The Effects of Environmental Conditions on Lipid Content and Composition in Five Microalgal Species

by

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This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Statement of Contribution

The thesis content and analysis resulting in this document are primarily the product of the intellectual efforts of Sarah Ruffell. However, multiple co-authors participated in the creation and publication of these chapters, and their contributions are explained in detail below.

The following chapters have been published or submitted for review in refereed journals:

Chapter 2

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Chapter 3

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Sarah Ruffell, Dr. Kirsten Müller, and Dr. Brendan McConkey developed the concepts for the chapters, with contributions from Sue Watson in Chapter 4. Sarah Ruffell also designed the experiments, and analyzed the results, in addition to writing and revising the manuscript. Dr. Kirsten Müller and Dr. Brendan McConkey also contributed significantly to the discussion of the results and review of the manuscript. Within Chapter 3, Sara Packull-McCormick assisted with experimental design, data collection and analysis. Within Chapter 4, Sue Watson was also an active contributor to the development of the concept of the paper, as well as participating in the discussion of results and reviewing of manuscript. Dr. Ken Stark's lab processed all of the algal

fatty acid samples in Chapters 2 to 4. Dr. Roland Hall's lab quantified the algal pigments discussed in Chapter 3.

Abstract

The objective of this thesis was to examine algal lipids for industrially relevant characteristics, in addition to the broader implications within the food web. A focus was placed on the pharmaceutical and aquaculture applications of these naturally synthesized fatty acids. Chapter 2 focused on the use of fatty acids as antibacterial agents in which multiple fatty acids were identified that were able to inhibit bacterial growth. Among the most effective fatty acids were 10:0, 16:1, 18:3(n-6), 20:4(n-6) and 22:2(n-6). Light cycle, light intensity, temperature and nutrients were manipulated to identify growth conditions that produced the greatest concentration of antibacterial fatty acids. Five microalgae were tested (*Goniochloris sculpta, Boekelovia hooglandii, Phaeodactylum tricornutum, Chloridella simplex,* and *Rhodella maculata*). *Phaeodactylum tricornutum* produced the largest total concentration of antibacterial fatty acids at 206 mg g^{-1} dry weight (dw), however *B. hooglandii* had the higher lipid productivity due to biomass productivity. For the 29 free fatty acids examined, on average, as the number of double bonds increased the degree of inhibition due to the acid increased. Through the demonstrated antibacterial effects of certain fatty acids, common structural characteristics could be linked to commercially valuable antibacterial properties. Chapter 3 investigated the species, *B. hooglandii,* in more detail to determine its potential use as aquaculture feedstock. This chapter examined the biomass productivity and nutritional profile in terms of protein, lipid (34 fatty acids), carbohydrates, and pigments (7 pigments) to establish whether this alga is a suitable feed. Within the aquaculture experiments performed in Chapter 3, the biomass productivity of *B. hooglandii* (0.52 g L^{-1} day^{-1} dw) was greater than three commonly used aquaculture algal feeds. In addition, the nutritional profile of this species was suitable for bivalve larvae and juvenile oysters. This experiment also identified trends and relationships within the

nutritional profile; for example, a strong correlation was identified between total protein and growth rate. Identification of new algal feeds and correlations among nutritional properties provides insight into the dynamic nature of the nutritional profile during culturing. In Chapter 4, emphasis was placed on identifying trends in omega-3 fatty acid behaviour in species under varying light, temperature, and nutrient conditions, and examining how these differences could impact the food web. An overall trend was observed where lower light intensity, continuous light, vitamin B_{12} supplementation, and reduced temperatures resulted in greater omega-3 production. In addition, the omega-3 content of the five microalgae studied contained a large proportion of EPA (>50% of total fatty acid content). Further research is required to determine if these trends are broadly applicable. Of the five algae studied, *G. sculpta* produced the high omega-3 content and biomass productivity under the same growth conditions, resulting in an algal strain that can act as a rich source of omega-three in food webs and for industrial purposes. Overall, this thesis highlights 3 factors that were the most influential on algal performance: growth condition, species and lipid structure. By understanding how these three factors influence each other, experimental conditions can be established that allow for the increased productivity of desirable fatty acids, and a greater appreciation of the role of these fatty acids in industry and ecosystems.

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With endless gratitude to my amazing family: my loving father, for his unwavering support, advice and infinite sense of humour; my wonderful mother, for her vision and inspiration; my beautiful sister, for her immeasurable patience and love; and finally, Pepper who continues to fill my life with joy.

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List of Abbreviations

14:0	Myristic acid
15:0	Pentadecanoic acid
16:0	Palmitic acid
16:1	Palmitoleic acid
16:2	Hexadecadienoic acid
16:3	Hexadecatrienoic acid
16:4	Hexadecatetraenoic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
18:2(n-6)	Linoleic acid
18:3	Linolenic acid
18:3(n-3)	α-Linolenic acid
18:3(n-6)	γ-Linolenic acid
18:4(n-3)	Octadecatetraenoic acid
20:4(n-3)	Eicosatetraenoic acid
20:4(n-6)	Arachidonic acid
20:5(n-3)	Eicosapentaenoic acid
22:6(n-3)	Docosahexaenoic acid
AA	Arachidonic acid
ALA	α-Linolenic acid
DHA	Docosahexaenoic acid
dw	Dry weight
CLSI	Clinical and Laboratory Standards Institute
CPCC	Canadian Phycological Culture Centre
DGDG	Digalactosyl diacylglycerol

EPA	Eicosapentaenoic acid
FA	Fatty acid
HPLC	High performance liquid chromatography
g	Gram
GC	Gas chromatography
h	hour
L	Liter
mg	Milligram
min	Minute
mL	Milliliter
MGDG	Monogalactosyl diacylglycerol
mM	Millimolar
MW	Molecular weight
MRSA	Methicillin-resistant *Staphylococcus aureus*
NI	No inhibition
nm	Nanometer
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PUFA	Polyunsaturated fatty acid
SAG	Culture Collection of Algae at Göttingen University
SFA	Saturated fatty acid
SQDG	Sulfoquinovosyl diacylglycerol
TSA	Trypticase soy agar medium
TSB	Tryptic soy broth
UTEX	University of Texas at Austin

Chapter 1: Introduction

1.1. Lipids and fatty acids

Lipids are defined as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" (Christie, 2012). Fatty acids are chains of hydrocarbons that consist of a carboxylic group and an aliphatic tail (Lawrence, 2008). They are identified based on the carbon chain length and number of double bonds. Thus, a fatty acid with 18 carbons and three double bonds would be identified as 18:3. A carbon chain with no double bonds is termed a saturated fatty acid (SFA), with one double bond it is a monounsaturated fatty acid (MUFA), and with two or more it is a polyunsaturated fatty acid (PUFA). Different PUFAs can have the same carbon length and number of double bonds. Hence, PUFAs are also identified based on the location of the closest double bond to the methyl terminal (Sahena et al., 2009). Additionally, fatty acids can be further categorized into groups based on a common structure. For instance, omega-3 fatty acids have a double bond that is three carbons away from the methyl terminal (Figure 1) (Sahena et al., 2009). This distinction is denoted using the notation (n-3) or ω3, for example α-linolenic acid (ALA) is known as 18:3(n-3) or 18:3ω3 (Yongmanitchai, 1991).

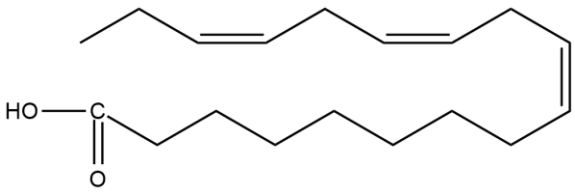

Figure 1. The structural diagram of ALA (modified from Bajpai, 1993).

1.1.1. Fatty acid synthesis pathways

The lipid synthesis pathway used by plants and vertebrates share many similarities, as well as significant differences (Wallis et al., 2002). Higher plants have Δ12 desaturase and Δ15 desaturase enzymes which allow them to synthesize 18:2(n-6) and 18:3(n-3) (Figure 2), whereas humans lack these two desaturases forcing them to rely on dietary sources for these two fatty acids (Schmid & Ohlrogge, 2002; Wallis et al., 2002). Once these fatty acids are ingested, humans are able to use other desaturases and elongases to synthesize a variety of fatty acids of different chain lengths and saturation. For example, the omega-3 fatty acid 20:4(n-6) can be synthesized from 18:2(n-6) via elongation of the carbon chain and increased unsaturation using Δ6 desaturase, elongase, and Δ5 desaturase (Wallis et al., 2002). From the omega-3 fatty acid 18:3(n-3), with progressive elongation and desaturation, a variety of fatty acids can be synthesized, including 20:5(n-3), and 22:6(n-3) (with the assistance of β-oxidation) (Wallis et al., 2002). Examining the pathway of fatty acid synthesis highlights the significant impact of certain fatty acids on fatty acids further along the synthesis pathway.

Within food webs, certain PUFAs are considered essential nutrients for consumers, due to the important role of these fatty acids in growth rates, egg production, and neural development within a population (Parrish, 2009). In addition, within humans the pathway used to convert 18:2(n-6) and 18:3(n-3) to 20:5(n-3) and 22:6(n-3), respectively, is not efficient and thus supplementing the diet with these fatty acids can help prevent fatty acid deficiencies (Surette, 2008). Primary producers have the ability to synthesize these fatty acids de novo, and act as a source of these essential fatty acids for consumers. Algae are examples of primary producers that can synthesize long chain fatty acids from 18:0, as seen in Figure 3. Thus, consumers can

selectively grazing on primary producers rich in essential fatty acids, in order to supplement their diet (Surette, 2008).

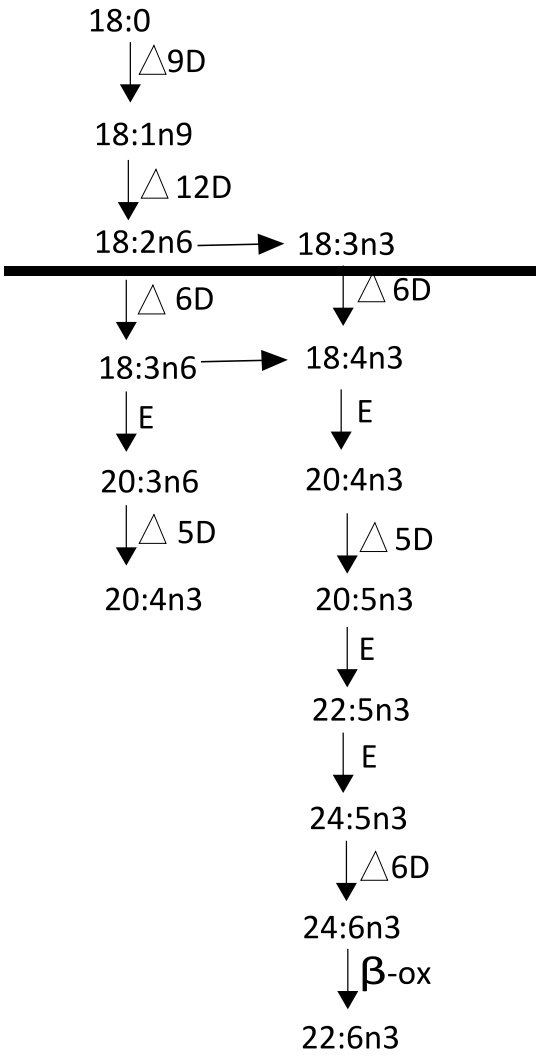

Figure 2. Fatty acid synthesis pathway involving plants (above line), and plants and humans (below line), where D represents desaturase, E represents elongase, and β-ox represents β-oxidation (modified from Wallis et al., 2002).

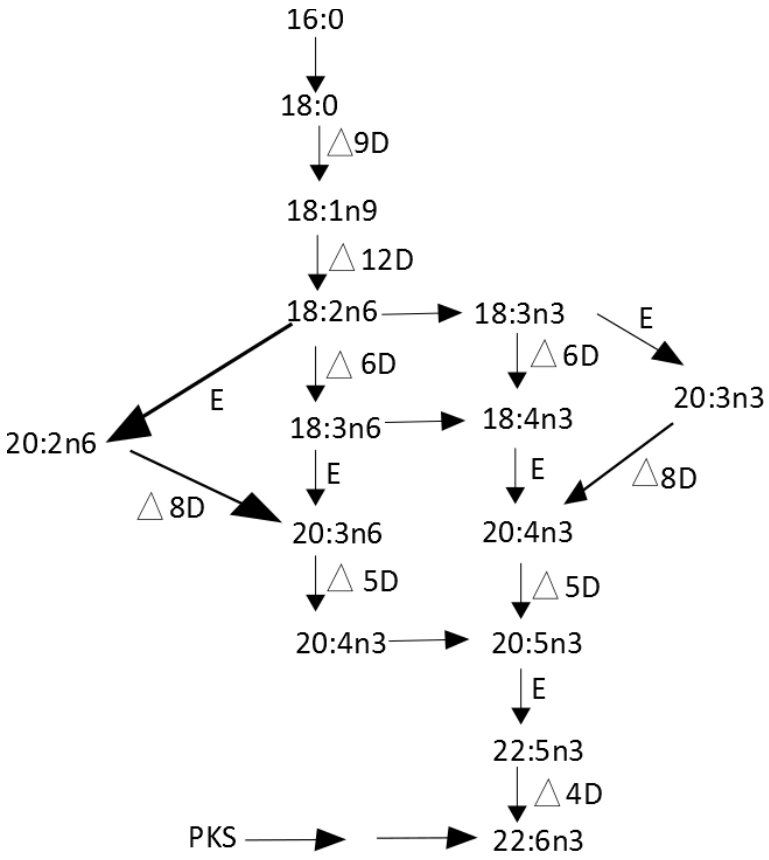

Figure 3. Fatty acid synthesis pathway in eustimatophytes, where D stands for desaturase and E represens elongase (modified from Harwood & Guschina, 2006).

1.1.2. The algal membrane

Photosynthesis is a process performed by plants that allows them to convert solar energy into glucose (Starr & Taggart, 1995). The initial steps in photosynthesis involve the capture of light and formation of ATP within the thylakoid membrane. This membrane consists of a lipid bilayer that has dynamic characteristics that allow it to modify its structure in response to environmental conditions (Starr & Taggart, 1995). Hence, the membrane must be strong and stable, while maintaining a certain level of fluidity in order to be able to adapt to protein

conformation, cell or organelle shape changes (Harwood & Russell, 1984). Most algal lipids are primarily in membranes and, under certain circumstances, in the cytoplasm as storage lipids (Wada & Murata, 2009). Polyunsaturated lipids are critical for synthesis of thylakoid membranes within the chloroplast and assembly of oligomeric components making up the photosynthetic structure (Harwood & Russell, 1984). Saturated fatty acid aliphatic chains, in the thylakoid membrane, result in a compact structure consisting of tightly packed, parallel fatty acids (Starr & Taggart, 1995). However, the rigid nature of the saturated structure is reduced when unsaturated fatty acids containing double bonds are present, resulting in a structure that is unable to pack as closely or tightly together, forming a membrane that is more fluid (Starr & Taggart, 1995).

Within the photosynthetic membrane, a variety of different lipid structures are present which play different structural roles. The thylakoid membrane, consists of glycerolipids, primarily monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG) and phosphatidylglycerol (PG), and proteins (Wada & Murata, 2009). The mitochondrial membrane is formed primarily from phospholipids [phosphatidylcholine (PC), PG, phosphatidylethanolamine (PE) and phosphatidylinositol (PI)] (Wada & Murata, 2009). Within an algal cell, there are a variety of lipids of different structures, with a variety of structural roles within the cell.

1.2 Algal fatty acids within a food web

Algal lipids play a variety of roles within a food web. For example, omega-3 fatty acids can be a source of essential nutrient for consumers (Guschina & Harwood, 2009). Essential fatty acids are compounds that are required by consumers that are unable to synthesize these compounds. Within the food web structure, as trophic level increases the proportion of individuals with the ability to synthesize essential fatty acids decreases (Kainz & Fisk, 2009).

These fatty acids enhance growth rates, survival and reproductive success for a range of consumers, including vertebrates and invertebrates. Many fatty acids are also precursors for eicosanoids, which assist in the regulation of physiological processes, such as spawning, egg-laying, and egg-production (Guschina & Harwood, 2009). Notably, some fatty acids can also function as semiochemicals, such as 18:3(n-3), and 20:5(n-3). However, further research is needed to understand the dynamic relationship between grazers, and the nutritional and semiochemical characteristics of certain fatty acids (Watson et al., 2009). The role of fatty acids as essential nutrients, eicosanoid precursors, and semiochemicals illustrates the complex nature of these fatty acids within a food web.

