this study proved that BBV systems built from recycled materials can deliver a stable current output over several weeks of operation. The aluminum used as anode did not negatively affect algal growth, and an algal bio-film formed on the anodic surface. The prototypes of the BBV systems have been presented at the World Ocean's Day in London (June 2017, UK) [19] and at the National Science Week in Canberra (August 2017, Australia) [20].

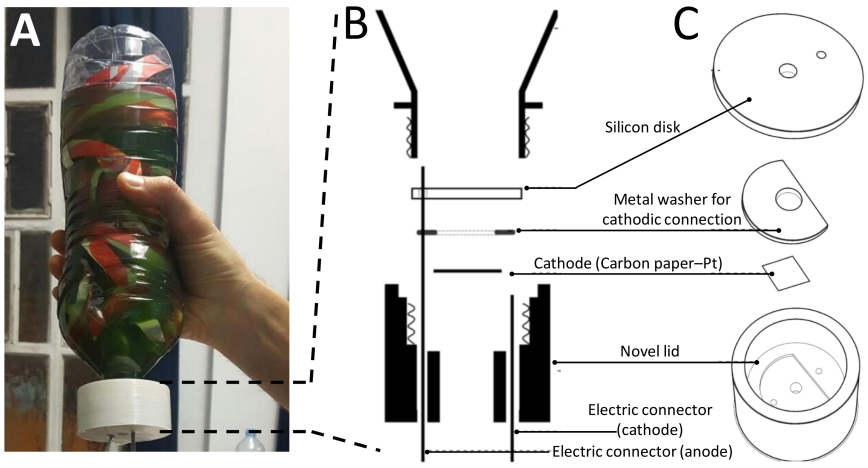

Figure 1. The Bio-Bottle-Voltaic (BBV) system. (**A**) The actual BBV system. (**B**) Schematic cross section of the components forming the lid of the BBV system. (**C**) A 3D semi-exploded view of the components forming the lid of the BBV system.

2. Materials and Methods

2.1. Building the BBV System

With the aim of creating BBV modules that can be fabricated with a limited budget, a system was designed that relied mostly on recycled materials available in almost every populated area. The system consists of recycled plastic 2 L bottles (polyethylene terephthalate—PET, ~200 cm^2 surface area), to house the algal cells and the anode, and a lid, hosting the cathode and the anodic/cathodic connectors necessary to operate the bioelectrochemical systems.

The anode was made by aluminum obtained from recycled drinking cans (three cans per each bottle). The lid was fabricated in the following manner. A mould was 3D printed, and the lid was cast into the mould using an epoxy resin. The lids contained a soft gasket made by a layer of silicone to provide sealing. A washer made from stainless steel grade 316 was used to provide electrical connection with the cathode. The anodic and cathodic connectors were made from threaded bars (5 mm in diameter, stainless steel grade 316).

The anode was fastened to the anodic connector with a M5 nut (stainless steel grade 316) and extended out of the lid of the device for connection to the external circuit.

The open-air cathode (5 mm diameter) was encased into the lid and fastened to the cathodic connector with an M10 washer (stainless steel grade 316). The cathodic connector extended out of the lid of the device for connection to the external circuit. The open-air cathode consisted of carbon paper, coated with a thin layer of platinum (3 mg of Pt per m^2, Alfa Aesar, Heysham, UK). The BBV system was connected to external copper wires to complete the circuit.

2.2. Culture Conditions and Biofilm Growth

C. sorokiniana (CCAP 211/8K) was obtained from the Culture Collection of Algae and Protozoa (Scottish Marine Institute, Oban, Scotland, UK). The cultures were prepared by inoculating the cells in modified Tris-Acetate Phosphate (TAP) medium [21] and were incubated in an illuminated (continuous light, 40–60 $\mu E \cdot m^{-2} \cdot s^{-1}$) incubator at 25 °C until the cells reached exponential growth phase. To initiate biofilm growth, planktonic cultures were concentrated by centrifugation (4000× *g*, 10 min), resuspended in 2 L of fresh medium to a concentration of 1 nmol·chlorophyll (Chl)·mL^{-1}, and inoculated in the 2 L PET bottle. The cultures (ca. 2.0 L) were allowed to settle and attach to the substrate material under static conditions. The chambers were manually agitated once per day and opened in non-sterile conditions once every week to permit gas exchange and cell sampling.

