A microalga and its microbiome: Diversity, variability and not just a question of B_{12}?

A thesis submitted to the University of Kent for the degree of MSc in Microbiology in the Faculty of Science, Technology and Medical studies. 2019 Georgina Alice Andrews

Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

Georgina Alice Andrews

10th June 2019

Acknowledgements

I dedicate this thesis to the memory of my Mum, who encouraged me to undertake this achievement and supported me in all that I did. I would like to thank my brother Geoffrey, my sisters Sally, Lizzie and Laura, my extended family and friends for all their support and encouragement. I would also like to thank AlgaeCytes Ltd for sponsoring this thesis and all the support from my colleagues there, and a special thanks to Gary Robinson and John Macdonald for their support, guidance and patience. I would also thank the University of Kent and the BBSRC.

Contents

Title page		i
Declaration		ii
Acknowledgements		iii
Contents		iv-vii
Abbreviations		viii
Abstract		ix
Chapter 1 Introduction		1-17
1.1	Microalgae	2
1.2	Omega 3's and 6's	3
1.3	Lipid biosynthesis	3-4
1.4	Culturing methods	4-6
1.5	Microalgal symbionts/interactions	6-7
1.5.1	Modes of interaction	7-9
1.5.2	Mutual benefits and requirements	9
1.5.3	Protection	10
1.5.4	Communication	10
1.5.5	Growth stimulation	10-11
1.5.6	Phytohormones	11
1.5.7	Nutrients and trace elements	11
1.5.8	Nitrogen	11-12
1.5.9	B –vitamins	13-17
1.6	Commercial applications	17
1.7	Aims and objectives	17
1.7.1	Aims	17
1.7.2	Objectives	17
Chapter 2 Materials and Methods		18-29
2.1.	Media and materials	19
2.1.1.	Growth media	19

2.1.2. Reagents 19

2.2. Equipment 19-23

2.2.1. Multicultivator MC1000 (MC) 19-20

2.2.2. Photobioreactor FMT 150 1L (1LPBR) 20-21

2.2.3. 100L flat panel photobioreactor (FPR) 21-22

2.2.4. 1000L Industrial Plankton industry reactor (IPR) 22-23

2.2.5. Handheld spectrophotometer 23

2.3. Microbial Cultures 23-25

2.3.1. ALG01 cultures 23

2.3.2. Agar cultures 23

2.3.3. Liquid cultures 24

2.3.4. ALG01 axenic cultures 24

2.3.5. Axenic supplementation 24

2.3.6. Bacterial preparation 24

2.3.7. Bacterial liquid cultures 24

2.3.8. Red, white and yellow bacterial tests 24

2.3.9. Isolation of bacterial colonies 24

2.3.10. Bacterial agar tests 25

2.4. Experimental design 25-27

2.4.1. Agar re-introduction tests bacteria 25

2.4.2. Agar re-introduction tests bacterial supernatant 25

2.4.3. Liquid reintroduction tests 25-26

2.4.4. Multi-unit run conditions 26

a) 1LPBR culture run conditions 26

b) FPR culture run conditions 26

c) IPR culture run conditions 26

2.4.5. Measuring bacterial levels over time from multiple units 26

2.4.6. Genetic identification of bacteria 26-27

2.4.7. Bioinformatic tools 27

a) Strain identification 27

b) Identifying gene presence 27-28

2.5. Analytical Procedures 28-29

2.5.1. Biomass harvesting 27

2.5.2. Dry weight 27

2.5.3. Dry sample preparation 27

2.5.4. Gram staining 28

2.5.5 Bacterial CFU/ml counts 28

2.5.6. ECHA Micro monitor Sig Nitrite bacterial test 28

2.5.7. Analytical Lipid extraction method 28

2.5.8. Analytical GC-MS 28-29

Chapter 3 Results 30-52

3.1. Growth of ALG01 under varying conditions and volumes 30-35

3.2. Axenic vs. Non-axenic 36-37

3.2.1 Agar cultures 36

3.2.2 Liquid cultures 36-37

3.3 Microbiome 38-50

3.3.1. Isolation and morphology 38-42

3.3.2 Reintroduction tests. 42

a) Agar tests 42-45

b) Liquid tests 45-50

3.3.3. ECHA Micro monitor Sig Nitrite bacterial test 50

3.4. Bioinformatics and molecular work 50-52

3.4.1 Cobalamin genes 52

3.4.2 Denitrification and ammonification 52

Chapter 4 Discussion 53-59

4.1. Growth of ALG01 under varying conditions and volumes 54

4.2. Axenic vs. Non-axenic 54-55

4.2.1 Agar cultures 54-55

4.2.2 Liquid cultures 55

4.3 Microbiome 55-57

4.3.1. Isolation and morphology 55-56

4.3.2 Reintroduction tests. 56-57

a) Agar tests 56

b) Liquid tests 56-57

4.4. Bioinformatics and molecular work 57-58

4.5. Industrial applications 58

4.6 Future experimentation 58-59

Final Conclusions 60

References 61-68

Appendix 69-70

Abbreviations

°C	Degrees centigrade
14:0	Myristic acid
14:1	Myristoleic acid
16:0	Palmitic acid
16:1	Palmitoleic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
1LPBR	Photobioreactor FMT 150 1L
22:0	Behenic acid
ABA	Abscisic acid
ALA 18:3	Alpha linoleic acid
ANOVA	Analysis of Variance
ARA 20:4	Arachidonic acid
ATP	Adenosine triphosphate
B_1	Thiamine
B_7	Biotin
B_{12}	Cobalamin
BBM	Bold modified basal broth
BBMA	BBM agar
CFU	Colony Forming Unit
CK	Cytokinin
CoA	Coenzyme A
DMA	Davis Minimal Agar

DNA	Deoxyribonucleic acid
EPA 20:5	Eicosapentaenoic acid
FFA's	Free Fatty Acids
FPR	100L flat panel photobioreactor
GA	Gibberellic acid
GC-MS	Gas Chromatography Mass Spectroscopy
GLA 18:3	Gamma linoleic Acid
IAA	Indole-3-acetic acid Auxin
IPR	1000L Industrial Plankton industry reactor
MC	Multicultivator MC1000
MRD	Maximum Recovery Diluent
PUFA pathway	Polyunsaturated fatty acids pathway
R2AA	R2A Agar
RPM	Revolutions Per Minute
SEM	Scanning electron microscope
SOP	Standard Operating Procedure
TCA cycle	tricarboxylic acid cycle
TEM	Transmission electron microscopy
TPP	Thiamine pyrophosphate
TSA	Tryptone Soya Agar
VF	Vibrioferrin
NA	Nutrient Agar
OD	Optical Density
YEA	Yeast Extract Agar

Abstract

Microalgae are a diverse group of phototrophic organisms, that have the potential to produce a wide range of products important in creating a sustainable future. Microalgae have been seen to have associated bacterial populations and often grow poorly without this interaction. There is much debate about the underlying mechanisms contributing to these relationships which are believed to be highly complex and specific, based on current findings. This may be due to many factors including the transfer of products such as cobalamin (B_{12}), carbon sources and nitrogen sources. The proprietary microalgae ALG01, as held by Algaecytes, is in the class of Eustigmatophyceae, which produces Eicosapentaenoic acid (EPA) an important omega-3 oil, needed by many living things, for growth and repair. ALG01 is grown phototrophically and is normally grown with associated microflora. The aim of this study was to investigate whether ALG01 could be grown axenically and to gain an insight into the role of the associated microflora.

In the present study, axenic ALG01 (devoid of microbiome) was shown not to grow well, with reduced chlorophyll content (qualitative observations only), like many other documented axenic strains. The addition of B_{12}, tryptone and glucose improved the growth of axenic cultures (approx. 50% increase) but did not return growth to normal levels when compared to cultures with the total microbiome. 87 morphologically different bacterial isolates were taken from an ALG01 culture through agar isolation, 83 were able to be subcultured, a further 3 were previously isolated from AlgaeCytes and all were tested against the axenic ALG01 on agar cultures. 65 showed a positive interaction, 18 showed no effect and none showed a detrimental effect. The addition of the bacteria only had a visible impact on algal growth on agar cultures where direct contact occurred. Bacterial additions into liquid algal cultures had no statistically significant effect on growth or lipid production when cultivated for 8 days, until the cultures entered stationary phase of growth within a small-scale system. Of the 90 strains tested the 10 with the most significant effect on agar were sequenced and were identified as *Pseudomonas, Pimelobacter, Brevundimonas, Microbacterium, Comamonas*, and *Sphingopyxis* species. Genomic analysis of these strains showed them to have a plethora of possible products that may underpin any interaction with ALG01 i.e. B_{12} production and denitrification capabilities. The growth of ALG01 axenically on a large scale is unrealistic and its microbiome has been shown to be more significant in the overall health and stability of the culture than initially thought. More investigation is needed into the dynamics of all the species within ALG01 cultures and our study reflects what others have found with other microbiome studies, namely that they are closely associated with the microalgae and are beneficial to growth, but their precise function is unknown. This has a large implication in the industrial uses of microalgae by being able to use the information for culture management, potential increase in product production, and as an indicator of culture health and performance.

Chapter 1

Introduction

1.1 – Microalgae

Algae are classified as any organism with chlorophyll *a* and an undifferentiated thallus (no roots, stems or leaves) (Richmond, 2008). Richmond (2008) states that 'in applied phycology the term microalgae refers to the microscopic algae *sensu stricto*, and the oxygenic photosynthetic bacteria, i.e. the cyanobacteria'. A statement that has since been used by many phycologists (Enzing *et al.* 2014, Ramanan *et al.* 2016). These organisms can be found across many environments, but primarily aquatic environments which can be either freshwater or saltwater (Richmond 2008). In recent years algal research had gained momentum, with investigations across this diverse group of organisms. Due to their diversity microalgae have extensive uses in industry and the capability of producing a wide range of products; these include and are not limited to fatty acids (Brennan, Owende 2010, D'Alessandro, Antoniosi Filho 2016; Spolaore *et al.* 2006), carbohydrates (Spolaore *et al.* 2006), animal feed *(Raja et al.* 2014; Raja *et al.* 2008), carotenoids (D'Alessandro, Antoniosi Filho, 2016) and health supplements(Raja *et al.* 2008; Spolaore *et al.* 2006). Each requiring a unique set of conditions to be able to exploit the production of these products. The proprietary microalgae ALG01, as held by AlgaeCytes Ltd., is in the class of Eustigmatophyceae, previously classified as Xanthophyceae (Richmond, 2008). This class of microalgae includes unicellular coccoid organisms that can also be found in freshwater and soil environments examples can be seen in Figure 1.1.

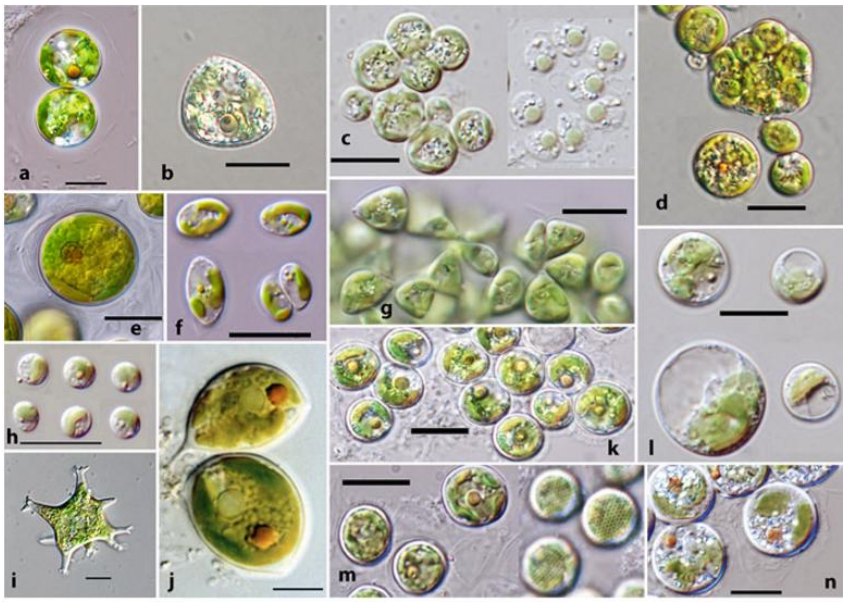


Figure 1.1 Light microscopy images representative of Eustigmatophyceae. A) *Chlorybotrys sp*. B) *Goniochloris sculpta* C) Unidentified strain. D) *Pseudellipsoidion edaphicum*. E) *Eustigmatos polyphem*. F) *Monodus unipapilla* G) Unidentified strain. H) *Nannochloropsis limnetica*. I) *Pseudostaurastrum sp.* J) *Characiopsis acuta*. K) Unidentified strain. L) Unidentified strain. M) Unidentified strain. N) Unidentified strain. Taken from Eliáš, M. *et al.* 2017.

The chloroplasts contain chlorophyll *a*; where the major light harvesting pigment is the carotenoid violaxanthin. This group produce small numbers of zoospores which have one or 2 apical flagella. They frequently have polygonal pyrenoids in the vegetative cells but not with the zoospores (Richmond, 2008; Eliáš, *et al*. 2017). Eustigmatophyceae are known to have a polysaccharidic cell wall and the eyespot is not enclosed in the chloroplast. There is little molecular information on this class; where there is it is mainly biased towards the species Nannochloropsis, and there is currently very little consensus on the genetic markers of this class of microalgae (Eliáš, *et al*. 2017). ALG01 is a potential player in the development of fatty acids as a source of Eicosapentaenoic acid, also known as EPA (Fig 1.2). This long chain unsaturated free fatty acid is an important omega-3 oil, needed by many living things, for growth and repair. Most mammals including humans cannot create Omega 3 and 6 and most acquire them from their diet in the form of fish. As fish stocks are dwindling an alternative source of the supplement is required. Acquiring such compounds from an algal source is not only a sustainable source but also a vegan one.

1.2 Omega 3's and 6's long chained fatty acids

Omega 3's and 6's are long chain fatty acids, longer than 18 carbons long that have the first double bond on the 3rd or 6th carbon on the chain from the methyl end of the chain (Milledge 2011). These oils are important for health, and have been noted to have beneficial effects in cardiovascular health, lowering cholesterol, lowering the risk of hypertension, gastrointestinal health, rheumatological health, neurological health, eye and skin health and can reduce inflammation (Simopoulos 2002; Simopoulos 2008; Yashodhara *et al.* 2009). As mammals are unable to synthesise them, they must gain them from their diets. Fish are the main source of omega oils and the stocks are dwindling (Adarme-Vega *et al.* 2012) and an alternative source is required. Acquiring them from an algal source is not only a sustainable one but also a vegetarian one. These oils are important for health for many reasons. There are links with Omega 3's and the stimulation of white blood cells, aiding in cardiovascular health, repression of autoimmune diseases, aiding in rheumatoid arthritis and also beneficial effects on asthma (Fernandes, Venkatraman 1993; Shahidi and Ambigaipalan 2018; Simopoulos 2002, Simopoulos 2008; Troyer, Venkatraman & Fernandes 1998; Yashodhara *et al.* 2009). There have been many other benefits described in the literature and others still under investigation.

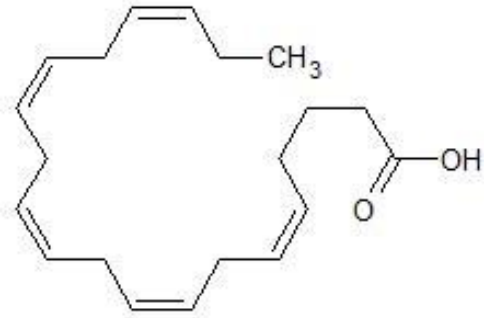


Figure 1.2: Structure of Eicosapentaenoic acid (EPA)

1.3 – Lipid biosynthesis

Microalgae have the ability to produce multiple long chain fatty acids. Each algal species having its own unique fatty acid profile based on the pathway present. The pathway depicted in Figure 1.3 is specific to the Eustigmatophyceae ALG01. This pathway is a representation and my not be complete. It was developed by AlgaeCytes through multiple testing methods. Products from photosynthesis and respiration are used within the chloroplasts to supply the fatty acid cycle, which creates the initial long chains from acetate transforming them into Coenzyme A (CoA) form to export them to the endoplasmic reticulum (Radakovits *et al.* 2010). There they go through the polyunsaturated fatty acids (PUFA) pathway which continually lengthens the carbon chain and adding double bonds. The longer chained fatty acids are then stored by the cell and can be used in the synthesis of membrane lipids (Mühlroth *et al.* 2013) The levels of free fatty acids (FFA's) can be affected by many aspects including as a survival technique from outside stressors such as nutrient limitation (Adarme-Vega *et al.* 2012; Cho *et al*. 2016; Huang *et al.* 2013; Widjaja *et al*. 2009), temperature (Adarme-Vega *et al.* 2012; Cho *et al*. 2016; Huang *et al.* 2013), pH (Cho *et al*. 2016) and carbon dioxide (Cho *et al*. 2016; Widjaja *et al*. 2009).

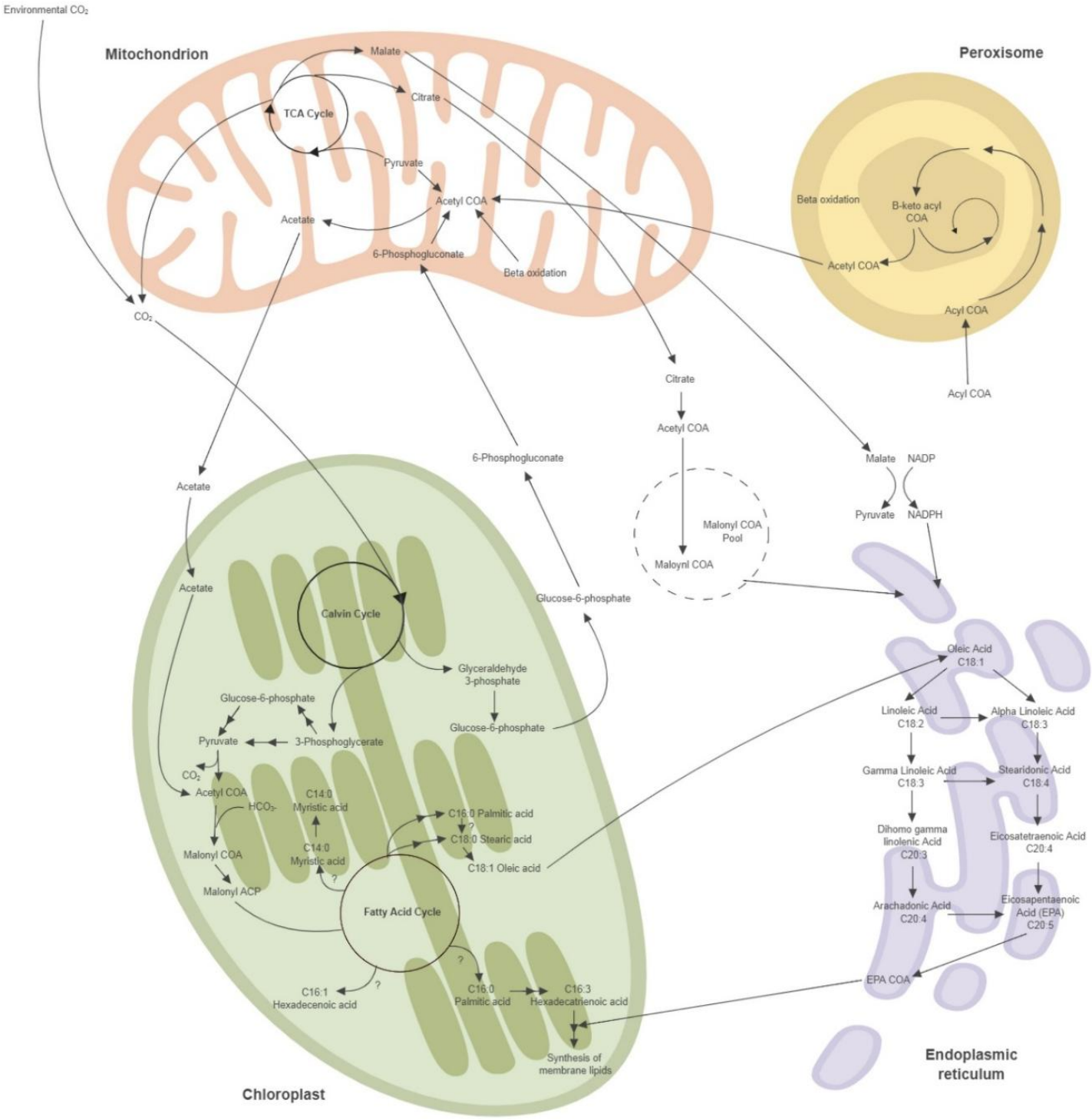


Figure 1.3: Fatty acid synthesis within the Eustigmatophyceae ALG01. Pathway developed from AlgaeCytes as a representation, not complete, adapted from Kegg map00061, Mühlroth *et al.* 2013 and Radakovits *et al.* 2010.

1.4 – Culturing methods

There are many ways in which algal cultures can be grown on a large scale. Open pond systems, raceway ponds, tubular photobioreactors, flat panel photobioreactors, vertical cylinders, and axenic photobioreactors (Christenson, Sims 2011; Richmond, 2008; Shen *et al.*, 2009). Each has positives and negatives. Photobioreactors are reactors which are used to grow phototrophs and also carry out photo-biological reactions (Richmond, 2008), open systems can be called photobioreactors but here we define photobioreactors as closed systems.

Cultivation of microalgae in open pond systems has been common in commercial applications (Christenson, Sims 2011; Shen *et al.*, 2009). These large outdoor systems are cost effective and require minimal management and maintenance. There are two main types of open pond systems: Raceway and circular (Christenson, Sims 2011; Richmond, 2008; Shen *et al.*, 2009). Raceways are moderate to shallow ditches, where water can flow in a loop, with a main motorised paddle to drive the movement and flow of water to allow for the transfer of nutrients and mixing of the culture itself (Fig 1.4a) (Shen *et al.*, 2009). Circular ponds are usually deep circular structures with a large motorised mixing rod that continually stirs the culture (Fig 1.4b) (Christenson, Sims 2011; Shen *et al.*, 2009). Open systems are good for species with a high growth rates and that have characteristics that enable them to survive extra environmental pressure as these systems are season dependent. Most microalgae cannot be grown in this manner due to the risk of contamination from fungi, bacteria, protozoa and other algal species (Richmond, 2008).

Closed photobioreactors can be used for most species due to the low risk of contamination due to it being a closed system, where conditions can be closely monitored (Richmond, 2008). Depending on how sophisticated the system is, most cultivation needs can be controlled automatically with little outside input. In some systems pH, nutrient addition, density monitoring, and harvests can be controlled remotely (Richmond, 2008; Shen *et al.*, 2009). This also helps reduce the risk of contamination in these systems. There are 3 main types of photobioreactors used: Cylinder, flat panel and tubular (Christenson, Sims 2011; Eriksen, 2008; Richmond, 2008; Shen *et al.*, 2009). Cylinder reactors are simple systems that are easy to use (Fig1.4c) (Richmond, 2008). They use air input from the base of the reactor to aid in not only gas transfer but also for mixing. Flat panel reactors are usually larger than that of cylinder reactors and can vary in size (Fig1.4d) (Shen *et al.*, 2009). These can be stacked for efficiency in use of space. The most common commercial bioreactor design is the tubular (Fig 1.4e) (Christenson, Sims 2011; Eriksen, 2008; Shen *et al.*, 2009). These can be built in modular form and are efficient in light utilisation. Each type of photobioreactor allows for better light transfer and has the ability to be used outdoor with natural light and inside with light supplementation, but at a higher cost to that of the open pond system.

Axenic bioreactors can also be used. This is where pure algal cultures can be grown in closed photobioreactor systems (Richmond, 2008). These systems can be grown heterotrophically or mixotrophically. This type of bioreactor is not widely used due to the ability of growing algae axenically (Richmond, 2008; Ramanan *et al.* 2016a). Most microalgae grow naturally with a large biome of other species, which are believed to be necessary for growth (Natrah *et al.* 2014; Ramanan *et al.* 2015). In laboratory and commercial conditions maintaining axenic algal cultures can be a challenge, both in the removal of other species and risk of contamination once a mono-species culture has been developed.

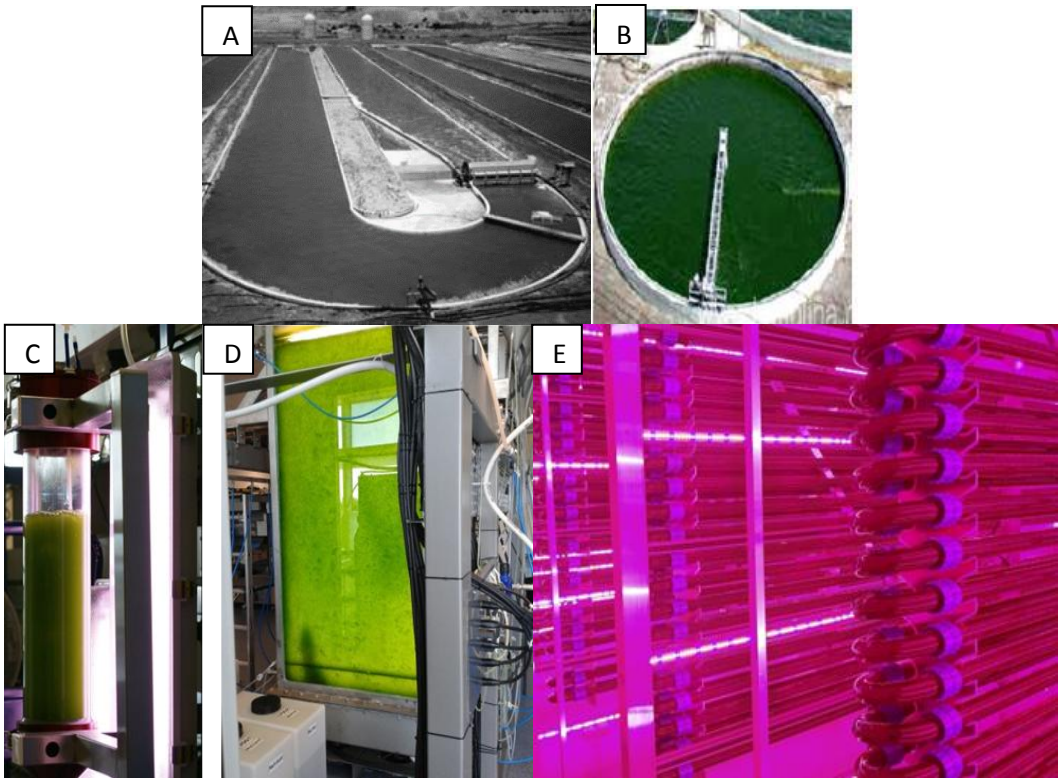


Figure 1.4: Different culturing strategies used on commercial scale. A) Raceway pond system (Richmond 2004), B) Circular pond system (Shen *et al.*, 2009), C) small vertical cylinder system (AlgaeCytes Ltd.), D) Flat panel reactor system (Algaecytes Ltd.), and E) Tubular reactor system (Algaecytes Ltd.).

1.5 – Microalgal symbionts/interactions

Symbiosis is defined as a close interaction between different organisms, where there is a close physical living association (Ferrière, Gauduchon & Bronstein 2007; Fulbright, *et al.* 2018; Leung, Poulin 2008; Moran 2007; Ramanan *et al.* 2016b). This can occur in three ways, mutualistic, commensal or parasitical. These are known as three distinctly different interactions but this terminology only really works in theory (Leung, Poulin 2008; Ramanan *et al.* 2016a). In reality these interactions are subtly different depending on how the interaction is oberved and when it has been observed (Leung, Poulin 2008; Miller, White & Boots 2006; Starr 1975).

Mutualism is described as a beneficail symbiosis where both individuals benefit from the interaction (Ferrière, Gauduchon & Bronstein 2007; Leung, Poulin 2008; Miller, White & Boots 2006; Moran 2007; Starr 1975). A good example of mutualism was presented by Croft *et al.* (2005) They presented data on mutualism in vitamin B_{12} auxotrophs, where bacteria supplied B_{12} in response to the algae supplying fixed carbon (Croft *et al.* 2005; Ramanan *et al.* 2016). Parasitism is the use of a host while causing detrimental effects such as reduced growth, damage and even death (Leung, Poulin 2008; Ramanan *et al.* 2016a). There have been very few studies on parasitism involving algal and bacterial relationships, those that have been conducted suggest bacteria use a cell lysis mechanism similar to that of a plant pathogen interaction (Arora *et al.* 2012; Ramanan *et al.* 2016; Wang *et al.* 2010). This mechanism involves cell lysis by the action of cellulases, chitinases, glycosidases, and other enzymes (Fuentes *et al.* 2016, Ramanan *et al.* 2016; Wang *et al.* 2010) Commensalism is defined as the symbiont gaining benefit at no cost or harm to the host (Leung, Poulin 2008; Moran 2007; Ramanan *et al.* 2016a). Distinguishing between mutualism and commensualism is near impossible as it is difficult to determine when an organism is benefitting or not from an exchange of products (Ramanan *et al.* 2016).There is only a subtle difference between commensalism, mutualism and parasitism, and are subject to semantics (Ferrière, Gauduchon & Bronstein 2007; Leung, Poulin 2008; Ramanan *et al.* 2016a), as if the circumstances or the environment change the relationship will change. This can be a result of a change within the lifecycle, a 3rd party addition or nutrient influx/decline (Miller, White & Boots 2006; Ramanan *et al.* 2016a). These changes can also change over time, whether that be evolutionary time or over the course of an organisnms own life cycle (Miller, White & Boots 2006).

In terms of microalgal interactions, each type of symbiosis is observed (Goecke *et al.* 2013; Kazamia *et al.* 2016; Kazamia *et al.* 2012; Natrah *et al.* 2014; Ramanan *et al.* 2015; Wang *et al.* 2016), and are highly complex. They use many modes of interaction involving many products being produced and transferred, communication and also direct attachment of bacteria to algal cells and endophytic bacteria (vector), which is summarised in Fig 1.5.