1.3 Commercial applications of algal fatty acids

1.3.1 Antibacterial effects of fatty acids

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an ongoing concern in medical institutions (Desbois et al., 2009). This type of bacteria has developed resistance to a variety of drugs, creating a challenge for researchers and the medical community (Desbois et al., 2009). The search for new sources of antibiotics has led to the discovery of antibiotic characteristics in algae (Desbois et al., 2009; Findlay & Patil, 1984; Ohta et al., 1994). Research on the diatom *Navicula delognei* demonstrated that the free fatty acids 18:4 and 16:4 have antibacterial effects on a variety of bacteria, including *Salmonella typhimurium, Staphylococcus aureus* and *S. epidermidis* (Findlay & Patil, 1984). Notably, 16:4 was as effective as ampicillin at inhibiting *S. typhimurium* (Findlay & Patil, 1984). During this same time period Lacey and Lord were able to show antibacterial properties of 18:3 on *S. aureus* (Lacey & Lord, 1981). In 1994, a study of 18:3 from *Chlorococcum* sp. demonstrated that low concentrations of this fatty acid were able to inhibit MRSA growth (Ohta et al., 1994).

Further research into the antibacterial properties of free fatty acids on MRSA demonstrated that 20:5, in *Phaeodactylum tricornutum*, can act as a successful antibiotic (Desbois et al., 2009). When compared with the common antibiotic ampicillin using disc diffusion, eicosapentaenoic acid (EPA) had a Minimum Bactericidal Concentration (MBC) of 40-80 µM while ampicillin had a MBC of 320-640 µM (Desbois et al., 2009). The fatty acid EPA can successfully inhibit gram positive human pathogens, such as *Bacillus cereus, B. weihenstephanensis, S. epidermidis, S. aureus*, and 2 MRSAs (MRSA252 and MRSA16a), and the gram positive fish/shellfish pathogen *Listonella anguillarum* (Desbois et al., 2009). The inhibitory nature of EPA towards these different bacteria is of high potential importance to the medical and aquaculture community (Desbois et al., 2009).

1.3.2. Aquacultural use of algal fatty acids

Microalgae contain a wide array of nutritional compounds that can be used for aquaculture purposes, such as proteins, lipids, pigments, and carbohydrates (Yaakob et al., 2014). For example as a source of natural pigments, algae can be used to improve the colouring, and in turn the market value, of consumer species. For instance, the diatom *Haslea ostrearia* is used to 'green' oysters, resulting in the gills and labial palp developing a blue-green pigment and oyster market value increasing by 40% (Spolaore et al., 2006). High synthesis rates of proteins, lipids, pigments, and/or carbohydrates allow algae to be a competitive feedstock in aquaculture. Traditionally within aquaculture, fishmeal was used as a protein source, however as the lack of sustainability and the presence of contaminants are growing concerns, demand for algae is increasing. In addition, algae are able to naturally synthesize essential fatty acids that increase the nutritional quality of the grazing organism (Yaakob et al., 2014). Due to the high nutritional

characteristics of algae, there is growing demand for microalgae within aquaculture (Yaakob et al., 2014).

1.3.3. Omega-3 fatty acids

1.3.3.1. The health benefits of omega-3s

Research has shown that omega-3 fatty acids play an important role in prenatal health, by assisting with the development of reproductive, nervous and optical systems. This is also true for infants, thus having a diet rich in omega-3s at a young age, as well as during pregnancy, is vital to a child's development (Yongmanitchai, 1991). Sufficient omega-3 intake is also important for adults; for example, clinical depression is linked to low omega-3 levels (Benatti, Peluso, Nicolai, & Calvani, 2004). A diet rich in omega-3 fatty acids has been linked to the prevention of retinal disease, cardiovascular disease, rheumatoid arthritis, Crohn's disease, and omega-3s are beneficial as anti-inflammatories, vasodilators, and hypolipidemic agents (Simopoulos, 1999; San Giovanni & Chew, 2005). An omega-3 enriched diet has demonstrated positive effects on human health, and the pharmaceutical and food industries are using algae as a source of these fatty acids (Simopoulos, 1999; Sidhu, 2003; San Giovanni & Chew, 2005; Lane et al., 2014).

1.3.3.2. Commercial demand for omega-3s

A report released in 2012 estimates that sales of omega-3 containing products will increase from $25.4 billion in 2011 to $34.7 billion in 2016, or 6% growth per annum (Leray, 2015). The Asia-Pacific market is quickly growing, becoming the second largest market behind North America (Leray, 2015). Over the next ten years, the following four markets will result in the continued growth of omega-3 products. The first is the increased demand for fortified infant formula, as a result of the increasing global population and expanding middle class in India and China (Leray, 2015). The second market is the increased demand for vegetarian algae-based

supplements, which is in part a result of India's growth (Leray, 2015). A third factor to consider is the expansion of generic products in the current markets, along with the introduction and creation of an omega-3 market in South America. Finally, the increased focus on high quality omega-3 supplemented pet foods will further increase the demand for omega-3s (Leray, 2015). As a result of this growth, there will be a greater market opportunity for algal products.

1.4. Choosing the appropriate algae

When selecting an algal species, a variety of factors must be taken into consideration. Since this project focuses on PUFA content, this needs to be a major factor to consider along with growth rate. Algal omega-3 content not only varies by phylum but also between genus and species. By closely examining the different algal groups, a better understanding of which groups show promise can allow for a more informed selection of an appropriate test organism. Lang et al. (2011) examined over 2000 strains of algae from the SAG culture collection. They determined that the Chlorophyceae typically contain very little omega-3s and the few that synthesize omega-3s produce primarily 18:3, with a third of species containing 5% or more of total FA content as 18:3 (Lang et al., 2011). However, an exception to this was the Chlorophyta, *Chlorococcum novae-angliae*, which had the second highest DHA level of the over 2000 strains analyzed, at 18.9% of total fatty acid content (Lang et al., 2011). They also noted that the Bacillariophyceae (diatoms) typically have high omega-3, in the form of EPA and sometimes DHA (Lang et al., 2011; Yongmanitchai, 1991). The DHA content was particularly high in Dinophyceae, Haptophyta and Euglenoids (with greater than 60% of strains containing at least 5% DHA), while EPA was generally quite high in Eustimatophyceae, Glaucophyta, Xanthophyceae and Rhodophyta (with greater than 81% of strains containing at least 10% or more of total FA as EPA) (Lang et al., 2011; Yongmanitchai, 1991). Of the entire SAG

collection studied, the Rhodophyta *Compsopogonopsis leptoclados* had the highest EPA content at 52.4% of total fatty acid content (Lang et al., 2011). During algal selection, taxonomy can provide guidance based on general trends in the different taxonomic groups, however fatty acid content still varies greatly among species.

1.5. Growth conditions

Appropriate growth conditions are vital to maximizing the growth rate of an algal species. This can become quite a challenge as the optimal conditions can vary significantly depending on the specific species. Understanding the impact of growth conditions on algal populations is key to understanding their role in a food web and utilizing a strain for industrial applications.

1.5.1. Temperature

Temperature is an important factor that influences the ability of an algal species to live in an ecosystem. For example, temperature can modify the rate of nutrient uptake and carbon absorption, which can result in a change in the organisms' physiology (Stengel et al., 2011). If the temperature is within an optimum range, the physiological changes that occur can be advantageous, such as increased growth rate, however if temperature moves out of this range, growth rate will suffer (Gotham & Rhee, 1981). In addition, the optimum temperature range can vary within class (Guscina & Harwood, 2012). When comparing two species within the Bacillariophyceae, *Detonula confervacea* shows optimum growth between 10-13°C while *Ditylum brightwellii* experiences optimum growth between 23-28°C (Eppley, 1972). Thus, temperature is a growth condition that can influence cell physiology and growth rate (Eppley, 1972; Gotham & Rhee, 1981; Stengel et al., 2011).

1.5.2. Light intensity and light:dark cycle

Light plays a key role in photosynthesis as the energy source responsible for photoautotrophic growth (Guschina & Harwood, 2012). The maximum growth rate of the algal culture is dependent on light conditions reaching saturation intensity. Higher light intensities result in photoinhibition due to damage to the chloroplast lamellae (Juneja et al., 2013). The rate of algal growth is also a function of algal species. A study working with the diatoms *Cyclotella meneghiniana* and *Stephanodiscus binderanus* determined that the light:dark (L:D) cycles resulting in optimal growth were 20:4 and 12:12, respectively (Sicko-Goad & Andresen, 1991). The light cycle has a major impact on an algal cell's access to light, in addition to their growth and viability. Studies have shown that longer photoperiods result in higher cell division (Foy et al., 1976; Juneja et al., 2013). However, to prevent photoinhibition from occurring, a dark cycle is required to allow for the complete conversion of captured photons to chemical energy (Juneja et al., 2013). Thus, the effects of light (intensity and cycle) must be taken into consideration when maximizing algal growth.

1.5.3. Nutrients

There are many different aspects within growth media that must be considered, including micronutrients, macronutrients, vitamins, and salinity (Stengel et al., 2011). Thus, for successful growth, all of these components must be present at appropriate concentrations within the media.

1.5.3.1. Nitrogen, Phosphorous and Silicate

To produce optimum growth, major limiting nutrients must co-exist in appropriate ratios. Nitrogen and phosphorous are common limiting nutrients, while silica is specifically limiting for diatoms (Graham & Wilcox, 2000; Juneja et al., 2013; Lavens & Sorgeloos, 1996). For example, for marine algae an appropriate estimated ratio is 106C:16N:1P, and for algae requiring silica a

ratio of 106C:16Si:16N:1P is close to optimal (Graham & Wilcox, 2000). As a baseline, studies have shown that freshwater algae typically require a minimum of 20 µg L^{-1} of phosphorous (Yongmanitchai, 1991). Notably, algae have the ability to store certain nutrients when growing in a nutrient rich environment, for example dinoflagellates have the ability to store phosphorous. This capability can influence the capacity of the algal cell to cope with nutrient depleted conditions (Graham & Wilcox, 2000). Thus, the nutritional requirements and access an algal cell has to limiting nutrients can greatly affect growth rate.

1.5.3.2. B Vitamins

Vitamin B has been shown to be an important growth factor for a large variety of algae (Panzeca et al., 2001). For example, *Ochromonas danica* is reliant on vitamins, including vitamin B, for rapid growth. The majority of dinoflagellates require B_{12} in their diets (Graham & Wilcox, 2000). In addition, if B_1 and B_{12} vitamins are combined with iron, a synergistic effect on growth is observed (Bertrand et al., 2007; Panzeca et al., 2001).

1.5.3.3. Iron

Iron has been shown to have a positive impact on phytoplankton growth, and has been linked to the production of algal blooms (Panzeca et al., 2001). A range of different factors can influence the effects of iron on algal growth, such as taxonomy, and trophic state. Research has shown that chrysophyceans have a high iron requirement relative to other algae (Graham & Wilcox, 2000). Another factor that influences the concentration of iron necessary for growth is whether the organism prefers eutrophic or oligotrophic environments (Moss, 1973). Taxonomy, and trophic state are just a few examples of factors that will influence the growth promoting properties of iron.

1.6. Manipulating fatty acid content

In order to maintain normal functioning under a variety of growth conditions, an algal cell will alter its fatty acid composition. Membrane fluidity is a principle characteristic of a cell that must be maintained to sustain normal operation. Fluidity can influence the ion permeability of a membrane and electron transport between photosynthetic complexes, directly impacting the photosynthetic efficiency of the cell (Morgan-Kiss et al., 2006; Guschina & Harwood, 2013). Thus, growth conditions can greatly influence the lipid composition of the algal species and affect cell functioning.

1.6.1. Effect of temperature on fatty acid profiles

The effect of temperature on fatty acid content and composition is relatively well understood in comparison to the effect of other abiotic factors. An algal cell responds in a variety of different ways to adapt to shifts in temperature. The cellular response involves modification to fatty acid chain lengths, degree of unsaturation, lipid class concentrations, and membrane lipid:protein ratio (Williams, 1998). The response will vary depending on the algae, however all responses are closely linked to the homeoviscosity principle which states that organisms will modify membrane composition to maintain a constant degree of fluidity (Williams, 1998). For example, algal species that live in low temperature habitats typically have membrane lipids with high unsaturated fatty acid content (i.e. low melting point fatty acids) and algae in high temperature habitats have highly saturated content (ie. high melting point fatty acids) (Harwood & Russell, 1984; Juneja et al., 2013). The ability of the algal cell to acclimatize to different temperatures allows for these species to function under a variety of different growth conditions.

1.6.2. Effect of salinity on fatty acid profiles

The relationship between fatty acid profiles in marine algae and NaCl is well established, however this is not the case with freshwater algae (Andersen, 2005; Zhila et al., 2011). In the freshwater Bacillariophyceae, *Navicula tennelloides*, increasing salinity from 0.5 to 1.7 M caused an increased lipid content but further increase to 2.5 M decreased lipid content (Al-Hasan et al., 1990). A study using two different strains of the freshwater chlorophyte, *Botryococcus braunii* (race-A), examined the impact of salt concentration on fatty acid content. Under high salt conditions, the UTEX strain exhibited decreased 18:1, while the Göttingen culture had increased the contentration of 18:1. For both strains the saturated fatty acid content decreased with increasing salinity, while the PUFA content increased. However, the extent to which PUFA increased varied significantly with the strain (Vazquez-duhalt & Arredondo-vega, 1991; Zhila et al., 2011). In comparison, marine algae have a higher salinity tolerance, thus an increase in lipid content is seen at much higher salinities. For example, the marine alga *Dunaliella salina* showed increased lipid content at 3.5 M NaCl, as compared to 0.5 M (Guschina & Harwood, 2006). These increased lipid concentrations could be the result of an osmotic response to liquid leaving the cell. To avoid damage to the cell from fluid loss, the cell wall compensates by increasing unsaturated fatty acid content and maintaining fluidity (Katz et al., 2007). Thus, more research is needed to look at the response of an algal cell to environmental conditions with respect to habitat and salinity concentrations.

1.6.3. Effect of light on fatty acid profiles

The presence of light promotes increased enzyme activity and MGDG production. Under light-limited conditions, there is an increase in total cellular content of polar lipids, MGDG and DGDG, and an increase in the proportion of PUFA content. This is hypothesized to be caused by

the increased proliferation of thylakoid membranes in reaction to the limiting light conditions, and has been demonstrated in green, red, and brown algae, as well as diatoms (Hamilton et al., 2015; Jacob-Lopes et al., 2008; Wada & Murata, 2009). During high light intensities, the algal cell will respond by increasing polar lipid or storage lipid content (Napolitano, 1994; Stumpf et al., 1987). Within the diatoms and marine algae, low light typically results in high EPA and low DHA levels (Thompson et al., 1990). The explanation for the positive effect of high light on DHA content is currently unknown. Typically, high light intensity causes the thylakoid membrane in chloroplasts to decrease, which would be expected to result in decreased DHA. However, it is hypothesized that the high DHA content is a result of increased DHA in other membranes (Tzovenis et al., 1997). Access to light and light intensity are factors that can greatly influence thylakoid synthesis, resulting in shifts in the fatty acid profile of the algal cell (Jacob-Lopes et al., 2008).

1.6.4. Effects of nutrients on fatty acid profiles

Nitrogen and phosphorous are limiting nutrients that are necessary for algal growth. However, deficiencies in nitrogen or phosphorous may produce desired results when high lipid content is required (Juneja et al., 2013). For example, under nitrogen deficient conditions, *Scenedesmus obliquus* exhibited decreased chlorophyll content, resulting in decreased levels of protein, polar lipids and PUFAs in the chloroplast membrane (Harrison et al., 1990; Piorreck & Pohl, 1984). However, nitrogen deficiency generally results in increased total lipid content (Guschina & Harwood, 2006). During nitrogen replete conditions PUFAs (notably C16 and C18) dominate and for some algae (*Scenedesmus* sp. and *Chlorella* sp.) EPA content increases, while for others (*B. braunii*, *Dunaliella bardawil* and *D. salina*) EPA content decreases (Piorreck & Pohl, 1984; Yongmanitchai & Ward, 1991). During phosphorous deficient growth conditions,

typically algal lipid content increases. For example, *S. obliquus* experiences an increase in lipid content by 10-30% under phosphorous deficient conditions (Tan & Lin, 2011). During these deficient conditions, the rate of phospholipid synthesis also decreases, while the synthesis rate of other lipids is unchanged (Harwood & Russell, 1984). A significant amount of PUFAs are observed within phospholipids in the membrane, thus a phospholipid decrease from phosphorous deficiency could have a deleterious effect on PUFA levels (Harrison et al., 1990). Thus, the effects of nitrogen and phosphorous concentration on the algal fatty acid profile, varies with the lipid type.