2.3. BPV Operation and Measurements

The voltages generated across a fixed external load (56 Ω) by the BBV systems and the abiotic BBV (i.e., BBV systems containing all the abiotic components, including the algal medium and operated without algal cells) were monitored every minute using a multi-channel ADC-20 high-resolution data logger (Pico Technology, St. Neots, UK). The devices were maintained in a cold frame (Figure S2) placed outdoors on a balcony of the Bernard Katz Building, University College London. The geographical coordinates are 51°31′28.1′′ N, 0°07′57.7′′ W, as shown in Figure S3.

The experimental run was 35 days long from 21 August 2017 to 24 September 2017. The ambient light provided a light photon flux, as shown in Figures S4 and S5. A small amount of tap water (2–5 mL) was added to the BBV systems every week to replace losses from sampling and eventual leaking.

The current output was calculated for the BBV systems and the abiotic BBV systems from Ohm's law, as shown in the Equation (1).

$$\text{Current(Ampere)} = \text{potential (Volt)}/\text{Resistance external (Ohm)} \quad (1)$$

On the basis of the current output, charge (Coulomb) accumulation was calculated by integrating the current output over time, as shown in the Equation (2).

$$\text{Charge(Coulomb)} = \text{Current(Ampere)} \times \text{time(second)} \quad (2)$$

2.4. Characterization of Algal Biofilm on the Anodic Surface and Algal Chlorophyll Content

Each aluminum anode was first washed in 10 L tap water to remove the cells resting on the anode surface, then transferred to a separate tray containing 2 L of water. The algal biofilm layers formed on the anode surface were scraped into the water until the aluminum was clean. The solution was mixed thoroughly to ensure the cells were evenly distributed, and samples were removed for spectrophotometric analysis. Cellular density was recorded by measuring the OD of each sample at 680 nm and 750 nm, with three technical repeats for each sample.

The amount of chlorophyll was measured by subtracting the 750 nm OD value from the 680 nm OD value and multiplying the total by 44.609. There was a strong correlation ($r^2 = 0.949$) in determining chlorophyll concentration between this method and the well-established chlorophyll quantification protocol, as described previously [22] (Figure S6).

2.5. Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine whether there were any significant differences between the means of independent (unrelated) groups of data. When the *p*-value is greater than 0.05, there is no statistically significant difference between group means. The complete results obtained from the ANOVA tests run in this study are shown in supplementary Tables S1 and S2. The results were calculated using online software [23].

Pearson Correlation Coefficient Calculator was used to measure the strength and direction of the relationship between two variables. When the R-value is >0, a positive correlation between the two variables is observed. The complete results obtained from the Pearson Correlation Coefficient Calculator run in this study are shown in supplementary Tables S3–S8. The results were calculated using online software [24].

3. Results

3.1. The Electrochemical Setup Used to Run the BBV Systems

The electrochemical setup was formed by wiring the BBV systems with a data logger connected to a computer for recording the data. For each BBV system, an external resistor (56 Ω) was placed in parallel with the data logger to permit current flow. The value of the external resistor was arbitrarily

chosen with the aim of performing a comparative investigation between the BBV systems. The overall electrochemical setup is schematically represented in Figure 2A. The complete experimental setup included two wired BBV systems (named BBV-1 and BBV-2, respectively) and two unwired bottles used as negative controls (named n.c.-1 and n.c.-2, respectively) (Figure 2B and Figure S3). The BBV-1 and BBV-2 were made using identical components. The unwired nature of those negative controls did not permit the measurement of any electrical output. These systems were used to determine the rate of algal biomass accumulation for the unwired BBV systems (n.c.-1 and n.c.-2) and compare it with the rate measured for the wired BBV systems (BBV-1 and BBV-2).