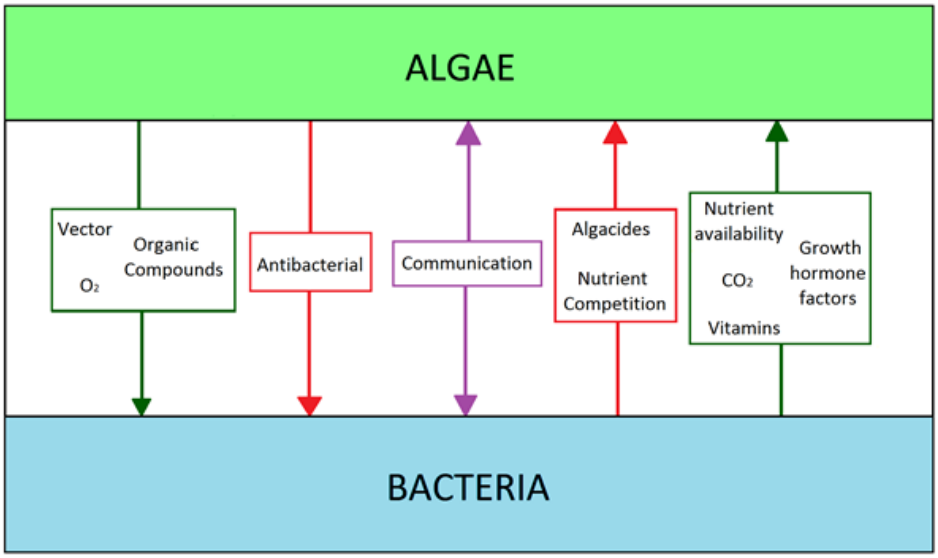


Figure 1.5: Summary of interactions that can occur between microalgae and bacteria. Red arrows negative interactions, green positive interactions and purple arrows can be positive or negative interactions. Direction of the arrow indicates direction of the interaction (Kazamia *et al.* 2016; Natrah *et al.* 2014; Tang, Koch & Gobler 2010; Wang *et al.* 2016).

1.5.1 – Modes of interaction

Microalgae and bacterial interactions have been a topic of discussion for many years. There is much debate on the underlying mechanisms which contribute to these relationships, and are believed to be highly complex and specific based on current findings (Natrah *et al.* 2014; Ramanan *et al.* 2015). Whether these relationships are species specific is still unknown, with some suggesting that association is reliant on the environment in which they are found and the extracellular products the microalgae produce (Goecke *et al.* 2013; Schwenk, Nohynek & Rischer 2014; Wang *et al.* 2016). Whether these are specific associations or just associations of pure convenience, the mechanisms involved are not fully understood. Initial research conducted on these interactions was concentrated on macroalgal species (Goecke *et al.* 2013). More recently the investigation into microalgal interactions has increased, with new bacterial species being identified and others being reclassified (Goecke *et al.* 2013; Natrah *et al.* 2014; Ramanan *et al.* 2016a). From those now documented it provides insight into the diversity and complexity of the relationships and the evolutionary drive towards them. Table 1.1 illustrates some of the documented interactions between algae and bacterial species.

Algal species	Bacterial species	Compound	Effect	Location	REFERENCE
Dunaliella bardawil	*Halamonas Sp.*	Siderophore	Makes iron more soluble for algal use.	Exophytic	Keshtacher-Liebson *at al.* 1995 Natrah *et al.* 2013
Amphidinium operculatum, *Porphyridium pupureum*	*Halamonas sp.*	Cobalamin (vitamin B_{12})	Most algal species cannot synthesise vitamin B_{12}. Essential for methionine pathway.	Exophytic	Croft *et al.* 2005, 2006 Natrah *et al.* 2013
Chlorella vulgaris	*Azospirillum brasilense*	Indole-3-acetic acid Siderophore	Increased algal growth. Siderophore mediated nitrogen fixation	Exophytic	Gonzalez – Bashan 2000 de-Bashan and Bashan 2008 Natrah *et al.* 2013 Fuentes *et al.* 2016, leyva *et al.* 2014
Neochloris oleoabundans *Scendedesmus sp*.	*Azotobacter vinelandii*	Siderophore	Siderophore mediated nitrogen fixation	Exophytic	Santos *et al.* 2014, Fuentes *et al.* 2016
Scrippsiella trochidea	*Marinobacter Roseobacter*	Vibrioferrin	Unusual marine Siderophore, making iron more soluble for algal use.	Exophytic	Amin *et al.* 2009 Fuentes *et al.* 2016
Botrycoccus braunii	*Rhizobium sp.*	Unknown	Enhanced algal growth in presence of bacteria.	Exophytic	Rivas *et al.* 2010 Natrah *et al.* 2013
Volvox carteri	*unidentified*	unknown	Unknown	Endophytic, found in cytoplasm and most abundant between the chloroplast and the plasmalemma	Cole, J.J. 1982

Table 1.1: Some known algae/bacterial relationships and compounds provided, adapted from Natrah *et al.* 2014

There are many different types of bacterial-microalgal relationships. Some bacteria are exophytic, they live closely with microalgae, whether they are found directly attached or on the algal sheath (Wang *et al.* 2016). Others are endophytic, living within the algae cells, causing a more direct transfer of products (Natrah *et al.* 2014). Yurchenko *et al.* 2018 confirmed endophytic bacteria present within a species of Eustigmatophyceae, suggesting a long term relationship which has evolved to be highly specific. Other bacteria appear to occur in the algal surroundings within the environment, without direct attachment (Kazamia *et al.* 2012), the resulting relationships being based on the ability of the microalgae and bacteria to produce external resources rather than their proximity (Amin *et al.* 2015). Each type of association would suggest finding similar bacterial communities associated in every location the microalgae are found. In some studies it is suggested that endophytic bacteria remain the same in both wild and laboratory kept cultures but external bacteria can vary greatly from the location in which they are found (Schwenk, Nohynek & Rischer 2014; Natrah *et al.* 2014), suggesting these relationships were evolved over time, due to exposure to each other rather than a specific need. The specificity of relationships have been investigated where an axenic culture of a microalga has had a non-native bacterium, which produces the same external resources and the original native bacterium, but the growth of the algae was not aided (Amin *et al.* 2015; Kazamia *et al.* 2016; Natrah *et al.* 2014). These findings suggests that though the interactions may have developed from exposure to a product over time it may evolve into a type of specificity (Kazamia *et al.* 2016). Yurchenko *et al.* (2018) studied a gene transfer event, that suggests a long-term partnership

between an Eustigmatophyte algae and a novel lineage of endosymbiotic bacteria. Emphasising the development of algal bacterial relationships over extended periods of time.

This microenvironment has been named the Phycosphere; first used in 1972 (Bell, Mitchell 1972) it was described as the zone that exists outside an algal cell or colony, extending outward for an undefined distance, which allows for the growth of bacteria through stimulation from extracellular products. This term was used sporadically at first with it gaining more precedence as more research into microalgae has occurred and can still be described as one of the most ignored bacterial habitats (Cho *et al.* 2015; Kim *et al.* 2014; Ramanan *et al.* 2015; Ramanan *et al.* 2016a; Sapp *et al.* 2007). A region that can host such intricate relationships which involve nutrient cycling and complex signalling deserve more investigation into understanding its mechanisms (Doucette 1995; Wang *et al.* 2010).

Bacterial cells can outnumber microalgae by a factor of between 10 and 1000 with highest bacterial numbers observed when the microalgae are in stationary phase when compared with the exponential growth phase (Natrah *et al.* 2014; Wang *et al.* 2016), and this trend is seen in both batch and semi-continuous cultures (Fig 1.6). This co-existence must be based on communication and the exchange of extracellular products (Natrah *et al.* 2014). When this communication fails or is interrupted the sensitive equilibrium is either pushed in favour of the bacteria or of that of the microalgae, and the relationship can turn from mutualistic to parasitic (Natrah *et al.* 2014; Wang *et al.* 2016) . If the microalgae are favoured the bacterial numbers fall and the microalgae cell density increases. If the bacteria are in favour they become dominant increasing their numbers and eventually start to attack the algal cells as a source of nutrients, thus eventually destroying the algal culture.

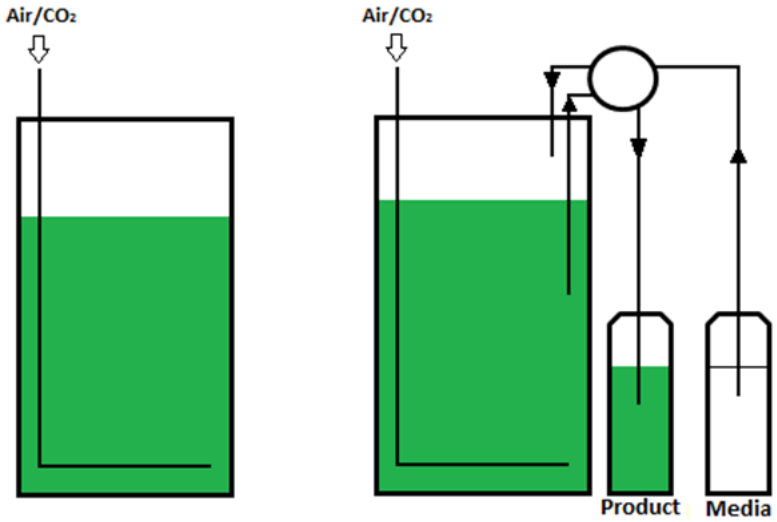


Fig 1.6: Schematic of a batch (A) and continuous (B) culturing method.

1.5.2 – Mutual benefits and requirements

Bacteria can stimulate algal growth and can achieve this in a variety of different ways; whether it is providing growth promoters, maintaining the environment or as a form of protection from other bacterial species (Wang *et al.* 2016). Bacteria will only produce extracellular products when it is beneficial to itself as it is metabolically expensive (Grant *et al.* 2014), therefore a mutualistic relationship must develop. This development must occur over time as either through exposure to a new source of nutrition or a growth aid that then develops into dependence (Amin *et al.* 2015; Goecke *et al.* 2013; Grant *et al.* 2014). Evolution must take place to create a strong association. Algae in return create a set of external conditions to promote growth and survival of the bacteria, which can include organic materials, protection, and a stable environment.

1.5.3 Protection

Some bacteria can provide protection to microalgae by limiting or preventing the growth of unwanted and harmful bacteria. This has been demonstrated using immobilised bacteria. *Chlorella vulgaris* and *Chlorella sorokiniona* grown in culture with immobilised *Azospirillum brasilerise* which had a probiotic effect by limiting the growth of unwanted and harmful bacteria and thus allowing the alga to grow more effectively (de-Bashan *et al.* 2004; Gonzalez & Bashan 2000; Hernandez *et al.* 2009; Wang *et al.* 2016). This type of protection can be achieved in many different ways, by the killing of unwanted bacterial cells by lysing them directly or indirectly by the release of lytic compounds, i.e. specific digestive enzymes, causing small changes in the microenvironment such as pH or by simply out competing for available nutrients (Natrah *et al.* 2014; Wang *et al.* 2016). The production of specific antibiotics can aid in protection by the involvement of disrupting compounds and pathways and thus contributing to cell death.

Microalgae can provide protection for bacteria in many ways. One is providing a physical barrier to damage which can be achieved in two ways, by enveloping the bacteria causing them to become endophytic, or the bacteria attach directly to the cell wall or sheath (Goecke *et al.* 2013; Grant *et al.* 2014; Ramanan *et al.* 2016b; Wang *et al.* 2016). *Volvox carteri* was found to contain endophytic bacteria (unidentified), they occurred throughout the cytoplasm but were highly abundant between the chloroplast and the plasmalema (Cole, 1982). It is thought that this mode of protection is what limits the creation of truly axenic microalgal cultures, but more investigation is still needed. They can also produce targeted antibiotic type compounds which can target specific unwanted bacteria and therefore reduces competition between bacterial species (Natrah *et al.* 2014). A *Nannochloropsis* species when grown with *Vibrio harveyi* produces compounds identified as being similar to terpenes and glycosides which inhibit the bacterial growth.

1.5.4 Communication

Communication between any organisms living in close proximity is highly important. Bacteria communicate with each other through quorum sensing (Bauer, Robinson 2002, Hughes, Sperandio 2008, Joint *et al.* 2002). Bacteria and microalgae communicate in many different forms (Kouzuma, Watanabe 2015; Natrah *et al.* 2014; Ramanan *et al.* 2015; Ramanan *et al.* 2016a) from interfering with quorum sensing to the production of stimulators and inhibitors (Bauer, Robinson 2002). Microalgae have the ability to produce compounds that interfere with quorum sensing by competing with bacterial compounds and either blocking or activating receptors causing the production of wanted compounds or reducing the levels of unwanted compounds. The most basic being able to control bacterial populations (Bauer, Robinson 2002; Joint *et al.* 2002). Bacteria appear to communicate with microalgae by the production of specific products that either activates or inhibits the release of algal product (Kazamia *et al.* 2016; Natrah *et al.* 2014; Tang, Koch & Gobler 2010; Wang *et al.* 2016). *Chlamydomonas reinhardtii* and *Chlorella sp.* have been seen to produce lumichrome, a derivative of vitamin B_2 (riboflavin), which interferes with bacterial signal receptors and has a stimulatory effect on quorum sensing. Much work has been conducted in understanding the effect bacteria and microalgae have on each other, but there is still much to learn (Amin *et al.* 2015; Bauer, Robinson 2002; Hughes, Sperandio 2008; Ramanan *et al.* 2016b)

1.5.5 Growth stimulation

The phycosphere can be varied, causing the activity within the microalgal surroundings to change depending on the extracellular products produced (Guo, Tong 2014; Helliwell *et al.* 2014; Natrah *et al.* 2014; Ramanan *et al.* 2015; Sapp *et al.* 2007). Microalgae can excrete carbon sources, in the form of old cell wall matrix which can include the provision of polysaccharides, ions and proteoglycans, external products, and other compounds into the surrounding environment (Natrah *et al.* 2014; Ramanan *et al.* 2015). In turn this can stimulate bacterial communities including bacterial DNA synthesis, enhancement of horizontal gene transfer and increased formation of bacterial biofilms (Natrah *et al.* 2014; Sapp *et al.* 2007). This carbon rich environment that is provided allows for stable communities to coexist. Quorum sensing is an important mechanism in bacteria that regulates multiple processes important for survival, some microalgae can produce compounds which can interfere with this process (Natrah *et al.* 2014; Ramanan *et al.* 2015). This is achieved by interfering with the signal receptor and/or the response regulators, which can up or down regulate the production of certain compounds, and thus effect the growth and development of

bacterial communities. Chlorella cultures were found to have 4 bacterial species and 1 fungus all living on the sheath of the algal cells. When these bacterial strains were reintroduced to an axenic Chlorella culture the microalga had higher long term chlorophyll content compared to the axenic culture alone (Wang *et al*. 2014). Sureshkumar *et al.* 2014, demonstrated that *Nannochlorpsis oculata* and *Chaetoceros calcitrans* had significantly higher growth when grown with a *Bacillus sp*. and a *Pseudomonas sp*. but the interactions are still unknown.

1.5.6 Phytohormones

It has been documented in some studied interactions that there is release of certain phytohormones from bacteria which aid in the growth of the microalga (Amin, Parker & Armbrust 2012; Cole 1982, Cooper, Smith 2015; Fukami, Nishijima & Ishida 1997; Kouzuma, Watanabe 2015). Indole-3-acetic acid also known as Auxin (IAA) in particular has been documented to have been produced in measureable numbers (Amin *et al.* 2015; Ramanan *et al.* 2016b). In the study conducted it was demonstrated that there was a direct exchange of IAA for organosulfur compounds between the microalga *Thalassiosira pseudonana* and a *Sulfitobacter* related bacteria (Amin *et al.* 2015), suggesting also a change in metabolism to be able to provide for each other's needs (Ramanan *et al.* 2016b). In another example, *Azospirillum brasilens* significantly increased the growth of *Chlorella vulgaris* by producing and releasing of IAA. Other phytohormones have also been found in microalgae, such as abscisic acid (ABA), gibberellic acid (GA), and cytokinin (CK). Whether these are produced by the algae themselves or by companion bacteria has not been fully investigated.

1.5.7 Nutrients and trace elements

Bacteria can also provide stability by providing access to certain nutrients and compounds such as CO_2 , trace elements and B vitamins (B_{12}, B1 and B7) (Amin *et al.* 2009; Helliwell *et al.* 2011; Helliwell *et al.* 2014; Kazamia *et al.* 2012; Sañudo-Wilhelmy *et al.* 2006). Bacteria, as they respire, remove unwanted excess $O_{2,}$, which in high levels can be toxic to microalgae, from the surrounding environment, releasing CO_2 through respiration, which in turn is available for the microalgae to use (Wang *et al.* 2016; Natrah *et al.* 2014). This in turn helps maintain the pH of the local environment and keep O_2 at levels that can be used by the microalgae but not at sufficient levels to poison the environment. Certain elements cannot be accessed by microalgae unless it is in a certain form (Amin *et al.* 2009; Kazamia *et al.* 2012; Schwenk, Nohynek & Rischer 2014). Bacteria can absorb those the algae cannot and change them into a format that is usable to the microalgae (Amin *et al.* 2009), for example, iron, cannot be accessed by microalgae directly and must be in a more complex form. Bacteria associated with microalgae have been documented to change the form of iron using different siderophores to make it soluble in water and more readily available to absorb. One such siderophore is vibrioferrin (VF) which has been documented as a product of a *Marinobacter* species in relation to the marine dinoflagellate *Gymnodinium catenatum* (Amin *et al.* 2009). Bacteria also aid microalgae in providing phosphorus and nitrogen which are essential nutrients needed for growth, development and production of some extracellular products (Kazamia *et al.* 2016). A *Pseudomonas* bacterium played a role in providing a temporary phosphorus reservoir for its host cyanobacteria *Microcystis aeruginosa* under phosphorus sufficient conditions (Wang *et al.* 2014). Algae in turn can produce many external products essential for bacterial growth. Such as an organic carbon source and amino acids (Natrah *et al.* 2014; Ramanan *et al.* 2015), though more research is currently needed to understand the exchanges between algae and bacteria.

1.5.8 Nitrogen

Nitrogen is a highly important factor in the growth and development of microalgae. It has links with both the Calvin cycle and the TCA cycle, which play essential roles within microalgal cells (Bolch, Subramanian & Green 2011; Kim *et al.* 2014; Teplitski, Rajamani 2011). The Calvin cycle, being a crucial part of photosynthesis, is responsible for the cellular synthesis of glucose and other storage products which cells rely on for survival (Richmond 2008; Teplitski, Rajamani 2011). The tricarboxylic acid (TCA) cycle is an essential part of respiration and the utilisation of products to fuel the electron transport chain and therefore produce cellular energy in the form of ATP (Richmond 2008). Nitrogen availability can have a large effect on the synthesis and turnover of amino acids; Turpin *et al.* 1988 observed a dramatic increase

in amino acid synthesis when N-starved algae had a nitrogen source re-introduced (Turpin *et al.* 1988). Some bacteria found associated with microalga are denitrifying and ammonia producing bacteria. These both reduce the available nitrogen source, usually nitrates and nitrites which are metabolically expensive for microalgae into the form of dinitrogen and ammonia (Peterson, *et al.* 2011). This allows the algae to utilise it more efficiently by aiding in the uptake process. There are many documented microalgae that utilise ammonia as a preferred nitrogen source, such as *Scenedesmus obliquus* (Chen *et al.* 2012), *Chlamydomonas reinhardii (Florencio* et al. *1983)* and the eustigmatophyceae *Nannochloris oculata* (Terlizzi *et al.* 1980). This process can also be beneficial when there is an oversupply of nitrate within the algal environment, making the microenvironment more suitable for the survival of the alga (Nils 2003). Whether this mechanism is to the direct benefit of the alga or the whole holobiont, it allows metabolic stability for the consortia.

Other types of bacteria found are nitrogen fixers, which convert inaccessible nitrogen sources into nitrate which can be utilised by the microalgae (Kim *et al.* 2014). A Rhizobium sp. found in association with *Botryococcus braunii*, the Rhizobium bacterium boosted the growth of *B. braunii* (Magdouli *et al.* 2016). A summary of denitrification and nitrogen fixing pathways is illustrated in Figure 1.7, indicating some of the main genes involved in this process.

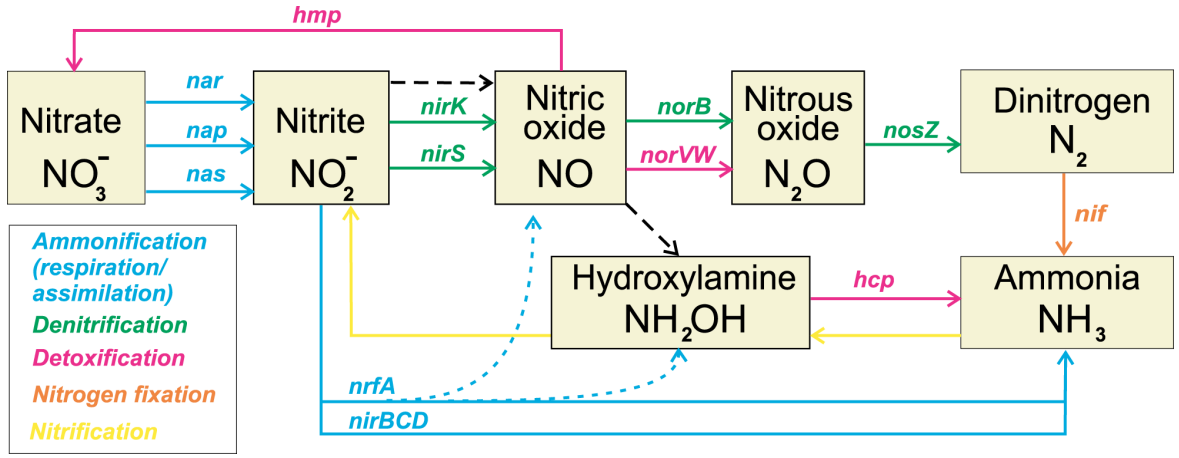


Figure 1.7: Ammonification, denitrification, detoxification, nitrogen fixation and nitrification pathways with associated genes. Adapted from Rodionov, *et al.* (2005).

1.5.9 B –vitamins

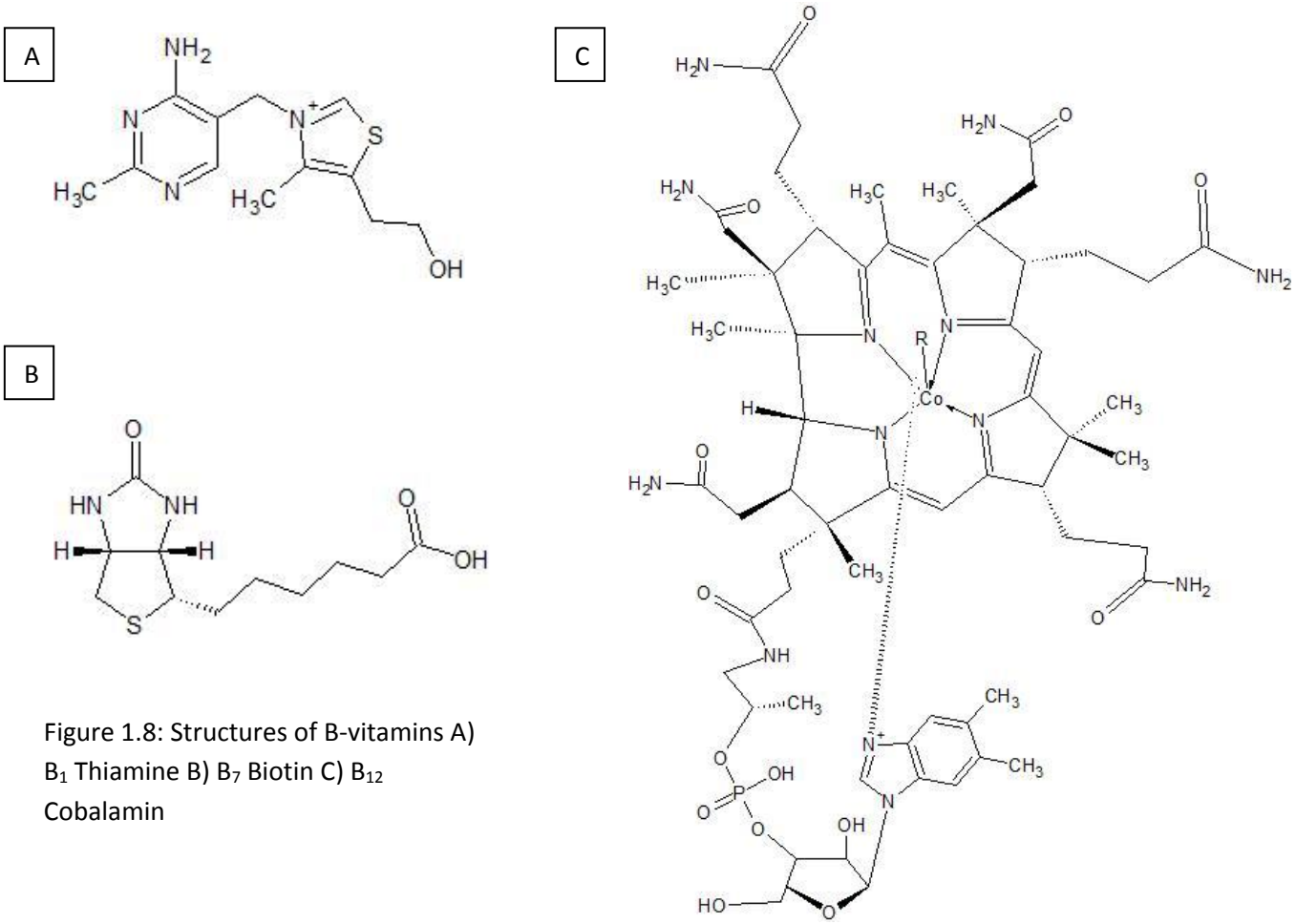


Figure 1.8: Structures of B-vitamins A) B_1 Thiamine B) B_7 Biotin C) B_{12} Cobalamin

B vitamins are important for the growth of many algal species, in particularly B_1, B_7, and B_{12}. Out of these three B vitamins, cobalamin (B_{12}) is the most sought after and it is estimated that 50% of algal species require it for sustained and healthy growth. Thiamine (B_1) and biotin (B_7) are also required for growth in approximately 22% and 5% of algal species respectively (Croft *et al.* 2005; Helliwell *et al.* 2011; Helliwell *et al.* 2014; Grant *et al.* 2014; Kazamia *et al.* 2012; Tang, Koch & Gobler 2010). Biotin is a cofactor for carboxylase enzymes, including acetyl co enzyme A (CoA), which are essential for fatty acid synthesis. Thiamine has a pivotal role in intermediary carbon metabolism (Croft *et al.* 2005); its active form Thiamine pyrophosphate (TPP) is essential for all organisms and is a cofactor for a number of enzymes involved in primary carbohydrate and branched amino acid metabolism *(*Croft *et al.* 2005). Cobalamin is a complex Co^{2+} containing modified tetrapyrrole that acts as a cofactor for enzymes involved in C1 metabolism and other certain radical reactions (Croft *et al.* 2005). Algal species can require one, two or all of these three B vitamins; there is no correlation between them for their need (Tang, Koch & Gobler 2010). Not all microalgae are auxotrophic for these vitamins but most will absorb them if they are made available (Croft *et al.* 2005; Rébeillé *et al.* 2007). *Lobomonas rostrata*, a known B_{12} dependant alga, and the rhizobium bacteria *Miesorhizobium loti* grew together well when no B_{12} or organic carbon source was present (Santos *et al.* 2014). *Thalassiosira pseudonanma* was stimulated by the addition of B_{12} or by live bacteria that synthesised the vitamin (Cole, 1982). *Chlamydomonas reinhardtii* when under temperature stress, growth was significantly improved when exposed to a B_{12} producing rhizobia (Magdouli *et al.* 2016). From metagenomic analysis it has been revealed that some bacterial genes that encode for B vitamins are closely linked with lipid production, and that some lipids found in microalgae may in fact be from the associated bacteria themselves (Goecke *et al.* 2013). The genes involved in the B_{12} synthesis pathway are described in Figure 1.9. There are two alternative routes in microbial de novo biosynthesis of vitamin B_{12}; the aerobic and anaerobic pathway (Fang, *et al.* 2017). These differ on the basis of the molecular oxygen requirement, and when the cobalt insertion occurs. Some cobalamin producing bacterial strains can also use the salvage pathway, where they can absorb corrinoids to synthesis B_{12} (Fang, *et al.* 2017). All methods leading to the

ultimate production of cobalamin, which is an important requirement for the production of methionine, an essential amino acid.