1.6.5. Effect of vitamin B_{12} on fatty acid profiles

Typically vitamin B_{12} (cobalamin), thiamine, or biotin may be required for growth (Andersen, 2005), though few algae require all three. A symbiotic relationship has been identified between bacteria and algae, with bacteria synthesizing cobalamin that is then taken up by auxotrophic algae and bacteria receiving fixed carbon (Croft et al., 2005). Certain algal species are able to synthesize cobalamin, while others require an exogenous source of this vitamin (Croft et al., 2005). For example, Yongmanitchai and Ward (1991) studied the effects of different culture conditions on growth rate and omega-3 content. Although, the addition of 100 ng L^{-1} of vitamin B_{12} did not significantly influence growth rate, EPA content increased by 65%. Further research is still needed in order to further explore the impact of cobalamin on algal lipid content.

1.7. Research objectives

The objectives of this thesis were to examine the industrial applications and food web implications of algal fatty acids. Chapter 2 investigates the antibacterial properties of algal lipids. Chapter 3 focuses on *B. hooglandii,* which consistently produced the greatest growth rate and

desirable lipid content, and the potential use of this alga within aquaculture. Chapter 4 examines the role omega-3 fatty acids play in the health of organisms within food webs, in addition to potential application in industry.

1.7.1. Chapter 2 objectives

Chapter 2 investigates the antibacterial characteristics of 29 pure fatty acids that are naturally synthesized by algae. Surface zone inhibition assays were performed to quantify the inhibitory nature of these fatty acids. In order for all of these commercial applications to be realized in the market, growth of algae containing high concentrations of these target fatty acids is key. Chapter 2 also identifies growth conditions that result in increased production of these target antibacterial fatty acids. Thus, by understanding the environmental conditions that result in high growth, algae can play a much greater role in the world of antibiotics.

Hypothesis: Polyunsaturated fatty acids are more inhibitory towards gram negative and gram positive bacteria, compared to monounsaturated and saturated fatty acids.

1.7.2. Chapter 3 objectives

Chapter 3 focuses on the nutritional profile and growth curve of one microalgal species, *B. hooglandii*, for potential aquaculture application. Through the analysis of protein, pigment, carbohydrate and fatty acid content, the appropriateness of this alga as a nutritional source can be identified. When combined with the growth curve of a culture, key times can be identified when biomass collection would be most appropriate for a specific aquaculture organism. In addition to this, Chapter 3 identifies trends between nutritional characteristics, and growth stages. This information can allow the aquaculture industry to make more informed decisions regarding potential nutritional compromises and appropriate sampling periods.

Hypothesis: The microalga *B. hooglandii* has a polyunsaturated fatty acid and protein content which would make it a suitable aquaculture feed for bivalve larvae.

1.7.3. Chapter 4 objectives

Chapter 4 examines the effects of light, temperature and nutrient conditions on omega-3 production. The adaptive mechanisms used by the cell to maintain function under a variety of growth conditions are identified and discussed in the context of omega-3 production

Hypothesis A: The higher light intensity of 130 µmoles m^{-2} s^{-1}, relative to 45 and 80 µmoles m^{-2} s^{-1}, will result in greater biomass productivity and total omega-3 fatty acid content for species with a light saturation point close to 130 µmoles m^{-2} s^{-1}.

Hypothesis B: Supplementation with vitamin B_{12} will result in greater omega-3 fatty acid productivity for the algal species that require vitamin B_{12}.

Hypothesis C: As temperature decreases relative to conditions analogous to the algal species natural habitat, omega-3 fatty acid content will increase, due to synthesis of unsaturated fatty acids needed to maintain membrane fluidity.

Chapter 2: Comparative assessment of microalgal fatty acids as topical antibiotics

This chapter contains material published in The Journal of Applied Phycology (Ruffell, S. E., Müller, K. M., & McConkey, B. J., 2015. Comparative assessment of microalgal fatty acids as topical antibiotics. *Journal of Applied Phycology*, DOI 10.1007/s10811-015-0692-4.)

2.1. Introduction

The increasing resistance of bacteria to traditional antibiotics has led to the search for new and more effective antibacterial agents (Desbois et al., 2009). Studies focusing on fatty acids as antibacterial agents have shown that certain bacteria have lower rates of resistance towards fatty acids compared to conventional antibiotics (Desbois et al., 2009; Desbois & Smith, 2010; Petschow et al., 1996). Algae have considerable potential as a source of antibacterial fatty acids due to a diverse fatty acid composition, high growth rate, high lipid content and the ability to grow under a range of conditions (Gong et al., 2011). In addition, research by Pratt (1942) demonstrated antibacterial properties in the green alga, *Chlorella vulgaris.* This led to further studies identifying similar properties in other algae and isolating fatty acids within algae as the main antibacterial agent (Desbois et al., 2009; Ohta et al., 1994; Sun et al., 2003). For example, using disc-diffusion Findlay and Patil (1984) noted that fatty acids 18:4 and 16:4 in the diatom *Navicula delognei* had antibacterial effects on a variety of bacteria, such as *Salmonella typhimurium, Staphylococcus epidermidis* and *S. aureus*. More recently, the omega-3 fatty acid 20:5(n-3) extracted from *P. tricornutum* (Phaeodactylaceae), was shown to be a successful antibiotic against MRSA (Desbois et al., 2009).

The purpose of this study was to identify highly antibacterial fatty acids in algae and create growth conditions suitable for accumulation of these fatty acids within the cell. The present study measured inhibition of bacterial growth as an indicator of the antibacterial activity of twenty-nine pure fatty acids present in algae on two common opportunistic pathogenic bacterial species, *Escherichia coli,* and *S. aureus*. *Escherichia coli* (gram-negative) can result in a variety of human diseases such as food poisoning, wound infection, septicemia, and endocarditis (Sussman, 1997). Strains of the gram-positive bacterium *S. aureus* are becoming increasingly resistant to commercial antibiotics (Desbois et al., 2009).

To study the effects of growth conditions on antibacterial fatty acid content, two marine algae *Rhodella maculata* (Rhodellophyceae) and *P. tricornutum* (Bacillariophyta), and three freshwater algae, *Boekelovia hooglandii* (Synurophyceae), *Goniochloris sculpta* (Xanthophyceae) and *Chloridella simplex* (Xanthophyceae) were selected. These five algae were chosen due to the expected high levels of fatty acids that may have antibacterial properties (Lang et al., 2011; Sheehan et al., 1998; Yongmanitchai, 1991; Desbois et al., 2009). The present study identified antibacterial fatty acids in algae as well as corresponding growth conditions that can be used to produce fatty acids for topical antibiotics.

2.2. Materials and methods

2.2.1. Bacterial strains and cultivation

The bacterial strains used in this study were *E. coli* (Migula) Castellani and Chalmers (ATCC 11303), and *S. aureus* Rosenbach (ATCC 6538) (provided by the Departmental Microbiology Culture Collection, University of Waterloo). The algae *B. hooglandii* Nicolai et Baas Becking (CPCC 484) (Synurophyceae) and *P. tricornutum* Bohlin (CPCC 162) (Bacillariophyta) were purchased from the Canadian Phycology Culture Collection (CPCC) at

the University of Waterloo (Waterloo, ON, Canada), and *G. sculpta* Geitler (SAG 29.96) (Xanthophyceae), *R. maculata* L. V. Evans (SAG 45.85) (Rhodellophyceae) and *C. simplex* Pascher (SAG 51.91) (Xanthophyceae) were purchased from the Culture Collection of Algae at Göettingen University (SAG) (Göttingen, Germany).

All the bacterial strains were sub-cultured three times as a purity check before beginning the experiment. This procedure was done by transferring one healthy colony to a new plate of media using streak plating. Once colonies were established, this was repeated two more times before the culture was used. Cultures were incubated in the dark in two Can-trol Environmental Systems Limited Model CES-37 (Markham, ON, Canada) on plates of Trypticase Soy Agar medium (TSA; Sigma-Aldrich) at 37°C (for *E. coli* and *S. aureus*). A colony was transferred to liquid medium, Tryptic Soy Broth (TSB), and incubated for 24h to use for spread plating.

2.2.2. Antibacterial assays

Fatty acids used in this experiment were purchased from Nu-Chek Prep Inc. (Elysian, MN, USA) and had a purity greater than 99%, with the exception of arachidonic acid with greater than 90% purity. Fatty acid stock solutions of 100 mg mL^{-1} were prepared by dilution with 100% ethanol. Certain fatty acids [10:0, 14:0, 16:0, 17:0, 18:0, 20:0, 22:1(n-9), 23:0, 24:0, and 24:1(n-9)] were heated to 50°C to improve solubility in alcohol. Seven millimeter diameter paper discs [Becton, Dickinson and Company (Mississauga, ON, Canada)] were loaded with appropriate volumes of stock solution to result in a disc containing 2000 µg, 800 µg, 250 µg or 25 µg of fatty acid. Tetracycline was used as a positive control and as a basis for comparison to determine how effective the fatty acids are relative to a typical dose of a common antibiotic (Heman-Ackah, 1976). A tetracycline stock solution of 50 mg mL^{-1} was diluted with sterile water and used to prepare 5 µg and 30 µg discs of tetracycline, the latter of which is the Clinical and Laboratory

Standards Institute (CLSI) standard (Clinical and Laboratory Standards Institute, 2006). Ethanol soaked discs were additionally prepared as solvent controls. Disk diffusion was selected as the test method to be consistent with standard methodology used by the CLSI for evaluating antibiotic effectiveness against bacteria (Sader et al., 2007).

Spread plating was used to disperse the bacteria on the plate. Loaded discs and bacterial plates were placed in a biosafety cabinet at room temperature until dry, then loaded discs were added to the bacterial plates. Bacteria were incubated in the dark, on plates of TSA media for 48 h at 37°C (for *E. coli* and *S. aureus*). Inhibition was quantified by measuring the zone of clearing around the discs to the nearest millimeter. The zone of inhibition was measured at 24 h and 48 h during incubation (Dawson et al., 2002; Naviner et al., 1999). All treatments were repeated 4 times.

2.2.3. Algal culture conditions

Algal cultures were grown in a Model E-36HO growth chamber (Percival Scientific, Inc., IA, United States) with 55 Watts PlusRite Compact Fluorescent FTL55/2G11/841 (CA, United States). Cultures were sub-cultured with new media every two weeks using aseptic technique. Pre-experiment growth conditions follow the methods described by the source culture collections for each alga. All stock cultures were grown in Erlenmeyer flasks in 50 mL of medium, not aerated/shaken, at 18 ± 1°C with a light intensity of 80 ± 5 µmol photons m^{-2} s^{-1}, and a 17h:7h light:dark cycle. The following media were used for culture maintenance: F/2 for *P. tricornutum* and *B. hooglandii* (Guillard, 1975), SAG Bold's Basal Medium (SAG BBM) for *G. sculpta*, SWES for *R. maculata* and ESP for *C. simplex* (Experimental Phycology and Culture Collection of Algae (SAG), 2014). When a growth condition was being varied within experiments all other parameters were kept at culture collection conditions.

For the growth condition experiments, sterile media was inoculated with cultures on Day 0 and culture optical densities were measured every two days over an 8 day period. On day 8 each algal sample was rinsed with dilute salt solution (9 g L^{-1} NaCl) two times and the rinsed pellet was stored at -80°C. The samples were then freeze dried using a Thermo Savant Model MODULYOD-115 (Holbrook, NY, US) prior to fatty acid analysis. Sampling was designed so algal cultures would be in exponential phase, however cultures could potentially be in a different growth phase due to changes in experimental growth conditions. Light absorbance measurements were taken on a Thermo Fisher Scientific Model Spectronic 200 spectrophotometer. *Phaeodactylum tricornutum* and *B. hooglandii* were measured at 600 nm, while *G. sculpta*, *R. maculata* and *C. simplex* were measured at 680 nm. Different wavelengths were optimal for the different species due to difference in pigmentation of the algae, with golden-brown algae measured at 600 nm (Yongmanitchai & Ward, 1991) and green algae measured at 680 nm (Jones et al., 2013). Standard curves were used to estimate culture density in g L^{-1} dry weight (dw). To create the standard curves algal samples at a range of densities were measured using absorbance and then samples were filtered and dried to establish a corresponding g L^{-1} dw. The following growth conditions were studied: light cycle (17h:7h and 24h:0h L:D), light intensity (45, 80 and 130 µmol photons m^{-2} s^{-1}) using a 55 Watts PlusRite Compact Fluorescent FTL55/2G11/841 (CA, United States), temperature (15°C, 20°C and 25°C), media type (SAG BBM, CPCC BBM, SWES, F/2 and ESP) and additional vitamin B_{12} (0, 10 and 100 ng L^{-1}). The growth of some algae is augmented by certain vitamins, notably vitamin B_{12}, where vitamin supplementation can allow for increased growth rates (Watanabe, 2005). The F/2 and SWES are saltwater media and include silica and soil extract, respectively (Guillard, 1975; SAG, 2014). The ESP is a freshwater medium containing soil extract and 0.1% proteose peptone (SAG, 2014). The CPCC BBM and

SAG BBM are freshwater media that differ in the trace metals, with respect to elements present and the concentration (Stein, 1980; SAG, 2014). The SAG BBM also contains a higher concentration of vitamins (Stein, 1980; SAG, 2014). All experiments were repeated four times.

The first component of this study was an antibacterial assay that tested the inhibitory effect of free fatty acids, whereas the second component of the study focused on growth conditions that increased the content of fatty acids in the form of triglycerides. For analysis, algal triglycerides, as well as fatty acid derivatives such as diglycerides and phospholipids, were converted to methylated fatty acids using the transesterification procedure described below.

2.2.4. Extraction and analysis of lipids

Extraction and analysis were performed by Dr. Ken Stark's lab (Waterloo, ON, Canada) using Zuñiga et al.'s (2012) method of 'direct transesterification with convectional heat'. Fatty acid content was quantified as fatty acid methyl esters and quantified using direct transesterification and gas chromatography. Derivatization occurred by adding 14% boron trifluoride in methanol and hexane to freeze dried algal powder, and the mixture was incubated at 90°C for 1 h (Zuñiga et al., 2012). Gas chromatography (Varian GC 3900) was used to quantify the fatty acid methyl esters (Zuñiga et al., 2012). The gas chromatograph used hydrogen gas as the carrier and flame ionization was used as a detector. A film capillary thickness of DB-FFAP 15 m x 0.10 mm i.d. x 0.10 µm was used as the separation column. Retention times were compared to standards in order to identify peaks (Metherel et al., 2013).

2.2.5. Statistical analysis

Algal culture biomass on day 0 and 8 were used to calculate the biomass productivity of an algal culture over 8 days. Biomass productivity was estimated using the equation Biomass

productivity (g L^{-1} day^{-1}) = (X_2-X_1) (t_2-t_1) $^{-1}$, where X_2 was day 8 biomass (g L^{-1}), X_1 was day 0 biomass (g L^{-1}), and t was the sample day (t_2=8 and t_1=0) (Hempel et al., 2012).

Statistical analyses were performed for antibacterial zone inhibition assays and algal growth conditions using SigmaPlot (San Jose, CA, US) and included independent t-tests or one-way ANOVAs, as appropriate. The t-test along with the Holm-Sidak method was used for light cycle data. The one-way ANOVA was used on the data from the inhibition assay, light intensity, temperature, media and vitamin B_{12} experiments. If the parametric one-way ANOVA failed the equal variance test or the normality test, the non-parametric Kruskal-Wallis One Way ANOVA on Ranks was used. The Kruskal-Wallis test was used when the residuals were not normally distributed and did not agree with the assumptions of the one-way ANOVA. When a one-way ANOVA showed a statistically significant difference, multiple comparisons were performed using the Holm-Sidak or Tukey test for parametric or non-parametric ANOVAs, respectively. Notation within Tables 3 through 7 follows the same notation to indicate statistically significant differences among treatments. Where statistically significant differences are present, each distinguishable group is given a letter (A, B or C). The superscript letters indicate the presence or absence of a significant difference between the data points with a single taxa and growth condition. Two values with the same superscript letter are not significantly different ($p>0.05$), while values with different superscript letters are significantly different ($p<0.05$). The sample size for each growth condition experiment was n=4 and repeats were placed in randomized locations within the incubator. Reported plus/minus values (±) refer to the standard deviation, based on the four repeats.