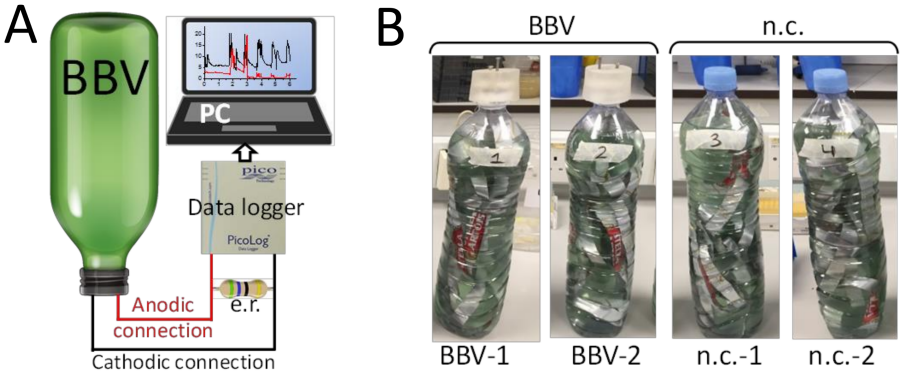

Figure 2. The experimental setup. (**A**) Schematic view of the experimental setup. The potential (mV) anode-to-cathode of the Bio-Bottle-Voltaic systems was measured by a data logger and recorded by a PC. An external resistor (e.r.) was placed in parallel with the data logger. (**B**) The experimental setup included two BBV systems (BBV-1 and BBV-2) and two unwired bottles as negative control (n.c-1 and n.c.-2).

3.2. Illustrative Electrical Output of the BBV Systems

Figure 3 shows a typical dataset collected over 24 h of operation. During the 24 h shown here, the light photon flux varied from 0 $\mu E \cdot m^{-2} \cdot s^{-1}$ (night-time) to 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ during the sunniest part of the day (Figure 3A). The orientation and geographical location where the experimental setup was placed (Figure S4) prevented direct exposure to sunlight and limited the light photon flux.

For the illustrative data displayed in Figure 3B, during the light–dark cycle, the current output varied from a minimum of 10–15 $\mu A \cdot bottle^{-1}$ to a maximum of 45–50 $\mu A \cdot bottle^{-1}$.

The other experimental system (BBV-2) gave a lower output. Figure S5 shows all the 35 cycles of 24 h each for both BBVs.

The current was calculated from the voltage by using the Equation (1). The minimum current output is referred to as the 'dark current' and is typically attributed to heterotrophic cellular metabolic activities (i.e., breakdown of stored carbon intermediates accrued during the light period). The difference between the maximum and the minimum is defined as the 'photo response' of the BBV system [9]. The trend of the daily variations of the light current output appeared to be in agreement with the variation of light photon flux observed in previous investigations ([11], Figure 3A).

To estimate the background abiotic current, two BBV systems were operated without algal cells for seven days. The results from these BBV systems operated without algal cells are shown in Figure S4. The yellow dotted line in Figure 3B shows the average current output recorded from those abiotic BBVs (~0.8 $\mu A \cdot bottle^{-1}$).

Figure 3 shows an illustrative example of the experimental data recorded for an algal BBV system over 24 h. The set of data for the abiotic BBV systems recoded over seven days (i.e., light photon flux and current output) is shown in Figure S4. The complete set of algal BBV systems data recoded over 35 days (i.e., light photon flux and current output) is shown in Figure S5.

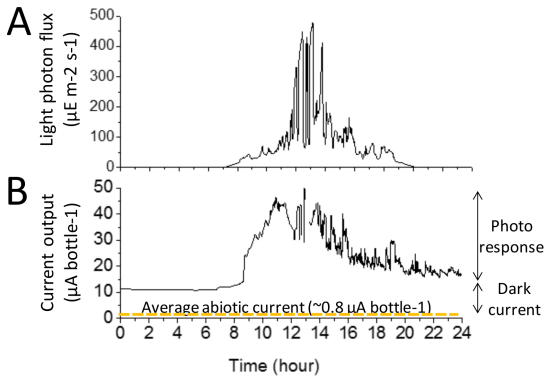

Figure 3. Electrical outputs of a BBV system. (**A**) Illustrative example of the light photon flux falling on the BBV system. (**B**) Illustrative example of the current output generated by a BBV system. The yellow dotted line shows the average current output for the abiotic BBV systems operated without algal cells.