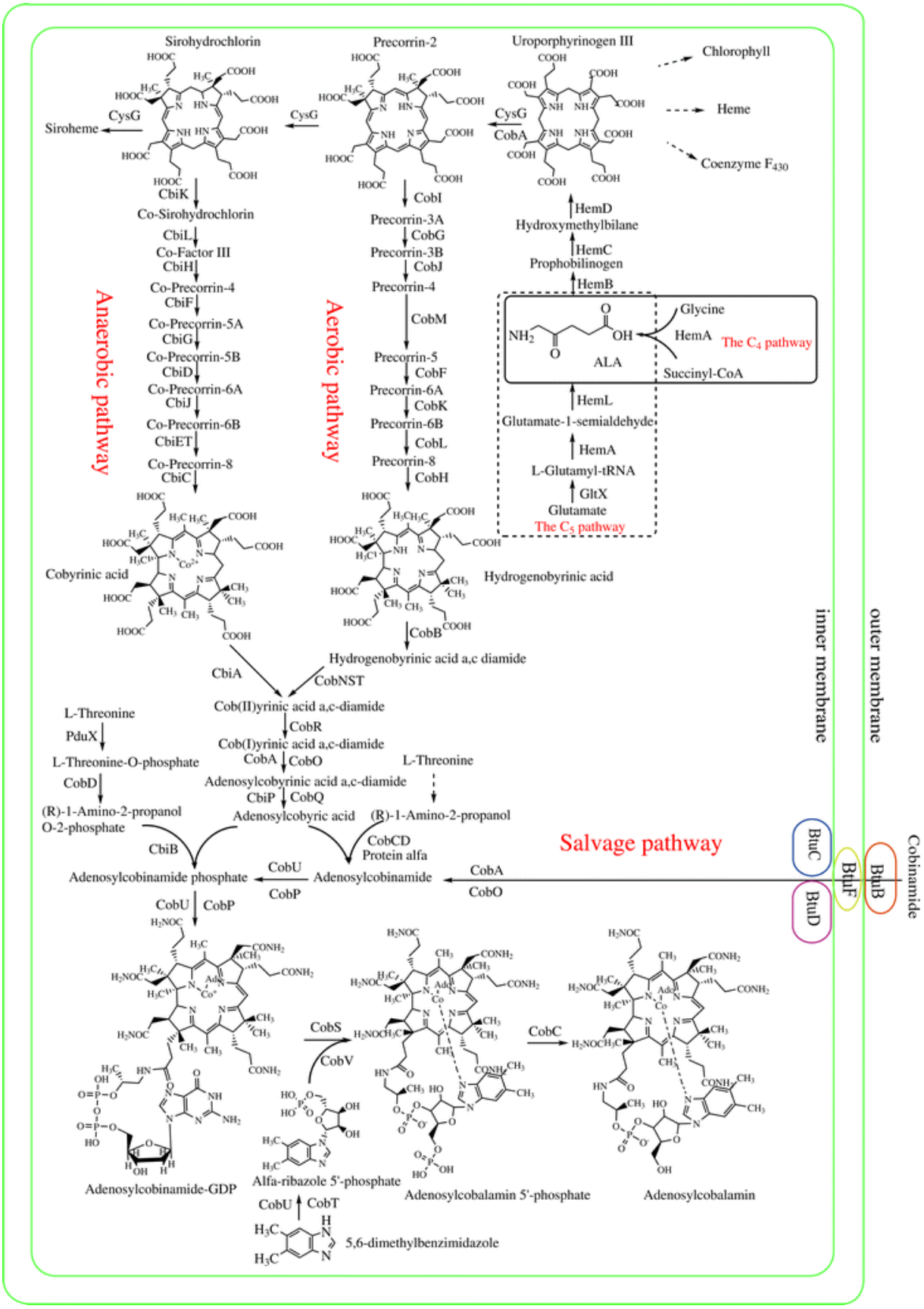


Figure 1.9: B_{12} synthesis pathway detailing genes involved. Adapted from Fang, *et al.* (2017).

In the case of B_{12} it has been identified that there is a link between auxotrophy with the presence of specific genes involved in the methionine pathway (Fig 1.10) (Croft *et al.* 2005; Helliwell *et al.* 2011; Helliwell *et al.* 2014; Kazamia *et al.* 2012). Methionine synthase can be produced by two different isoforms of the same gene *metE* and *metH*. *MetE* is the B_{12}-independent form of methionine synthase and can be used to produce methionine in the absence of B_{12}. In some species that have been analysed, those that are B_{12} auxotrophic either have a silenced form of *metE*, pseudogenes or the gene is completely missing (Helliwell *et al.* 2011; Helliwell *et al.* 2014). Fig 1.11 shows the variability of functional *metE* genes found in 4 species of microalgae. This explains the presence of B_{12} auxotrophy across several lineages but not being able to be accounted to a single evolutionary event, as different strains of the same species can have different B_{12} dependencies. Those species which are B_{12}-independent that have been assessed, have both genes present, and enables them to utilise any cobalamin when present (Croft *et al.* 2005; Kazamia *et al.* 2012). This may explain where the bacterial relationships for B_{12} dependent species have evolved. The over use of the *metH* gene over long periods of time silencing the *metE* gene, causing mutations and deletions to occur much more frequently in that genetic area and therefore the loss of the gene (Croft *et al.* 2005; Helliwell *et al.* 2011; Helliwell *et al.* 2014; Kazamia *et al.* 2012).

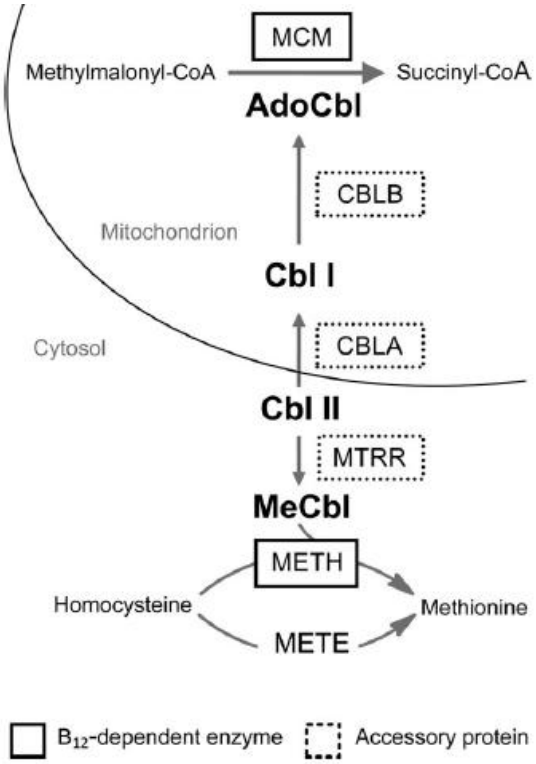


Figure 1.10: Diagram illustrating the intracellular metabolism of B_{12} in eukaryotes (Helliwell *et al.* 2011). *metE* catalyses the same reaction as *metH* but does not require the presence of B_{12} as a cofactor. Both use methyltetrahydrofolate as the methyl donor, but for *metH* cobalamin transfers the methyl group to the substrate. AdoCBl – adenosylcobalamin; MeCbl – methylcobalamin. CB I and CB II refer to the oxidation state of the cobalt ion within B_{12}.

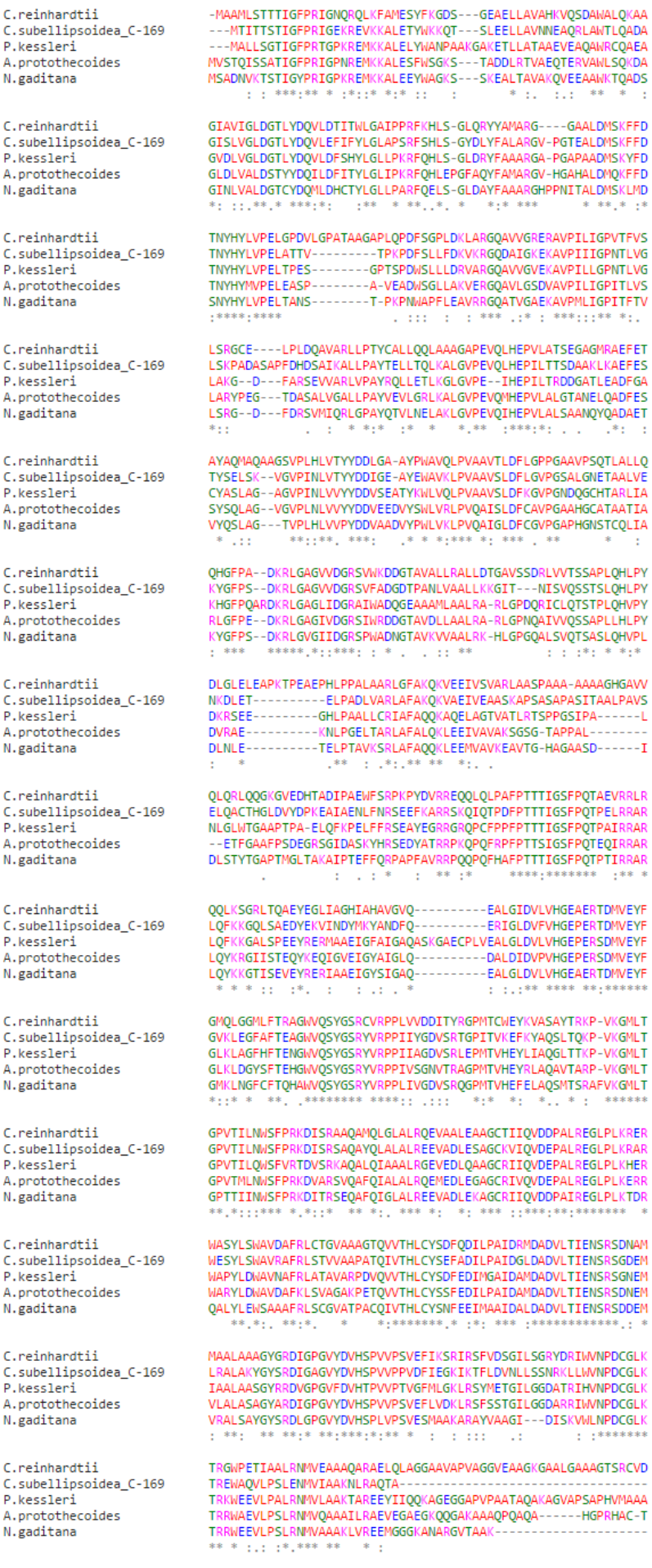


Figure 1.11: Clustal omega analysis of five *MetE* proteins. There are both conserved and variable regions.

Coding letters indicate the amino acid present. Colouration is the property of that amino acid:

- Red: Small hydrophobic (including aromatic-Y),
- Blue: Acidic,
- Magenta: Basic -H,
- Green: Hydroxyl + sulfhydryl +amine + G,
- Grey: Unusual amino acids.

The under alignment score symbols are:

- An asterisk * - indicating positions which have a single, fully conserved residue,
- A colon : - indicating conservation between groups of very similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix)
- A Period . - indicating conservation between groups of weakly similar properties (scoring =< 0.5 and > 0 in the Gonnet PAM 250 matrix)

Sequences were obtained through the NCBI database (Blanc *et al.* 2012; Carpinelli *et al.* 2014; Gao *et al.* 2014; Merchant *et al.* 2007; Ota *et al.* 2016) and analysed through Clustal Omega (Goujon *et al.* 2010, Sievers *et al.* 2011)

More widespread analysis into the interaction between microalgae and bacteria needs to be conducted, in particularly looking into the extracellular products exchanged and the B_{12} *metE*/*metH* gene presence (Helliwell *et al.* 2014; Helliwell *et al.* 2011). The majority of work already conducted has been on macroalgae, and that conducted on microalgae has been focused on marine species (Goecke *et al.* 2013). The work into these relationships has provided insight into novel bacterial species and clades which have now been documented (Goecke *et al.* 2013; Wang *et al.* 2016). Also the link that some of the microalgal lipids may be in fact produced by the bacteria warrants further investigation into the development of axenic lines and therefore the overall relationship between microalgae and bacteria (Helliwell *et al.* 2011; Goecke *et al.* 2013; Wang *et al.* 2016).

1.6 Commercial applications

Microalgae have many commercial applications, each product having its own challenges in production (Christenson, Sims 2011; Fuentes *et al.* 2016; Milledge 2011; Wang *et al.* 2016; Wijffels, Barbosa & Eppink 2010). Each type of microalga will produce different products and require a unique set of optimised growth conditions. Not all products that can be obtained from microalgae are sustainable or economically feasible (Wang *et al.* 2016; Wijffels, Barbosa & Eppink 2010). Amongst the documented problems is that of biofilms and monocultures (Christenson, Sims 2011). Monocultures cause problems through the risk of contamination from unwanted bacteria, other microalgal strains and cyanobacteria (Christenson, Sims 2011; Kouzuma, Watanabe 2015). Introducing other bacteria and algae into the system will cause competition which can reduce the overall culture integrity. Biofilms cause not only an integrity issue but that of an operational one (Lutzu and Dunford 2018; Spolaore *et al.* 2006; Ramanan *et al.* 2015). The excessive build-up of biofilms can cause issues of culture movement, self-flocculation and light penetration. Being able to control the microbiome and components within the Phycosphere allow a great control on the commercialisation of cultures with respect to prolonging culture health (Christenson, Sims 2011).

There are many documented products from microalgae such as pigments (D'Alessandro, Antoniosi Filho 2016; Spolaore *et al.* 2006), biofuels (Brennan, Owende 2010; Wijffels, Barbosa & Eppink 2010), fatty acids (D'Alessandro, Antoniosi Filho 2016; Spolaore *et al.* 2006), carbohydrates (Spolaore *et al.* 2006), animal feeds (Raja *et al.* 2014) and high value chemicals (Milledge 2011; Wijffels, Barbosa & Eppink 2010). One of particular interest is that of omega 3's and reducing the requirement from the already dwindling fish stocks (Adarme-Vega *et al.* 2012; Spolaore *et al.* 2006)

1.7 Aims and objectives

To gain understanding of the relationship and necessity of the microbiome associated with the proprietary algal strain ALG01 will allow for a better overall understanding of its microenvironment and also the alga itself. This knowledge will allow for higher understanding in its cultivation and the utilisation of this relationship to benefit productivity and commercial use.

1.6.1 Aims

- Growth of the microalgae to obtain bacterial free cultures and maintaining its growth.
- Cultivation and identification of the associated bacterial strains - phenotypic and molecular characterisation of the isolated strains.
- Effect of exogenous B_{12} addition – establish whether B_{12} is 'sufficient' for the growth of the microalgae.
- Yield analysis and optimisation of target omega-3 fatty acids – manipulation of the light and cultivation conditions in the presence and absence of the associated bacteria and B_{12}

1.6.2 Objectives

- To identify the natural bacterial flora necessary for the optimised growth of the target microalgae and what factors they provide.
- To investigate what factors determine recognition, specificity and a sustained interaction.

Chapter 2

Materials and Methods

2.1. Media and materials

All media was prepared by dissolving the media components in distilled water and autoclaving at 121°C at 15psi for 15mins. For agar media, the components were heated to dissolve before autoclaving. Agar media was poured aseptically within a laminar flow system before use.

2.1.1. Growth media

- Bold modified basal broth (BBM), 20mlL^{-1} BBM stock (Sigma) pH 4.5.
- BBM agar (BBMA), 20mlL^{-1} BBM stock (Sigma) and 20gL^{-1} agar (Sigma) pH 4.5-5 before buffering with 1M $NaHCO_3$ to pH 7.
- BBM with supplements. 20mlL^{-1} BBM stock (Sigma), yeast extract 2g/L (Sigma), Tryptone 0.5g/L (Sigma) and Vitamin B_{12} 0.2g/L (Sigma) buffered with 1M $NaHCO_3$ to pH 7.
- Tryptone Soya Agar (TSA), 40gL^{-1} (Sigma) pH 7.3.
- Yeast Extract Agar (YEA), 23gL^{-1}(Sigma) pH 7.2.
- Nutrient Agar (NA), 23gL^{-1} (Sigma) pH 6.8.
- R2A Agar (R2AA), 18.12gL^{-}1 (Sigma) pH 7.2.
- Davis Minimal Agar (DMA), 26.6gL^{-}1 (Sigma) pH 7.0.

2.1.2. Reagents

All chemicals were supplied by Sigma Aldrich

- Maximum recovery diluent (MRD), 9.5gL^{-1}(Sigma) pH 7.0. A protective and isotonic diluent containing peptic digest of animal tissue and sodium chloride for maximal recovery of micro-organisms and non-selective.
- Nitrite reducing bacterial detection kit (Micro monitor sig nitrite bacterial tests) was supplied by ECHA microbiology.
- Ethanol
- Palin test kits - Standard method Reference 4500-Nitrate-E, and 4500-P-C.

2.2. Equipment

2.2.1. Multicultivator MC1000 (MC)

A Photon Systems Instruments (PSI) small bioreactor consisting of 8 vessels each with a capacity of 70ml, submerged into a thermo-controlled water bath (Fig.2.1.A). Each vessel was independently illuminated by white light at a maximum of 1000µmol/m^2/s. All hydrated aeration from air provided via an air pump passed through a water bottle to hydrate the air and reduce evaporation and to mix cultures. The unit software measured optical density (OD) at 680nm and 720nm at 10 minute intervals. Data was downloaded from the unit via software to PC in CSV file format; full details are available at: http://www.psi.cz/products/photobioreactors/multi-cultivator-mc-1000 (Photons systems instruments, 2017a).

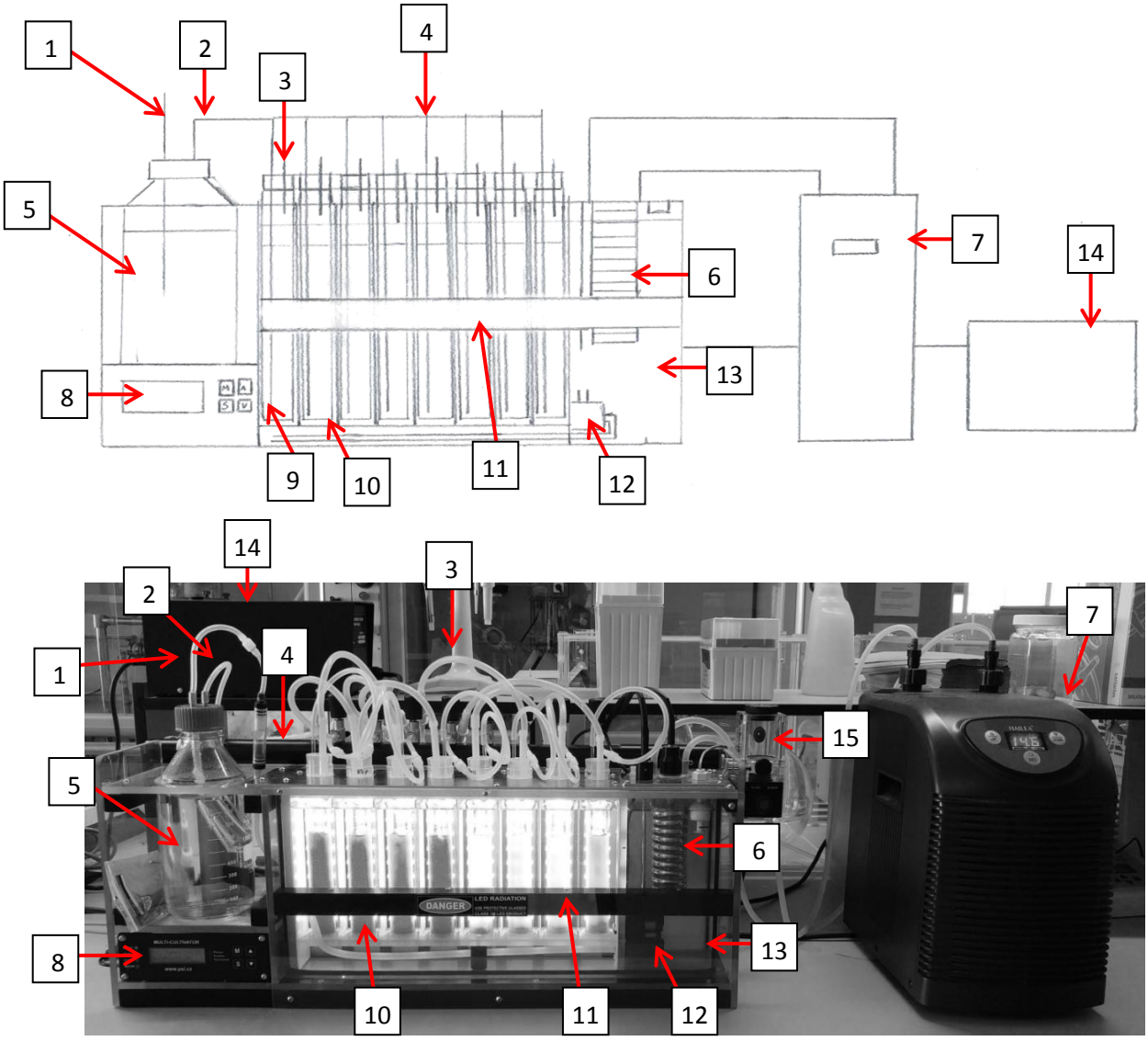


Figure 2.1: MC. 1) Airline in, 2) airline to vessels, 3) vessel exhaust, 4) aeration bar, 5) humidifier bottle, 6) heating and cooling coil, 7) cooler unit , 8) control panel, 9) airline rods, 10) cultivation vessel, 11) sensor bar, 12) water bath pump, 13) water bath, 14) power pack, 15) cold water store (Photons systems instruments, 2017a).

2.2.2. Photobioreactor FMT 150 1L (1LPBR)

A PSI 1L size photobioreactor, consisted of a 1L glass cultivation vessel that was placed within the main device; which had fully automated readings (Fig 2.2.A). Temperature, light and aeration was fully controlled by the software with pH, OD, and quantum yield (QY) measurements taken every 10 minutes and recorded to the devices computer, which can be accessed via USB. Adjustable light panel of both white and red light available with a maximum of 1000µmol/m^2/s. Aeration through a gas mixing system that allows for control of the volume of air per minute and the addition of CO_2 at a percentage in air. The aeration was passed through a humidifier bottle to reduce evaporation of the culture. Full details are available at: http://www.psi.cz/products/photobioreactors/photobioreactor-fmt-150. (Photons systems instruments, 2017b).

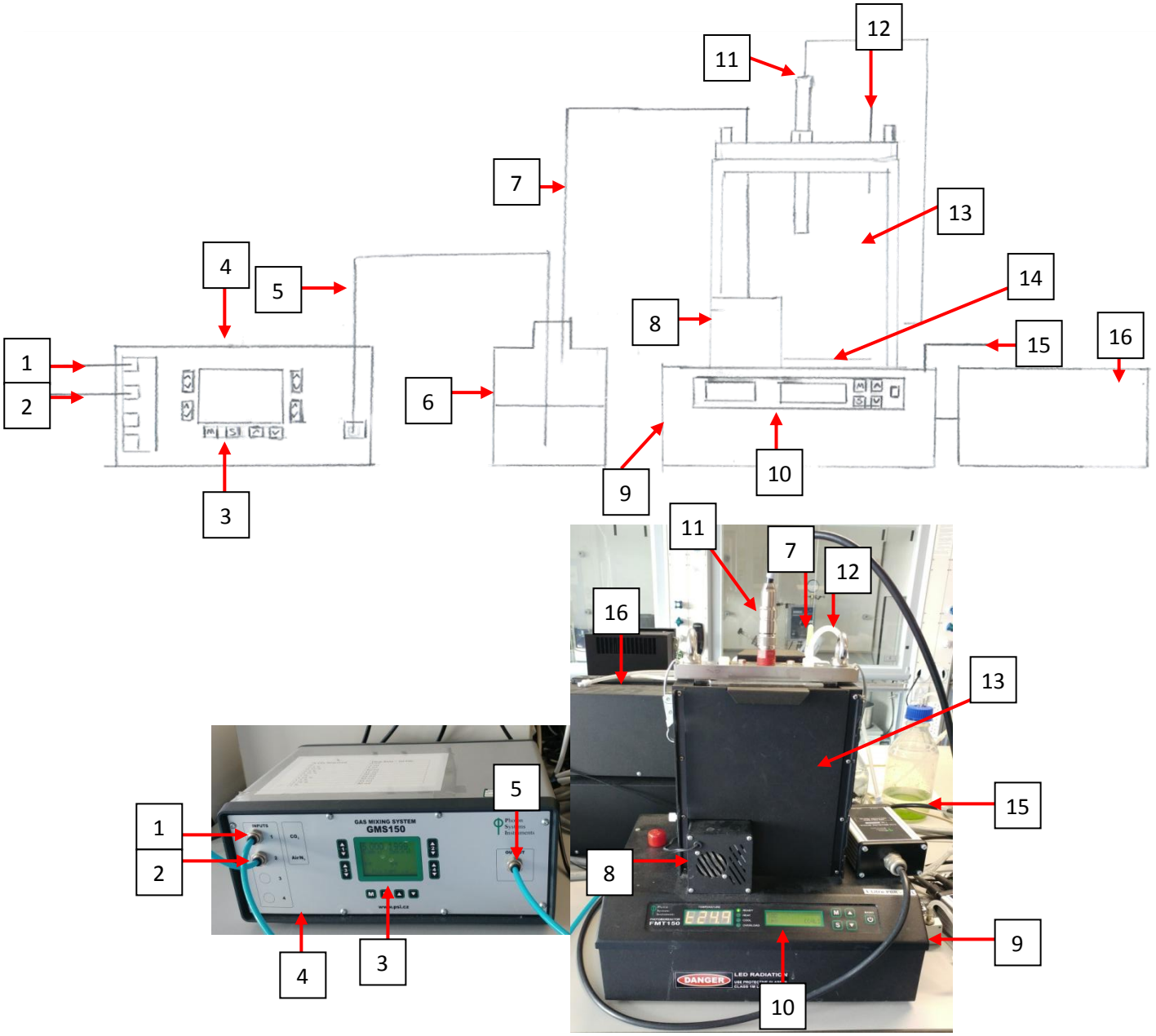


Figure 2.2: 1LPBR 1) CO_2 line in, 2) airline in, 3) control panel, 4) gas mixer, 5) airline out, 6) humidifier bottle 7) airline to unit, 8) measurement reader, 9) 1LPBR main unit, 10) control panel, 11) pH and temperature probe, 12) exhaust, 13) cultivation vessel, 14) aeration bar, 15) PC connection, 16) power pack (Photons systems instruments 2017b).

2.2.3. 100L flat panel photobioreactor (FPR)

A flat panel photobioreactor designed by AlgaeCytes Ltd. (Fig 2.3.A). Aeration rods at the base of the reactor vessel to allow for good air distribution and mixing. It has extending light arms either side of the reactor to reduce and increase light intensity of red LED panels. The fan is located at one end to move air and enable good cooling of the reactor. All samples were manually taken for measurements of pH and OD using a handheld spectrophotometer.

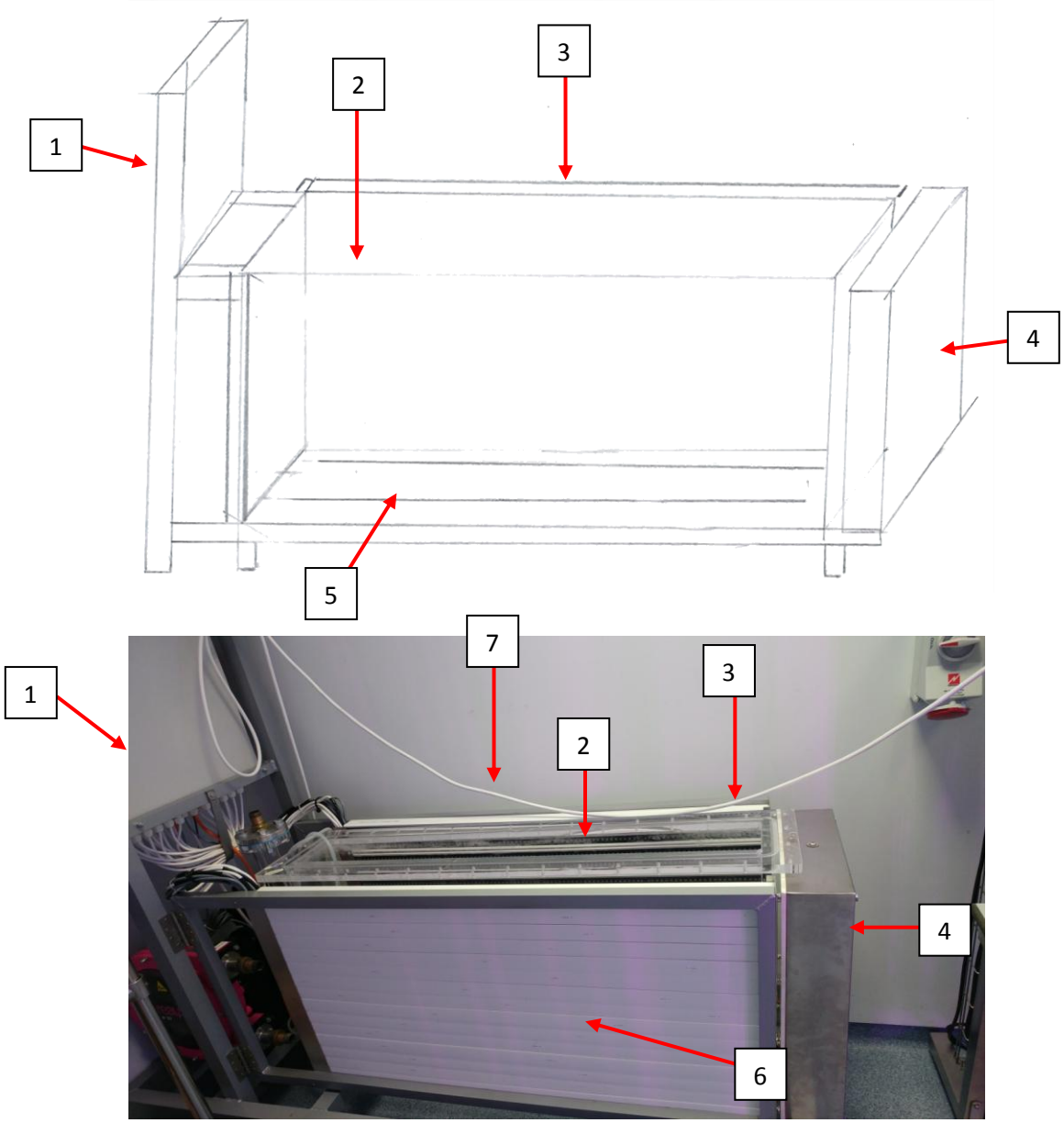


Figure 2.3: FPR. 1) Control panel, 2) 100L vessel, 3) left light panel, 4) cooling fan, 5) aeration bars, 6) right light panel, 7) aeration line.