2.3. Results and Discussion

The antibacterial activity of twenty-nine free fatty acids was determined and the growth conditions (light cycle and intensity, temperature, nutrients, and temperature) of the five algae species investigated were manipulated to increase the content of inhibitory fatty acids in these algae. Of the twenty-nine fatty acids studied, thirteen were inhibitory towards growth of gram-positive *S. aureus* at 24 and 48 h, whereas three fatty acids [10:0, 22:2(n-6) and 18:3(n-3)] were inhibitory towards the growth of gram-negative *E. coli* and only during the first 24 h (Table 1). This is supported by studies demonstrating that gram-negative bacteria are much more resistant to antibiotics than gram-positive (Kabara et al., 1972; Russel, 1991). Some studies have suggested that gram-negative bacteria's low inhibition is a result of higher resistance to compounds that can influence the electron transport chain (Nagaraja, 1995). Others hypothesized that this resistance is a result of lipopolysaccharides in the cell wall of gram-negative bacteria that prevent the fatty acids from influencing the membrane (Kabara et al., 1972).

Table 1 displays the response of *S. aureus* to candidate inhibitory fatty acids, with 250 µg of 20:4(n-6) creating the largest inhibition zone of 25.2 mm, exceeding that of 30 µg of tetracycline (19.3 mm) ($p<0.001$). Fatty acids 16:1 and 18:3(n-6) at 250 µg had comparable zones of inhibition as compared to the 30 µg tetracycline control, with inhibition zones of 19.4 mm and 18.8 mm respectively. Two fatty acids, 18:2(n-6) and 22:6(n-3) had a reduced inhibition zone at 800 µg compared to 30 µg tetracycline, but not significantly different than 5 µg of tetracycline (14.7 mm), with 17 mm and 15.3 mm inhibition zones respectively. All 5 of the above fatty acids were inhibitory at 24 and 48 h.

The resistance of bacteria to a particular fatty acid is dependent on the bacteria being assayed and the fatty acid chemical structure characteristics. This was shown previously in a

Table 1. The lowest fatty acid dose (25 µg, 250 µg, 800 µg or 2000 µg) and resulting growth inhibition zone (mm, in brackets) for two representative species of bacteria.

Bacteria/ Lowest effective dose (µg)	*Escherichia coli*		*Staphylococcus aureus*	
Fatty Acids[a]	24 h	48 h	24 h	48 h
10:0	2000 (10.3±1.3)	NI	800 (9.8±1.7)	800 (8.3±0.5)
12:0	NI	NI	2000 (9.5±0.6)	NI
14:0	NI	NI	NI	NI
16:0	NI	NI	NI	NI
17:0	NI	NI	NI	NI
18:0	NI	NI	NI	NI
20:0	NI	NI	NI	NI
23:0	NI	NI	NI	NI
24:0	NI	NI	NI	NI
14:1	NI	NI	2000 (11.5±2.4)	2000 (13.5±0.6)
16:1	NI	NI	250 (19.4 ± 2.8)	800 (18.4±3.6)
18:1(n-7)	NI	NI	NI	NI
18:1(n-9)	NI	NI	NI	NI
20:1(n-9)	NI	NI	NI	NI
22:1(n-9)	NI	NI	NI	NI
24:1(n-9)	NI	NI	NI	NI
18:2(n-6)	NI	NI	800 (17.0±1.4)	800 (16.3±1.0)
18:3(n-6)	NI	NI	250 (18.8±2.4)	250 (16.0±2.9)
20:2(n-6)	NI	NI	NI	NI
20:3(n-6)	NI	NI	800 (11.5±1.0)	800 (10.8±0.5)
20:4(n-6)	NI	NI	250 (25.3±3.8)	250 (19.5±2.4)
22:2(n-6)	800 (10.5±1.3)	NI	NI	NI
22:4(n-6)	NI	NI	250 (8.8±0.5)	2000 (9.0±1.2)
22:5(n-6)	NI	NI	250 (12.5±1.0)	800 (11.5±0.6)
18:3(n-3)	25 (8.5±1.0)	NI	250 (13.1±1.4)	250 (12.0±1.4)
20:3(n-3)	NI	NI	2000 (8.5±0.6)	NI
20:5(n-3)	NI	NI	250 (13.6±1.4)	250 (11.4±0.8)
22:5(n-3)	NI	NI	800 (9.3±0.5)	2000 (9.3±0.5)
22:6(n-3)	NI	NI	800 (15.3±2.2)	800 (15.0±1.4)

[a] Arachidonic acid is >90% purity; all other fatty acids are > 99% purity

study by Galbraith et al. (1971), which identified a positive correlation between a fatty acid's inhibitory effect, chain length and number of double bonds. Within the present study, as double bonds increased from 18:1 to 18:3, 20:1 to 20:5 or 22:1 to 22:6 the inhibitory response of *S. aureus* shifted from no inhibition to varying levels of inhibition. In addition, degree of unsaturation may account for the effectiveness of some of the most antibacterial fatty acids in this study 22:2(n-6), 18:3(n-6) and 20:4(n-6). The high level of inhibition may be a function of both long carbon chain length and number of double bonds. Within the monounsaturated fatty acids, 16:1 was highly effective as an antibacterial agent, which is an exception to the positive correlation between inhibition, carbon chain length and unsaturation. The fatty acid 16:1 has previously been identified as an effective antibiotic and is naturally excreted by the skin of mice as a topical antibiotic (Katsuta et al., 2005). Another exception to this correlation occurs within the saturated fatty acids 10:0 and 12:0, which have been shown to be strongly inhibitory in previous reports (Desbois & Smith, 2010) as well as the current study. The variation in inhibition between fatty acids of different chain length and unsaturation has also been linked to solubility and lipophilicity (Galbraith et al., 1971). However, mechanisms underlying the antibacterial properties of fatty acids are still unclear. One hypothesis is that the cell membrane is a primary focus of the antibacterial activity of a fatty acid, as a result of the decreased electron transport chain and oxidative phosphorylation in the presence of fatty acids (Desbois & Smith, 2010).

Concentrations of these antibacterial fatty acids in algae can be increased by manipulating growth conditions. The effect of growth conditions (light intensity, light cycle, temperature, vitamins B_{12}, and media) on fatty acid content in algae was investigated for the five selected strains. Light cycle (24h:0h and 17h:7h), as shown in Table 2, was tested to determine its effect on the cellular content of the subset of fatty acids with antibacterial activity identified within this

study (referred to herein as target fatty acids). Within the light cycle experiment, *P. tricornutum* produced the greatest total target fatty acid content at 24 h light with 64.1 mg g^{-1} dw. Twenty-four hour light produced higher total target fatty acid content in four of the five tested algae, with *R. maculata* as the exception. Of the algae tested, *P. tricornutum* was influenced the most with an increase in total target fatty acid content of 80%. This was mainly due to the fatty acid 16:1, which was the most abundant, making up ~ 96-98% of total target fatty acid content. In addition to higher total target fatty acid content, twenty-four hour light produced the highest 20:4(n-6) content of the tested growth conditions with 7.66 mg g^{-1} dw in *R. maculata*.

The effect of light intensity was also investigated over a range of 45 to 130 µmol photons m^{-2} s^{-1} (Table 3). Similar to the light cycle experiment, *P. tricornutum* had the highest total target fatty acid content of 182 mg g^{-1} dw at 80 µmol photons m^{-2} s^{-1}; the other four tested algae had higher total target fatty acid content at the lowest intensity. In addition, the highest 18:3(n-6) content of all tested growth conditions was 2.00 mg g^{-1} dw in *P. tricornutum* at the lowest light intensity.

Photoperiod can play a large role in the lipid composition of photosynthetic algae (Sukenik & Carmeli, 1990). For example, twenty-four hour light caused the total target fatty acid content in 4 of the 5 algae to increase, primarily due to increased 16:1 content. This agrees with a study by Sukenik and Carmeli (1990) demonstrating that in the presence of light algae synthesize triacylglycerols (notably 16:0 and 16:1) and in the absence of light algal cell division occurs and lipids are metabolized for cellular maintenance. Under the lowest light intensity the highest total target fatty acid in four of the five tested algae occurred, largely as a consequence of 16:1 production. Typically, under lower light conditions the algal cells synthesize more thylakoid membrane to increase light capture, resulting in increased PUFAs (Wada & Murata, 2009;

Table 2. The target and total target fatty acid content of the five test algae to two different light cycles, 17h:7h and 24h:0h (light: dark). T-tests and post-hoc testing were performed to determine statistically significant changes.

	Light Duration	Target Fatty acid (mg g^{-1} dw)					Total Target FA (mg g^{-1} dw)[a]	% of Total FA[b]
		C10:0	C16:1	C18:3(n-6)	C20:4(n-6)	C22:2(n-6)		
Boekelovia hooglandii	17 h	0.013 ± 0.015	20.5 ± 3.0^{Bc}	0.108 ± 0.021	0.19 ± 0.09	0.077 ± 0.078	20.9 ± 3.1^{B}	23.8
	24 h	0.052 ± 0.006	28.7 ± 2.5^{A}	0.226 ± 0.037	0.71 ± 0.15	0.022 ± 0.011	29.7 ± 2.5^{A}	23.6
Phaeodactylum tricornutum	17 h	0.017 ± 0.009	34.1 ± 4.5^{B}	0.488 ± 0.090	1.03 ± 0.24	0.033 ± 0.017	35.7 ± 4.8^{B}	39.1
	24 h	0.004 ± 0.002	63.1 ± 5.3^{A}	0.440 ± 0.025	0.53 ± 0.04	0.037 ± 0.009	64.1 ± 5.2^{A}	41.8
Goniochloris sculpta	17 h	0.005 ± 0.004	0.5 ± 0.1	0.100 ± 0.013	1.59 ± 0.41	0.112 ± 0.021	2.3 ± 0.5	5.1
	24 h	0.003 ± 0.003	0.6 ± 0.1	0.247 ± 0.055	3.65 ± 1.04	0.072 ± 0.019	4.6 ± 1.2	8.7
Rhodella maculata	17 h	0.003 ± 0.004	1.9 ± 0.5	0.172 ± 0.040	7.81 ± 1.36	0.051 ± 0.027^{A}	9.9 ± 1.1	20.0
	24 h	0.011 ± 0.006	1.0 ± 0.1	0.130 ± 0.017	7.66 ± 1.08	0.010 ± 0.002^{B}	8.8 ± 1.2	29.7
Chloridella simplex	17 h	0.002 ± 0.002	36.6 ± 1.9^{B}	0.179 ± 0.022	3.08 ± 0.29	0.046 ± 0.016	39.9 ± 2.2^{B}	28.9
	24 h	0.050 ± 0.006	41.3 ± 1.0^{A}	0.315 ± 0.026	4.32 ± 0.16	0.018 ± 0.002	46.0 ± 1.1^{A}	34.2

[a] includes five target FAs; [b] Total Target FAs as % of total FA; [c]A, B, and C represent statistically significant differences between light duration within a given species

Table 3.The target and total target fatty acid content in five different algae to three light intensities,45, 80and 130 µmol photons m^{-2} s^{-1}. One-way ANOVAs and post-hoc testing were performed to determine statistically significant changes.

	Light[a] intensity	Target Fatty acid (mg g^{-1} dw)					Total Target FA (mg g^{-1} dw)[b]	% of Total FA[c]
		C10:0	C16:1	C18:3(n-6)	C20:4(n-6)	C22:2(n-6)		
Boekelovia hooglandii	45	0.0130 ± 0.0059	49.3 ± 6.6	0.75 ± 0.27	2.7 ± 1.2	0.062 ± 0.011^{Ac}	68 ± 7	21.2
	80	0.0156 ± 0.0142	36.6 ± 12.2	0.19 ± 0.30	0.6 ± 1.1	0.018 ± 0.008^{B}	38 ± 14	22.8
	130	0.0172 ± 0.0170	48.6 ± 12.1	0.52 ± 0.34	1.7 ± 1.1	0.022 ± 0.004^{B}	51 ± 14	23.6
Phaeodactylum tricornutum	45	0.0090 ± 0.0048	134.2 ± 4.6	2.00 ± 0.06	1.8 ± 0.1	0.018 ± 0.007	138 ± 5^{B}	44.8
	80	0.0161 ± 0.0117	178.4 ± 82.2	2.45 ± 1.09	1.5 ± 0.6	0.031 ± 0.015	182 ± 84^{A}	45.3
	130	0.0063 ± 0.0044	129.5 ± 26.9	1.54 ± 0.98	0.9 ± 0.6	0.020 ± 0.006	132 ± 28^{B}	45.9
Goniochloris sculpta	45	0.0054 ± 0.0028	0.8 ± 0.1	0.33 ± 0.04	9.9 ± 1.3	0.049 ± 0.017	11 ± 1	15.6
	80	0.0142 ± 0.0152	0.8 ± 0.1	0.33 ± 0.02	9.1 ± 1.7	0.032 ± 0.012	10 ± 2	14.6
	130	0.0081 ± 0.0022	0.5 ± 0.4	0.40 ± 0.21	7.2 ± 3.1	0.039 ± 0.006	8 ± 3	13.3
Rhodella maculata	45	0.0012 ± 0.0008	1.3 ± 0.1^{Ad}	0.14 ± 0.01	5.3 ± 0.6^{A}	0.008 ± 0.003^{B}	7 ± 1^{A}	23.3
	80	0.0007 ± 0.0005	1.0 ± 0.1^{B}	0.15 ± 0.02	3.2 ± 0.4^{B}	0.007 ± 0.002^{B}	4 ± 1^{B}	20.3
	130	0.0003 ± 0.0004	1.0 ± 0.2^{AB}	0.13 ± 0.09	2.6 ± 2.1^{B}	0.018 ± 0.004^{A}	4 ± 2^{B}	16.2
Chloridella simplex	45	0.0051 ± 0.0012	43.7 ± 3.0	0.24 ± 0.01	4.6 ± 0.3	0.028 ± 0.014	49 ± 3	34.8
	80	0.0178 ± 0.0132	41.7 ± 3.8	0.24 ± 0.02	4.7 ± 0.4	0.050 ± 0.029	47 ± 4	31.2
	130	0.0168 ± 0.0047	35.5 ± 4.9	0.24 ± 0.04	4.1 ± 0.7	0.027 ± 0.011	40 ± 6	28.7

[a] ± 5 µmol photons m^{-2} s^{-1}; [b] includes five target FAs [c] Total Target FAs as % of total FA; [d]A, B, and C represent statistical significant differences between groups

Guschina & Harwood, 2009). However, if light intensity is so low it is inhibiting growth, fatty acids may shift to storage lipids, predominantly monounsaturated and saturated fatty acids. Thus, light can play a major role in the content of a cell and 24 h exposure or low light intensity can be used to produce higher content of the most effective antibacterial fatty acids.

Nutrients play a substantial role in the lipid content and growth of algal cells. In Table 4, a variety of different media types were compared to determine which recipe created the highest target fatty acid content. This part of the study focused on the influence of a medium recipe as a whole and therefore changes in algal response are not identified with changes to a specific nutrient, with the exception of vitamin B_{12}, which was tested independently. Comparing all species, *Chloridella simplex* synthesized the greatest total target fatty acid content at 91.4 mg g^{-1} dw in ESP medium, making up 41.7% of the total fatty acid content. Within a given species, higher target fatty acid content was achieved in the marine organisms' *P. tricornutum* and *R. maculata* when grown in marine media. For example, the total target fatty acid content was 65% greater in F/2 compared to CPCC BBM in *P. tricornutum*. The freshwater alga *C. simplex* showed the same trend in freshwater media, with a 120% increase from F/2 to ESP. Notably, the freshwater alga *B. hooglandii* showed the opposite trend of *C. simplex,* with higher total target fatty acid content in marine media. *Goniochloris sculpta* had no significant differences in fatty acid production with different media.