3.3. Characterisation of the Electrical Output

During the experimental run, the cumulative daily photon flux (yellow bars) varied from 1.58 E·m^{-2}·day^{-1} (5 September 2017) to 7.65 E·m^{-2}·day^{-1} (26 August 2017). The average daily temperature (blue line) ranged between a minimum of 10.9 °C (19 September 2017) to a maximum of 22.0 °C (28 August 2017) (Figure 4A).

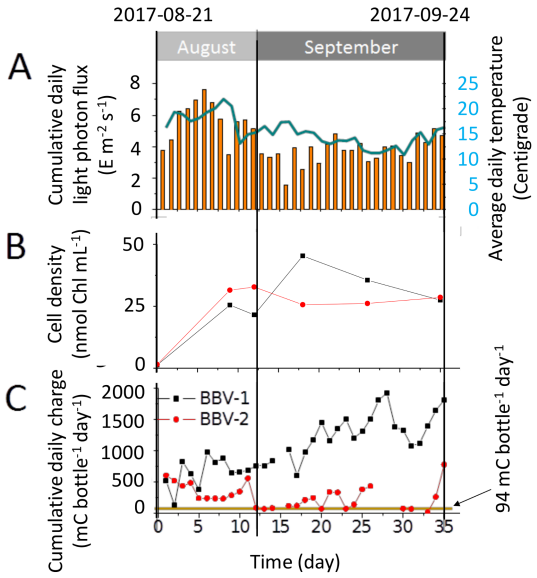

Figure 4. Performance of the BBV systems during the entire experimental run of 35 days (**A**) Cumulative daily light photon flux (yellow bars) falling on the BBV systems and average daily temperature (blue line). (**B**) Growth curve over 35 days for the cells of *Chlorella sorokiniana* inoculated into the BBV systems. (**C**) Daily charge accumulation by the BBV systems inoculated with *C. sorokiniana* over 35 days. The yellow dotted line shows the average abiotic charge accumulation per day for a BBV system operated with medium only.

During the 35-day experimental period, the growth of the algal culture in the BBV systems was assessed by measurement of chlorophyll concentration. The chlorophyll content increased from the initial inoculum of ~1 nmol·Chl·mL^{-1} to 27–29 nmol·Chl·mL^{-1} (Figure 4B).

The daily cumulative charge generated by the BBV-1 rose over time from the initial 519 mC·bottle^{-1}·day^{-1} to 1928 mC·bottle^{-1}·day^{-1}. By contrast, the BBV-2 displayed a more modest

increase from the initial 609 mC·bottle^{-1}·day^{-1} to a maximum of 777 mC·bottle^{-1}·day^{-1} (Figure 4C). The estimates of daily cumulative charge were derived from the data of current output shown in Figure S5, using the Equation (2). The average current output for the BBV-1 and BBV-2 systems were ~13.2 µA·bottle^{-1} and ~3.6 µA·bottle^{-1}, respectively.

3.4. Biomass Accumulation in the BBV Systems

An equal amount of *C. sorokiniana* (~1 nmol·Chl·mL^{-1}) was inoculated in each of the four bottles (BBV-1/2 and n.c.-1/2). The growth curves obtained by sampling the algal suspension for the BBV systems (BBV-1 and BBV-2) and the unwired negative controls (n.c.-1 and n.c.-2) appeared to be comparable to each other, as shown in Figure 5A. For both groups, a stationary phase was reached 10–12 days after inoculation. The algal cells in the BBV and n.c. systems reached a maximum average chlorophyll density of 35.5 ± 9.9 nmol·Chl·mL^{-1} and 30.6 ± 5.6 nmol·Chl·mL^{-1}, respectively. These values were not significantly different (Anova $p = 0.603$; Table S1).