2.2.4. 1000L Industrial Plankton industry reactor (IPR)

Fully automated 1000LPBR from Industrial Plankton Ltd. is a bioreactor 1000L in size which was easily controlled via the control panel (Fig2.4.A). It measured OD, water level, temperature and pH automatically and adjusts the pH by controlling the input CO_2. Sampling and harvesting were controlled via pumps operated via the control panel allowing for easy use. The unit was also self cleaning and has specific modifications for the use of AlgaeCytes Ltd. Full details are available at: https://industrialplankton.com/productsPage.php (Industrial plankton, 2016).

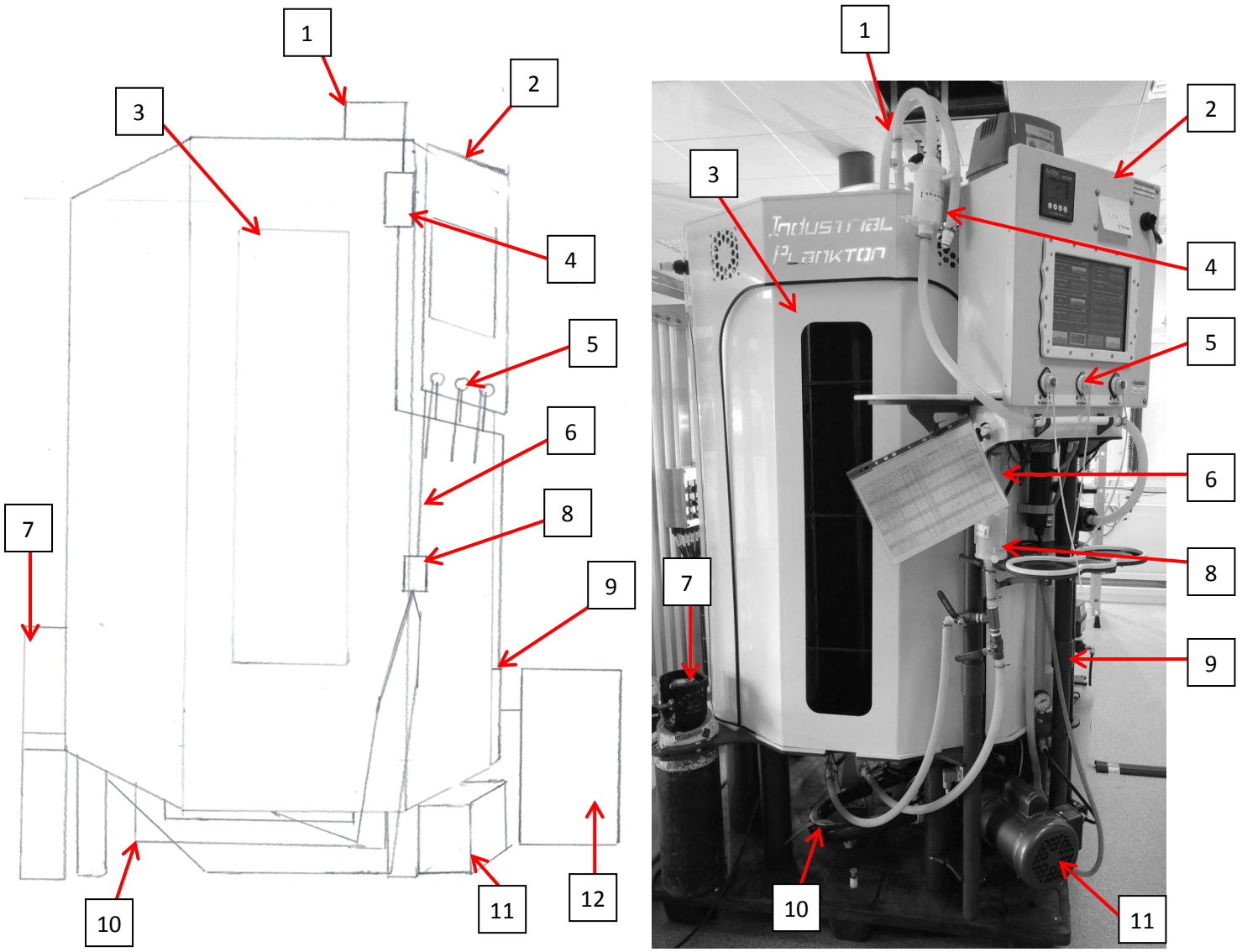


Figure 2.4: IPR, semi –automated system. 1) Water inlet, 2) control panel, 3) culture view window, 4) water filter, 5) nutrient port pumps, 6) air and CO_2 line 7) CO_2 bottle, 8) air filter, 9) UV sterilisation for water, 10) circulation tube, 11) Circulation pump, 12) water cooler (Industrial Plankton, 2016).

2.2.5. Handheld spectrophotometer

A Palintest photometer 7100, capable for use with all palintests for water testing including phosphate levels, nitrate levels, turbidity and optical density at 450nm, 500nm, 550nm, 570nm, 600nm, 650nm (https://www.palintest.com/en/products/photometer-7100).

2.3. Microbial Cultures

2.3.1. ALG01 cultures

ALG01 was grown and maintained in both liquid BBM flask cultures and on BBMA cultures.

2.3.2. Agar cultures

Agar cultures were streaked onto BBMA, and kept under constant white light at 10-50 $\mu molm^{-2}s^{-1}$ at 20°C with no additional aeration, as per the culture maintenance at AlgaeCytes Ltd.. The lower temperature is to allow for slower growth and long term cultures.

2.3.3. Liquid cultures

Liquid cultures were kept at 25°C under 24 hour white light at 100-200µmol$m^{-2}s^{-1}$ on rota shakers at 120RPM with no additional aeration.

2.3.4. ALG01 axenic cultures

ALG01 axenic lines were created at AlgaeCytes Ltd. and held as part of the main microalgal collection. Here we define axenic algal cultures are those that are of a single algal species that may contain reduced levels of bacteria (below 10^4 CFU/ml). These were created using a series of antibiotic cocktails over several algal culture generations. A mix of Kasugamycin (100mg/L), Cefotaxime (100mg/L), Gentamycin (500mg/L) and Ciprofloxacin (500mg/L) were used concurrently and individually.

2.3.5. Axenic supplementation

Axenic cultures were grown under standard BBM media and under BBM media with supplementation.

2.3.6. Bacterial preparation

Bacteria were isolated from algal cultures, that had been grown for at least 2 weeks in growth medium maximum recovery diluent (MRD) (OD at least 1.0 at 650nm), by conducting a serial dilution series (10^{-2}-10^{-6}), using MRD, and spread plating on a suitable agar (TSA, YEA, NA, BBA, R2AA and DMA). Samples were incubated at multiple temperatures (20°C, 30°C and 37°C) for 3-7 days. Once cultures had developed they were stored at 4°C until required. Only culturable bacteria were investigated.

2.3.7. Bacterial liquid cultures

Bacterial isolates previously isolated from algal cultures on agar were aseptically transferred using a sterile loop into sterile 50ml glass vessels containing MRD, and incubated at 30°C for 3-7 days for good dense growth (OD 2.0 at 650nm). Afterwards the cultures were stored at 4°C for maximum of 7 days until required. Sub-culturing of the cultures occurred as required to maintain the stock bacterial isolates for use. Fresh cultures were created through subculture 24 hours prior to use to allow for young bacterial cultures that were not under stress.

2.3.8. Red, white and yellow bacterial tests

a) Re-establish agar slants of red, yellow and white bacteria

Samples of Red (1a) white (1B) and yellow (1C) held at AlgaeCytes were obtained in the form of slants. Aseptically a sample of each was taken using a sterilised metal 1ml loop as streaked on fresh yeast extract agar using standard microbiological methods. Plates were then incubated at 30°C for 3 days.

b) Red (1a) white (1B) and yellow (1C) bacteria growth on BBM/minimal media

Plates of red (1a) white (1B) and yellow (1C) bacteria were taken out of storage and left to adjust to room temperature. Using a sterile 1ml loop under aseptic conditions, a sample of each bacterium was streaked diagonally across BBM agar and incubated at 20°C, 30°C and 37°C for 7 days. Cultures were observed for growth daily through visible inspection of the plates.

2.3.9. Isolation of bacterial colonies

A 1ml sample of ALG01 underwent a serial dilution using MRD to promote bacterial growth, and was spread plated under aseptic conditions using a standard microbiology method. Dilutions were spread plated on TSA, YEA, NA, BBA, R2AA and DMA and incubated at 20°C, 30°C and 37°C for up to 7 days.

From the sample produced from culture dilutions, each different type of colony formed on the agar was re-streaked and isolated purely on the respected media and at the temperature it had been previously grown on. The samples were numbered, logged and incubated for up to 7 days until good growth had occurred. Bacterial cultures were re-streaked until certain it was a mono culture and stored at 4°C until required for further analysis.

2.3.10. Bacterial agar tests

Each bacterial isolate was patch tested against each type of agar used at 3 different temperatures to establish more data on the bacterial isolates before genetic testing. Each bacterium was streaked under standard patch test conditions using a grid of 24, on BBMA, YEA, NA, TSA, DMA and R2A, at 20°C, 30°C, and 37°C. Plates were incubated for 3-7 days and observed for growth, colouration and any other details relevant for identification.

2.4. Experimental design

2.4.1. Agar re-introduction tests bacteria

ALG01 was re-streaked from an agar culture on BBM agar from AlgaeCytes Ltd. main collection. Bacteria cultures isolated previously were grown overnight in liquid culture (and MRD) at appropriate temperatures (20°C, 30°C and 37°C). The bacteria were then washed in BBM 3 times and then re-suspended in BBM to make an OD of 0.5 at 650nm. A sterile 1ml loop was then used to streak the bacteria from the edge of the plate toward the algal streak to prevent cross contamination. Samples were all prepared under aseptic technique and conducted in duplicates.

2.4.2. Agar re-introduction tests bacterial supernatant

ALG01 was re-streaked from an agar culture on BBM agar from AlgaeCytes Ltd. main collection. Bacterial cultures isolated previously were grown overnight in liquid culture (and MRD) at appropriate temperatures (20°C, 30°C and 37°C). The bacterial cultures were then centrifuged at 13200 RPM for 5 minutes; the supernatant was transferred to a new vessel. This was repeated 3 times or until there was no visible bacterial pellet. A sterile 1ml loop was then used to streak the bacterial supernatant from the edge of the plate toward the algal streak to prevent cross contamination. Samples were all prepared under aseptic technique and conducted in duplicates. This was to assess if the bacteria were producing any extra cellular products that would enhance microalgal growth.

2.4.3. Liquid reintroduction tests

From the agar reintroduction tests isolates observed to impact algal growth significantly by promoting or inhibiting growth were tested in liquid cultures. 3 sets of experiments were conducted: dosing levels, individual reintroduction and bacterial mixes.

All followed the following protocol: The bacterial cultures isolated previously were grown overnight in liquid culture (MRD) at the appropriate temperatures (20°C, 30°C and 37°C). The bacteria were then washed through centrifugation at 13000rpm and resuspension in BBM 3 times and then re-suspended in BBM to make an OD of 1.0 at 650nm. The multi-cultivator unit was set up as standard for ALG01 cultures (AlgaeCytes Ltd. SOP) under 175µmol/m^2/s with a constant photoperiod at 25°C under Bold modified Basal broth (BBM). Algal cultures were started with at an OD of 0.2 at 725nm .

For dosing level tests bacterial cultures were added to the algal cultures at 0.1ml, 1ml, and 2ml, volumes. For reintroduction tests 0.1ml of each bacterial culture was added into separate algal cultures. For bacteria mixes reintroduction tests 0.1ml of a bacterial mix was added to an algal culture.

Mixes	Bacterial isolates used	Reason
1	01-1a, 01-1b and 01-1c	Originally isolated by AlgaeCytes Ltd. 2013
2	01-14, 01-16 and 01-19	All isolated on TSA, and had an effect on agar cultures
3	01-14, 01-16, 01-19, 01-29, 01-58, 01-64 and 01-87	All new isolates that had an effect on agar cultures
4	01-1a, 01-1b, 01-1c, 01-14, 01-16, 01-19, 01-29, 01-58, 01-64 and 01-87	All bacterial isolates observed to have an effect on agar culture tests
5	01-14, 01-58 and 01-64	All isolated at 37°C

Table 2.1: Description of mixes used in the liquid reintroduction mixes test.

For all reintroduction liquid tests each test culture was grown until the end of log growth, lipids and growth were analysed for the effect of the bacteria.

2.4.4. Multi-unit run conditions

The following conditions were used on the units held at AlgaeCytes Ltd. under the standard operating procedures (SOP's) of the company. These experiments were with ALG01 under varying units to compare the growth, lipid production and bacterial number fluctuations.

a) Photobioreactor FMT 150 1LPBR (1LPBR) Culture run conditions

Set up as per the AlgaeCytes Ltd. SOP. 175µmol/m^2/s white light with a constant photoperiod at 25°C under Bold modified Basal broth (BBM). Aeration at 1000ml/min with CO_2 at 0.25%, algal cultures were started with at an OD of 0.2 at 725nm at a starting pH of 7.0.

b) 100L flat panel photobioreactor (FPR) Culture run conditions

Set up as per the AlgaeCytes Ltd. SOP. 400µmol/m^2/s red light (6 of 14 blocks of dark red LEDs) with a constant photoperiod at room temperature (24-29°C) under Bold modified Basal broth (BBM). Starting pH of 7.0, with harvests conducted on the growing culture. Harvest volume was replaced with distilled water mixed with fresh nutrients.

c) 1000L Industrial Plankton industry reactor (IPR) Culture run conditions

Set up as per the AlgaeCytes Ltd. SOP. White light at 10-12 light panels on at 25°C. pH controlled with the addition of CO_2 into the air line with the max pH of 7.8. Harvests conducted on the growing culture. Harvest volume was replaced with distilled water mixed with fresh nutrients.

2.4.5. Measuring bacterial levels over time from multiple units

2ml samples were taken daily from 1LPBR runs, FPR runs and IPR runs and stored at 2-4°C for up to 4 days before analysis (it was noted at this storage temperature the samples did not change significantly when stored up to a maximum of 4 days). Samples underwent a series dilution (10^{-2}-10^{-6}) in MRD for spread plating to allow for single colony formation for total colony forming unit analysis of the culture. Analyses were conducted by counting total single colonies at a suitable dilution to allow for the count to fall between 30-300 colonies for accuracy.

$$Colonies\ counted\ \times dilution = colony\ forming\ units\ per\ ml$$

2.4.6. Genetic identification of bacteria

The following bacterial isolates were chosen for genetic identification because they were observed to have an effect on algal growth during the agar test or were the originally isolated bacteria from ALG01 from AlgaeCytes Ltd. 01-1a, 01-1b, 01-1c, 01-14, 01-16, 01-19, 01-29, 01-58, 01-64 and 01-87.

Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1). Samples were prepared by growing 'fresh' 24 hour field plates of

each bacterial isolate, taking the culture via a sterile loop into the vials provided by Microbes NG (Microbes NG, 2018) containing a storage solution and beads. These were sent to Microbes NG for genome analysis. DNA sequences were analysed by both Microbes NG and through NCBI blast alignment tool (Altschul, *et al.* 1990).

2.4.7. Bioinformatic tools

Many bioinformatic tools were used to analyse the genome data obtained through Microbes NG. Each isolate was analysed for identification, and if any denitrification or B_{12} synthesis genes were present. If denitrification and B_{12} genes are present it could explain association of the microbiome.

a) Strain identification

Each sequence was identified by Microbes NG against their own database and also through NCBI blast alignment tool using the FASTA formatted whole genome tolls provided by Microbes NG to compare results (MicrobesNG, 2018).

b) Identifying gene presence

The sequences that were identified via the NCBI database were not all fully annotated. Alternative full genomes with annotation were then selected to be critiqued. Those selected were the closest relative species available through the NCBI. Those genomes are listed in table 2.2 below.

Species	NCBI ID	Reference
Pseudomonas aeruginosa PAO1	2603714	Stover *et al.* 2000.
Pimelobacter simplex	259593	Shtratnikova, *et al.* 2015.
Brevundimonas sp. EAKA	13676	Tully *et al.* 2018.
Microbacterium hominis	35569	Tan-Guan-Sheng Adrian *et al.* 2016.
Comamonas thiooxydans	36735	Ma *et al.* 2009.
Sphingopyxis terrae	14711	Ohtsubo *et al.* 2016.

Table 2.2: List of genomes investigated with the associated NCBI ID numbers. These known genomes were searched to identify key cobalamin synthesis, denitrification and nitrogen fixing genes.

The pathways involved with B_{12} production, ammonification and denitrification were investigated and the following genes were selected for investigation within the bacterial genomes:

Gene	Description	Pathway	Figure ref
cobO	cob(I)alamin adenosyltransferase	Cobalamin synthesis	1.9
cobS	cobalamin synthase	Cobalamin synthesis	1.9
cysG	siroheme synthase	Cobalamin synthesis	1.9
cbiA	cobyrinic acid a,c-diamide synthase	Cobalamin synthesis	1.9
cbiK	cobalt chelatase	Cobalamin synthesis	1.9
cobC	cobalamin biosynthetic protein CobC	Cobalamin synthesis	1.9
cobU	nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase	Cobalamin synthesis	1.9
narL	two-component response regulator NarL	Ammonification	1.7
napB	cytochrome C protein NapB	Ammonification	1.7
nirS	nitrite reductase	Denitrification	1.7
narG	nitrate reductase A subunit alpha	Ammonification	1.7
nosZ	nitrous-oxide reductase	Denitrification	1.7
norB	nitric oxide reductase subunit B	Denitrification	1.7
nrfA	ammonia-forming cytochrome c nitrite reductase	Ammonification	1.7
hcp	protein S-nitrosylase	Detoxification	1.7
norV	anaerobic nitric oxide reductase flavorubredoxin	Detoxification	1.7
norW	NADH:flavorubredoxin reductase	Detoxification	1.7
nirK	NirK nitrite reductase	Denitrification	1.7
nirB	nitrite reductase catalytic subunit NirB	Ammonification	1.7
nirC	nitrite transporter NirC	Ammonification	1.7
nirD	nitrite reductase subunit NirD	Ammonification	1.7

Table 2.3: List of genes selected and the pathways they are associated with.

2.5. Analyses

2.5.1. Biomass harvesting

Liquid cultures were harvested by decanting vessels into sterile 50ml falcon tubes taking care to transfer all material and centrifuged at 4000RCF for 5 minutes in a Heraeus Biofuge. The supernatant was removed carefully leaving the resultant pellet. If multiple pellets were created from large samples these would be combined and re-centrifuged to remove as much water as possible.

2.5.2. Dry weight

Harvested culture pellets were transferred to pre-weighed weigh boats and dried within a vacuum oven at 40°C under 800-1000mBar (Gallenkamp) of pressure for a minimum of 12 hours until a constant dry weight was reached. The dry weight was calculated by subtracting the original mass of the weigh boat from the final mass of the weigh boat plus biomass, taking into account the original volume.

2.5.3. Dry sample preparation

Dried algal samples were transferred into a mortar and ground into a fine powder using a pestle. Samples were placed into a clean 10ml glass universal and stored at -20°C until samples were required.

2.5.4. Gram staining

Performed as per standard procedure as described in Beveridge (2001). During gram staining, cell sizes were measured using a standard camera set up and analysed via image J.

2.5.5. Bacterial CFU/ml counts

A sample of algal culture underwent a serial dilution series (10^{-2}-10^{-6}), using MRD, and spread plating on a YEA agar. Samples were incubated 30°C for 3 days, and colonies were counted in the range of 30-300 for accuracy. Taking into account the plate dilution the colony forming unit (CFU) per ml is calculated.

2.5.6. ECHA Micro monitor Sig Nitrite bacterial test

Following the procedure for ECHA nitrite tests, 2ml of a fresh 24h bacterial sample was added to a 10ml glass vessel containing an orange gel, which in the presence of ammonia turns pink in colouration due to an indicator. The vessels were sealed and incubated at 30°C for up to 5 days. A positive result of the presence of denitrifying bacteria is indicated by the orange colour turning pink and bubbles appearing. The gel contains potassium nitrite which can be reduced to ammonia and nitrogen.

If the pH of bacterial sample is very alkaline it will turn the gel pink immediately and if the bacterial sample is too acidic that any ammonia produced in the reaction will be neutralised and no colour change will be observed, and a positive result is indicated by gas formation.

This is a qualitative indicator of the presence of denitrifying bacteria, not definitive results, but allowing the information to aid in the genetic work conducted, indicating which strains that you would expect to have denitrifying genes present.

2.5.7. Analytical Lipid extraction method

To extract lipids from dried algal samples, a 8-10mg homogenized sample of the algal biomass was carefully weighed out into a 2ml GC vial and labelled with a sample number. Each sample should be analysed in duplicate as a minimum. The weight of the sample to the nearest 0.1mg was recorded. 50 µL of a tridecanoic acid standard (2 mg/mL) in methanol was added to the sample, followed by 225 µL of chloroform: methanol (2:1, v/v). 225 µL of 30% hydrochloric acid: methanol solution was added and the vial sealed tightly. The sealed vessels were immediately placed into a pre heated oven at 70°C ± 2.5°C for 1 hour. After 1 hour in the oven, the vials were removed and allowed to cool to room temperature for at least 15 minutes. After cooling, 1.0 mL HPLC grade hexane was added to the vial and vortexed well for 1 minute to mix the contents well. The sample was then left to stand at room temperature undisturbed for at least 10 minutes. The upper phase of the sample was transferred to a clean labelled 2.0ml GC vial, and sealed. Sample was then analysed via GCMS immediately or frozen until analysis could be performed.

2.5.8. Analytical GC-MS

All lipid samples were run on a gas chromatography instrument (Agilent 6890A GC system) with mass spectroscopy detector (MS) using a variable split-flow injector with a split/splitless inlet using a capillary column (Supelco GC Column – SP2560) fused silica length 100 m, internal diameter 0.25 mm, film thickness 0.2 µm. The carrier gas was helium at a flow rate of 1.0ml/minute with a split ratio of 10:1. The injector temperature was set to 260°C. The syringe was set to conduct 6 hexane washes pre sample and 3 sample washes before injection of 1µl. The run time was 55 minutes with a solvent delay of 11 minutes. The oven temperature after sample injection was 140°C for 5 minutes, increasing to 240°C at a rate of 4°C/minute and holding 240°C for 25 minutes. The retention time of EPA was at 36.2 minutes.

This method is semi-quantitative as a standard calibration curve using a FAME kit is set up allowing the responses to be quantified, via the computer system associated with the GCMS using the program Chem Station.

Compound	Abbreviation	Compound	Abbreviation
Myristic acid	14:0	Linoleic acid	18:2
Myristoleic acid	14:1	Gamma linoleic Acid	GLA 18:3
Palmitic acid	16:0	Alpha linoleic acid	ALA 18:3
Palmitoleic acid	16:1	Behenic acid	22:0
Stearic acid	18:0	Arachidonic acid	ARA 20:4
Oleic acid	18:1	Eicosapentaenoic acid	EPA 20:5

Table 2.4: List of compounds analysed via GC-MS and abbreviations

Chapter 3

Results

3.1. **Growth of ALG01 under varying conditions and volumes**

To begin understanding the growth of ALG01 and any associated bacteria, growth curves were analysed from multiple systems at AlgaeCytes Ltd. The 1L photobioreactor (1LPBR), Flat panel 100L photobioreactor (FPR) and industrial plankton 1000L photobioreactor (IPR) were all set up as per the standard procedure AlgaeCytes Ltd. The FPR and IPR systems were run as continuous systems with regular input of media, and harvests occurring. The growth of the algae in these systems was measured manually with a hand held spectrophotometer, whereas the 1LPBR has its own automatic OD measurement system. The 1LPBR was also run as a batch system with the only nutrients added at the start of the run with no additional supplementation. All systems were run at room temperature (25°C) in BBM media. The 1LPBR and IPR were conducted under white light whereas the FPR was conducted under red light. Bacterial levels were measured through total colony forming units (CFU) counts throughout the runs. Figures 3.1, 3.2 and 3.3 illustrate this.

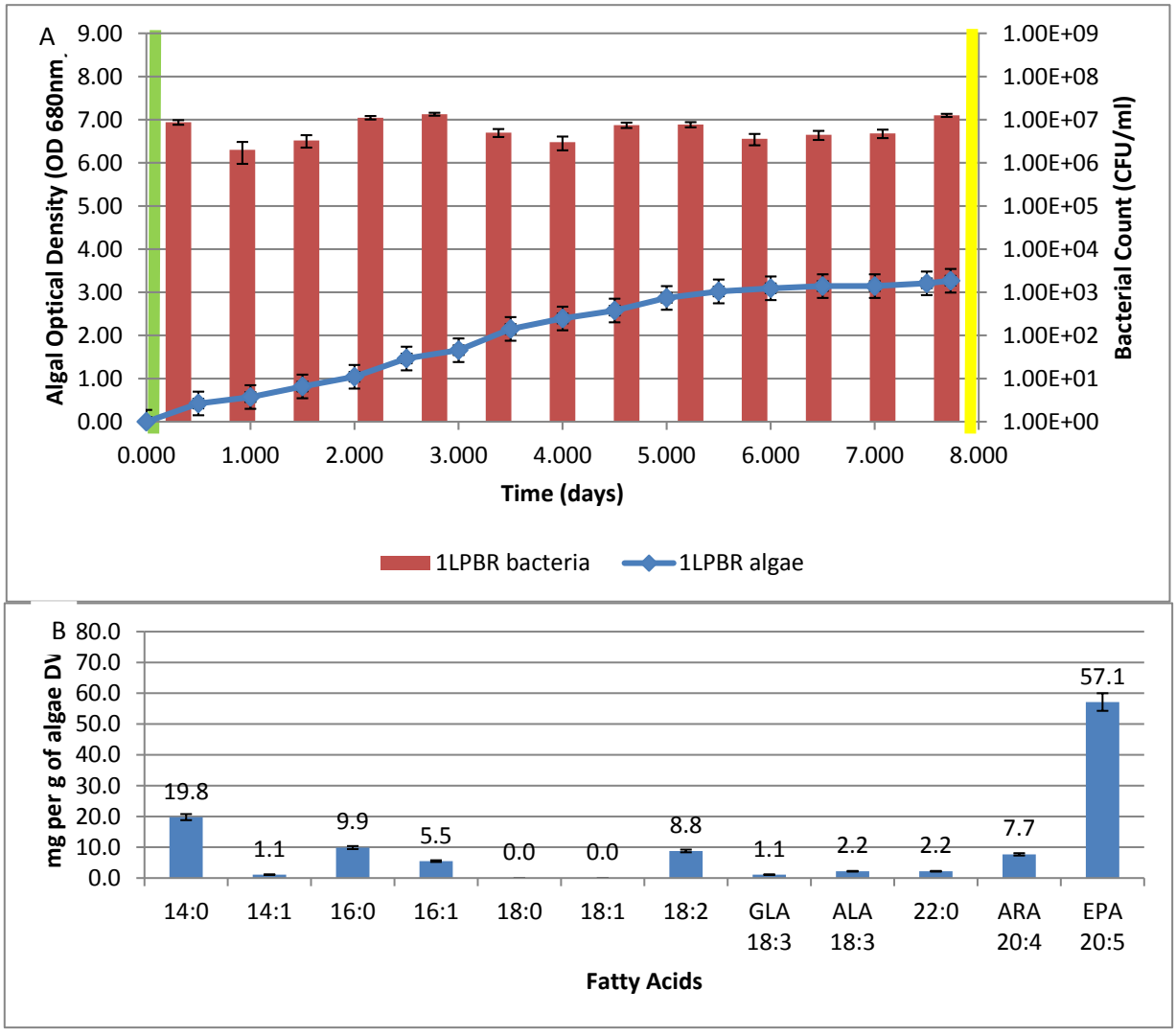


Figure 3.1: Average run within the 1L Photobioreactor (1LPBR) system under standard growth conditions. A) Algal growth curves measured in optical density at 680nm, with fluctuating bacterial numbers measured in total colony forming unit counts (CFU/ml) from daily samples with standard deviation. Lines indicate nutrient addition of 1L BBM (green) and harvests of 1L volume (yellow) that took place B) Average lipid profile at harvest of the system in mg/g of dried algal biomass. 14:0 - Myristic acid, 14:1 - Myristoleic acid, 16:0 - Palmitic acid, 16:1 - Palmitoleic acid, 18:0 - Stearic acid, 18:1 - Oleic acid, 18:2 - Linoleic acid, GLA 18:3 - Gamma linoleic Acid, ALA 18:3 - Alpha linoleic acid, 22:0 - Behenic acid, ARA 20:4 - Arachidonic acid, EPA 20:5 - Eicosapentaenoic acid