The effect of vitamin B_{12} was tested at 3 doses (0, 10 and 100 ng L^{-1}) as shown in Table 5. The highest total target fatty acid content was in *P. tricornutum*, with 52.4 mg g^{-1} dw in medium enriched with 100 ng L^{-1} vitamin B_{12}. Within *B. hooglandii*, *P. tricornutum* and *G. sculpta*, the addition of vitamin B_{12} (10 or 100 ng L^{-1}) significantly increased the 20:4(n-6)

Table 4. The target and total target fatty acid content of the five test algae under different nutrient conditions: marine media (F/2), freshwater (CPCC BBM and SAG BBM), freshwater with soil extract (ESP) and marine with soil extract (SWES).

	Media type	Target Fatty acid (mg g^{-1} dw)					TotalTarget FA (mg g^{-1}	% of Total FA[b]
		C10:0	C16:1	C18:3(n-6)	C20:4(n-6)	C22:2(n-6)		
Boekelovia hooglandii	F/2	0.025 ± 0.008^{Ac}	40.0 ± 2.2^{A}	0.05 ± 0.01^{B}	0.07 ± 0.02^{C}	0.023 ± 0.015	40.2 ± 2.3^{A}	26.9
	CPCC BBM	0.007 ± 0.007^{B}	28.6 ± 5.9^{B}	0.41 ± 0.15^{A}	1.09 ± 0.39^{B}	0.012 ± 0.006	30.3 ± 6.5^{B}	21.2
	ESP	0.010 ± 0.008^{B}	30.1 ± 1.7^{B}	0.51 ± 0.04^{A}	1.96 ± 0.27^{A}	0.015 ± 0.008	32.6 ± 1.7^{B}	18.2
Phaeodactylum tricornutum	F/2	0.005 ± 0.006	22.3 ± 9.8	0.12 ± 0.04^{A}	0.28 ± 0.08^{A}	0.060 ± 0.022^{A}	22.7 ± 0.2^{A}	24.8
	CPCC BBM	0.003 ± 0.003	13.6 ± 1.8	0.08 ± 0.02^{AB}	0.13 ± 0.03^{B}	0.040 ± 0.017^{AB}	13.8 ± 1.9^{B}	21.6
	ESP	0.001 ± 0.002	14.8 ± 1.3	0.06 ± 0.01^{B}	0.11 ± 0.02^{B}	0.019 ± 0.005^{B}	15.0 ± 1.4^{B}	30.5
Goniochloris sculpta	F/2	0.029 ± 0.004	1.9 ± 0.2^{A}	0.36 ± 0.07	2.12 ± 0.22^{B}	0.077 ± 0.042	4.9 ± 0.5	3.32
	CPCC BBM	0.002 ± 0.002	0.8 ± 0.1^{B}	0.60 ± 0.24	4.94 ± 2.25^{A}	0.031 ± 0.018	6.4 ± 2.6	9.19
	SAG BBM	0.004 ± 0.004	0.8 ± 0.1^{B}	0.40 ± 0.01	3.46 ± 0.10^{A}	0.026 ± 0.004	4.7 ± 0.2	7.9
Rhodella maculata	F/2	0.003 ± 0.004	1.7 ± 1.0	0.18 ± 0.03	5.32 ± 1.63^{A}	0.055 ± 0.024^{B}	7.3 ± 2.1^{A}	14.1
	CPCC BBM	0.008 ± 0.010	2.6 ± 2.4	0.14 ± 0.10	1.45 ± 0.45^{B}	0.350 ± 0.212^{A}	4.5 ± 2.8^{B}	6.32
	ESP	0.003 ± 0.002	1.0 ± 0.3	0.07 ± 0.07	1.68 ± 1.42^{B}	0.026 ± 0.01^{B}	2.8 ± 1.8^{C}	13.1
	SWES	0.011 ± 0.020	2.2 ± 0.2	0.31 ± 0.04	5.47 ± 0.90^{A}	0.030 ± 0.009^{B}	8.0 ± 1.1^{A}	17.2
Chloridella simplex	F/2	0.002 ± 0.001^{B}	39.9 ± 3.9^{B}	0.24 ± 0.03^{B}	1.40 ± 0.15^{B}	0.053 ± 0.026	41.6 ± 4.0^{B}	40.4
	CPCC BBM	0.003 ± 0.003^{B}	45.6 ± 1.9^{B}	0.31 ± 0.03^{B}	1.64 ± 0.16^{B}	0.043 ± 0.025	47.6 ± 2.0^{B}	38.6
	ESP	0.017 ± 0.009^{A}	87.8 ± 7.7^{A}	1.05 ± 0.10^{A}	2.49 ± 0.19^{A}	0.039 ± 0.006	91.4 ± 8.0^{A}	41.7

[a] includes five target FAs; [b] Total Target FAs as % of total FA; [c] A, B, and C represent statistical significance

content. For example, on addition of 10 ng L^{-1} vitamin B_{12}, the cellular content of 20:4(n-6) in *P. tricornutum,* The effect of vitamin B_{12} was tested at 3 doses (0, 10 and 100 ng L^{-1}) as shown in Table 5. The highest total target fatty acid content was in *P. tricornutum*, with 52.4 mg g^{-1} dw in medium enriched with 100 ng L^{-1} vitamin B_{12}. Within *B. hooglandii*, *P. tricornutum* and *G. sculpta*, the addition of vitamin B_{12} (10 or 100 ng L^{-1}) significantly increased the 20:4(n-6) content. For example, on addition of 10 ng L^{-1} vitamin B_{12}, the cellular content of 20:4(n-6) in *P. tricornutum, B. hooglandii* and *G. sculpta* increased by 68%, 322% and 560%, respectively. The study's highest overall content of 10:0 and 20:4(n-6) was in *G. sculpta* in response to culture collection media enriched with 100 ng L^{-1} of vitamin B_{12} with 0.348 and 25.1 mg g^{-1} dw, respectively.

Nutrient composition (in the form of media and vitamin supplements) can result in algal stress that can lead to reduced growth and fatty acids being directed to more saturated fatty acids (Guschina & Harwood, 2009; Wada & Murata, 2009). Within this study, three of the algae exhibited an increase in PUFA content when growing in media analogous to the natural habitat (for example, freshwater algae in freshwater medium). This suggests that the algae were under minimal stress, resulting in greater fatty acid accumulation in the thylakoid membrane as PUFAs (Wada & Murata, 2009). The freshwater alga, *B. hooglandii*, was an exception with higher total target fatty acid content in marine medium, predominantly as 16:1 content. Higher 16:1 content from growth in marine media could be due to increased stress in the freshwater alga, resulting in a shift in fatty acid composition to storage lipids rich in monounsaturated fatty acids. The addition of vitamin B_{12} to media resulted in three test algae experiencing significantly increased 20:4(n-6) content. Although the mechanism is not known, the results indicate that vitamin B_{12}

Table 5. The target and total target fatty acid content to media enriched with 0, 10 and 100 ng L^{-1} of vitamin B_{12} in five algae. Statistical analysis: one-way ANOVAs and post-hoc testing were performed.

	Vitamin B_{12}[a]	Target Fatty acid (mg g^{-1} dw)					Total Target FA (mg g^{-1} dw)[b]	% of Total FA[c]
		C10:0	C16:1	C18:3(n-6)	C20:4(n-6)	C22:2(n-6)		
Boekelovia hooglandii	0	0.018 ± 0.010^{Ad}	39.8 ± 5.5	0.16 ± 0.07^{C}	0.35 ± 0.17^{C}	0.038 ± 0.009	40.3 ± 5.6^{B}	28.6
	10	0.014 ± 0.002^{A}	44.2 ± 3.1	0.56 ± 0.050^{A}	1.48 ± 0.13^{A}	0.030 ± 0.004	46.3 ± 3.3^{A}	21.6
	100	0.005 ± 0.004^{B}	37.5 ± 2.9	0.44 ± 0.05^{B}	1.12 ± 0.13^{B}	0.029 ± 0.002	39.1 ± 3.0^{B}	21.8
Phaeodactylum tricornutum	0	0.010 ± 0.038	33.7 ± 10.6^{AB}	0.28 ± 0.09	0.41 ± 0.12^{C}	0.046 ± 0.031	35.7 ± 11.6^{B}	38.5
	10	0.007 ± 0.006	30.5 ± 8.2^{B}	0.30 ± 0.09	0.69 ± 0.20^{B}	0.065 ± 0.025	31.6 ± 8.5^{B}	31.8
	100	0.003 ± 0.002	50.7 ± 4.4^{A}	0.67 ± 0.06^{A}	0.97 ± 0.09^{A}	0.020 ± 0.003	52.4 ± 4.5^{A}	37.5
Goniochloris sculpta	0	0.003 ± 0.003^{B}	0.9 ± 0.3^{B}	0.18 ± 0.03^{B}	3.18 ± 0.20^{B}	0.154 ± 0.102	4.4 ± 0.6^{B}	7.08
	10	0.117± 0.064 ^{B}	2.3 ± 0.2^{A}	1.28 ± 0.36^{A}	21.03 ± 3.02^{A}	0.362 ± 0.131	25.1 ± 3.7^{A}	12.1
	100	0.348 ± 0.166^{A}	3.6 ± 1.9^{A}	1.36 ± 0.40^{A}	25.13 ± 8.00^{A}	0.418 ± 0.200	30.9 ± 10.2^{A}	12.2
Rhodella maculata	0	0.008 ± 0.012	1.8 ± 2.3	0.10 ± 0.12^{B}	5.28 ± 8.10	0.081 ± 0.12	7.2 ± 8.8	14.7
	10	0.005 ± 0.006	0.8 ± 0.1	0.05 ± 0.02^{A}	1.52 ± 0.70	0.404 ± 0.006	2.4 ± 0.8	10.6
	100	0.001 ± 0.001	1.0 ± 0.2	0.06 ± 0.02^{A}	3.75 ± 2.12	0.029 ± 0.013	4.9 ± 2.4	15.7
Chloridella simplex	0	0.002 ± 0.002	44.7 ± 6.0	0.27 ± 0.04	3.53 ± 0.58	0.052 ± 0.029	48.5 ± 6.6	33.8
	10	0.004 ± 0.004	37.9 ± 4.9	0.23 ± 0.04	3.10 ± 0.35	0.059 ± 0.013	41.3 ± 5.3	32.2
	100	0.003 ± 0.002	44.0 ± 3.1	0.25 ± 0.03	3.55 ± 0.20	0.067 ± 0.032	47.8 ± 3.3	33.2

[a] ng L^{-1}; [b] includes five target FAs; [c] Total Target FAs as % of total FA; [d]A, B, and C represent statistical significance

supplementation is beneficial for fatty acid synthesis. In this study nutrients greatly influenced algal fatty acids and could potentially result in greater antibacterial fatty acids within the cell.

Temperature was the final growth condition investigated (Table 6). At 15°C *P. tricornutum* had the study's overall highest total target fatty acid content of 206.1 mg g^{-1} dw, the vast majority of which was 16:1 (203.3 mg g^{-1} dw). The total target fatty acid content in *B. hooglandii* and *P. tricornutum* more than doubled, increasing by 39.1 mg g^{-1} dw ($p<0.05$) and 109 mg g^{-1} dw ($p<0.001$), respectively at 15°C compared to 25°C. The mid-range temperature of 20°C produced the lowest total target fatty acids levels for four of five algae, with *C. simplex* as the exception. In general, there appears to be higher fatty acid accumulation at 15°C and 25°C, with a reduction in fatty acid content at the intermediate temperature of 20°C.

When an environment's temperature decreases the algal cells fatty acid composition typically shifts to greater PUFA content (Hu, 2007). This adaptation reduces the rigidity of the cellular membrane to allow for increased fluidity and prevent cellular damage (Nishida & Murata, 1996). Of the 5 growth conditions tested, temperature resulted in the greatest total target fatty acid content of 206.1 mg g^{-1} dw at 15°C in *P. tricornutum*, however the biomass productivity of this strain is low at 15°C (see section 4.3.3.). This suggests that at 15°C *P. tricornutum* is under stress, resulting in high lipid accumulation and low growth (Guschina & Harwood, 2009). Also, within the temperature experiment 20°C produced the lowest total target fatty acid content in four of the five algae. This could be a result of stress caused by a temperature change from culture maintenance (18°C) to experimental conditions (25°C or 15°C). The change from 18°C to 20°C may not have resulted in a significant stress response because temperature variation causes the two conditions to overlap (ie. 18 ± 1°C vs. 20 ± 1°C). Thus,

Table 6. The target and total target fatty acid content of different temperature (15°C, 20°C and 25°C) in five algae. Statistical analysis: one-way ANOVAs and post-hoc testing were performed.

	Temperature	Target Fatty acid (mg g^{-1} dw)					Total Target FA (mg g^{-1} dw)[a]	% of Total FA[b]
		C10:0	C16:1	C18:3(n-6)	C20:4(n-6)	C22:2(n-6)		
Boekelovia hooglandii	15°C	0.031 ± 0.053	68.7 ± 32.3^{A}	0.48 ± 0.30^{A}	0.4 ± 0.6^{B}	0.195 ± 0.028^{A}	69.8 ± 3.3^{A}	33.8
	20°C	0.013 ± 0.015	20.5 ± 3.0^{B}	0.11 ± 0.02^{B}	0.2 ± 0.1^{C}	0.077 ± 0.078^{B}	20.9 ± 3.1^{B}	23.8
	25°C	0.033 ± 0.022	28.4 ± 4.3^{B}	0.42 ± 0.05^{A}	1.8 ± 0.2^{A}	0.068 ± 0.034^{B}	30.7 ± 4.5^{B}	19.4
Phaeodactylum tricornutum	15°C	0.002 ± 0.002^{Bc}	203.3 ± 43.0^{A}	1.86 ± 0.82^{A}	0.9 ± 0.4^{B}	0.055 ± 0.014	206.1 ± 44.2^{A}	50.1
	20°C	0.017 ± 0.009^{A}	34.1 ± 4.5^{B}	0.49 ± 0.09^{B}	1.0 ± 0.2^{B}	0.033 ± 0.017	35.7 ± 4.8^{C}	39.1
	25°C	0.018 ± 0.005^{A}	91.6 ± 9.8^{AB}	1.43 ± 0.15^{A}	3.8 ± 0.3^{A}	0.044 ± 0.014	96.8 ± 10.1^{B}	47.0
Goniochloris sculpta	15°C	0.003 ± 0.004^{B}	1.0 ± 0.1^{B}	0.25 ± 0.08^{B}	3.8 ± 0.3^{B}	0.068 ± 0.005^{B}	5.1 ± 0.5^{B}	9.64
	20°C	0.005 ± 0.004^{B}	0.52 ± 0.1^{B}	0.10 ± 0.01^{C}	1.6 ± 0.4^{C}	0.112 ± 0.021^{A}	2.3 ± 0.5^{C}	5.08
	25°C	0.025 ± 0.004^{A}	2.0 ± 0.7^{A}	1.63 ± 0.60^{A}	19.3 ± 5.1^{A}	0.130 ± 0.039^{A}	23.0 ± 6.2^{A}	13.5
Rhodella maculata	15°C	0.003 ± 0.002	1.8 ± 0.6	0.24 ± 0.07^{A}	8.6 ± 2.5^{B}	0.036 ± 0.006	10.7 ± 3.1	25.2
	20°C	0.003 ± 0.004	1.9 ± 0.5	0.17 ± 0.04^{B}	7.8 ± 1.4^{C}	0.051 ± 0.027	9.9 ± 1.1	20.0
	25°C	0.006 ± 0.004	2.0 ± 1.3	0.24 ± 0.04^{A}	9.7 ± 1.6^{A}	0.026 ± 0.012	12.0 ± 2.8	22.5
Chloridella simplex	15°C	0.007 ± 0.011	46.6 ± 17.8	0.24 ± 0.09	2.2 ± 0.8	0.031 ± 0.013	49.1 ± 19.0	38.7
	20°C	0.002 ± 0.002	36.7 ± 1.9	0.18 ± 0.02	3.1 ± 0.3	0.047 ± 0.016	39.9 ± 2.2	28.9
	25°C	0.013 ± 0.012	34.2 ± 3.8	0.37 ± 0.05	4.1 ± 0.4	0.049 ± 0.022	38.7 ± 4.2	33.9

[a] includes five target FAs; [b] Total Target FAs as % of total FA; [c]A, B, and C represent statistical significance

temperature should be carefully selected to produce an alga with significantly higher cellular content of the most antibacterial fatty acids.