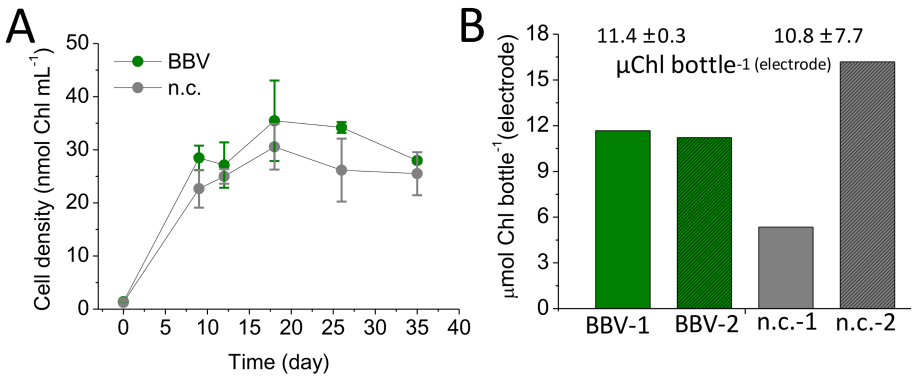

Figure 5. Algal cell growth. (**A**) Growth curves of *C. sorokiniana* for the wired BBV systems (green line) and for the unwired negative control (grey line) over the entire experimental run (35 days). (**B**) Chlorophyll amounts (µmol Chl) derived from the algal cells attached to the anode into the BBV systems (green bars) and from the algal cells attached to the anode into the unwired negative control (grey bars).

When the algal biofilm layer formed over the anodic surface was considered (Figure S1), the BBV systems 1 and 2 were found to be quite similar to each other, with a total chlorophyll content of 8.9 and 8.6 µmol·Chl·bottle^{-1}, respectively. By contrast, in the negative control systems, the density of biofilm on the anodic surface ranged from 4.1 to 12.4 µmol·Chl·bottle^{-1} for the n.c.-1 and n.c.-2, respectively (Figure 5B). When the average was considered (11.4 ± 0.3 µmol·Chl·bottle^{-1} and 10.8 ± 7.7 µmol·Chl·bottle^{-1}), no significant difference was observed (Anova $p = 0.918$; Table S2).

4. Discussion

This study demonstrates a modular method for conducting biophotovoltaic experiments using a widely available microalga (*C. sorokiniana*) and recycled materials (plastic bottles and aluminum from drinking cans). The long-term growth experiment (35 days) offers insights into the behavior of a BBV device under outdoor environmental conditions during the summer (2017) in a temperate location (London, UK).

The electrical output of the BBV systems (cumulative daily charge) in response to light intensity (cumulative daily photon flux) was examined for cultures that had reached a stationary phase in the growth curve (older than 12 days) (Figure 6A). On the basis of the Pearson analysis, it was observed that there was some degree of positive correlation between the amount of light falling on the bottle and the electrical output for both BBV systems, with R values of 0.166 and 0.323 for BBV-1 and BBV-2, respectively (Tables S3 and S4). It is important to note that, although the electrical output of the

BBV-1 varied substantially from that of the BBV-2 system (Figure 4C), the slopes of the regression lines fitting the data points in Figure 6A were very similar to each other (67.9 and 66.5 for BBV-1 and BBV-2, respectively).

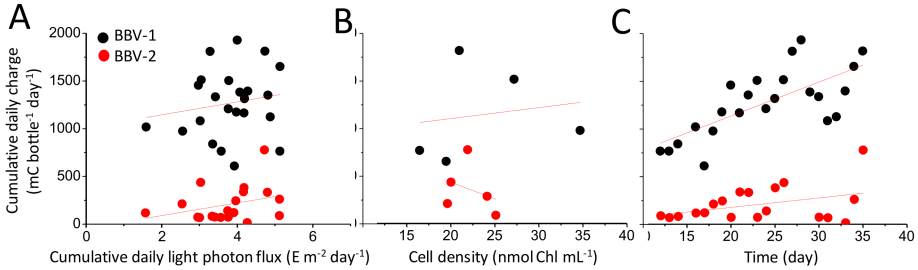

Figure 6. Charge accumulation versus light photon flux, cell density, and time. (**A**) The cumulative daily charge for two BBV systems (BBV-1 is shown in black and BBV-2 is shown in red) is plotted against the cumulative daily light photon flux. (**B**) The cumulative daily charge for two BBV systems is plotted against the cell density. The measurements were taken on different days. (**C**) The cumulative daily charge for two BBV systems is plotted against the time. The above data relate to the steady state only (from day 12 to day 35).