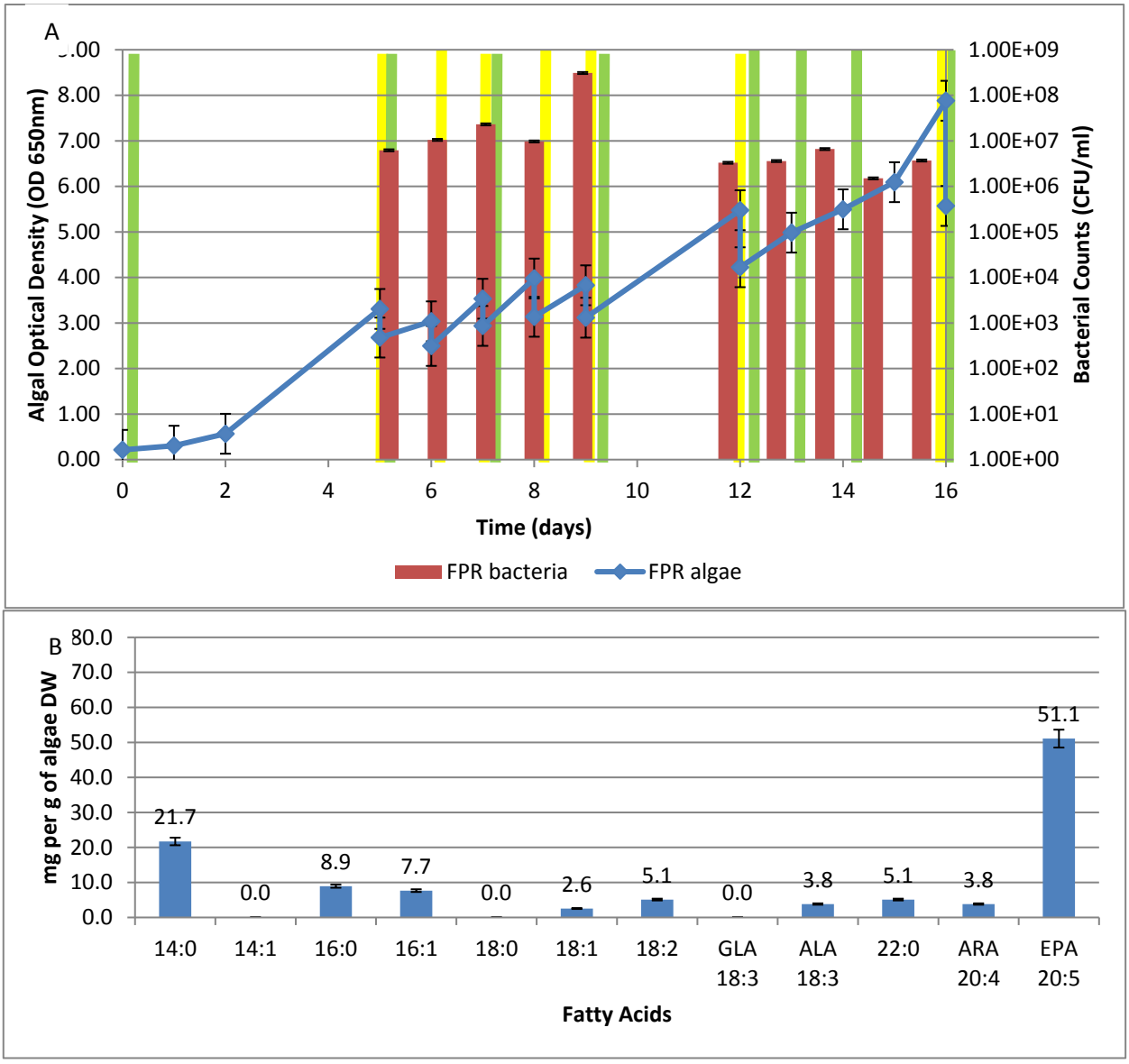


Figure 3.2: Average run within the Flat Panel photobioreactor (FPR) system. A) Algal growth curves measured in optical density at 650nm, with fluctuating bacterial numbers measured in total colony forming unit counts (CFU/ml) from daily samples with standard deviation. Lines indicate nutrient addition (green) of 5L of concentrated BBM and harvests (yellow) of 20L of culture, which was replaced with 15L of distilled water. B) Average lipid profile at harvests of the system in mg/g of dried algal biomass. 14:0 - Myristic acid, 14:1 - Myristoleic acid, 16:0 - Palmitic acid, 16:1 - Palmitoleic acid, 18:0 - Stearic acid, 18:1 - Oleic acid, 18:2 - Linoleic acid, GLA 18:3 - Gamma linoleic Acid, ALA 18:3 - Alpha linoleic acid, 22:0 - Behenic acid, ARA 20:4 - Arachidonic acid, EPA 20:5 - Eicosapentaenoic acid

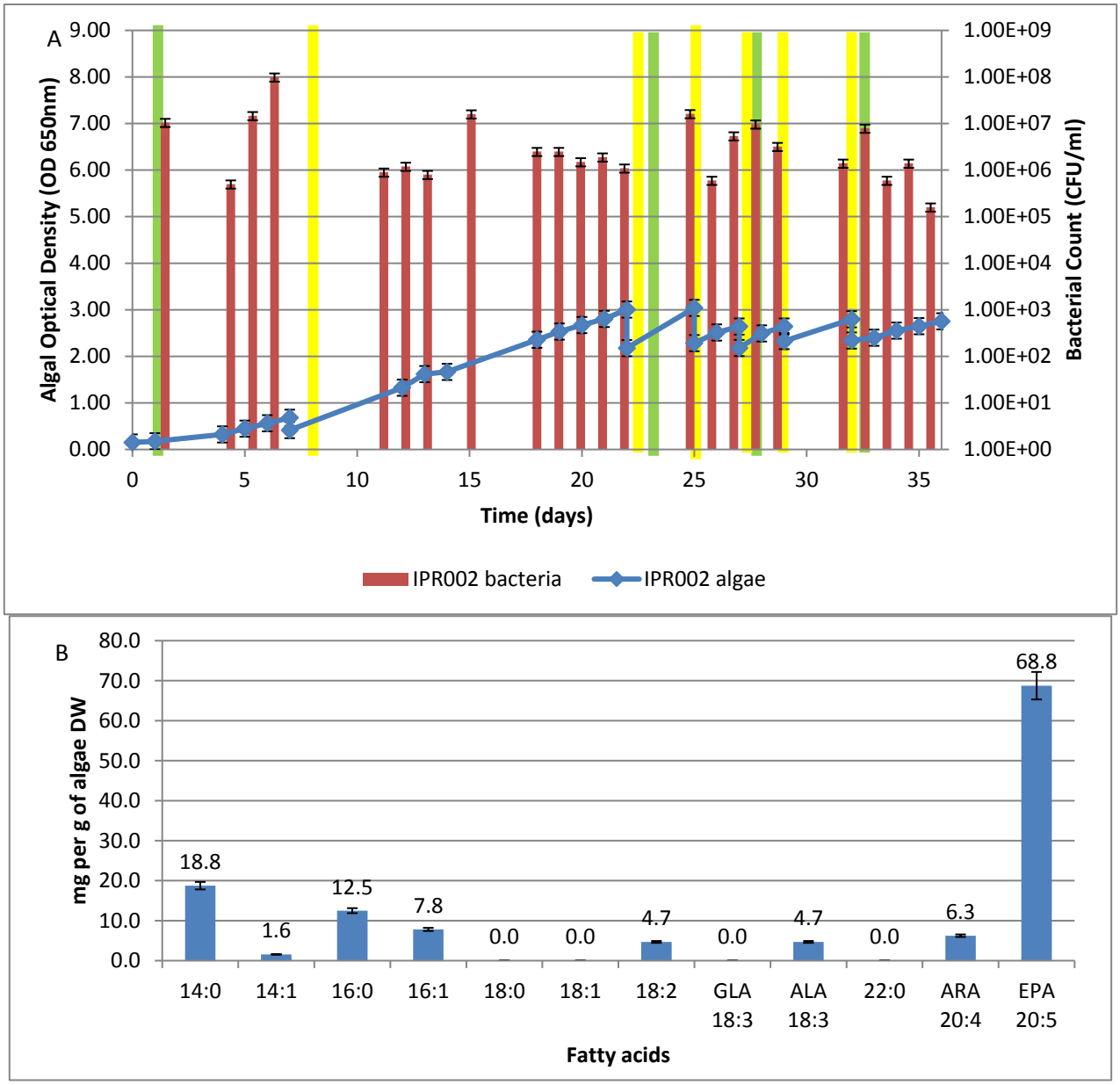


Figure 3.3: Mean run within the Industrial Plankton Industry reactor (IPR) system (n=3). A) Algal growth curves measured in optical density at 650nm, with fluctuating bacterial numbers measured in total colony forming unit counts (CFU/ml) from daily samples with standard deviation. Lines indicate nutrient addition (green) of 10L of concentrated BBM and harvests (yellow) of 130L of culture, volume was replaced with 120L of distilled water B) Mean lipid profile at harvests of the system in mg/g of dried algal biomass (n=6). 14:0 - Myristic acid, 14:1 - Myristoleic acid, 16:0 - Palmitic acid, 16:1 - Palmitoleic acid, 18:0 - Stearic acid, 18:1 - Oleic acid, 18:2 - Linoleic acid, GLA 18:3 - Gamma linoleic Acid, ALA 18:3 - Alpha linoleic acid, 22:0 - Behenic acid, ARA 20:4 - Arachidonic acid, EPA 20:5 - Eicosapentaenoic acid

The growth of ALG01 within the FPR and IPR systems are similar, as they are similar systems. The algal growth in the 1LPBR is different due to being a batch system. The bacterial growth curves are different, with the larger systems experiencing 1-fold (FPR) and 2-fold (IPR) higher bacterial fluctuations. Measured in total colony forming units (CFU/ml), there was no pattern in the bacterial numbers during algal growth, with total bacterial levels rising and falling with no external input.

In Figure 3.4 it illustrates the algal growth curves and associated total bacterial counts for the 1LPBR, FPR and IPR over the first 8 days of growth. The algal growth is between the 1LPBR and FPR systems during the first 8 days of growth, which is also reflected in the mean doubling times (Table 3.1). The IPR, being the largest system, had a long lag phase. The lipid profiles from each system are not significantly different as illustrated in Figure 3.5 with a P-value >0.05.

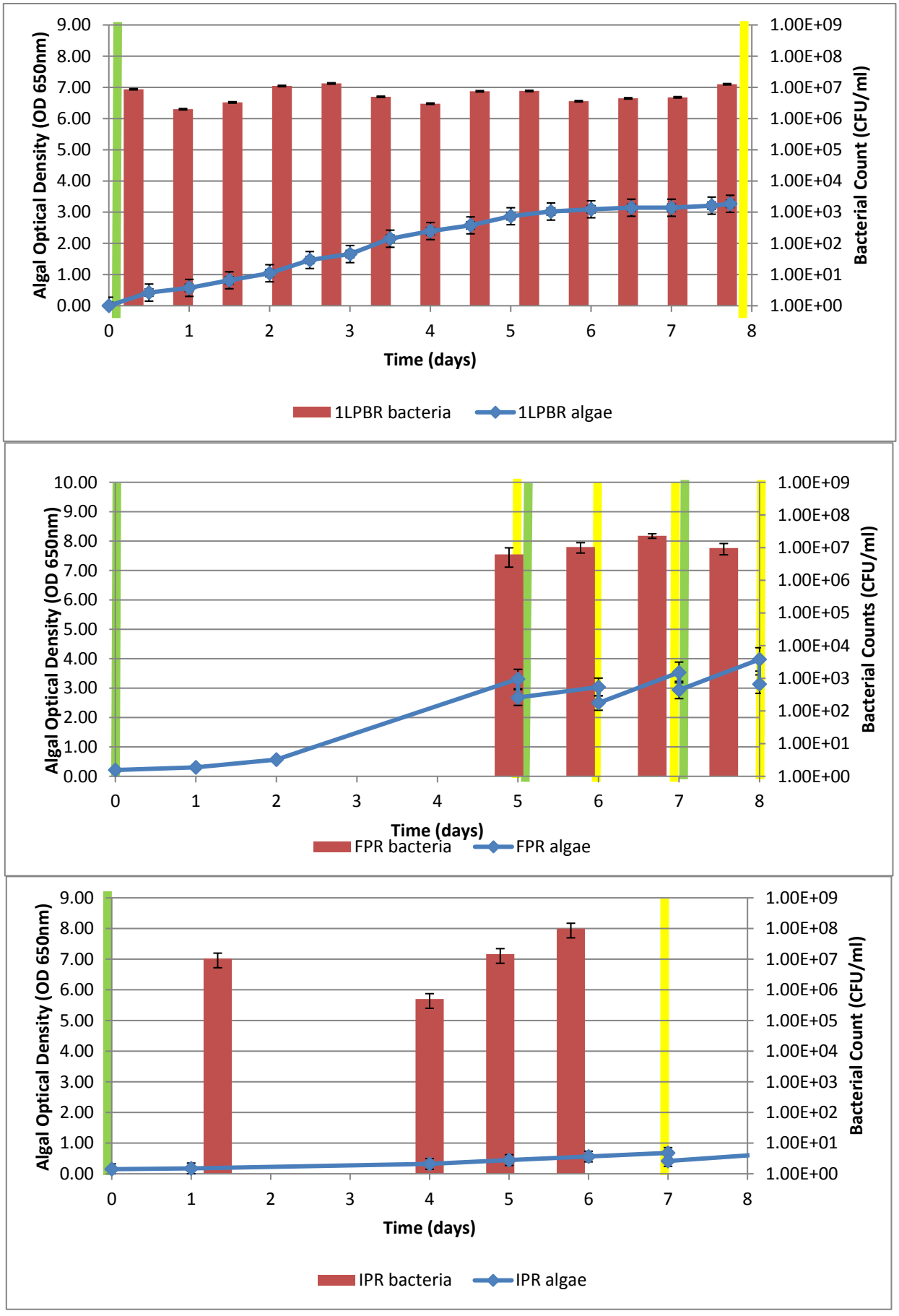


Figure 3.4: Comparison of 1LPBR, FPR and IPR algal growth curves and daily bacterial numbers over the first 8 days of growth under standard conditions (as stated in 2.4.4). The growth of the algae and the bacteria over this time is different between the 3 different photobioreactors.

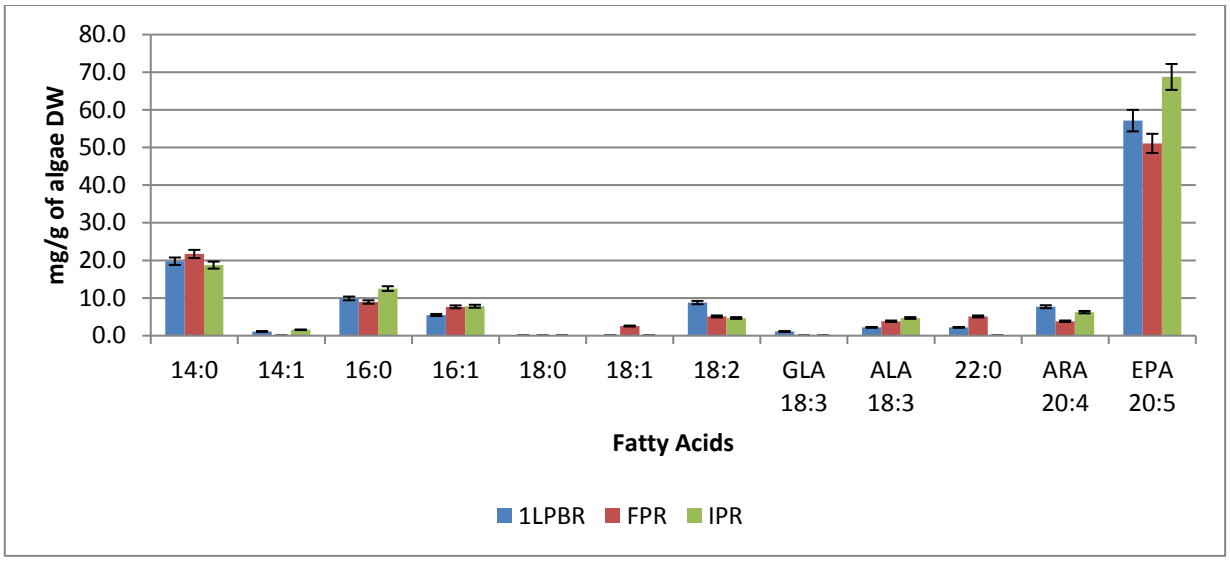


Figure 3.5: Mean lipid profiles of the 1LPBR, FPR and IPR (n=2, n=4, n=6). 14:0 - Myristic acid, 14:1 - Myristoleic acid, 16:0 - Palmitic acid, 16:1 - Palmitoleic acid, 18:0 - Stearic acid, 18:1 - Oleic acid, 18:2 - Linoleic acid, GLA 18:3 - Gamma linoleic Acid, ALA 18:3 - Alpha linoleic acid, 22:0 - Behenic acid, ARA 20:4 - Arachidonic acid, EPA 20:5 - Eicosapentaenoic acid

Unit	Mean Doubling Time during growth (days)	Dry weight (g/L)	EPA (mg/g of algal biomass)
1LPBR	1.52	1.39	57.1
FPR	1.26	1.06	55.56
IPR	3.21	0.20	54.28

Table 3.1: Average mean doubling times during growth phase, dry weight and EPA of the 1LPBR, FPR and IPR. EPA yields for the 1LPBR are at the final harvest, for the FPR and IPR EPA yields are average across all harvests conducted.

The variability of bacterial counts is not limited to different systems. In Figure 3.6 the total bacterial counts of multiple 1LPBR runs under identical conditions are shown. Each highly different from each other, with varying degrees of fluctuation. From no change through to 2-fold variations; indicating that the bacterial levels seem to be independent to the external inputs and algal growth. Bacterial growth must be influenced by internal dynamics and pressures that were not measured here.

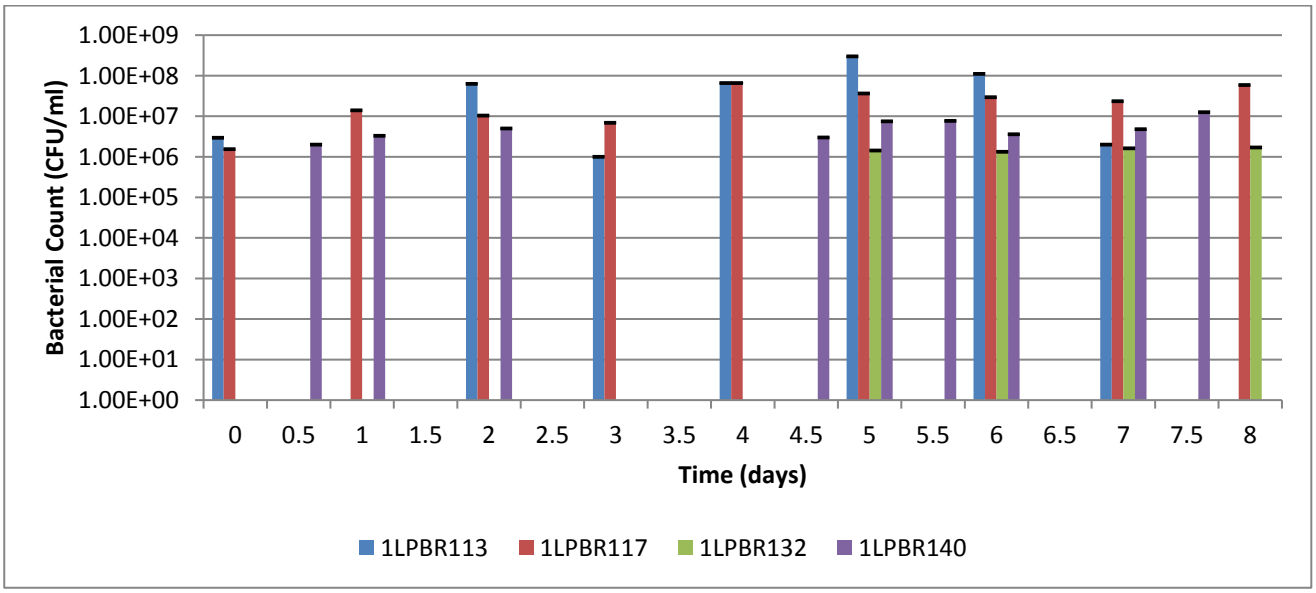


Figure 3.6: Bacterial levels measured over time course of multiple 1LPBR runs. Each 1LPBR run was identical, with very differing total culture bacterial numbers.

3.2. Axenic vs. Non-axenic

3.2.1 Agar cultures

When looking into the effects of a microbiome, understanding what happens when that microbiome is removed is important to gain insight into the possible interactions taking place. The growth of ALG01 is different between a culture with its full biome and without. Axenic cultures grow much slower and with less chlorophyll and pigmentation (Figure 3.7) than that of a non-axenic culture. Improving the growth of the axenic culture was achieved by adding supplements into the media. By adding glucose, tryptone and B_{12} into the media increased both the growth and colouration of the axenic line but is still slower and weaker in health than the non-axenic culture.

Figure 3.7: Growth comparison between ALG01 A) a culture with its full microbiome B) an axenic culture [The growth of the axenic culture was observed to be the same at day 33] and C) an axenic culture with supplements (glucose, tryptone and B_{12}). Growth period being A) 21 days B) 23 days C) 33 days.

The algae were also tested with the addition of each of the supplements individually and no difference of growth was observed when tryptone and B_{12} were added to the cultures individually. As adding supplements into the agar did not improve the axenic cultures to 'normal' levels of growth (qualitative results only) indicates that the bacteria are providing the alage with more than just nutrition.

3.2.2 Liquid cultures

Within the Multicultivator system (MC) the axenic and non-axenic cultures were grown in separate vessels to observe the differences within a liquid system. They were grown at room temperature (25°C), under 175µmol/m^2/s white light in BBM media. Within these liquid cultures the growth curves of the axenic and non-axenic cultures were not significantly different. Figure 3.8 shows that both curves are very similar measuring similar optical densities (OD). The main difference observed was that the cultures with the full microbiome had higher levels of self-flocculation occurring, which can be observed in Figure 3.8 as the drops in OD (at time points at day 3 and day 4.5), and required higher levels of mixing to maintain the culture in liquid. This was achieved by each culture vessel being manually mixed via inversion on a daily basis, the experimental set up can be observed in Figure 3.9. This is unlike the agar equivalent where the non-axenic culture was much more stable in its growth. Table 3.2 illustrates the growth and lipid yields for both the axenic and non-axenic cultures, including final media levels, pH and mean doubling time. All of the measured factors were highly similar.

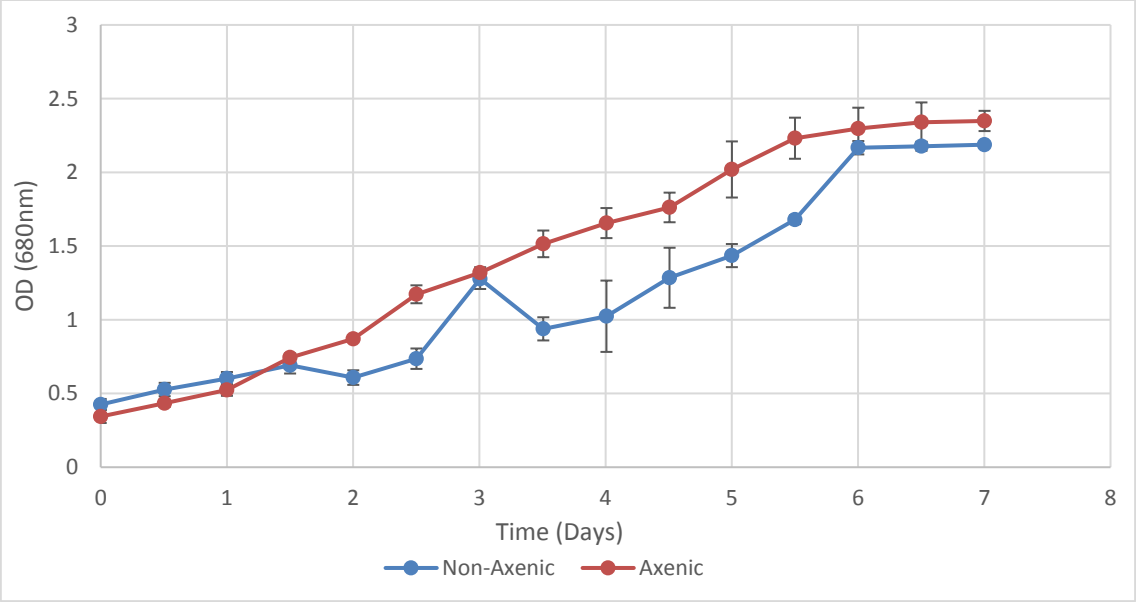


Figure 3.5: Growth comparison between non-axenic and axenic liquid cultures under standard conditions. Self-flocculation can be observed as the drops in OD, which is then seen to increase due to manual inversion mixing of each culture.

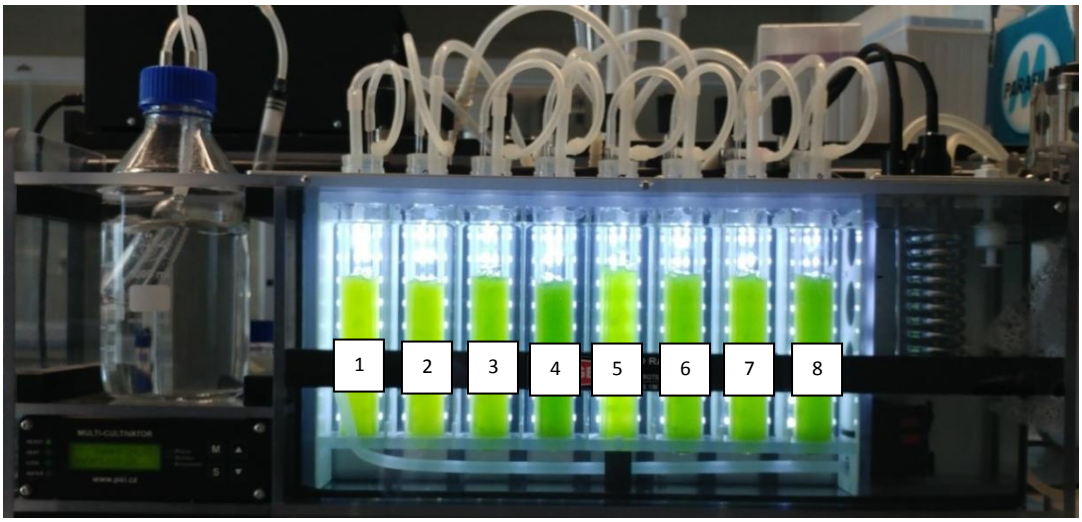


Figure 3.9: Example of liquid culture tests within the multicultivator system (MC). Here a test of axenic vs non-axenic cultures is depicted, Vessels 1-4 non-axenic and 5-8 axenic (left to right).

	pH at Harvest	Final media nitrate level (mg/L)	Final media phosphate level (mg/L)	Mean doubling time (days)	Harvest biomass weight (g/L)	Standard deviation	EPA yield (mg/g of dry algal biomass)	Standard deviation
Axenic	9.93	29.0	95.0	2.77	0.77	0.04	59.48	0.59
Non-axenic	10.17	17.0	124.0	2.87	0.65	0.05	57.53	2.34
P-value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05		> 0.05	

Table 3.2: Average results at harvest for axenic and non-axenic cultures including p-value significance from a one way ANOVA analysis.

3.3 Microbiome

3.3.1. Isolation and morphology

To understand more about the microbiome present in ALG01 cultures, bacteria were isolated by creating a dilution series (10^{-2}-10^{-6}) of algal culture within maximum recovery diluent (MRD). This was used to promote all bacterial types and to aid the growth of any weak or damaged bacterial cells. Each dilution was spread plated on multiple agar types and at multiple temperatures to ensure that the total culturable microbiome was captured. It is noted that those isolated bacteria were part of the culturable microbiome and do not include any unculturable bacteria. Yeast extract agar (YEA); nutrient agar (NA); bold modified basal broth agar (BMMA); tryptone soya agar (TSA); Davis minimal agar (DMA); and R2 agar (R2A) were used at 20°C, 30°C and 37°C. This was to capture as much of the majority and the diversity of the microbiome as possible. From each plate created, any visibly differing colonies in morphology were independently collected and sub-cultured onto the same conditions they were originally isolated from.

	YEA	NA	BBMA	TSA	DMA	R2AA
20°C	4	6	0	5	1	6
30°C	13	4	9	5	6	10
37°C	5	5	0	9	2	3
Total	22	15	9	19	9	19

Table 3.3: The number of morphologically different microorganisms isolated under the different condition types. YEA – yeast extract agar, NA – nutrient agar, BBMA – bold modified basal agar, TSA – tryptone soya agar, DMA – Davis minimal agar, R2A – R2 agar.

87 bacterial isolates were collected from across the conditions as illustrated in Table 3.3. Three additional previously isolated bacteria were obtained from AlgaeCytes Ltd as they had been isolated at an earlier time, from an ALG01 culture in 2013. Of the total 90 isolated only 83 were successfully subcultures on, onto the same conditions in which they were first isolated. Indicating that some damaged and unculturable bacteria were also captured using this method.

Each isolate was then tested on each agar type (YEA, NA, BBMA, TSA, DMA, and R2A) and at each temperature (20°C, 30°C and 37°C). This was to gain understanding of morphology and growth for each isolate to ensure that they were not an artefact of isolation. It was also to check for duplicate any potential isolation (Table 3.4). Each isolate also underwent gram-stain analysis and cell sizing (Table 3.5) to gain further information about bacterial distribution and duplication.

Bact. no.	20	30	37	20	30	37	20	30	37	20	30	37	20	30	37	20	30	37
01-1A	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes
01-1B	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-1C	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-1	Yes	No	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No	No
01-4	Yes	Yes	Yes	No	No	No	No	Yes	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes
01-5	No	No	No	No	No	Yes	No	No	Yes	No	No	No	No	No	No	Yes	Yes	Yes
01-6	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes
01-7	No	Yes	No	No	No	Yes	Yes	No	Yes	No	No	No	No	Yes	No	No	No	No
01-8	Yes	Yes	No	No	No	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes	No	Yes
01-10	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes
01-11	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
01-12	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
01-13	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
01-14	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
01-16	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
01-17	Yes	Yes	Yes	No	No	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No	No	Yes	Yes
01-18	Yes	Yes	No	Yes		Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
01-19	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
01-20	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-21	Yes	Yes	Yes	Yes	No	No	Yes	No	No	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
01-22	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
01-23	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
01-24	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
01-25	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-26	No	Yes	No	No	No	No	No	No	No	No	Yes	No	No	No	Yes	Yes	Yes	Yes
01-27	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-28	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-29	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-30	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-35	No	No	No	No	No	No	No	No	Yes	No	No	No	No	Yes	No	No	No	No
01-36	No	No	No	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes	No	No	No	No
01-37	No	Yes	No	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes
01-38	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No
01-39	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-40	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	No	No	Yes	Yes
01-41	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-42	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
01-43	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
01-44	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-45	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-46	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-47	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-48	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-49	Yes	Yes	No	No	No	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No
01-50	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes	No	No	No	Yes	Yes	Yes
01-51	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
01-53	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
01-54	No	Yes	No	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes

	YEA			BBMA			NA			TSA			DMA			R2AA		
Bact. no.	20	30	37	20	30	37	20	30	37	20	30	37	20	30	37	20	30	37
01-55	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-56	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-57	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-58	No	No	No	No	Yes	No	Yes	Yes	Yes	No	Yes	No	No	No	No	No	Yes	No
01-59	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-60	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Yes	No	No	No	Yes	Yes	Yes
01-61	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	Yes
01-63	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes
01-64	No	Yes	Yes	No	No	No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
01-65	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
01-66	No	Yes	Yes	No	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes
01-67	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
01-68	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-69	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-70	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-71	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-72	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-73	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No	No	No	No	No	No	Yes	Yes	Yes
01-74	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-75	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-76	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
01-77	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-78	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-79	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-80	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-81	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-82	No	Yes	Yes	No	No	No	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	No	Yes	Yes
01-83	No	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes
01-84	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-85	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-86	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-87	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-88	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-89	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
01-90	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table 3.4: Summary of bacterial isolate agar tests. Growth was attempted for each bacterial isolate against 6 different medias (YEA, BBMA, NA, TSA, DMA and R2AA) at 3 different temperatures (20°C, 30°C and 37°C). A No indicates that there was no visible growth over 5-7 days whereas a Yes indicates that growth occurred.