In conclusion, this study identified multiple algal fatty acids that have potential use as a topical antibiotic. In addition, growth conditions were established that enhance algal content of the antibacterial fatty acids that caused the greatest inhibition. Fatty acids 10:0 (capric acid), 16:1 (palmitoleic acid), 18:3(n-6) (gamma-linolenic acid; GLA), 20:4(n-6) (arachidonic acid) and 22:2(n-6) (docosadienoic acid) were the most effective antibacterial fatty acids. *Phaeodactylum tricornutum* had the highest total target fatty acid content at 206.1 mg g^{-1} dw, at growth conditions of 15°C, 17h:7h, 80 µmol photons m^{-2} s^{-1} in F/2 medium. However, *P. tricornutum* has a low biomass productivity (0.048 g L^{-1} day^{-1} dw), whereas *B. hooglandii* has the highest biomass productivity (0.38 g L^{-1} day^{-1} dw) of the 5 algae (Ruffell et al., submitted; Chapter 4) and a total target fatty acid content of 32.6 mg g^{-1} dw in ESP medium at 17h:7h, 18°C, and 80 µmol photons m^{-2} s^{-1}. The marine alga *P. tricornutum* has potential as a source of antibiotics if it is initially cultured in ideal growth conditions, and then shifted to conditions suitable for accumulation of antibacterial fatty acids, such as 20°C, 16h:8h, and Mann and Myers medium (Yongmanitchai & Ward, 1991). Microalgae can grow under a range of growth conditions, can contain high concentrations of fatty acids, and can have a high growth rate (Gong et al., 2011). Thus, they provide a sustainable source of a variety of antibacterial fatty acids that could potentially be used as topical antibiotics.

Chapter 3: Nutritional characteristics of the potential aquaculture feed species, *Boekelovia hooglandii*

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3.1. Introduction

Approximately, 1 000 tonnes of algal biomass are used annually for aquaculture feed (Hemaiswarya et al., 2011), primarily for culturing larvae, juvenile finfish, and raising zooplankton as feed for juvenile animals (Priyadarshani & Rath, 2012). There are numerous factors that are considered by the aquaculture industry when selecting microalgae for aquaculture feed. These can include the size of the algal cells, nutrient composition, digestibility, pigment content, and growth rate of the candidate algae (Glaude & Maxey, 1994). However, algal cell size is the main determinant in the ability of an aquaculture species to be able to feed effectively on phytoplankton (Glaude & Maxey, 1994). For instance, planktivore rotifers intended as feed for marine finfish graze on microalgae of between 10 and 20 µm, whereas larval mollusks and shrimp can more easily digest cells that are less than 10 µm (Glaude & Maxey, 1994). Furthermore, the nutritional profile of the algal feed is dependent on the cellular content of proteins, fatty acids, pigments, and carbohydrates (Glaude & Maxey, 1994). Within the present study, the term nutritional profile refers to the concentration of fatty acids, proteins, carbohydrates, and pigments within the algal cell. This profile can impact the growth of the

aquaculture species to which this algal feed is being applied (Renaud et al., 1999). For example, bivalve larvae have increased growth when fed an algal diet rich in protein and moderate levels of lipids and carbohydrates (Renaud et al., 1999). On the other hand, microalgae with high carbohydrate concentrations and moderate levels of PUFAs, such as omega-3 fatty acids, have been used for culturing juvenile oysters and larval scallops (Brown et al., 1997). Notably, essential omega-3 fatty acids have been linked to the maintenance of homeostasis, neural development and cardiovascular health in vertebrates (Tocher, 2015) leading to better growth of those organisms. In addition, carotenoid pigments can enhance the color of certain aquatic organisms, thus increasing the commercial value (Spolaore et al., 2006). For example, tissue deposits of carotenoid pigments can be converted into vitamin A and can increase the pigmentation of the muscle in salmonids and carp, this in turn increases the market value of these fish (Spolaore et al., 2006; Tanumihardjo, 2011). Lastly, the growth rate of an algal species plays a critical role in the large-scale production of these organisms as aquaculture feed. For instance, if an important nutritional component (such as proteins, fatty acids, carbohydrates, or pigments) is abundant during high growth rates, this will result in an increased yield of the nutritional constituent. For example, the concentration of fatty acids should be considered with algal growth rate to determine the overall fatty acid productivity (Huerlimann at al., 2010). As a result, the composition and growth rate of different algal feeds are important factors for the aquaculture industry to consider when identifying the most suitable algal species (Brown et al., 1997).

The growth phase of a species can greatly influence the concentration of nutritional components in the algal cell (Fernández-Reiriz et al., 1989). As an algal batch culture grows, it proceeds through four key phases: lag, exponential, stationary, and finally a death phase. The nutritional composition of algal cells in culture can shift depending on the conditions during

these different phases. For example, lipids typically increase during the exponential and late stationary phases (Siron et al., 1989; Huerlimann, 2010; Chiu et al., 2009) and carbohydrates also increase as the culture shifts from lag to stationary phase (Chu et al., 1982; Fernández-Reiriz et al., 1989; Zhu et al., 1997). Furthermore, several studies have demonstrated that protein concentrations can be variable according to culture conditions (Chu et al., 1982; Fidalgo et al., 1998; Fernández-Reiriz et al., 1989; Zhu et al., 1997). For example, cell protein concentration may stay the same throughout all growth phases, decrease between the exponential and stationary phases (Fidalgo et al., 1998; Fernández-Reiriz et al., 1989; Zhu et al., 1997), or increase between the exponential and stationary phases (Fernández-Reiriz et al., 1989). Lastly, Dawczynski et al. (2007) noted that microalgae contain the full complement of essential amino acids needed for aquaculture feedstock. By examining previous literature, it is clear that the nutritional composition of the algal cell is heavily influenced by a variety of factors, such as growth phase and algal species.

The nutritional profile of an algal strain can vary over time and is dependent on the taxonomic class, genus and species (Fernández-Reiriz et al., 1989). For example, lipid and carbohydrate content tends to increase as the culture proceeds from lag to stationary phase in diatom species such as *P. tricornutum* and *Chaetoceros calcitrans (*Fernández-Reiriz et al., 1989). In contrast, in the raphidiophyte *Heterosigma akashiwo,* peak lipid and carbohydrate concentrations occur during exponential phase, followed by a continual decline (Fernández-Reiriz et al., 1989). In addition, Fernández-Reiriz et al. (1989) noted that protein concentration tends to increase from lag to stationary growth phases for certain diatoms and chrysophytes (e.g. *C. calcitrans*), but typically decreases from lag to stationary phases for species within the class Chlorodendrophyceae (e.g. *Tetraselmis suecica*). Therefore, identifying the nutritional

composition of various algal species at different growth phases can enable the aquaculture industry to identify the species and the growth conditions that are most advantageous for aquaculture feed and when best to collect biomass for this purpose.

The species *B. hooglandii* M.F.E. Nicolai & L.G.M. Baas-Beck (Synurophyceae) has considerable potential for use in the aquaculture feed industry. *Boekelovia hooglandii* was identified as having a high omega-3 fatty acid content, the ability to grow in a wide range of temperatures, and a tolerance to a range of salinities (Barclay et al., 1991). The algal species *B. hooglandii* has been classified as marine (Throndsen, 1996), however this species can be observed in inland saline waters and grows well in waters with intermediate conductivity (Barclay et al., 1991). In addition, this organism should be easily digestible by larval mollusks and shrimp (planktivores) due to the small size (6 µm mean diameter) and absence of a cell wall (Microalgal Technology Research Group, 1986). The present study examines the suitability of this species as an aquaculture feed and also evaluates the nutritional composition and changes in the concentrations of fatty acids, proteins, carbohydrates, and pigments through the different growth phases. In addition, these changes were examined to identify patterns over the growth phases of this organism. Information from this study can aid the aquaculture industry in making informed decisions to improve the nutritional properties and productivity of the algae used for aquaculture feed.

3.2. Materials and Methods

3.2.1. Algal stock culture conditions

The microalga *B. hooglandii* (Synurophyceae) M.F.E. Nicolai & L.G.M. Baas-Beck (CPCC 484) was obtained from the Canadian Phycological Culture Centre (CPCC) at the

University of Waterloo (Waterloo, ON, Canada). The algal stock cultures were supplemented with new F/2 medium every two weeks in a 1:1 ratio. Stock cultures were grown in a Model-36HO (Percival Scientific, Inc., IA, United States) growth chamber under a light cycle of 17h:7h light:dark with an intensity of 80 ± 5 µmoles m^{-2} s^{-1} using 55 Watts PlusRite Compact Fluorescent FTL55/2G11/841 (CA, United States), at a temperature of 18 ±1°C. The F/2 medium was used in accordance with the SAG culture collection F/2 recipe, and was being used by the culture collection as the base medium to grow *B. hooglandii*. The recipe consisted of 0.075 g L^{-1} $NaNO_3$, 0.005 g L^{-1} NaH_2PO_4 x H_2O, 0.03 g L^{-1} Na_2SiO_3 x $9H_2O$, 1 ml L^{-1} vitamin solution, 1 ml L^{-1} micronutrient solution and 905 ml L^{-1} filtered artificial seawater (SAG, 2015). SAG (2015) provides the recipes for vitamin and micronutrient solutions.

3.2.2. Media type experiment

In order to determine growth conditions that result in higher growth rates and essential fatty acid production, *B. hooglandii* was grown in a number of media types. Three media recipes were compared: a saltwater medium [F/2 (Guillard, 1975)], a freshwater medium [BBM (Stein, 1980)], and a freshwater medium with soil extract [ESP (SAG, 2015)]. Culture collection conditions were followed (17:7 light:dark cycle, light intensity 80 ± 5 µmoles m^{-2} s^{-1} using 55 Watts PlusRite Compact Fluorescent FTL55/2G11/841 (CA, United States), F/2 medium and 18 ± 1°C), except when a media type was being tested at which time culture collection conditions were maintained except for use of a different medium (SAG, 2015). Biomass was estimated from optical density at 600 nm, measured using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific). A standard curve was used to estimate culture density in gram dw per liter. In order to create a standard curve, algal samples at a range of densities were measured using absorbance, and then filtered and dried to establish the respective gram dw per liter concentrations. Biomass

was estimated using OD every other day for 8 days, at which point approximately 30 mg dw biomass was collected for fatty acid analysis. Due to the higher levels of biomass and fatty acid content of *B. hooglandii* in ESP medium, this medium was used for the remainder of experiments. Light and temperature conditions used for generating the growth curve and for nutritional analysis remained at 18ºC, 80 µmoles m^{-2} s^{-1} light intensity, and 17h:7 h light cycle. The ESP recipe consisted of 0.2 g L^{-1} KNO_3, 0.02 g L^{-1} K_2HPO_4, 0.02 g L^{-1} $MgSO_4$ x $7H_2O$, 30 ml L^{-1} low clay soil extract, 0.1% proteose peptone, and 5 ml L^{-1} micronutrient solution (SAG, 2015).

3.2.3. Growth curve conditions

Inoculations of algal cells were added to 4 L flasks each containing 2 L of fresh ESP media on Day 0, at an initial concentration of 0.469 ± 0.068 g L^{-1}. Biomass (g L^{-1}) was estimated based on optical density at 600 nm using a spectrophotometer (Thermo Fisher Scientific, Model-Spectronic 200) three times per week over the course of the 70 day experiment. Once optical density was measured, algal culture was collected and aliquots were used for nutritional analyses (refer to `3.2.6. Nutritional profile` for volumes). Based on preliminary growth curves, analyses of fatty acids, carbohydrates, and proteins were performed on day 0 and the last day of each growth phase [lag (day 0-23), exponential (24-35), stationary (36-49), and death (50-70)]. Samples were additionally analyzed at either the mid-point of a stage (lag, exponential, stationary, and death) or approximately weekly if the growth phase was greater than two weeks long. The lag phase exhibited very little observable growth and thus was not analyzed at the mid-point. Pigment samples were analyzed on day 0 and the final day of each growth phase. Overall, nine time points were analyzed for protein, fatty acid, and carbohydrate (day 0, 23, 30, 35, 44, 49, 56, 63, and 70). This experiment was performed in four replicates.

3.2.4. Growth phase identification

The growth curve was plotted using biomass (g L^{-1}) on a log scale, and the log-linear portion was identified as the exponential phase of the curve. To calculate biomass (g L^{-1}), a standard curve was constructed that related biomass to optical density. The standard curve was constructed using the absorbance values of a culture of *B. hooglandii* at 600 nm under a range of known culture densities (0.1 – 1.0 g L^{-1}). Biomass of the culture was measured by collecting, drying, and then weighing the algae to establish grams dw per liter. Using the known biomass of the culture and the absorbance of the dilutions, a line of best fit was calculated and used to determine the biomass of the samples. The exponential phase and changes in culture density were used to identify the lag, exponential, stationary and death phases.

3.2.5. Statistical analysis

To determine growth rate the biomass (g L^{-1}) was plotted over time on a log scale graph and an exponential curve was fit to the data. The slope parameter 'b' on the equation $y=ae^{bt}$ was used to estimate the growth rate (day^{-1}). Biomass in the late exponential phase (days 33 and 35) was used to calculate the biomass productivity (g L^{-1} day^{-1} dw). Biomass productivity was estimated using the equation Biomass productivity (g L^{-1} day^{-1}) = (X_2-X_1) $(t_2-t_1)^{-1}$, where X_2 was day 35 biomass (g L^{-1}), X_1 was day 33 biomass (g L^{-1}), and t was the sample day (t_2=35 and t_1=33) (Hempel et al., 2012).

SigmaPlot (SanJose, CA, USA) was used for paired t-test comparisons, and SPSS (Armonk, NY, USA) was used for the linear regression analysis. Paired t-tests were used to compare the cell's physiological characteristics at different growth phases. The r-squared values were calculated for variable pairs, and any comparisons with r-squared values greater than 0.5 were analyzed by linear regression. The notation within Tables 7 to 12 was used to indicate

whether values were significantly different. Superscript letters indicate whether values are significantly different from one another using different letters ($p<0.05$), and values with the same letter were not significantly different ($p>0.05$). The letters compare values within a single taxa and growth condition. The data range indicated with the plus-minus sign (±) refers to the standard deviation (n=4).

3.2.6. Nutritional profile

Cultures of *B. hooglandii* were sampled for protein, pigment, fatty acid, and carbohydrate analysis. An estimated 120 mg dw of biomass was required for the protein (5 mg), fatty acid (10 mg), carbohydrate (5 mg) and pigment (100 mg) analysis. A minimum equivalent of 120 mg dw of biomass was collected, using culture density to calculate the volume required for minimum biomass, i.e. day 0 (400 ml), day 23 (300 ml), day 30 (150 ml), day 35 (50 ml), day 44 (50 ml), day 49 (50 ml), day 56 (50 ml), day 63 (50 ml), and day 70 (100 ml). Over the 70 day period a total of 800 ml of culture was removed from the initial 2 L of culture. The algal samples collected were rinsed with a solution (9 g NaCl in 1 L deionized water), stored at -80°C, and freeze dried prior to analysis. Blanks were used to account for the effect of salt residue on overall sample weight. For protein analysis, microalgal proteins were extracted using a 24% trichloroacetic acid bath (Slocombe et al., 2013). The protein precipitate was resuspended in 0.5 ml of Lowry reagent (48:1:1; 2% (w/v) Na_2CO_3 anhydrous in 0.1 N NaOH: 1% (w/v) NaK Tartrate tetrahydrate: 0.5% (w/v) $CuSO_4$ x $5H_2O$ in H_2O). The solution was then incubated at 55°C for 3 h, followed by centrifugation at 15,000 x g for 20 min. Proteins were then quantified using the Lowry assay according to Slocombe et al. (2013). Optical density at 600 nm was measured using a spectrophotometer (Thermo Fisher Scientific, Model-Spectronic 200) and was

used to determine protein content. A calibration curve using bovine serum albumin (BSA) stock solution (200 mg ml^{-1}) was generated to estimate protein concentrations (Slocombe et al., 2013).