The correlation between the cell density (amount of Chl·mL^{-1}) and the electrical output of the BBV systems (cumulative daily charge) was marginally positive for the BBV-1 (R = 0.167, Table S5) and negative for the BBV-2 (R = −0.323, Table S6), with the slope of the regression lines fitting the data point in Figure 6B displaying a positive value for the BBV-1 (11.5) and a negative one for the BBV-2 (−35.1). These results suggest that the charge accumulation is independent of cell density during the stationary phase. In other words, when the cell culture has reached a steady state, other factors (e.g., light photon flux, formation of algal biofilm on the anodic surface, etc.) might influence the rate of charge accumulation.

When the culture age versus the electrical output was considered, the slope of the regression lines fitting the data point in Figure 6C for the BBV-1 system (35.5) was ~3.5 times bigger than for the BBV-2 system (9.9). In both cases, the correlation was positive, with a moderate coefficient for the BBV-1 (R = 0.706, Table S7) and a weaker value for the BBV-2 (R = 0.382, Table S8).

The increase in current during the experimental run, once the algal culture was at a steady state, may be due to enhanced biofilm formation of algal cells on the anodic surface. This would be in agreement with the findings reported by McCormick et al. 2011 [11]. However, this hypothesis seems to be contradicted by the data presented in Figure 5B (green bars), where the amount of chlorophyll extracted from the algal biofilm formed over the anodic surface of the BBV-1 (8.9 = µmol·Chl·bottle^{-1}) was almost identical to the figure observed for the BBV-2 (8.6 µmol·Chl·bottle^{-1}). Nevertheless, as the systems were non-axenic, the anode in the BBVs may have been colonized by different consortia of microalgae and bacteria. Therefore, while the two BBV systems may have a similar number of algal cells, the BBV-1 system might contain more electrogenic bacteria [2]. To validate this hypothesis, the level of bacterial contamination and the physical properties of the biofilm (e.g., adhesion and cohesion) need to be assessed. Future work is required to characterize the microbial consortium of the biofilm covering the aluminum anode and identify the population of bacteria and algae colonizing the surface. In addition, to understand the population dynamics within the BBV system better, it would be necessary to measure how the composition of the population of cells changes over time.

The aluminum anode appears to be compatible with the growth of photosynthetic microorganisms and the formation of biofilms (Figure S1). In previous studies, biofilms of *Synechocystis* and *C. sorokiniana* have been cultivated photoautotophically on metallic surfaces, for example on a layer of carbon [25] and using stainless steel woven meshes [26].

Furthermore, the formation of biofilm from mixed cultures of microorganisms obtained from seawater inoculum on aluminum surfaces has been reported recently [27]. Observations over the course of the experiments presented here indicated no difference in the growth patterns between the wired (BBV-1/2) and the unwired (n.c.-1/2) bottles (Figure 5). This suggests that harvesting electrons does not compromise the accumulation of biomass in the liquid culture.

Over the course of the experiment, a thick green biofilm was also observed on the inner surface of the plastic bottles. Because of the non-axenic nature of the experiment, we expect the biofilm to be composed of an algal-bacterial consortium. Biofilm formation on PET plastic by bacteria that secrete exopolysaccharides and attract microalgae [28] could affect the electrical output of the system, as the cells on the surface would absorb light but not directly contribute to electricity formation, as they are not in contact with the anode.

From the electrical output results (Figure 4), it can be seen that the BBV-1 performed better than the BBV-2. There are two potential reasons for this: biological or electrical. No significant difference in cell growth measured as chlorophyll content for both cell suspension (Anova $p = 0.603$, Table S1) and anodic biofilm (Anova $p = 0.918$, Table S2) was noted over the duration of the experiment (Figure 5). Therefore, we believe that a physical impediment (e.g., electrical imperfection in the wiring of the systems) may have caused a problem with the electrical output.