Bact. no.	Gram result	shape	Size (µm)	Bact. no.	Gram result	shape	Size (µm)	Bact. no.	Gram result	shape	Size (µm)
01-1A	+	Coccobacillus	0.5-1	01-30	+	Bacillus	0-10	01-64	-	Bacillus	0.2-1.5
01-1B	-	Bacillus	2	01-35	+	Bacillus	1-5	01-65	+	Bacillus	0.5-1.3
01-1C	+	Coccus	0.7-1.2	01-36	-	Bacillus	1-5	01-66	+	Bacillus	0.3-1.1
01-1	-	Bacillus	2.5-4.5	01-37	-	Coccus	0.5-1	01-67	+	Bacillus	0.5-2
01-4	+	Bacillus	1-2	01-38	-	Coccobacillus	0.1-7	01-68	+	Bacillus	0.5-2
01-5	+	Bacillus	1.5-2.5	01-39	+	Coccus	0.5-3	01-69	-	Coccobacillus	0.4-1.9
01-6	+	Bacillus	1.5-2.5	01-40	+	Bacillus	0.7-2.5	01-70	+	Coccus	0.4-1.8
01-7	-	Coccus	1-2	01-41	+	Coccobacillus	0.5-3	01-71	+	Coccobacillus	0.4-1.4
01-8	-	Bacillus	1-5	01-42	+	Bacillus	0.6-3	01-72	+	Coccobacillus	0.5-2.3
01-10	+	Bacillus	1-10	01-43	-	Bacillus	1-4	01-73	+	Coccus	0.7-3.4
01-11	-	Bacillus	2-10	01-44	+	Coccus	0.7-2.3	01-74	+	Coccobacillus	0.3-1.8
01-12	+	Coccus	0.5-2	01-45	+	Bacillus	0.5-2.5	01-75	+/-	Coccus	0.4-2.2
01-13	-	Bacillus	1-5	01-46	+	Bacillus	1-3	01-76	-	Bacillus	0.3-1.8
01-14	+	Bacillus	0.5-3	01-47	+	Bacillus	1-2.5	01-77	+	Bacillus	0.2-1.5
01-16	+	Bacillus	1-4	01-48	+	Bacillus	0.7-2.6	01-78	+	Bacillus	0.5-2.4
01-17	-	Bacillus	0.5-3.5	01-49	-	Bacillus	0.8-3	01-79	+	Bacillus	0.3-2.6
01-18	+	Coccus	0.5-2	01-50	+	Bacillus	1-3.3	01-80	+	Coccus	0.3-2.4
01-19	+	Coccobacillus	0.5-3.5	01-51	+	Coccus	0.5-2	01-81	+/-	Bacillus	0.3-2.6
01-20	+	Coccobacillus	0.5-2	01-53	+	Bacillus	0.7-3.4	01-82	+/-	Coccobacillus	0.5-1.5
01-21	-	Coccobacillus	0.4-2	01-54	+	Bacillus	1-3	01-83	+	Bacillus	1-2.5
01-22	+	Coccobacillus	0.5-2	01-55	-	Coccus	0.7-1.5	01-84	+	Bacillus	0.4-1.5
01-23	+	Bacillus	0.5-4	01-56	+	Coccus	0.6-1.4	01-85	+/-	mixed positive rods negative coccus	1.5-4.5
01-24	-	Coccus	0.3-1.5	01-57	+	Bacillus	0.5-3.6	01-86	+	Bacillus	0.5-2.5
01-25	+	Bacillus	0.5-2.5	01-58	+	Bacillus	0.5-3.5	01-87	+	Coccobacillus	0.5-2.3
01-26	+	Bacillus	1-3	01-59	+	Coccus	0.6-1.4	01-88	+	Coccus	0.7-2.4
01-27	-	Bacillus	0.5-2	01-60	+	Bacillus	0.6-3.1	01-89	+	Coccobacillus	0.2-2.7
01-28	+	Bacillus	0.5-2	01-61	-	Bacillus	0.8-2.8	01-90	+/-	mixed positive rods negative coccus	0.3-3
01-29	+	Bacillus	0.5-2	01-63	-	Coccobacillus	0.7-1.6				

Table 3.5: Gram-stain results with cell shape and size of cells. Phenotypic assignment for shape: Bacillus- rod shaped, coccus – spherical shaped. Size measurements were conducted through using images taken at the same distance; using the software Image J. 10-20 cells were measured to acquire the size measurements

83 had different profiles when considering morphology, growth characteristics and Gram-stain results. There was a mix of Gram-positive and Gram-negative bacteria, and also a mix of bacterial cell shape (Table 3.5). 2 isolates (01-85 and 01-90) appear to be mixed cultures from the Gram-stain, shape and size. From the above data there appears to be no duplication in isolates based on phenotypic analysis and cannot be confirmed without genetic analysis.

3.3.2 Reintroduction tests.

a) Agar tests

Each of the 83 isolates was tested against ALG01 on BBMA agar to observe the effect on its growth, to whether it is a positive or negative effect, on a medium that has no carbon source. Non-axenic ALG01 was used, due to the very limited growth of axenic ALG01 on agar, and the assumption that increasing the amount of a specific bacterium would have the same effect on both axenic and non-axenic cultures. Tables 3.6 and 3.7 illustrate this. Table 3.6 illustrates the scoring used in Table 3.7 with examples of reintroduction agar plates. Many of the bacterial isolates grew on the BBM agar in the presence of ALG01, and over half of the bacteria had a positive effect on algal growth. None were observed to have a negative effect on algal growth. Indicating that there is a positive and beneficial interaction between the microbiome and ALG01.

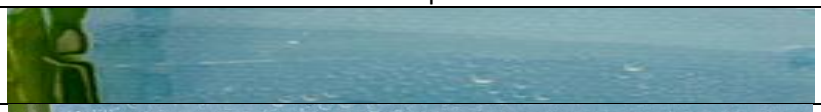
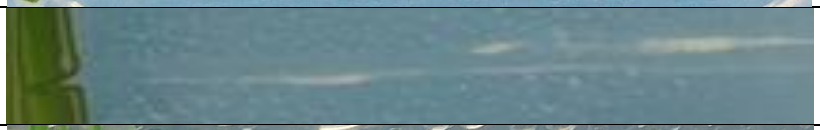
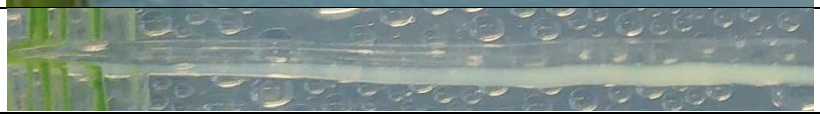
Bacteria		
Score	Description	Example
+	Minimal growth	
++	Low level growth along streak or at one end	
+++	Growth along whole streak or moderate growth at one end	
++++	Extensive growth along streak or one end	
Algae		
Score	Description	Example
+	Minimal growth at contact point	
++	Growth at contact point	
+++	Growth of algae starting to extend up bacterial streak	
++++	Extensive growth extending up bacterial streak. Empty cell indicates no growth observed	

Table 3.6: Scoring of the algal and bacterial growth for reintroduction agar plates with examples of growth shown.

	Bacterial growth		Algal growth	
Bact. no.	repeat 1	repeat 2	repeat 1	repeat 2
01-1A	+	++		
01-1B	+	++		
01-1C	+	++		+
01-1		+		
01-4	+	+		
01-5	+	+	+	
01-6	+	+		+
01-7	++	+	+	
01-8	++	++	+	
01-10	+		+	
01-11	+	+		+
01-12	+	+	+	
01-13	+			
01-14	+	+	++	++
01-16	++	+++	++	++++
01-17		+		
01-18	++	++	++	+
01-19	+	++	++	+++
01-20	++	+++		+
01-21	+	+		+
01-22	+	+	+	+
01-23	++	+		+
01-24	++	+++	+	
01-25	++	+++		++
01-26	+	+	+	+
01-27	++	++		
01-28	+	+++	+	+
01-29	+++	++	++++	
01-30	++++	++++	+	+
01-35	+	+	+	+
01-36	++	++	+	+
01-37	+	++	+	++
01-38	+	+		+
01-39	+++	++		+
01-40	+			
01-41	++	++		+
01-42	+			
01-43	+	+		+
01-44	++	++		+
01-45	++	++		+
01-46	+++	++	+	++
01-47	+++	+++	+	++

	Bacterial growth		Algal growth	
Bact. no.	repeat 1	repeat 2	repeat 1	repeat 2
01-48	++	++	+	+
01-49	+	+		
01-50	+	+	+	
01-51	+	+	+	
01-53	++	++		+
01-54	+	+	+	
01-55	+	+		
01-56	+++	++		
01-57	+++	+++	++	
01-58	+	+	++	+++
01-59	++	++	+	+
01-60	+	+	+	
01-61	+	+	+	
01-63	+	+		
01-64	+	+	++++	+
01-65	++	++	++	+
01-66	+		++	
01-67		+		+
01-68	+	++	++	+
01-69	++	++	+	+
01-70	+	+		+
01-71	+	+		
01-72	+	+	+	+
01-73		+		+
01-74	+	++	+	+
01-75	+++	+++	+	+
01-76	+	+	++	+
01-77	++	++	+	+
01-78	++	++	+	++
01-79	+	+	+	+
01-80	++++	+++	++	+
01-81	+	+	+	+
01-82	++	++	+	+
01-83				
01-84	+	+	+	+
01-85	+	+	+	++
01-86	+	++	+	+
01-87	+	+++	+	+++
01-88	+	+	++	
01-89	+		+	
01-90	+	+		+

Table 3.7: Bacterial isolate re-introduction to ALG01 BBM agar plates. Results + (little growth) to ++++ (dense growth). Empty cell indicates no growth observed. Plates incubated for 5-14 days under 100µmol/m^2/s white light at 25°C.

Due to the growth of the bacteria on media without a carbon source in the presence of the alga; the bacterial isolates were tested against the carbon free medium BBMA. This was against BBMA on its own, BBMA with a carbon source in the form of glucose and BBMA with ALG01. This was to test if the bacteria could grow on media without a carbon source, and if they would grow with a readily available source or are specific to ALG01.

Bact. no.	BBMA	BBMA + Glucose	BBMA + ALG01
01-1A	No	Yes	Yes
01-1B	No	Yes	Yes
01-1C	No	Yes	Yes
01-1	No	No	Yes
01-4	No	No	Yes
01-5	No	No	Yes
01-6	No	No	Yes
01-7	No	No	Yes
01-8	No	No	Yes
01-10	No	Yes	Yes
01-11	No	No	Yes
01-12	No	No	Yes
01-13	No	No	Yes
01-14	No	No	Yes
01-16	No	No	Yes
01-17	No	No	Yes
01-18	Yes	No	Yes
01-19	No	Yes	Yes
01-20	Yes	No	Yes
01-21	Yes	No	Yes
01-22	No	No	Yes
01-23	Yes	Yes	Yes
01-24	Yes	Yes	Yes
01-25	No	No	Yes
01-26	No	Yes	Yes
01-27	Yes	No	Yes
01-28	Yes	No	Yes
01-29	No	No	Yes
01-30	No	No	Yes
01-35	No	Yes	Yes
01-36	Yes	Yes	Yes
01-37	Yes	Yes	Yes
01-38	No	No	Yes
01-39	Yes	No	Yes
01-40	No	No	Yes
01-41	No	No	Yes
01-42	No	No	Yes
01-43	No	No	Yes
01-44	No	Yes	Yes
01-45	No	Yes	Yes
01-46	No	Yes	Yes
01-47	Yes	Yes	Yes

Bact. no.	BBMA	BBMA + Glucose	BBMA + ALG01
01-48	Yes	No	Yes
01-49	No	Yes	Yes
01-50	No	No	Yes
01-51	No	No	Yes
01-53	Yes	Yes	Yes
01-54	Yes	Yes	Yes
01-55	No	No	Yes
01-56	Yes	No	Yes
01-57	Yes	No	Yes
01-58	No	No	Yes
01-59	No	Yes	Yes
01-60	Yes	No	Yes
01-61	No	No	Yes
01-63	No	No	Yes
01-64	No	No	Yes
01-65	No	No	Yes
01-66	No	No	Yes
01-67	No	No	Yes
01-68	No	Yes	Yes
01-69	No	No	Yes
01-70	No	No	Yes
01-71	No	No	Yes
01-72	No	No	Yes
01-73	No	No	Yes
01-74	No	Yes	Yes
01-75	Yes	Yes	Yes
01-76	Yes	Yes	Yes
01-77	No	No	Yes
01-78	Yes	No	Yes
01-79	No	No	Yes
01-80	Yes	No	Yes
01-81	No	No	Yes
01-82	No	No	Yes
01-83	No	No	No
01-84	No	No	Yes
01-85	No	No	Yes
01-86	No	Yes	Yes
01-87	Yes	No	Yes
01-88	No	No	Yes
01-89	No	Yes	Yes
01-90	No	Yes	Yes

Table 3.8: Growth of each bacterial isolate on bold basal modified agar (BBMA), BBMA with glucose and BBMA with ALG01, incubated at 20°C, under 100µmol/m^2/s white light for 3-7 days. Most of the bacteria did not grow on just BBMA as it has no carbon source present. More isolates grew on BBMA when glucose had been added as a carbon source. The majority of bacterial isolates grew on BBM in the presence of ALG01 indicating that the alga is providing more than just an available carbon source.

Looking more closely at the growth of the bacterial isolates (Table 3.8), the vast majority (61 of the 83) did not grow on BBMA agar. This is most likely due to a lack of a carbon source within the media. When a carbon source was added in the form of glucose more isolates were able to grow on the BBMA but still 57 did not. When ALG01 was added to the media all but 1 bacterial isolate grew, indicating that ALG01 is providing a carbon source. For those bacterial isolates that grew in the presence of the alga but did not with just a carbon source indicates that the algae is providing more than just a carbon source for the bacteria to thrive. The supernatant of the bacterial cultures was also tested against the growth of ALG01, no effect was observed indicating close contact is needed for the exchange of products between the alga and the bacterium.

b) Liquid tests

From the agar tests 10 bacterial isolates were chosen for further testing. These 10 were identified as having either the largest effect on algal growth or were the 3 original isolates held at Algaecytes Ltd. 01-1a, 01-1b, and 01-1c, originally isolated from Algaecytes Ltd. 01-14, 01-16, 01-19, 01-29, 01-58, 01-64, and 01-87 were seen to have the largest effect on agar. They also had varying effects of growth indicating some may have autotrophic capabilities (Table 3.8).

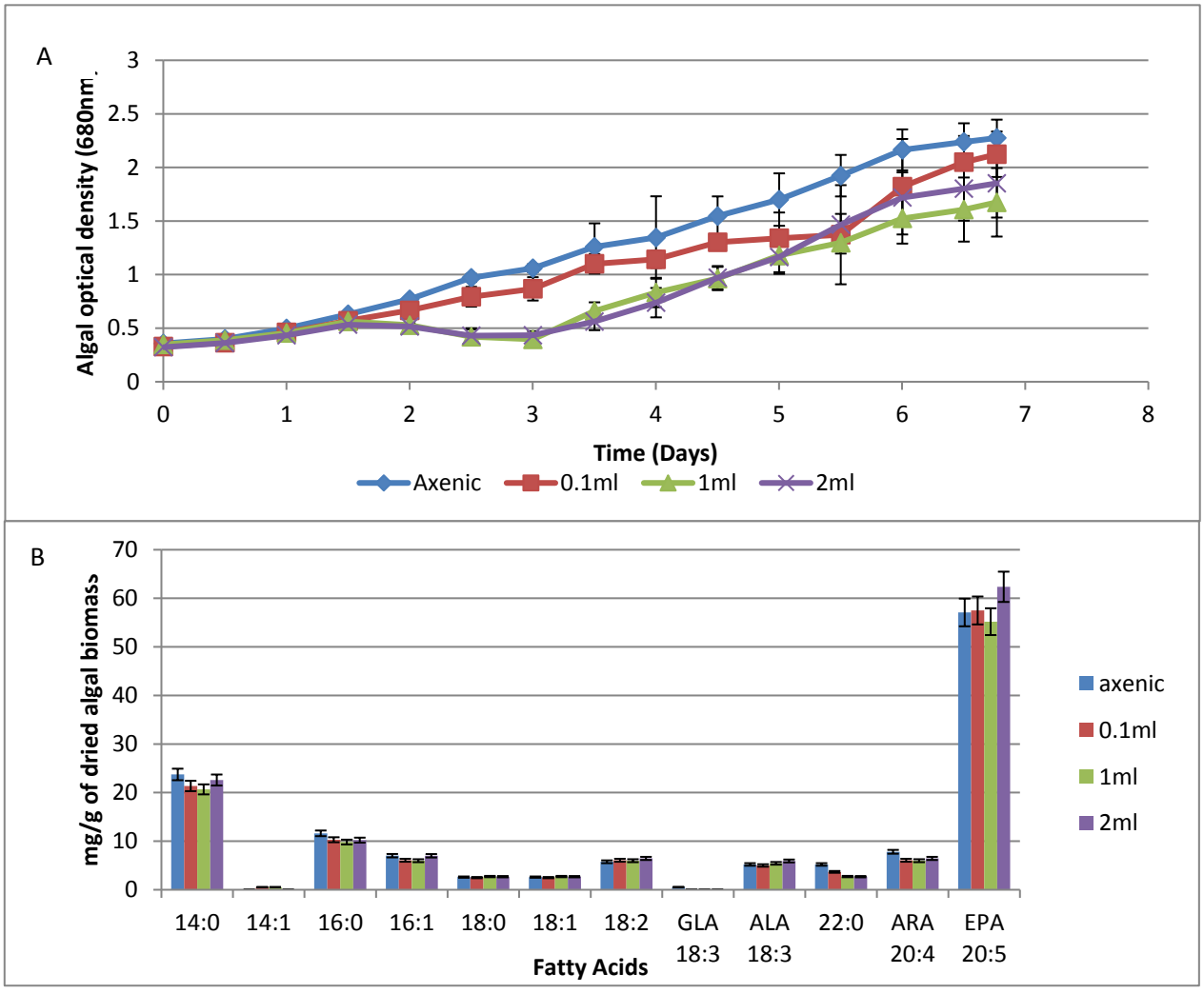


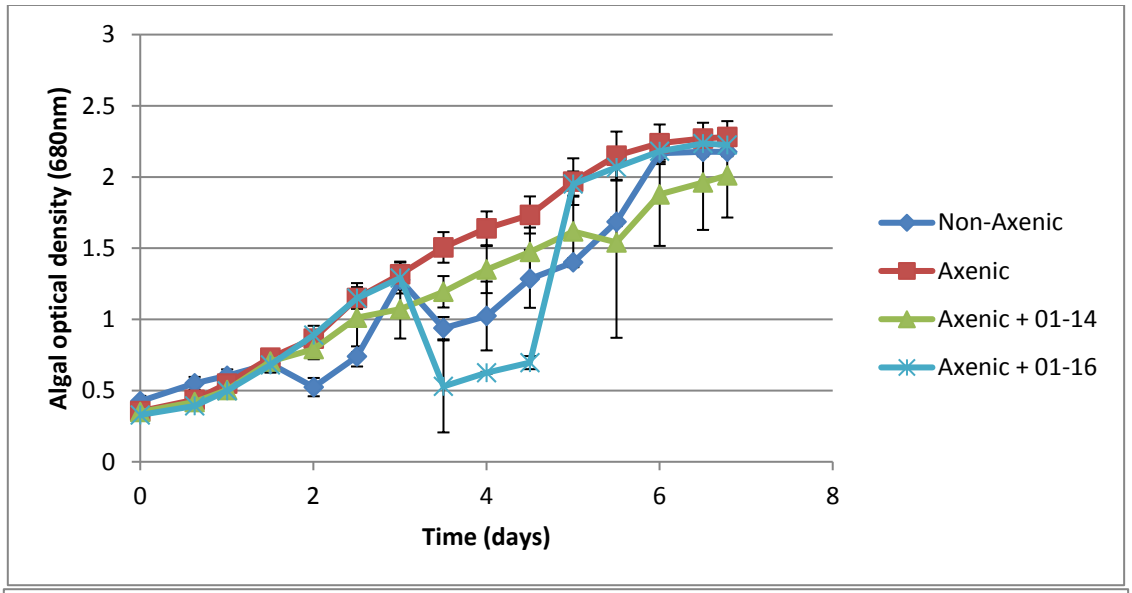
Figure 3.10: Dosing level tests, different volumes of bacterial culture 01-64 added to ALG01 culture. Bacterial culture added at equivalent of approximately 8 x 10^8 CFU/ml A) growth curve of bacterial isolate reintroduction dose test B) associated average lipid profiles of each dosing level. Error bar of standard deviation. 14:0 - Myristic acid, 14:1 - Myristoleic acid, 16:0 - Palmitic acid, 16:1 - Palmitoleic acid, 18:0 - Stearic acid, 18:1 - Oleic acid, 18:2 - Linoleic acid, GLA 18:3 - Gamma linoleic Acid, ALA 18:3 - Alpha linoleic acid, 22:0 - Behenic acid, ARA 20:4 - Arachidonic acid, EPA 20:5 - Eicosapentaenoic acid

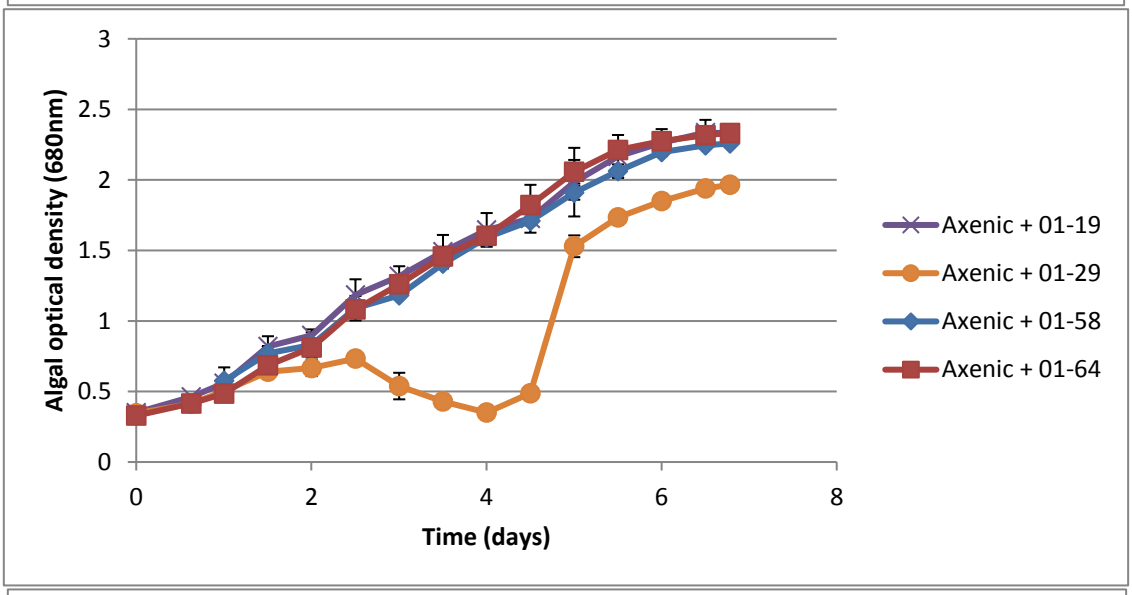
Firstly, the level of bacterial addition was tested to observe if the initial dose of bacteria would have an effect on the growth and lipid production of ALG01 axenic liquid cultures. The bacterial isolate that had the largest effect on the agar cultures, 01-64, was used as this would potentially have the largest effect on the liquid cultures. Different volumes of the bacterial culture which had been prepared to be an OD of 1.0 at 650nm were added to algal cultures within the multicultivator system. From Figure 3.10 and Table 3.9 it can be observed that in terms of final yields or EPA and mean doubling time there are no real differences between treatments. In terms of self-flocculation and dry weight yields it can be observed that the higher the initial bacterial levels are the lower the harvest dry weight is with a higher occurrence of early self-flocculation of the algae.

	Mean Doubling Time during growth (days)	Dry weight (g/L)	EPA (mg/g of algal biomass)
Axenic	2.52	0.74	57.09
0.1ml addition	2.49	0.71	57.49
1ml addition	2.96	0.57	55.19
2ml addition	2.66	0.60	62.36
P value	>0.05	>0.05	>0.05

Table 3.9: Average mean doubling times, dry weight and EPA yields for each dosing volume used. No significant difference between the additions in terms of yields and growth, P values obtained via one way ANOVA.

After the dosage was determined each bacterial isolate chosen was tested with an ALG01 axenic liquid culture within the MC system, to observe it effects. The growth curves are illustrated in Figure 3.11. From Table 3.7 there is no significant difference between each addition in terms of growth and EPA production. There is a significant difference during the growth of the cultures which is that the addition of 01-16 and 01-29 caused higher levels of self-flocculation, which could be from changes in internal conditions, changing the charge of the algal cells or attachment off the bacterial cells to the algae.





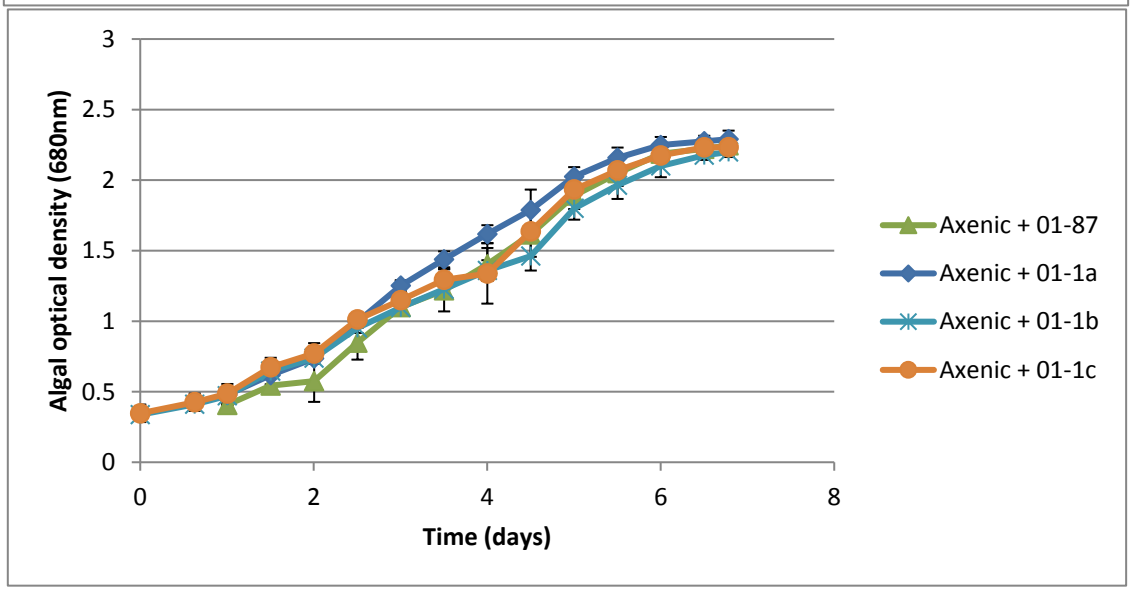


Figure 3.11: Growth curves of all reintroduction tests. All curves similar with 01-16 and 01-29 having higher levels of self-flocculation which was controlled with daily manual inversions. N=4, error bars indicate standard deviation.