The fatty acid content was analyzed by Dr. Ken Stark's lab (Waterloo, ON, Canada) as fatty acid methyl ester equivalents, which were derivatized using direct transesterification and quantified by gas chromatography as described in Zuñiga et al. (2012). Fatty acids were derivatized by adding 300 µl hexane and 1 ml 14% boron triflouride in methanol to freeze dried algae, followed by incubation for 60 min at 90°C (Zuñiga et al., 2012). Then samples were dried under nitrogen, and the resulting fatty acid methyl esters were dissolved in 65 µl of heptane and quantified using gas chromatography (Varian GC 3900), using hydrogen as the carrier gas and flame ionization as the detection method (Zuñiga et al., 2012). A split ratio of 200:1 and temperature of 250°C was set on the injector. A DB-FFAP 15 m x 0.10 mm i.d. x 0.10 µm film thickness capillary column was used as the separation column. To identify peaks, retention times were compared with external standards (GLC-462, GLC-569; Nu-Chek Prep, Elysian, MN) containing 28 fatty acid methyl esters of equal concentration by weight (Zuñiga et al., 2012).

For carbohydrate analysis, extraction of carbohydrates from algal biomass was performed using a sulfuric acid-UV method according to Albalasmeh et al. (2013). Algal biomass (5 mg) was combined with 3 ml concentrated sulfuric acid, rapidly mixed for 30 s, followed by cooling on ice for 2 min. Absorbance was then measured using UV light at 315 nm using a UV spectrophotometer to determine carbohydrate concentration (Albalasmeh et al., 2012). A glucose calibration curve was used to establish the corresponding carbohydrate concentration for an absorbance value.

Algal samples for pigment analysis were stored in the dark at -20°C (Thomas et al., 2013). In preparation for HPLC pigment analysis, freeze dried samples were extracted using an 80:15:5 mixture by volume of acetone:methanol:water and incubated at -20°C for 24 h (Thomas et al., 2013). The extract was filtered using a 0.22 µm polytetrafluoroethylene syringe filter, dried under a stream of nitrogen gas and re-suspended in 500 µL of injection solution (70:25:5 acetone:ion pairing reagent:methanol by volume) before HPLC analysis (Thomas et al., 2013). The HPLC analysis was performed by Dr. Roland Hall's lab (Waterloo, ON, Canada). To separate pigment compounds, a gradient of two mobile phases was used. Mobile phase A was composed of 90:10 v:v of methanol:ion pairing reagent, and mobile phase B was composed of 73:27 v:v of methanol: acetone (Thomas et al., 2013). The ion pairing reagent consisted of 0.75 g tetrabutylammonium acetate and 7.7 g ammonium acetate. Pigment analysis was performed using Symmetry C18 columns (3.5 µm) on a Waters HPLC reverse-phase system. Calibration curves were conducted for all pigments [including β-carotene, chlorophyll (*a, b,* and *c*3), diadinoxanthin, echinenone, fucoxanthin, lutein, myxoxanthophyll, phaeophytin *a*, and zeaxanthin], and Sudan II was used as an internal standard for all samples (Thomas et al., 2013).

3.3. Results and Discussion

Within this study, experiments on the effect of media types were performed in order to identify a media resulting in greater biomass productivity (g L^{-1} day^{-1} dw) and fatty acid content, compared to the reference medium (F/2) used to grow *B. hooglandii* in the culture collection. For the most productive medium, trends in the algal nutritional profile and growth rate were established over the four growth phases (lag, exponential, stationary, and death). This study focused on the main cellular characteristics, such as total protein, fatty acid, pigment, and growth rate, in addition to highlighting certain fatty acids and pigments of commercial interest.

Emphasis was placed on highly correlated characteristics, based on the coefficient of determination using r-squared values greater than 0.9. In addition to the primary findings discussed in this Chapter, a complete list of identified correlations is provided in Appendices section 'A.1. Chapter 3 Supplementary data'. Once culture conditions resulting in a stable algal population were established, additional studies were performed to explore nutrient composition over different phases of growth.

3.3.1 Algal growth

Growth media can have a large impact on fatty acid concentration and biomass productivity (Guschina & Harwood, 2006). The halotolerant alga, *B. hooglandii*¸ was grown in a variety of freshwater and marine media types [ESP (marine with soil extract), BBM (freshwater), and F/2 (marine)]. We noted that the media recipes being compared are quite different in nutrient composition and concentration, and the effect of individual components has not been investigated. Of the media studied, the greatest total omega-3 fatty acid content (62.4 mg g^{-1}) and biomass productivity (0.38 g L^{-1} day^{-1}) by *B. hooglandii* were in samples grown in ESP medium (Table 7). The halotolerant alga, *B. hooglandii,* has been identified in saline ponds containing humic acids (Barclay et al., 1991). This could partly explain the greater biomass productivity of the culture in the marine medium containing 0.1% proteose peptone and soil extract. Notably, soil extracts contain an array of micro-nutrients, macro-nutrients, vitamins and humic acids that can increase the growth of certain algae (Andersen, 2005). In addition, researchers have observed an increase in the abundance of *B. hooglandii* with increasing dissolved organic carbon concentration (Barclay et al., 1991). Thus, the presence of these nutrients within the medium may have resulted in the far greater biomass production in the ESP medium. There are a few potential concerns with use of ESP medium in industrial scale-up, notably the higher cost of

Table 7. The effects of five different culture collection media, F/2, BBM, and ESP, on the total fatty acid content and omega-3 fatty acid content of B. hooglandii *(17h:7h, 80 ± 5 µmoles m^{-2} s^{-1}, and 18 ± 1°C).*

Media	Biomass productivity (g L^{-1} day^{-1} dw)	Omega-3 fatty acid production (mg g^{-1}dw)								Total omega -3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
		16:4 (n-3)	18:3 (n-3)	18:4 (n-3)	20:3 (n-3)	20:4 (n-3)	20:5 (n-3)	22:5 (n-3)	22:6 (n-3)		
F/2	0.145 ± 0.005^{C}	0.372± 0.061^{B}	0.055± 0.012^{B}	0.829± 0.070^{B}	0.020± 0.011	0.0227± 0.0078^{B}	0.687± 0.093^{B}	0.0115± 0.0085^{B}	0.351± 0.014^{B}	2.35± 0.09^{B}	149± 7^{B}
BBM	0.264 ± 0.018^{B}	0.130± 0.110^{A}	0.630± 0.200^{A}	14.9 ± 4.9^{A}	0.021± 0.013	0.094± 0.027^{A}	31.0± 12.1^{A}	0.169± 0.057^{A}	8.91± 4.02^{A}	56.1± 21.4^{A}	143± 37^{AB}
ESP	0.382 ± 0.012^{A}	0.121± 0.114^{A}	0.666± 0.068^{A}	14.7 ±1.8^{A}	0.042± 0.017	0.072± 0.016^{A}	36.0± 5.6^{A}	0.203± 0.012^{A}	10.6± 2.1^{A}	62.4± 9.7^{A}	180± 14^{A}

[a]A, B, and C indicate statistically significant differences between groups (refer to 3.2.5 Statistical analysis)

proteose peptone compared to other organic nitrogen sources and the unpredictable nutrients within soil extracts (Andersen, 2005) which may need to be addressed. Due to the higher levels of biomass and fatty acid content of *B. hooglandii*, ESP medium was used for the remainder of the project. After identifying a media type resulting in a relatively high biomass productivity for *B. hooglandii,* this study further examined the specific growth phases.

Throughout the lag, exponential, stationary, and death phase of a culture, the growth characteristics of the algal cell culture continually shifted (Figure 4). The log-linear portion of the growth curve, when plotted on a log scale, was identified as the exponential phase of the curve (Day 24-35). The region prior to the exponential phase was identified as the lag phase (Day 0-23). Subsequently, the stationary phase was identified as the region of the curve after the exponential phase but before biomass loss (Day 36-49). Finally, the portion of the curve with continually decreasing biomass was identified as the death stage (Day 50-70). The lag phase exhibited minimal growth, however at the end of the exponential phase, the growth rate peaked (0.17 ± 0.02 h^{-1}) as shown in Figure 5. Additionally, at the end of stationary phase the greatest culture density occurred at 4.89 ± 0.51 g L^{-1} (Fig. 4). These observations are consistent with nitrogen or nutrient deficiency in the stationary phase, which may have resulted in reduced growth and a shift away from protein synthesis (Markou et al., 2012). In addition to biomass and growth rate, other physiological characteristics including pigment, fatty acid, protein, and carbohydrate content also shift throughout the growth phases.

3.3.2 Algal pigments

In this study, the highest total pigment concentration in *B. hooglandii* occurred at the end of the lag phase and in exponential phase, with 0.058 ± 0.034 mg g^{-1} and 0.042 ± 0.028 mg g^{-1},

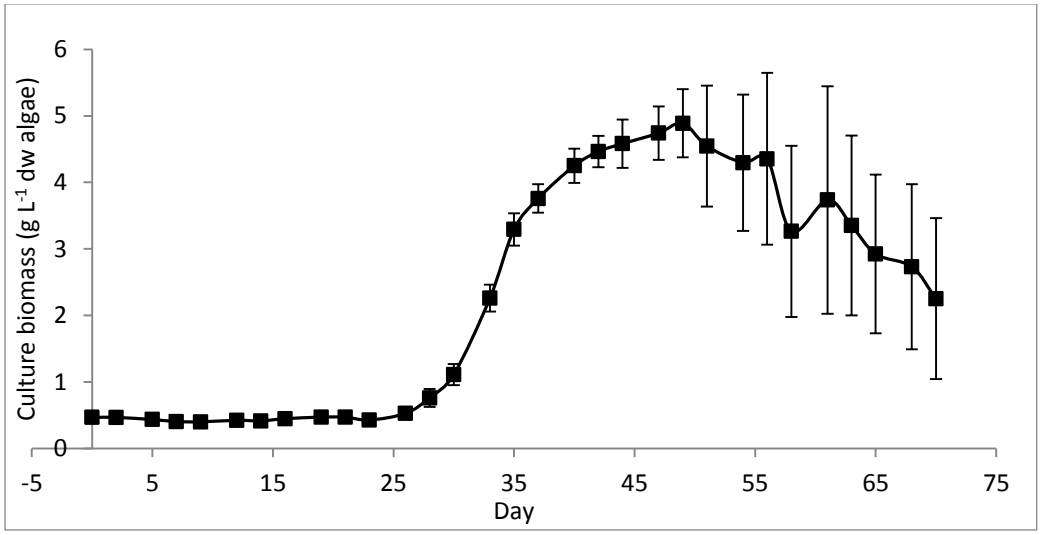

Figure 4. Algal biomass concentration (g L^{-1} dw algae) throughout growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70). Error bars indicate standard deviation (n=4).

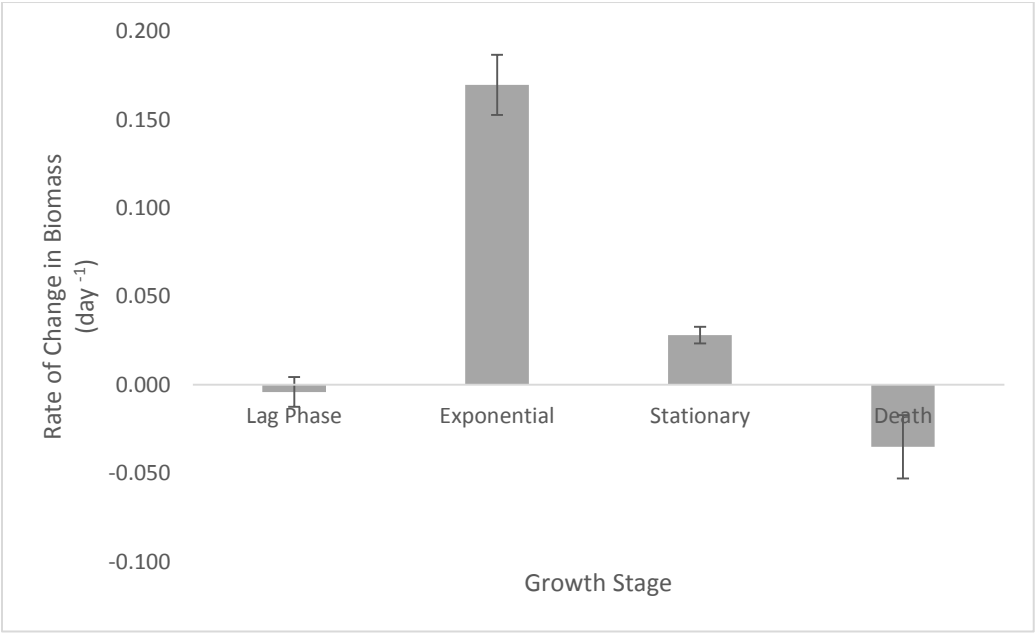

Figure 5. Algal rate of change of biomass (day^{-1}) on the final day of each growth phase, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70). Error bars indicate standard deviation (n=4).

respectively (Table 8). Of the pigments analyzed, four out of seven (fucoxanthin, diatoxanthin, diadinoxanthin, and chlorophyll *a*) peaked at the end of the lag phase. However, this peak remained stable and was not significantly different from the concentration at the end of exponential phase. The drop in pigment concentration after exponential phase may be due to reduced nutrients and increased stress in stationary and death phase (Harket et al., 1996). As a culture ages and nutrient limitation occurs with increasing cell density, stress during the stationary and death phases can result in pigment loss. For example, Harker et al. (1996) demonstrated that as stress on a culture increases, pigment concentration decreases, and cells begin to bleach. This loss of cell pigment, due to stress, is possibly linked to oxidative degradation within the cell (Harker et al., 1996). Over the different growth phases pigment concentration varied, and correlations formed between different algal pigments.

In addition to the increased cellular concentration in algal pigments during exponential phase, correlations were present among individual pigments. Of the pigments studied, chlorophyll *a* had the greatest positive correlation with total pigment concentration ($r=0.962$, $p=0.009$) as expected since chlorophyll *a* is the primary pigment in algae (Stace, 1989). Fucoxanthin was also strongly correlated with total pigment, with an r value of 0.897 ($p=0.039$). Of all pigments measured, chlorophyll *a* and fucoxanthin were present in the highest proportions within the algal cell, as seen in Table 8. Chlorophyll *a* and fucoxanthin are the major pigments in Chrysophyceae of which *B. hooglandii* is a member (Stace, 1989; Thomas et al., 2013). The high cellular content of both could explain the correlation with total pigment. Of the nutritional components analyzed, correlations were not only present between individual pigments, but also individual fatty acids.