The geographical location of the experimental run may have influenced the outcome of the experimental run described here. The BBV systems were installed in a built-up location, with 51°31′28.1′′ N, 0°07′57.7′′ W orientation (Figure S3). Shading from surrounding buildings may have offered protection from weather variation to the system. However, during the period of measurements, light levels did not exceed 500–600 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Figures S4 and S5), whereas, during summer, it might be expected that the system would be exposed to light photon flux up to 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ [29]. In addition, ambient temperatures from a nearby weather station (NW3) reported an average temperature in August of 18.0 °C, as opposed to 14.2 °C in September [30]. *C. sorokiniana* grows optimally at temperatures around 38 °C, and the temperature has been shown to have an influence on productivity [31]; therefore, despite the greater amount of sunshine in August (5.7 ± 1.3 $E \cdot m^{-2} \cdot day^{-1}$) compared to September (3.8 ± 0.8 $E \cdot m^{-2} \cdot day^{-1}$), the relatively low temperatures will have led to a low specific growth rate for this alga.

While the BBV has advantages in terms of sustainability due to the use of recycled materials, accessibility, and modularity, a number of limitations of the system were noted. In Figure S5, the signal in the BBV-2 system (red trace) experienced several disconnections, therefore further work on maintaining a stable system for the electrical setup is required. Gas exchange was not optimal within the BBV, and the static nature of the system and the use of the sealed cap may mean that the cells could become carbon-limited. Further improvement in the design of the BBV will target this limitation. The starter cultures were prepared in TAP medium, and acetate is normally exhausted after 2–3 days under laboratory conditions [32], meaning that the primary mode of growth in the BBV system should be autotrophic, with CO_2 as the primary carbon source. Manual agitation of the bottles was performed during the experiment, which would not be very practicable on a larger scale. It was not possible to measure CO_2 concentrations in the liquid, but it would be expected that the rate of diffusion would be influenced by the diffusion coefficient between the liquid and the biofilm on the anode, as well as by the concentration difference between each of the phases in the system, i.e., the headspace, the liquid, and the biofilm.

5. Conclusions

The prototypes of BBV construction described in this investigation have an important advantage over previous experimental BPV devices. The previous devices generally operate using customised components [33], which might be expensive and difficult to be reproduced by third parties. Our BBV device is built inside a recycled plastic bottle and uses, as anode, widely available aluminum obtained from standard drinking cans. With this configuration, our BBV reached a maximum current output

of 2.25–2.5 $mA \cdot m^{-2}$. A larger current output (40–80 $mA \cdot m^{-2}$) was reported when *C. vulgaris* was incorporated into highly customized porous ceramic anodes [34].

A better understanding of the role of biofilm formation on the anode and of the physical connection will allow to explain some of the variation in the electrical output between devices and potentially pave the way for the creation of an optimised prototype with enhanced electrical output.

Our BBV systems have the potential to be a valuable platform for cost-effective investigations and as an educational toolkit in schools. In addition, with an overall average current output variation from 13 $\mu A \cdot bottle^{-1}$ (BBV-1) to 4 $\mu A \cdot bottle^{-1}$ (BBV-2) (Figure S5B), the BBV systems described here have the potential for running applications with a very low current consumption, such as environmental sensors [35].

Supplementary Materials: The supplementary materials are available online at http://www.mdpi.com/2079-7737/7/2/26/s1: Figure S1: The aluminium anode, Figure S2: Top view of the actual experimental setup, Figure S3: Geographical location, Figure S4: Electrical output of abiotic BBV systems during 7 days of experimental run, Figure S5: Electrical output of BBV systems during the entire experimental run of 35 days, Figure S6: Optical density vs chlorophyll extraction; Table S1: ANOVA test for the difference between the maximum of chlorophyll density reached in samples of cell suspension taken from BBV systems and the unwired negative controls, Table S2: ANOVA test for the difference between the total chlorophyll concentration accumulated on the anodic biofilm of BBV systems and the unwired negative controls, Table S3: correlation coefficient for the cumulative daily light photon flux versus the cumulative daily charge for the BBV-1, Table S4: correlation coefficient for the cumulative daily light photon flux versus the cumulative daily charge for the BBV-2, Table S5: correlation coefficient for the cell density versus the cumulative daily charge for the BBV-1, Table S6: correlation coefficient for the cell density versus the cumulative daily charge for the BBV-2, Table S7: correlation coefficient for the time versus the cumulative daily charge for the BBV-1, Table S8: correlation coefficient for the time versus the cumulative daily charge for the BBV-2.

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