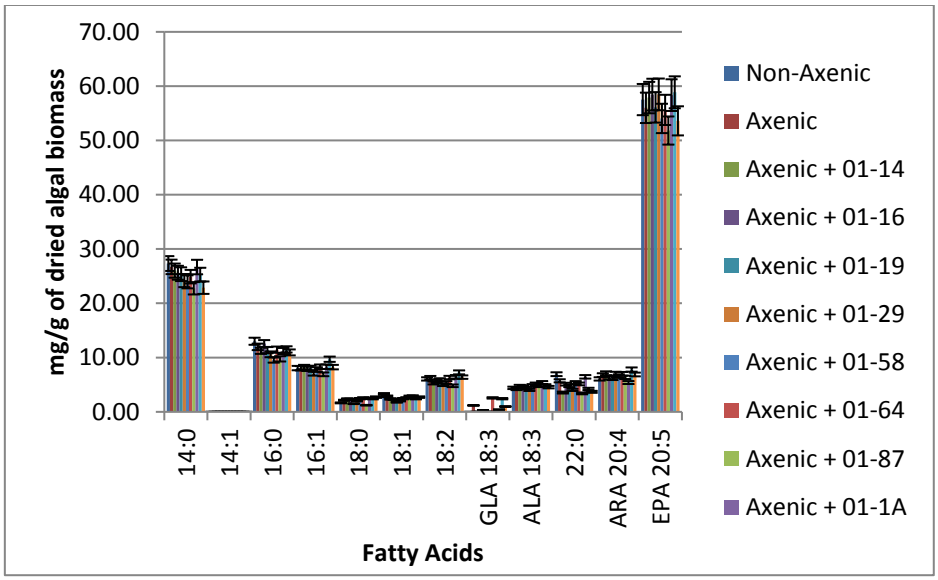


Figure 3.12: Average lipid profiles for bacterial reintroductions tests. Error bars indicate standard deviation. 14:0 - Myristic acid, 14:1 - Myristoleic acid, 16:0 - Palmitic acid, 16:1 - Palmitoleic acid, 18:0 - Stearic acid, 18:1 - Oleic acid, 18:2 - Linoleic acid, GLA 18:3 - Gamma linoleic Acid, ALA 18:3 - Alpha linoleic acid, 22:0 - Behenic acid, ARA 20:4 - Arachidonic acid, EPA 20:5 - Eicosapentaenoic acid. there is no real difference between additions

	Mean Doubling Time during growth (days)	Dry weight (g/L)	EPA (mg/g of algal biomass)
Non-Axenic	2.26	0.65	57.53
Axenic	1.92	0.75	59.48
Axenic +01-14	2.51	0.59	57.90
Axenic + 01-16	2.35	0.78	56.09
Axenic + 01-19	2.30	0.66	54.40
Axenic + 01-29	2.52	0.65	58.44
Axenic + 01-58	2.45	0.73	58.48
Axenic + 01-64	2.06	0.70	53.25
Axenic + 01-87	2.09	0.89	57.17
Axenic + 01-1A	1.84	0.84	58.35
Axenic + 01-1B	2.32	0.58	54.05
Axenic + 01-1C	1.97	0.68	51.82
P value	>0.05	>0.05	>0.05

Table 3.10: Mean doubling time, dry weight and EPA yields of each bacterial isolate addition. No real difference is observed between treatments in terms of growth and lipid production, with a p value of > 0.05.

Biome	Composition	Rationale
1	1a, 1b, 1c	Originally isolated by Algaecytes Ltd.
2	14, 16, 19	All isolated using TSA plates
3	14, 16, 19, 29, 58, 64, 87	all but the original 3
4	All 10	full tested biome
5	14, 58, 64	all originally isolated at 37°C

Table 3.11: Externally added bacterial biome and rationale for its choice.

Table 3.11 Illustrates the different bacterial mixes chosen, considering the whole microbiome presence within the algae culture; multiple strains working together may produce an effect on liquid cultures. Figure 3.13 shows the growth curves and lipid profiles of these mixes.

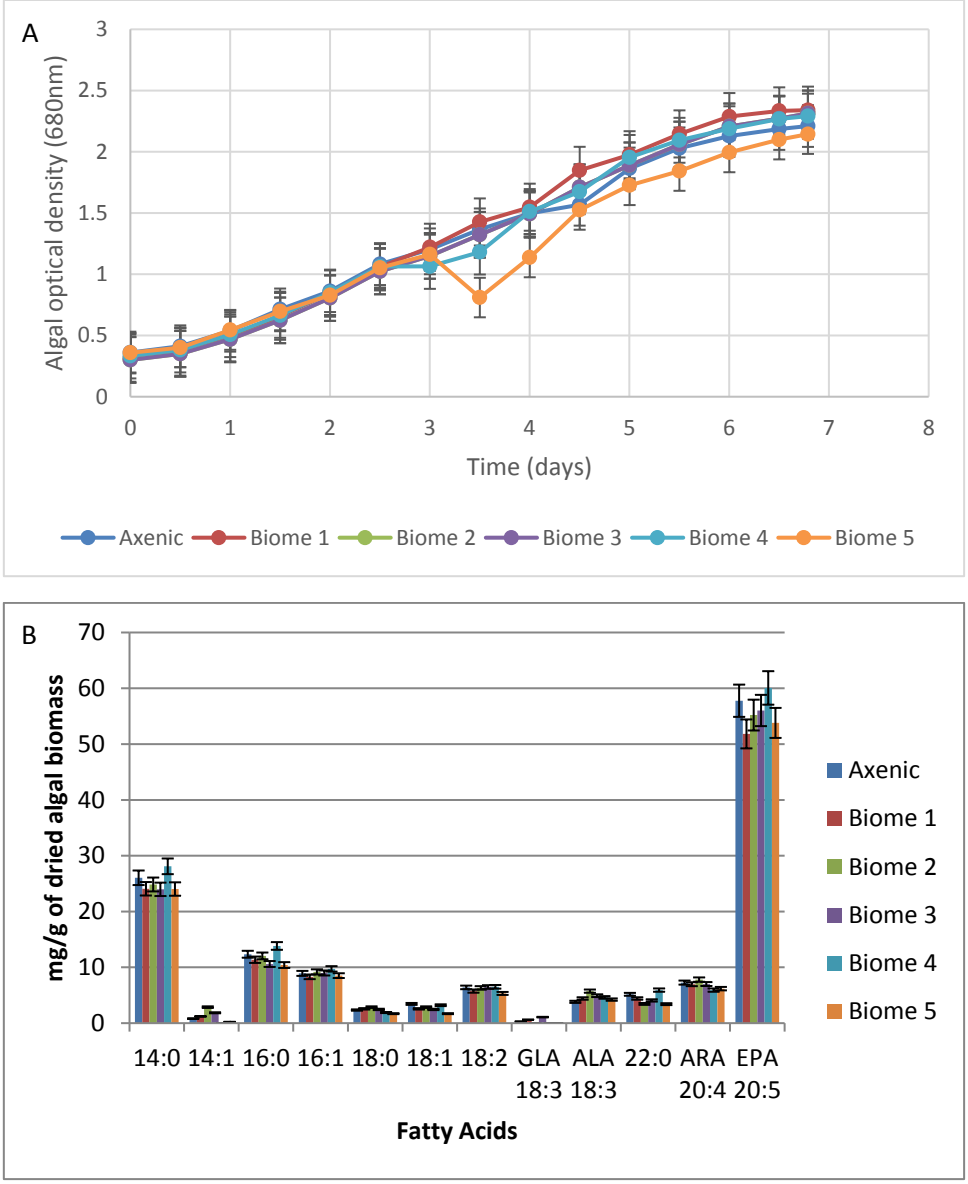


Figure 3.13: A) average growth curve of bacterial mixes reintroduction tests B) associated average lipid profiles of each bacterial mix addition. No real difference is observed between treatments in terms of growth and lipid production. 14:0 - Myristic acid, 14:1 - Myristoleic acid, 16:0 - Palmitic acid, 16:1 - Palmitoleic acid, 18:0 - Stearic acid, 18:1 - Oleic acid, 18:2 - Linoleic acid, GLA 18:3 - Gamma linoleic Acid, ALA 18:3 - Alpha linoleic acid, 22:0 - Behenic acid, ARA 20:4 - Arachidonic acid, EPA 20:5 - Eicosapentaenoic acid. there is no real difference between additions

	Mean Doubling Time during growth (days)	Dry weight (g/L)	EPA (mg/g of algal biomass)
Axenic	1.92	0.75	59.48
Axenic + mix 1	2.51	0.59	57.90
Axenic + mix 2	2.35	0.78	56.09
Axenic + mix 3	2.30	0.66	54.40
Axenic + mix 4	2.52	0.65	58.44
Axenic + mix 5	2.45	0.73	58.48
Axenic + 01-64	2.06	0.70	53.25
P value	>0.05	>0.05	>0.05

Table 3.12: Mean doubling time, dry weight and EPA yields of each bacterial mix addition. No real difference is observed between treatments in terms of growth and lipid production.

As seen in Figure 3.13 there is no significant difference between the mixes and the control in terms of growth and lipid production. There was also no significant difference between replicates.

The differences observed between the liquid and agar reintroduction tests may be due to the different culture dynamics in the different states and with the agar cultures there was direct contact between the algae and bacterial cultures whereas in liquid the proximity may not be as direct.

3.3.3. ECHA Micro monitor Sig Nitrite bacterial test

Denitrification and ammonification can be tested with the use of a test for ammonia and nitrogen production. As the bacteria breakdown the nitrites in the growth medium, ammonia and nitrogen is produced the pH change can be detected and the bubbles formed can be observed. The 10 bacterial isolates were tested to see if any were denitrifying/ammonia producing bacteria using the ECHA testing kits. All isolates were observed to have a positive result.

Sample	Score Colour (NH_3)	Score Bubbles (N_2)	Result
01-1a	****	****	Positive
01-1b	**	**	Positive
01-1c	****	****	Positive
01-14		**	Positive
01-16	**	**	Positive
01-19	**	*	Positive
01-29	***	****	Positive
01-58		****	Positive
01-64		****	Positive
01-87		***	Positive

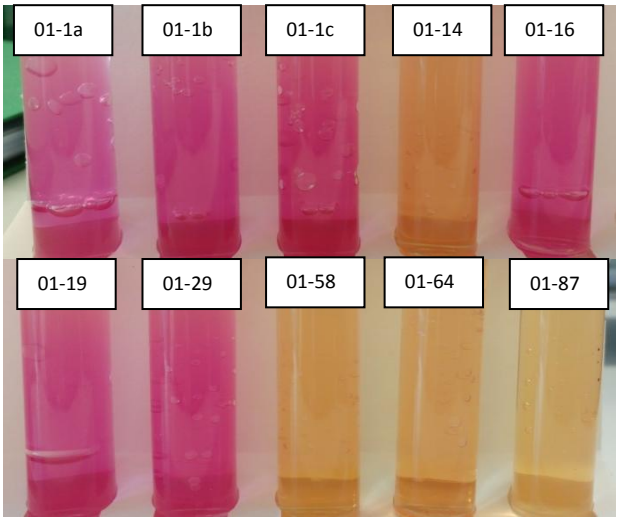


Table 3.13 Results from ECHA Micro monitor Sig Nitrite bacterial test Samples. * 0-24% pink colouration and gas bubbles, ** 25-49% pink colouration and gas bubbles, *** 50-74% pink colouration and gas bubbles, **** 75-100% pink colouration and gas bubbles. Colouration that did not turn pink but produced bubbles are acidic causing the ammonia produced to be neutralised and cannot turn the gel pink. And Figure 3.14. in ECHA Micro monitor Sig Nitrite bacterial test. From top left to right 01-1a, 01-1b, 01-1c, 01-14, 01-16. Bottom left to right 01-19, 01-29, 01-58, 01-64, 01-87. All samples show pink colouration or bubbles indicating a positive result.

3.4. Bioinformatics and molecular work

The 10 chosen bacteria in Table 3.11 were sent for sequencing through microbes NG. the results are displayed in the Table below, displaying identification through both the microbes NG database and the NCBI blast database.

Bacterial isolate	Microbes NG	% match	NCBI blast	NCBI ID	% match
ALG01-1a	*Pseudomonas aeruginosa*	91.21	*Pseudomonas aeruginosa DN1*	341318	75.0
ALG01-1b	*Pseudomonas aeruginosa*	92.64	*Pseudomonas aeruginosa*		89.0
ALG01-1c	*Pseudomonas aeruginosa*	87.59	*Pseudomonas aeruginosa PA96*	237015	88.0
ALG01-14	*Nocardioides sp.*	8.08	*Pimelobacter simplex strain VKMAc-2033D*	259593	87.0
ALG01-16	*Brevundimonas subvibrioides*	14.43	Brevundimonas sp. *DS20*	257504	87.0
ALG01-19	*Nocardioides sp.*	7.52	*Pimelobacter simplex strain VKMAc-2033D*	259593	86.0
ALG01-29	*Microbacterium testaceum*	7.91	*Microbacterium hominis strain SJTG1*	422004	46.0
ALG01-58	*Brevundimonas subvibrioides*	13.66	*Brevundimonas sp. DS20*	257504	88.0
ALG01-64	*Rubrivivax gelatinosus*	3.18	*Comamonas thiooxydans*	36735	11.0
ALG01-87	*Sphingopyxis alaskensis*	25.40	*Sphingopyxis terrae NBRC/5098 strain 203-1*	14711	82.0

Table 3.14: identification of the chosen 10 bacterial isolates. Identification through Microbes NG database from bacterial sequencing and also analysed via NCBI blast tool using the full FASTA files proved by Microbes NG. Percentage matches quoted for each, based on how much the query sequence matches the database sequences.

For the investigation into the presence of any cobalamin, and denitrification genes the identification of each isolate based in the NCBI blast results were used as the identities regardless that most were below a 90% match. As the identified were not all complete and fully annotated genomes, the nearest complete annotated genomes were used and are detailed below.

NCBI blast	NCBI ID	% match	Genomes assessed	NCBI ID
Pseudomonas aeruginosa DN1	341318	75.0	*Pseudomonas aeruginosa PAO1*	2603714
Pseudomonas aeruginosa PA96	237015	89.0	*Pseudomonas aeruginosa PAO1*	2603714
Pseudomonas aeruginosa PA96	237015	88.0	*Pseudomonas aeruginosa PAO1*	2603714
Pimelobacter simplex strain VKMAc-2033D	259593	87.0	*Pimelobacter simplex VKMAc-2033D*	259593
Brevundimonas sp. *DS20*	257504	87.0	*Brevundimonas sp. EAKA*	13676
Pimelobacter simplex strain VKMAc-2033D	259593	86.0	*Pimelobacter simplex VKMAc-2033D*	259593
Microbacterium hominis strain SJTG1	422004	46.0	*Microbacterium hominis*	35569
Brevundimonas sp. DS20	257504	88.0	*Brevundimonas sp. EAKA*	13676
Comamonas testosterone P19	297416	11.0	Comamonas *testosterone TK102*	859
Sphingopyxis terrae NBRC/5098 strain 203-1	14711	82.0	*Sphingopyxis terrae NBRC/5098 strain 203-1*	14711

Table 3.15: Bacterial isolate identification through Blast and the genomes used to investigate gene presence.

3.4.1 Cobalamin genes

Cobalamin being a potential factor in microalgal bacterial interactions, specific genes from the pathway were chosen to be searched for. The genes below were chosen from the pathway outlined in figure 1.9.

	cobO	cobS	cysG	cbiA	cbiK	cobC	cobU
Pseudomonas aeruginosa	NP_249963.1	YP_791964.1	NP_251301.1			NP_249967.1	NP_249970.1
Pimelobacter simplex strain							
Brevundimonas sp.		KDP95196.1					
Microbacterium hominis strain				AUG28220.1			
Comamonas testosteroni		SUY79311.1					
Sphingopyxis terrae							

Table 3.16: Genes found within each of the identified bacterial isolates that are involved in the cobalamin synthesis pathway. Data obtained from published genomes (Ma *et al.* 2009; Ohtsubo *et al.* 2016; Shtratnikova*, et al.* 2015; Stover *et al.* 2000; Tan-Guan-Sheng Adrian *et al.* 2016; Tully *et al*. 2018). Ticks indicate presence, cell left blank indicate absence or not yet annotated.

3.4.2 Denitrification and ammonification

As nitrogen plays a large role in the growth in microalgae, the denitrification and nitrogen fixing pathways were investigated in each of the bacterial genomes. The genes below were chosen from the pathway outlined in figure 1.7, with emphasis on the denitrification and ammonification pathways, based on the results of the sig nitrite tests (3.3.3). The genomes searched were those described in Table 3.15

	Pseudomonas aeruginosa PAO1	*Pimelobacter simplex strain VKMAc-2033D*	*Brevundimonas sp. EAKA*	*Microbacterium hominis strain*	*Comamonas testosteroni TK102*	*terrae NBRC/5098 strain 203-1*
narL	NP_252568.1	AIY17653			SUY80016.1	
napB	NP_249864.1				GAW76468.1	
nirS	NP_249210.1					
narG	NP_252564.1					
nosZ	NP_252082.1					
norB	NP_249215.1					
nrfA	WP_004419064.1					
hcp	YP_263147.1	AIY19362				
norV	YP_005208834.1					
norW	YP_005208835.1					
nirK	YP_262569.1					
nirB	NP_250472.1					
nirC	NP_249208.1					
nirD	NP_250471.1					

Table 3.17: Genes found within each of the identified bacterial isolates that are involved in ammonification and denitrification pathway. Data obtained from published genomes (Ma *et al.* 2009; Ohtsubo *et al.* 2016; Shtratnikova*, et al.* 2015; Stover *et al.* 2000; Tan-Guan-Sheng Adrian *et al.* 2016; Tully *et al*. 2018). Blue genes are part of the ammonification pathway, Pink are part of the denitrification pathway.

Chapter 4

Discussion

4.1. Growth of ALG01 under varying conditions and volumes

The growth of ALG01 is highly variable across different systems, of different size and control. There can be some similarities between the growth of ALG01 within the different systems when only looking at specific time points but overall those similarities are not reliable. These differences seen can be attributed to the differences in size of the cultures grown as well as the difference in maintenance of these cultures. The associated microbiome, in particular the associated bacterial levels fluctuate highly also. When ALG01 is grown repeatedly in the same system it is predictable and reproducible, this is due to the ability to treat the cultures the same in terms of nutrient additions and harvesting. Small scale cultures are more easily controlled and are reproducible due to their small volumes. Larger systems can cause changes in the culture dynamics, such as rates of gas transfer, distribution of media and higher risks of biofilm. Contamination will also increase at higher volumes due to a reduced ability to keep items sterile. The levels of associated bacteria on the other hand still remains highly variable even within the same system which is grown under the same conditions. With each experiment resulting in highly variable bacterial levels this indicated that the bacterial levels seem to be independent to the external inputs and algal growth.

System	Volume	Light µmol/m^2/s	Aeration	CO_2	OD (680nm)	Dry weight (g/L)	EPA	pH	Batch or Continuous	Length of run (Days)
Multi-cultivator	8 X 70ml	175	Unknown flow rate	N/A	0.3-2.3	1.0	56.4	6-10	Batch	8
1LPBR	1L	175	1000ml/min	0.25%	0.5-3.2	1.2	57.1	6-10	Batch	8
100L FPR	100L	350	Unknown flow rate	To pH adjust	0.2-7.8	1.0-4.0	55.6	7-8.5	Continuous	16
1000L IPR	1000L	1540	Unknown flow rate	To pH adjust	0.15-2.75	1.0	54.3	7-8.5	Continuous	35

Table 4.1: Summary of system conditions.

Fulbright *et al.* (2018) observed similar bacterial fluctuations in the associated biome of the Eustigmatophyceae, *Nannochloropsis salina*. In the study they looked at multiple sized photobioreactor systems, at small (5ml, 1L, 2L and 4L), medium (20-60L) and large (200L) volumes. They stated that there were noticeable changes in the bacterial communities measured over a series of sequential large-scale batch cultivations that had similar algal growth rates. Fulbright states that the complex ecosystem that exists in microalgal cultures requires investigation and understanding to allow for reproducible successful large scale and industrial microalgal cultivation. From the results only a small proportion of the ALG01 microbiome has been characterised and the role each play specifically is not yet determined. Understanding the microbiome allows not necessarily better control of the phycosphere, but better identification of algal culture health and what the presence of certain bacterial isolates indicate.

4.2. Axenic vs. Non-axenic

4.2.1 Agar cultures

The impact of the bacterial association can be investigated by removing its presence from the microalga. When ALG01 was grown without the presence of its microbiome (axenically), there was very little growth compared with a culture of the alga with its full biome over the same time period. Rivas *et al.* (2010) also states that from their experimentation the observations confirm those in the literature that *Botryococcus braunii* grows significantly slower under axenic conditions.Growth of *Chlorella* was also seen to be less than that of non-axenic, indicating the bacteria influenced the metabolism (Cho *et al.* 2015). It has been previously documented that the associated biome can provide multiple factors that aid growth including nitrogen, phytohormones and essential vitamins (Amin, Parker and Armbrust 2012; Cole 1982; Smith 2015). Cultures when grown with suplementation with glucose, tryptone and cobalamin (B_{12}) the growth of axenic

ALG01 is improved to approximtely 50% of a non-axenic culture (estimations based on qualitative results), but is not returned to normal levels of growth when compared to cultures with the total microbiome. This indicated that the bacteria are potentially providing more than just nutrition to the microalgae. Croft *et al*. (2014) observed that many cultures, after removing the associated bacteria from the microalgae, without further supplementation, inparticular B_{12}, there was a marked decline in health and cell death leading to total culture death. In the test performed they used the results to provide evidence for B_{12} dependancy in those species, but it also indicates the difficulties in producing axenic cultures. *Scenedesmus bicellularis* grew better when grown with its associated bacteria when compared with the axenic growth (Mouget *et al*. 1995).

4.2.2 Liquid cultures

Within liquid cultures, established axenic cultures grow as well as non-axenic cultures and experience less self-flocculation, though the differences are not statistically different (From qualitative data). It has been found before that some bacterial association can increase algal flocculation. Kouzuma *et al.* (2015) stated that when a *Nannochloropsis* species has *Escherichia coli* coated in a cationic polymer added to the culture experienced enhanced aggregation of algal cells causing betting flocculation. Which would aid in the dewatering and harvesting of cultures by using an endogenous bacterium.

4.3 Microbiome

Microbes NG	NCBI blast	Collins (2017) BBSRC Flip Report
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	Methylophilus
Nocardioides sp.	*Pimelobacter simplex strain*	Sphingopyxis
Brevundimonas subvibrioides	*Brevundimonas sp.*	Rhizobiaceae
Microbacterium testaceum	*Microbacterium hominis strain*	Pelomonas
Rubrivivax gelatinosus	*Comamonas testosteroni*	Emticicia
Sphingopyxis alaskensis	*Sphingopyxis terrae*	Alphaproteobacteria
		Delftia
		Acinetobacter
		Uncultured

Table 4.2 Summary of species identified from this study and from a personal communication from Collins (2017) BBSRC Flexible Interchange Programme report.

4.3.1. Isolation and morphology

The isolation of multiple bacterial species from within the microalgal cultures has a lot of bias, testing in this manner only takes into account those culturable bacteria that are able to grow and sustain under the conditions chosen (Amin *et al.* 2015). This does not include the unculturable or those damaged during culturing. To get a full microbiome analysis complex molecular analysis is required. Collins (2017) who worked on ALG01 cultures, using a commercially available genomic DNA extraction kit was able to identify 13 different bacteria across 3 different sample sets. 1 experimental culture, 1 stock flask culture, and bacterial isolates previously isolated by AlgaeCytes Ltd. From Table 4.2 shows the variability in identification using 2 different methods.

Molecular analysis conducted by Fulbright *et al.* (2018) where 275 samples were analysed for bacterial identification. Using a culturing method does limit the analysis conducted but still gives insight into those species present. 87 morphologically different bacterial isolates were taken from an ALG01 culture, where only 83 were able to be subcultured. A further 3 that were previously isolated by AlgaeCytes Ltd. were also used in the analysis. Using multiple isolation temperatures and media allowed for a more varied culturable analysis than others have conducted. Through multiple testing they were thought to all be separate species, though not confirmed through genetic analysis.

Some of the bacterial isolates were observed to be able to grow on minimal media without an available carbon source. This indicates that some of the bacteria are potentially autotrophic. All the isolates are were able to grow on the minimal media in the presence of the microalgae. This suggests that the algae are providing the bacteria with a carbon source. This with the algae not growing as well in the absence of the bacteria this suggests that there is mutualism between the bacteria and the microalgae. Croft *et al*. (2014) also observed that there was no carbon source in the culture media and the bacteria grew alongside the microalgae, presumably using the products of photosynthesis to grow. Indicating a mutualistic relationship between the bacteria, *Halomonas* sp. and the microalgae species *Amphidinium operculatum* and *Porphyridium purpureum*.

4.3.2 Reintroduction tests.

To test if the isolated bacterial isolates had any significant effect on the growth of the microalgae when reintroduced to a culture. Many others have demonstrated an effect being seen in algal growth with bacterial reintroduction. Multiple methods are used but the most effective have appeared to be direct contact reintroduction via agar cultures or through direct liquid interaction (Kazima *et al.* 2012; Natrah *et al.* 2014; Wang *et al.* 2016). Reintroducing the bacteria isolated back into axenic cultures can give evidence if the species are benefitting cultures or are opportunistic strains that provide no use for the algae.

a) Agar tests

Algal cultures when grown in direct contact with each of the bacterial isolates isolated from ALG01, 65 displayed a positive interaction with distinct algal growth along the bacterial culture line. 18 of the isolates showed no effect on the algal cultures but there were no isolates that showed a negative effect. Those that have little or no effect may have been species that have been introduced to the culture when first isolated under laboratory conditions, is a contaminant or an artefact from the original environment. These bacteria do not help or hinder the growth of ALG01 and can be assumed that they are not required by the culture for survival.

The supernatant of the bacterial isolates was also tested against the microalgae to identify if these algal promoting properties were from compounds naturally excreted by the bacteria without external effect from the alga itself. None tested showed any effect on algal growth suggesting that if any products are being released by the bacteria itself, it is only in the presence of the algae. This indicates a mutualistic relationship. It has been previously noted that bacteria will only produce extracellular products when it is beneficial to itself as it is metabolically expensive (Grant *et al.*2014). Of the 86 isolates tested, 7 showed the highest effect on the growth of the microalga were chosen alongside the 3 isolated previously by AlgaeCytes Ltd. for further testing in liquid cultures. The 3 previously isolated were chosen as an indicator for eventual genetic analysis, as they had been identified previously.

b) Liquid tests

During the liquid reintroduction tests there was no significant difference in the growth and lipid production of ALG01 under any of the bacterial additions. Those reintroduced were the 10 that showed the highest effects during the agar trials or were previously isolated and held at AlgaeCytes Ltd. Cultures with specific bacterial isolates added were observed to have more self-flocculation occurring, but this was not significant and not the same for every addition.

Bacterial mixes were also reintroduced to the cultures to replicate the effect of the total biome but only with those species that were observed to have influenced the algal growth in the agar trials. Mixes where chosen based on effect on the algae, and the isolation conditions. These were observed to also have no significant effect on the algal growth, and the growth of all the mixes used and the axenic culture were very similar.

The differences observed between the liquid and agar reintroduction tests may be due to the different culture dynamics in the different states. Within the agar cultures there was direct contact between the algae and bacterial cultures which would allow for a more direct line of communication, potentially through

direct attachment allowing for direct transfer of materials and products. Whereas in the liquid cultures the proximity may not be as direct and more planktonic, as it is a more fluid and dynamic culture, where bacterial cells may not directly attach to the ALG01 cells which would make for a lesser amount of close communication and the interaction be less distinct. Thus, causing the interaction to be unobservable. Sureshkumar *et al*. (2014) has observed this in the growth of *Nannochloropsis oculata*was. The growth of the algal culture was significantly increased when growth in the presence of *Bacillus sp*. and *Pseudomonas sp*. when compared to the control.

4.4. Bioinformatics and molecular work

6 genera of bacteria were identified based on molecular analysis of the 10 bacterial strains analysed for reintroduction into the cultures (*Pseudomonas*, *Pimelobacter*, *Brevundimonas*, *Microbacterium*, *Comamonas* and *Sphingopyxis)*. In other phycosphere studies some of these species have been found and seem to common place within microalga cultures. In particular *Pseudomonas* (Cole, 1982; Goecke *et al.* 2012; Mouget *et al*. 1995; Natrah *et al.* 2013; Wang *et al.* 2014), Brevundimonas (Goecke *et al.* 2012; Natrah *et al.* 2013), and *Microbacterium* (Natrah *et al.* 2013; Wantanabe *et al.* 2005), have all been previously identified. It is unknown from this study if these bacteria would be found with ALG01 in its natural habitat or if the association has occurred through contamination or artefacts from the initial isolation (Schwenk, Nohynek and Rischer 2014). It has been seen that there are differences between the associated bacteria between differently isolated wild samples and between different laboratory samples (Natrah *et al.* 2014). It is essential to look at whether these associations are accidental through random meeting or out of necessity, though this would be difficult to distinguish. In the case of ALG01 it can be observed that the association has become a requirement. Creating an axenic ALG01 showed cobalamin was a requirement for the alga as the health of ALG01 did not improve without the addition of B_{12}. (Bolch, Subramanian and Green 2011; Kim *et al* 2014; Treplitski, Rajamani 2011). Microalgae are known to utilise nitrate and nitrite as a source which is metabolically expensive but are able to use ammonia much more efficiently and will preferentially use if available (Peterson, *et al.* 2011). The bacteria can be theorised as providing these essential elements for growth and survival in exchange for photosynthetic products such as a carbon source (Natrah *et al.* 2014; Ramanan *et al.* 2015).

strain	B_{12}	Denitrification	Ammonification	Antibiotic	Other
Pseudomonas aeruginosa	B_{12} producer	Yes	Yes	Pyocyanin	Siderophore (pyoverdine)
Pimelobacter simplex strain	Unknown	Unknown	Uses NH_4	Related to species that can produce sandramycin	n/a
Brevundimonas sp.	Unknown	Some species are known	Yes	Unknown	n/a
Microbacterium hominis strain	Uses B_{12}	An associated species is	Yes	Unknown	n/a
Comamonas testosteroni	B_{12} producer	Denitrifying potential	Some species are known	Unknown	n/a
Sphingopyxis terrae	Uses B_{12}	Yes	Unknown	Unknown	n/a

Table 4.3 Summary of bacterial strains B_{12} denitrification and ammonification ability. Unknown indicates that the genomes have not yet been fully mined and annotated.

From the identified strains it is documented that some are known B_{12} / denitrifying/ and antibiotic producers. Genetic analysis of the bacterial isolates indicates some have specific genes that are associated with the cobalamin synthesis pathway and the denitrification/ammonification pathways. This indicates that these bacterial strains are present for a particular reason, whether to provide the alga with vitamin B_{12}, a more available nitrogen source in the form of ammonia, to protect the alga from unwanted bacterial species through the secretion of antibiotics or just to help maintain the stability of phycosphere as a whole.

For all the strains investigated none indicated having all the genes required for the full pathways for B_{12} synthesis and denitrification/ammonification (Table 3.16 and 3.17). This could suggest that the genes present are significantly different, have yet to be annotated, they have a different pathway for synthesis or are not able to produce all the products of the pathway. Three of the isolates were identified to having some of the denitrifying and ammonification genes present and are documented within the literature to having this potential. This was confirmed with the ECHA sig nitrite tests, meaning that they are providing a usable form of nitrogen for the microbiome. Whether that be nitrogen to supply the microbiome and support the holobiont through maintenance of the environment, a source of nitrogen for other bacterial strains to utilise or ammonia for the microalga to use more efficiently. Denitrifying ability and ammonification being as important as B_{12} production, which can suggest that the holobiont requires a multitude of diversity in bacterial species to maintain a healthy phycosphere.

4.5. Industrial applications

Giordano and Wang (2018) stated that Algal commercial cultivation is often conducted empirically, without a full understanding of the physiology behind it. Understanding the microalgal phycosphere could allow for a fundamentally more reliable use of microalgae for the production of high value chemicals. These organisms are thought of as a single entity not as part of an ecosystem. These microenvironments hold such a multitude of organisms, each having numerous complex communications within a varying and unpredictable environment. The dynamics of bacterial and microalgal interactions is a complex changing relationship that based on influences of the environment can naturally move between mutualism, commensalism and parasitism (Leung, Poulin 2008).

Gaining insight into the bacteria that live within these microenvironments could be the key to the commercialisation of many species which are yet to be utilised. Many species that are developed are chosen as they are thought to be monoculture or have the ability to be grown as a monoculture, but with the presence of a microbiome heterotrophic and mixotrophic growth is near impossible. The presence of an organic carbon source would cause an uncontrollable proliferation of bacteria, and cause the ultimate crash of a culture (Giordano and Wang, 2018; Subashchandrabose*, et al.* 2011). Large scale production of microalgae also has the added challenge of potential contamination, as it is noted that the larger the scale of photobioreactor the risk of contamination increases due to challenge of sterility at large scales.

Having a detailed molecular analysis of the microbiome could allow for better culture management (Fulbright, *et al.* 2018); in the potential increase in product production and as an indicator of culture health and performance. It has been observed that there are major shifts in the microbiome composition in different systems. Gaining an understanding of what drives these differences and their functionality on the culture could be crucial in the understanding of the cultures and therefore the large scale algal cultivation (Fulbright, *et al.* 2018; Giordano and Wang, 2018). The presence or absence of certain species could have large implications on cultures and whether that bacteria has a direct impact on the algal productivity or as a tool for predicting the performance of a culture would allow for better algal culture screening and reduce the risk of losses due to a culture crash or low performance, saving time and monies for companies if they could predict the behaviour of their organisms.

4.6 Future experimentation

To understand the relationship between ALG01 and its microbiome, further testing could be carried out.

- Metagenomics of microbiome throughout production

A full molecular analysis of a complete culture to analyse bacteria found within ALG01 cultures, analyse any species specific fluctuations throughout growth and commercial production.

- ALG01 – sequencing to see what its capability is

A full genomic analysis of ALG01 to analyse its genetic capability and potential responses to the environment. Including if it contains the METE and METH genes, and therefore gain more understanding of its B_{12} requirements and biosynthetic and assimilatory capability.

- SEM and TEM microscopy for more detailed visualisations

SEM to observe the location of associated bacteria, free floating or attached to the algal cells, with looking into samples from multiple time points within culture growth to observe any changes. TEM to observe any endophytic bacteria and changes in the algae's internal structures throughout its growth. Using uranyl acetate and lead acetate to enhance image quality, and immunostaining to enhance organelle imaging.

- Analysis of small molecules

Look into small molecule synthesis, to identify any small molecules produced by the associated bacteria in isolation and within co-cultures of ALG01, and if these change over time. Molecules to be potentially found including:-

  - Antibiotics
  - Siderophores
  - B_{12}
  - Plant hormones

Initially using LC-MS to identify any products being produced, focusing on those made in larger quantities, then using radioactive labelling to identify the uptake and movement of molecules within the phycosphere.

Final Conclusions

The growth of microalgae cannot be looked at as just the microalga itself but as an entire ecosystem, following the importance of the concept of the holobiont. The phycosphere is comprised of many species, all of which interact in a complex manner where most interactions cannot be measured directly. Trying to maintain an axenic culture at scale is highly improbable and not cost effective. The impact on growth and overall product development from an axenic culture is costly, and the implications of growing a not truly axenic culture under heterotrophic and mixotrophic conditions could have devastating results on the culture, and ultimately culture death. Gaining an understanding of the ecosystem within a culture is more beneficial to enable knowledge of growth, health and an ability to predict how the culture will react to outside influences. The bacteria found in the phycosphere are highly diverse and provide not only a source of vitamin B_{12} but also a nitrogen source and potentially compounds to protect the algae from invading species (antibiotics). In return the microalga provides a carbon rich environment where the bacteria can thrive. A more in-depth knowledge of the microalga and its phycosphere will lead to better large scale culture maintenance and in turn a better yield and quality of high value products.

References

Adarme-Vega, T.C., Lim, D.K., Timmins, M., Vernen, F., Li, Y., Schenk, P.M. (2012), Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. *Microbial Cell Factories.* 11(1) 96

Altschul, S., Gish, W., Miller, W., Myers, E., Lipman, D. (1990) Basic local alignment search tool. *Journal of Molecular Biology*. 215(3) 80360-2

Amin, S., Hmelo, L., Van Tol, H., Durham, B., Carlson, L., Heal, K., Morales, R., Berthiaume, C., Parker, M., Djunaedi, B. (2015) Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature.* 522(7554) 98-101.

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

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

Arora, M., Anil, A.C., Delany, J., Rajarajan, N., Emami, K., Mesbahi, E. (2012) Carbohydrate-degrading bacteria closely associated with *Tetraselmis indica*: influence on algal growth. *Aquatic Biology*, *15(*1) 61-71

Atlas, R.M. (2010) *Handbook of microbiological media,* CRC press.

Bauer, W.D., Robinson, J.B. (2002) Disruption of bacterial quorum sensing by other organisms. *Current Opinion in Biotechnology,* 13(3) 234-237

Bell, W., Mitchell, R. (1972) Chemotactic and growth responses of marine bacteria to algal extracellular products. *Biological Bulletin,* 143(2) 265-277

Beveridge, T.J. (2001) Use of the Gram stain in microbiology. *Biotechnic & Histochemistry,* 76(3) 111-118.

Blanc, G., Agarkova, I., Grimwood, J., Kuo, A., Brueggeman, A., Dunigan, D.D., Gurnon, J., Ladunga, I., Lindquist, E., Lucas, S. (2012) The genome of the polar eukaryotic microalga *Coccomyxa ubellipsoidea* reveals traits of cold adaptation. *Genome Biology,* 13(5) R39

Bolch, C.J., Subramanian, T.A., Green, D.H. (2011) The toxic dinoflagellate *Gymnodinium Catenatum* (dinophyceae) requires marine bacteria for growth. *Journal of Phycology,* 47(5) 1009-1022

Brennan, L., Owende, P. (2010) Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews,* 14(2) 557-577

Carpinelli, E.C., Telatin, A., Vitulo, N., Forcato, C., D'Angelo, M., Schiavon, R., Vezzi, A., Giacometti, G.M., Morosinotto, T., Valle, G. (2014) Chromosome scale genome assembly and transcriptome profiling of *Nannochloropsis gaditana* in nitrogen depletion. *Molecular Plant,* 7(2) 323-335

Chen, S.Y., Pan, L.Y., Hong, M.J., Lee, A.C., (2012) The effects of temperature on the growth of and ammonia uptake by marine microalgae. Botanical Studies, 53(1) 125-133

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

Cho, D.H., Ramanan, R., Heo, J., Shin, D.S., Oh, H.M., Kim, H.S., (2016) Influence of limiting factors on biomass and lipid productivities of axenic Chlorella vulgaris in photobioreactor under chemostat cultivation. Bioresource technology, 211, 367-373

Christenson, L., Sims, R. (2011) Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnology Advances,* 29(6) 686-702

Cole, J.J. (1982) Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecology and Systematics,* 13(1) 291-314

Collins, C. (2017) BBSRC Flexible Interchange Programme (FLIP) report.

Cooper, M.B., Smith, A.G. (2015) Exploring mutualistic interactions between microalgae and bacteria in the omics age. *Current Opinion in Plant Biology,* 26, 147-153

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

D'Alessandro, E.B., Antoniosi Filho, N.R. (2016) Concepts and studies on lipid and pigments of microalgae: A review. *Renewable and Sustainable Energy Reviews,* 58, 832-841

de-Bashan, L.E., Bashan, Y. (2008) Joint immobilization of plant growth-promoting bacteria and green microalgae in alginate beads as an experimental model for studying plant-bacterium interactions. *Applied and Environmental Microbiology,* 74(21) 6797-6802

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

Doucette, G.J. (1995) Interactions between bacteria and harmful algae: a review", *Natural Toxins,* 3(2) 65-74

ECHA microbiology (2017) "SigTests: Sig Nitrite" Available: https://www.echamicrobiology.com/products/test-kits/sig-nitrite accessed: 24/12/17

Eliáš, M., Amaral, R., Fawley, K.P., Fawley, M.W., Němcová, Y., Neustupa, J., Přibyl, P., Santos, L.M., Ševčíková, T. (2017) Eustigmatophyceae Chapter 11 in Archibald, J., Simpson A., Salmovits, C., (eds) Handbook of the protists. Springer.

Enzing, C., Ploeg, M., Barbosa, M., Sijtsma, L., (2014) Microalgae – based products for the food and feed sector: an outlook for Europe. JRC Scientific and Policy Reports European Commission.

Eriksen, N.T. (2008) The technology of microalgal culturing. *Biotechnology Letters,* 30(9) 1525-1536

Fang, H., Kang, J., Zhang, D. (2017) Microbial production of vitamin B 12: a review and future perspectives. Microbial Cell Factories, 16(1) 15

Fernandes, G., Venkatraman, J.T. (1993) Role of omega-3 fatty acids in health and disease. *Nutrition Research,* 13, S19-S45

Ferrière, R., Gauduchon, M., Bronstein, J.L. (2007) Evolution and persistence of obligate mutualists and exploiters: competition for partners and evolutionary immunization. *Ecology Letters,* 10(2) 115-126

Florencio, F.J., Vega, J.M., (1983) Utilization of nitrate, nitrite and ammonium by Chlamydomonas reinhardii. Planta, 158(4) 288-293

Fuentes, J.L., Garbayo, I., Cuaresma, M., Montero, Z., González-del-Valle, M., Vílchez, C. (2016) Impact of Microalgae-Bacteria Interactions on the Production of Algal Biomass and Associated Compounds. *Marine drugs,* 14(5) 100

Fukami, K., Nishijima, T., Ishida, Y. (1997) Stimulative and inhibitory effects of bacteria on the growth of microalgae. *Live Food in Aquaculture* Springer, 185-191

Fulbright, S.P., Robbins-Pianka, A., Berg-Lyons, D., Knight, R., Reardon, K.F. & Chisholm, S.T. 2018, Bacterial community changes in an industrial algae production system. *Algal research*, Vol. 31, pp.147-156.

Gao, C., Wang, Y., Shen, Y., Yan, D., He, X., Dai, J., Wu, Q. (2014) Oil accumulation mechanisms of the oleaginous microalga *Chlorella protothecoides* revealed through its genome, transcriptomes, and proteomes. *BMC genomics,* 15(1) 1

Giordano, M., Wang, Q. (2018) Microalgae for Industrial Purposes. *Biomass and Green Chemistry,* Springer, 133-167

Goecke, F., Thiel, V., Wiese, J., Labes, A., Imhoff, J.F. (2013) Algae as an important environment for bacteria–phylogenetic relationships among new bacterial species isolated from algae. *Phycologia,* 52(1) 14-24

Gonzalez, L.E., Bashan, Y. (2000) Increased growth of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense. Applied and Environmental Microbiology,* 66(4) 1527-1531

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., Lopez, R. (2010) A new bioinformatics analysis tools framework at EMBL–EBI. *Nucleic acids research,* 38(2) W695-W699

Grant, M.A., Kazamia, E., Cicuta, P., Smith, A.G. (2014) Direct exchange of vitamin B_{12} is demonstrated by modelling the growth dynamics of algal–bacterial cocultures. *The ISME journal,* 8(7) 1418-1427

Guo, Z., Tong, Y.W. (2014) The interactions between *Chlorella vulgaris* and algal symbiotic bacteria under photoautotrophic and photoheterotrophic conditions. *Journal of Applied Phycology,* 26(3) 1483-1492

Helliwell, K.E., Scaife, M.A., Sasso, S., Araujo, A.P., Purton, S., Smith, A.G. (2014) Unravelling vitamin B_{12}-responsive gene regulation in algae. *Plant Physiology,* 165(1) 388-397

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

Hernandez, J., de-Bashan, L.E., Rodriguez, D.J., Rodriguez, Y., Bashan, Y. (2009) Growth promotion of the freshwater microalga Chlorella vulgaris by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. *European Journal of Soil Biology, 4*5(1) 88-93

Huang, X., Huang, Z., Wen, W., Yan, J., (2013) Effects of nitrogen supplementation of the culture medium on the growth, total lipid content and fatty acid profiles of three microalgae (Tetraselmis

subcordiformis, Nannochloropsis oculata and Pavlova viridis). Journal of applied phycology, 25(1) 129-137

Hughes, D.T., Sperandio, V. (2008) Inter-kingdom signalling: communication between bacteria and their hosts. *Nature Reviews Microbiology,* 6(2) 111-120

Industrial Plankton. (2016) "1000L Industrial Plankton industry reactor". Available: https://industrialplankton.com/productsPage.php. Last accessed 24/12/17

Joint, I., Tait, K., Callow, M.E., Callow, J.A., Milton, D., Williams, P., Camara, M. (2002) Cell-to-cell communication across the prokaryote-eukaryote boundary. *Science (New York, N.Y.),* 298(5596) 1207

Kazamia, E., Czesnick, H., Nguyen, T.T.V., Croft, M.T., Sherwood, E., Sasso, S., Hodson, S.J., Warren, M.J., Smith, A.G. (2012) Mutualistic interactions between vitamin B_{12}-dependent algae and heterotrophic bacteria exhibit regulation. *Environmental microbiology,* 14(6) 1466-1476

Kazamia, E., Helliwell, K.E., Purton, S., Smith, A.G. (2016) How mutualisms arise in phytoplankton communities: building eco-evolutionary principles for aquatic microbes. *Ecology Letters,* 19(7) 810-822

Keshtacher-Liebso, E., Hadar, Y., Chen, Y. (1995) Oligotrophic Bacteria Enhance Algal Growth under Iron-Deficient Conditions. *Applied and Environmental Microbiology,* 61(6) 2439-2441

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

Kouzuma, A., Watanabe, K. (2015) Exploring the potential of algae/bacteria interactions. *Current Opinion in Biotechnology,* 33, 125-129

Leung, T., Poulin, R. (2008) Parasitism, commensalism, and mutualism: exploring the many shades of symbioses", *Vie et Milieu,* 58(2) 107

Lutzu, G.A., Turgut, N.D., (2018) Interactions of microalgae and other microorganisms for enhanced production of high-value compounds. *Frontiers in bioscience (Landmark edition)*, *23*, 1487-1504

Ma, Y.F., Zhang, Y., Zhang, J.Y., Chen, D.W., Zhu, Y., Zheng, H., Wang, S.Y., Jiang, C.Y., Zhao, G.P., Liu, S.J., (2009) The complete genome of *Comamonas testosteroni* reveals its genetic adaptations to changing environments. *Applied Environmental Microbiology*., 75(21), 6812-6819

Magdouli, S., Brar, S., Blais, J. (2016) Co-culture for lipid production: Advances and challenges. *Biomass and Bioenergy,* 92, 20-30

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Marechal-Drouard, L., Marshall, W.F., Qu, L.H., Nelson, D.R., Sanderfoot, A.A., Spalding, M.H., Kapitonov, V.V., Ren, Q., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S.M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C.L., Cognat, V., Croft, M.T., Dent, R., Dutcher, S., Fernandez, E., Fukuzawa, H., Gonzalez-Ballester, D., Gonzalez-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P.A., Lemaire, S.D., Lobanov, A.V., Lohr, M., Manuell, A., Meier, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J.V., Moseley, J., Napoli, C., Nedelcu, A.M., Niyogi, K., Novoselov, S.V., Paulsen, I.T., Pazour, G., Purton, S., Ral, J.P., Riano-Pachon, D.M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S.L., Allmer, J., Balk, J., Bisova, K., Chen, C.J., Elias, M., Gendler, K., Hauser, C., Lamb, M.R., Ledford, H., Long, J.C., Minagawa, J., Page, M.D., Pan, J., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A.M., Yang, P., Ball, S., Bowler, C., Dieckmann, C.L., Gladyshev, V.N., Green, P.,

Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R.T., Brokstein, P., Dubchak, I., Goodstein, D., Hornick, L., Huang, Y.W., Jhaveri, J., Luo, Y., Martinez, D., Ngau, W.C., Otillar, B., Poliakov, A., Porter, A., Szajkowski, L., Werner, G., Zhou, K., Grigoriev, I.V., Rokhsar, D.S., Grossman, A.R. (2007) The Chlamydomonas genome reveals the evolution of key animal and plant functions. *Science (New York, N.Y.),* 318(5848) 245-250

MicrobesNG. (2018) MicrobesNG. Available: https://microbesng.uk/. Last accessed 10/1/18

Milledge, J.J. (2011) Commercial application of microalgae other than as biofuels: a brief review. *Reviews in Environmental Science and Bio/Technology,* 10(1) 31-41

Miller, M.R., White, A., Boots, M. (2006) The evolution of parasites in response to tolerance in their hosts: The good, the bad, and apparent commensalism. *Evolution,* 60(5) 945-956

Moran, N.A. (2007) Symbiosis as an adaptive process and source of phenotypic complexity. *Proceedings of the National Academy of Sciences of the United States of America,* 104(1) 8627-8633

Mouget, J.-., Dakhama, A., Lavoie, M.C., de la Noüe, J. (1995) Algal growth enhancement by bacteria: Is consumption of photosynthetic oxygen involved? *FEMS Microbiology Ecology,* 18(1) 35-43

Mühlroth, A., Li, K., Røkke, G., Winge, P., Olsen, Y., Hohmann-Marriott, M., Vadstein, O., Bones, A., (2013) Pathways of lipid metabolism in marine algae, co-expression network, bottlenecks and candidate genes for enhanced production of EPA and DHA in species of Chromista. *Marine drugs*, 11(11) 4662-4697

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

Nils, R.P., (2003) Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. *Limnology and Oceanography*, 48(1) 93-105

Ohtsubo, Y., Nonoyama, S., Nagata, Y., Numata, M., Tsuchikane, K., Hosoyama, A., Yamazoe, A., Tsuda, M., Fujita, N., Kawai, F., (2016) Complete genome sequence of *Sphingopyxis terrae strain* 203-1 (NBRC 111660), a polyethylene glycol degrader. *Genome Announcements*. 4(3) 00530-16

Ota, S., Yoshihara, M., Yamazaki, T., Takeshita, T., Hirata, A., Konomi, M., Oshima, K., Hattori, M., Bisova, K., Zachleder, V., Kawano, S. (2016) Deciphering the relationship among phosphate dynamics, electron-dense body and lipid accumulation in the green alga *Parachlorella kessleri. Scientific Reports,* 6, 25731

Palintest – water analysis technologies (2017) Photometer 7100. Standard methis ref. 450-nitrate-E and 4500-P-C. Available: https://www.palintest.com/en/products/photometer-7100 Last accessed 24/12/17

Peterson, C.G., Daley, A.D., Pechauer, S.M., Kalscheur, K.N., Sullivan, M.J., Kufta, S.L., Rojas, M., Gray, K.A., Kelly, J.J., (2011) Development of associations between microalgae and denitrifying bacteria in streams of contrasting anthropogenic influence. *FEMS Microbiology Ecology*, 77(3) 477-492.

Photons systems instruments, (2017a) Multicultivator MC-1000. Available: http://www.psi.cz/products/photobioreactors/multi-cultivator-mc-1000. Last accessed 24/12/17

Photons systems instruments, (2017b) Photobioreactor FMT 150 1L. Available: http://www.psi.cz/products/photobioreactors/photobioreactor-fmt-150. Last accessed Last accessed 24/12/17.

Radakovits, R., Jinkerson, R.E., Darzins, A., Posewitz, M.C., (2010) Genetic engineering of algae for enhanced biofuel production. *Eukaryotic cell*, 9(4) 486-501

Raja, R., Hemaiswarya, S., Kumar, N.A., Sridhar, S., Rengasamy, R. (2008) A perspective on the biotechnological potential of microalgae, *Critical Reviews in Microbiology,* 34(2) 77-88

Raja, R., Shanmugam, H., Ganesan, V. & Carvalho, I. (2014) Biomass from microalgae: An overview. *Oceanography*, 2(1) 118

Ramanan, R., Kang, Z., Kim, B., Cho, D., Jin, L., Oh, H., Kim, H. (2015) Phycosphere bacterial diversity in green algae reveals an apparent similarity across habitats. *Algal Research,* 8, 140-144

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

Rébeillé, F., Ravanel, S., Marquet, A., Mendel, R.R., Smith, A.G., Warren, M.J. (2007) Roles of vitamins B_5, B_8, B_9, B_{12} and molybdenum cofactor at cellular and organismal levels. *Natural Product Reports,* 24(5) 949-962

Richmond, A. ed., (2008) Handbook of microalgal culture: biotechnology and applied phycology. John Wiley & Sons.

Rivas, M.O., Vargas, P., Riquelme, C.E. (2010) Interactions of *Botryococcus braunii* cultures with bacterial biofilms", *Microbial Ecology,* 60(3) 628-635

Rodionov, D.A., Dubchak, I.L., Arkin, A.P., Alm, E.J., Gelfand, M.S. (2005) Dissimilatory Metabolism of Nitrogen Oxides in Bacteria: Comparative Reconstruction of Transcriptional Networks. *Public Library of Science.*

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

Sañudo-Wilhelmy, S., Gobler, C., Okbamichael, M. & Taylor, G. 2006, "Regulation of phytoplankton dynamics by vitamin B_{12}", *Geophysical Research Letters,* vol. 33, no. 4.

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

Schwenk, D., Nohynek, L., Rischer, H. (2014) Algae–bacteria association inferred by 16S rDNA similarity in established microalgae cultures. *Microbiology Open,* 3(3) 356-368

Shahidi, F., Ambigaipalan, P., (2018) Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. *Annual Review of Food Science and Technology*, 9(1)

Shen, Y., Yuan, W., Pei, Z., Wu, Q., Mao, E. (2009) Microalgae mass production methods. *Transactions of The ASABE,* 52(4) 1275-1287

Shtratnikova, V.Y., Schelkunov, M.I., Pekov, Y.A., Fokina, V.V., Logacheva, M.D., Sokolov, S.L., Bragin, E.Y., Ashapkin, V.V., Donova, M.V. (2015) Complete genome sequence of steroid-transforming *Nocardioides simplex* VKM Ac-2033D. *Genome announcements*, 3(1) e01406-14

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D., Higgins, D.G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology,* 7, 539

Simopoulos, A.P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & Pharmacotherapy,* 56(8) 365-379

Simopoulos, A.P. (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine (Maywood, N.J.),* 233(6) 674-688

Smith, A.G., Croft, M.T., Moulin, M., Webb, M.E. (2007) Plants need their vitamins too. *Current Opinion in Plant Biology,* 10(3) 266-275

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. (2006) Commercial applications of microalgae. *Journal of Bioscience and Bioengineering,* 101(2) 87-96

Starr, M.P. (1975) A generalized scheme for classifying organismic associations. *Symposia of the Society for Experimental Biology,* 1-20

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406(6799) 959

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

Sureshkumar, S., Jasmin, B., Rahiman, K.M., Mohammed, A.H. (2014) Growth enhancement of micro algae, Chaetoceros calcitrans and Nannochloropsis oculata, using selected bacterial strains. *International Journal of Current Microbiology and Applied Sciences,* 3(4) 352-359

Tang, Y.Z., Koch, F., Gobler, C.J. (2010) Most harmful algal bloom species are vitamin B_1 and B_{12} auxotrophs. *Proceedings of the National Academy of Sciences of the United States of America,* 107(48) 20756-20761

Tan-Guan-Sheng Adrian, P.W., Tan, J.W.C., Wai-Fong Yin, K.G.C., (2016) Draft genome sequence of *Kocuria rhizophila* strain TPW45, an actinobacterium isolated from freshwater. *Journal of Genomics*, 4, 16

Teplitski, M., Rajamani, S. (2011) Signal and nutrient exchange in the interactions between soil algae and bact Terlizzi Jr, D.E., Karlander, E.P., (1980) Growth of a coccoid nanoplankter (Eustigmatophyceae) from the Chesapeake Bay as influenced by light, temperature, salinity and nitrogen source in factorial combination 1. Journal of phycology, 16(3) 364-368eria. *Biocommunication in Soil Microorganisms* Springer, 413-426

Troyer, D.A., Venkatraman, J.T., Fernandes, G. (1998) Effects of calorie restriction and ω-3 dietary fat on aging in short-and long-lived rodents. *Age,* 21(4) 175

Tully, B.J., Graham, E.D., Heidelberg, J.F., (2018) The reconstruction of 2,631 draft metagenome-assembled genomes from the global oceans. *Scientific Data*, 5, 170203

Turpin, D.H., Elrifi, I.R., Birch, D.G., Weger, H.G., Holmes, J.J. (1988) Interactions between photosynthesis, respiration, and nitrogen assimilation in microalgae. *Canadian Journal of Botany,* 66(10) 2083-2097.

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

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

Watanabe, K., Takihana, N., Aoyagi, H., Hanada, S., Watanabe, Y., Ohmura, N., Saiki, H., Tanaka, H. (2005) Symbiotic association in Chlorella culture. *FEMS Microbiology Ecology,* 51(2) 187-196

Widjaja, A., Chien, C.C. and Ju, Y.H., 2009. Study of increasing lipid production from fresh water microalgae Chlorella vulgaris. Journal of the Taiwan Institute of Chemical Engineers, 40(1) 13-20

Wijffels, R.H., Barbosa, M.J., Eppink, M.H. (2010) Microalgae for the production of bulk chemicals and biofuels. *Biofuels, Bioproducts and Biorefining,* 4(3) 287-295

Yashodhara, B.M., Umakanth, S., Pappachan, J.M., Bhat, S.K., Kamath, R., Choo, B.H. (2009) Omega-3 fatty acids: a comprehensive review of their role in health and disease. *Postgraduate Medical Journal,* 85(1000) 84-90

Appendices

Blast alignment of known genes against Microbes NG sequences

Gene	Species	Accession number	Description
cobO	Pseudomonas sp.	32561033	cob(I)yrinic acid a,c-diamide adenosyltransferase
cobS	*Escherichia coli*	946520	cobalamin synthase
cysG	*Escherichia coli*	947880	fused siroheme synthase 1,3-dimethyluroporphyriongen III dehydrogenase and siroheme ferrochelatase/uroporphyrinogen methyltransferase. YraL is homologous to the C-terminal methyltransferase domain of CysG. cysG is transcribed from the nirB operon and from cysG promoters.
cbiA	*Yersinia enterocolitica*	4713594	cobyrinic acid a,c-diamide synthase
cbiK	*Yersinia enterocolitica*	4713584	cobalt chelatase
cobC	*Flavobacterium columnare*	34303201	alpha-ribazole phosphatase
cobU	*Escherichia coli*	912633	adenosylcobinamide kinase/adenosylcobinamide phosphate guanyltransferase
narL	*Escherichia coli*	7153481	two-component response regulator NarL; phosphorylated by NarQ; activates transcription of nitrate and nitrite reductase genes and represses transcription of fumarate reductase
napB	*Escherichia coli*	946698	The napB gene encodes the diheme cytochrome c550 protein which is complexed with NapA in the periplasm; it receives electrons from the membrane-bound proteins and passes them to NapA.
nirS	*Escherichia coli*	946698	The napB gene encodes the diheme cytochrome c550 protein which is complexed with NapA in the periplasm; it receives electrons from the membrane-bound proteins and passes them to NapA.
narG	*Escherichia coli*	945782	nitrate reductase 1, alpha subunit. Induced by anaerobiosis plus nitrate. The subunit of nitrate reductase A is the actual site of nitrate reduction and also contains the molybdenum cofactor .
nosZ	*Pseudomonas aeruginosa*	879824	nitrous-oxide reductase
norB	*Pseudomonas aeruginosa*	882193	nitric oxide reductase subunit B
nrfA	*Escherichia coli*	948571	cytochrome c552 nitrite reductase
hcp	*Escherichia coli*	946592	protein S-nitrosylase
norVW	*Escherichia coli*	914713	anaerobic nitric oxide reductase transcriptional regulator
nirK	*Pseudomonas sp.*	32564345	nitrite reductase, copper-containing

Table AP1: Summary of genes used to interrogate sequences from microbes NG with the associated NCBI accession numbers

Cobalamin genes

Bacterial isolate	cobO	cobS	cysG	cbiA	cbiK	cobC	cobU
ALG01-1a	✓	✓					
ALG01-1b	✓	✓					
ALG01-1c	✓	✓					
ALG01-14							
ALG01-16	✓	✓					
ALG01-19		✓					
ALG01-29	✓	✓					
ALG01-58		✓					
ALG01-64	✓	✓					
ALG01-87	✓	✓					

Table AP2: genes found within each of the tested isolates that are involved in the cobalamin synthesis pathway. Ticks indicate presence, cell left blank indicate absence.

Denitrification and ammonification

Bacterial isolate	narL	napB	nirS	narG	nosZ	norB	nrfA	hcp	norVW	nirK
ALG01-1a	✓	✓	✓	✓	✓	✓				
ALG01-1b	✓	✓	✓		✓	✓				
ALG01-1c	✓	✓	✓	✓	✓	✓				
ALG01-14			✓		✓					
ALG01-16			✓		✓					
ALG01-19	✓	✓	✓	✓						
ALG01-29		✓	✓							
ALG01-58			✓							
ALG01-64		✓		✓						
ALG01-87	✓	✓								

Table AP3: genes found within each of the tested isolates that are involved in ammonification and denitrification pathway. Ticks indicate presence, cell left blank indicate absence.