3.3.3 Algal fatty acid composition

Algal fatty acids vary significantly from one growth phase to the next and from species to species (Borowitzka & Moheimani, 2013). The highest total fatty acid concentration observed in this study occurred at the end of the stationary phase, at 271 ± 21 mg g^{-1} (Fig. 6). The high fatty acid content exhibited in the stationary phase may be the result of nitrogen deficiency (Markou et al., 2012). In addition, the increase in total fatty acid content is consistent with numerous other studies of microalgae such as *P. tricornutum, D. tertiolecta*, and *I. galbana*, which have shown that lipid concentration in algae increases during the lag and stationary phases (Siron et al., 1989; Huerlimann, 2010; Chiu et al., 2009; Fidalgo et al., 1998). However, Barclay et al. (1991) observed different changes in lipids where the lipid concentration increased during the exponential and stationary phases in *B. hooglandii*. Overall, the total lipid content was greatest in stationary phase, however the proportion of the individual fatty acids varied with growth phase. Throughout the different algal growth phases, certain fatty acids were present in consistently high concentrations relative to the other fatty acids analyzed (Figure 7). The relatively high proportions of these compounds allows them to be used as estimates of the concentration of their structural group, including 16:1 for total fatty acid, 16:0 for SFA, and 20:5(n-3) for PUFA content. Of the thirty-four fatty acids analysed, 16:1 was present in the greatest amounts (Table 9), at a level consistently higher than that of total SFAs and PUFAs combined. This high concentration has been shown in other algal species such as *Isochrysis zhangjiangensis* (Chrysophyceae), with 16:1 being a major component of lipids (Huang & Cheung, 2011). As a result of the high proportion of 16:1 (28.5 to 48.0% of total fatty acid content, depending on sample day), the composition correlates significantly with the total fatty acid concentration ($r=0.941$, $p=0.000$). In addition, 16:1 has been identified as a precursor for the

Table 8. The pigment content (μg g^{-1} dw) of B. hooglandii *during the four growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70).*

Day	Fucoxanthin	Diatoxanthin	Diadinoxanthin	Zeaxanthin	Chlorophyll c	Chlorophyll a	β- carotene	Total Pigment
0	15.5 ± 6.4	0.133 ± 0.063	6.92 ± 2.42	0.94 ± 0.21	1.25 ± 1.19	5.79 ± 1.25	0.082 ± 0.041	30.6 ± 7.0
23	22.7 ± 12.2	0.303 ± 0.212	9.02 ± 4.82	3.83 ± 2.89	2.82 ± 2.46	18.9 ± 12.1	0.191 ± 0.061	57.7 ± 34.0
35	13.2 ± 9.0	0.122 ± 0.231	7.59 ± 2.74	5.23 ± 0.97	0.29 ± 0.74	15.3 ± 13.9	0.240 ± 0.050	41.9 ± 27.6
49	7.57 ± 1.75	n.d.[a]	7.20 ± 1.36	2.45 ± 0.43	1.68 ± 1.91	5.00 ± 0.77	0.121 ± 0.124	24.0 ± 1.8
70	3.37 ± 1.88	n.d.	3.77 ± 0.47	1.25 ± 0.36	0.31 ± 0.29	3.00 ± 0.42	n.d.	11.7 ± 2.4

[a] not detected

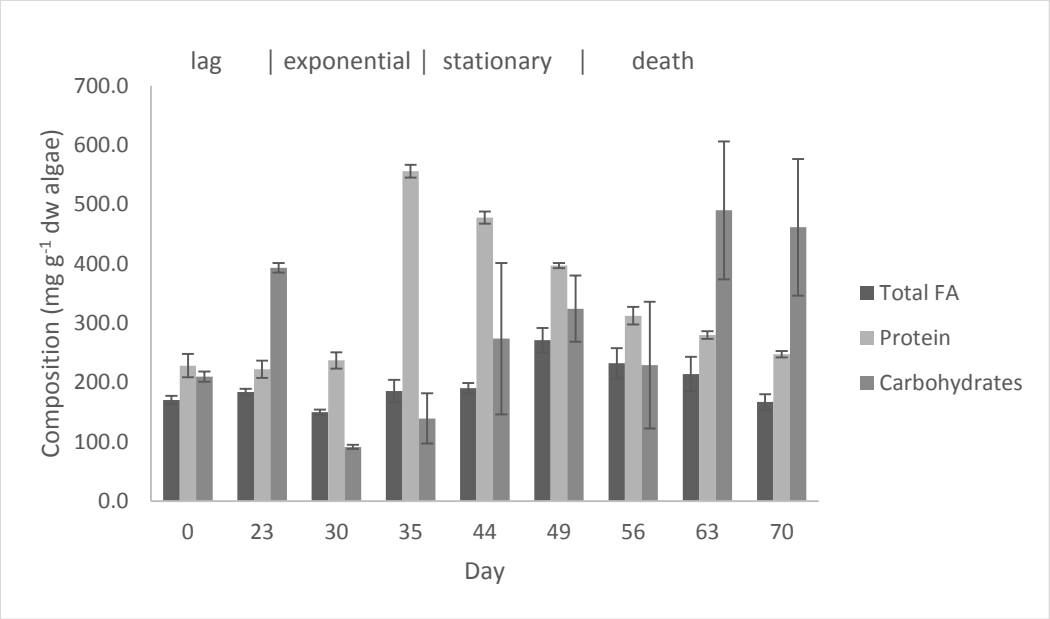

Figure 6. Biochemical composition of the algal cell through growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70). Error bars indicate standard deviation (n=4).

PUFA synthesis pathway (Beaudoin, 2000), and hence the high 16:1 concentration in *B. hooglandii* would contribute to a higher PUFA content (Sheehan et al., 1998). Another example of a fatty acid being an accurate indication of the fatty acid group includes palmitic acid (16:0) and total SFA; which contributes to 64-75% of the total SFA content (Table 10). This finding is in agreement with Goss and Wilhelm (2009), who demonstrated that C16 is commonly present in higher concentrations in algae, relative to other fatty acids. The saturated fatty acid 18:0 was the only other fatty acid to have a strong correlation with the total SFA concentration (r=0.955, p=0.000). Eicosapentaenoic acid [20:5(n-3); EPA] is another example of a highly abundant fatty acid, making up ~ 80.9% of the total PUFA content. Not surprisingly, EPA was strongly correlated with total omega-3 (r=0.996, p=0.000) and total PUFA (r=0.986, p=0.000) concentrations, as seen in Tables 11 and 12. Of the PUFAs, EPA was present in the highest proportions within the class Chrysophyceae, of which *B. hooglandii* is a member (Borowitzka &

Moheimani, 2013). The high EPA content characteristic of this taxonomic group would explain the correlations among EPA, total omega-3 and total PUFA concentration (Borowitzka & Moheimani, 2013). In addition to the influence of 16:1, 16:0 and EPA on different fatty acid groups, a variety of other correlations were identified among fatty acids and other cellular characteristics, such as pigments.

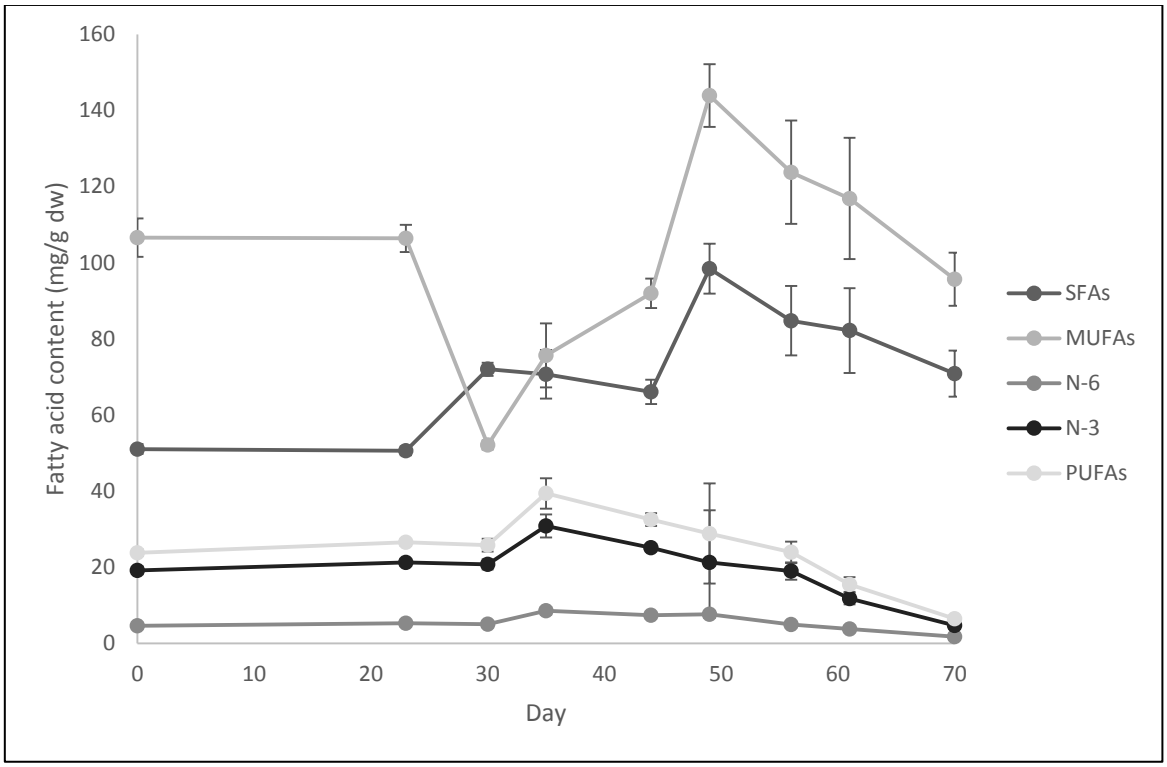

Figure 7. The fatty acid composition of the algal cell through growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70). Error bars indicate standard deviation (n=4)..

Table 9. The monounsaturated fatty acid content (mg g^{-1} dw) of B. hooglandii *during the four growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70).*

Day	C12:1	C14:1	C16:1	C18:1(n-7)	C18:1(n-9)	C20:1(n-9)	C22:1(n-9)	C24:1(n-9)
0	0.0062±0.0018	0.0313±0.0009	81.9±3.7	5.53±0.50	11.3±0.9	0.177±0.007	0.0311±0.0009	0.808±0.009
23	0.0090±0.0013	0.0310±0.0079	88.1±2.8	5.55±0.48	11.8±0.7	0.197±0.005	0.0310±0.0029	0.886±0.009
30	0.0420±0.0200	0.0311±0.0027	42.8±0.8	3.54±0.13	4.01±0.37	0.104±0.023	1.29±0.03	0.301±0.055
35	0.0262±0.0113	0.0401±0.0222	67.5±7.7	1.83±0.11	5.08±0.54	0.111±0.071	0.717±0.043	0.347±0.066
44	0.0119±0.0044	0.0482±0.0181	83.1±3.5	1.76±0.07	6.24±0.15	0.0545±0.0094	0.480±0.105	0.298±0.063
49	0.0097±0.0096	0.0545±0.0050	128±7	2.59±0.22	11.7±0.9	0.0434±0.0051	0.484±0.133	0.350±0.050
56	0.0095±0.0033	0.0454±0.0076	109±12	3.73±0.35	9.92±1.02	0.0551±0.0201	0.461±0.050	0.305±0.029
63	0.0123±0.0075	0.0280±0.0110	102±14	4.31±0.59	9.26±1.29	0.0597±0.0016	0.510±0.123	0.366±0.046
70	0.0148±0.0038	0.0552±0.0122	79.4±4.4	3.77±0.60	8.05±2.09	0.0942±0.0342	0.881±0.111	0.389±0.067

Table 10. The saturated fatty acid content (mg g^{-1} dw) of B. hooglandii *during the four growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70).*

Day	C10:0	C12:0	C14:0	C16:0	C17:0	C18:0	C20:0	C22:0	C23:0	C24:0
0	0.0273 ±0.0018	0.0421 ±0.0370	8.57 ±0.11	33.0 ±1.3	1.69 ±0.09	1.57 ±0.09	0.185 ±0.017	0.219 ±0.013	0.0187 ±0.0073	1.73 ±0.03
23	0.0253 ±0.0111	0.0570 ±0.0293	9.16 ±0.46	35.8 ±0.9	1.80 ±0.06	1.77 ±0.06	0.198 ±0.020	0.150 ±0.013	0.0207 ±0.006	1.96 ±0.05
30	0.0534 ±0.0391	0.218 ±0.040	15.0 ±0.4	48.5 ±1.3	2.27 ±0.04	2.60 ±0.42	0.293 ±0.059	0.610 ±0.032	0.0731 ±0.0233	2.48 ±0.16
35	0.0531 ±0.0134	0.210 ±0.053	15.7 ±0.6	44.9 ±4.3	3.57 ±0.36	2.35 ±0.67	0.230 ±0.100	0.517 ±0.088	0.0653 ±0.0432	3.03 ±0.28
44	0.0402 ±0.0312	0.0961 ±0.0232	13.6 ±0.4	44.2 ±2.3	3.39 ±0.13	1.92 ±0.16	0.175 ±0.014	0.401 ±0.111	0.0211 ±0.0130	2.27 ±0.09
49	0.0631 ±0.0212	0.116 ±0.019	18.1 ±1.3	69.7 ±4.8	3.76 ±0.21	3.53 ±0.20	0.215 ±0.016	0.395 ±0.051	0.0244 ±0.0133	2.52 ±0.19
56	0.0271 ±0.0134	0.0906 ±0.0053	14.4 ±1.3	61.8 ±7.0	2.37 ±0.23	3.27 ±0.39	0.196 ±0.032	0.240 ±0.040	0.0180 ±0.0181	2.17 ±0.22
63	0.0263 ±0.0082	0.0900 ±0.0260	13.6 ±0.9	61.4 ±9.4	1.53 ±0.20	2.63 ±0.47	0.208 ±0.046	0.377 ±0.081	0.0140 ±0.0134	2.29 ±0.22
70	0.0239 ±0.0069	0.123 ±0.058	11.0 ±1.2	51.2 ±4.2	0.459 ±0.017	2.55 ±0.39	0.218 ±0.022	0.640 ±0.068	0.0166 ±0.0027	1.84 ±0.22

Table 11. The omega-6 fatty acid content (mg g^{-1} dw) of B. hooglandii *during the four growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70).*

Day	C20:3(n-6)	C20:4(n-6)	C22:2(n-6)	C22:4(n-6)	C22:5(n-6)
0	0.141±0.221	2.15±0.09	0.0901±0.0260	0.126±0.013	0.0621±0.0265
23	0.354±0.200	2.30±0.07	0.0904±0.0294	0.125±0.012	0.0754±0.0191
30	0.184±0.035	1.59±0.09	0.218±0.040	0.272±0.069	0.106±0.043
35	0.386±0.061	3.20±0.20	0.0984±0.0501	0.212±0.052	0.0711±0.0720
44	0.374±0.036	2.70±0.15	0.0664±0.0191	0.741±0.535	0.0363±0.0019
49	0.385±0.055	2.90±0.25	0.0611±0.0063	0.452±0.484	0.0450±0.0101
56	0.245±0.039	1.65±0.20	0.0510±0.0340	0.266±0.332	0.0320±0.0110
63	0.226±0.072	1.06±0.20	0.0693±0.0632	0.390±0.291	0.0355±0.0253
70	0.129±0.049	0.424±0.031	0.0443±0.0041	0.0185±0.0013	0.0148±0.0073

Table 12. The omega-3 fatty acid content (mg g^{-1} dw) of B. hooglandii *during the four growth phases, lag (day 0-23), exponential (day 24-35), stationary (day 36-49), and death (day 50-70).*

Day	C16:4(n-3)	C18:3(n-3)	C18:4(n-3)	C20:3(n-3)	C20:4(n-3)	C20:5(n-3)	C22:5(n-3)	C22:6(n-3)
0	0.263±0.141	0.186±0.009	0.234±0.006	0.0200±0.0055	0.165±0.002	16.1±0.9	0.215±0.015	1.98±0.09
23	0.222±0.115	0.216±0.016	0.252±0.006	0.0351±0.0110	0.180±0.002	17.9±0.8	0.228±0.011	2.25±0.10
30	0.612±0.099	0.229±0.059	0.291±0.036	0.0734±0.0213	0.269±0.020	17.6±1.2	0.176±0.019	1.51±0.04
35	0.571±0.058	0.511±0.067	0.380±0.057	0.0470±0.0141	0.614±0.181	25.3±2.3	0.374±0.077	3.06±0.34
44	0.292±0.046	0.347±0.026	0.429±0.025	0.0333±0.0174	0.656±0.085	20.8±0.8	0.179±0.049	2.42±0.07
49	0.340±0.111	0.347±0.024	0.706±0.030	0.0416±0.0140	0.788±0.054	16.1±13.9	0.201±0.027	2.72±0.14
56	0.284±0.066	0.221±0.017	0.548±0.062	0.0211±0.0040	0.501±0.084	15.6±1.9	0.143±0.010	1.66±0.19
63	0.418±0.153	0.191±0.064	0.368±0.033	0.0441±0.0140	0.326±0.070	9.30±1.10	0.078±0.006	1.01±0.12
70	0.467±0.032	0.0461±0.0041	0.188±0.007	0.0202±0.0088	0.116±0.014	3.46±0.24	0.0314±0.0065	0.400±0.081

3.3.4 Correlation between pigments and fatty acids

Within this study, correlations were identified between pigments and fatty acids. For example, the omega-3 fatty acid EPA was positively correlated with β-carotene. This correlation may be a consequence of the role of omega-3 fatty acids, and hence EPA, in the thylakoid membrane. Under environmental conditions that enhance growth, the thylakoid membrane contains increased content of PUFA-rich lipids (that assist with membrane synthesis) and pigment-protein complexes are also formed, from carotenoids and chlorophyll (Guschina & Harwood, 2006; Takaichi, 2011). However, under stressful growth conditions (such as nutrient limitation or high light intensity), pigment and PUFA concentrations in the thylakoid membrane drop, and fatty acids shift to storage lipids, which are rich in MUFAs and SFAs (Guschina & Harwood, 2006; Markou et al., 2012). This response may explain the correlation between EPA and β-carotene; however, it does not explain why there is not a strong correlation among the other PUFAs and pigments. Notably, the omega-3 fatty acid 22:5(n-3) was also strongly positively correlated with β-carotene ($r=0.930$, $p=0.022$).

3.3.5 Algal protein content

Algal protein content can be greatly influenced by growth phase and environmental conditions. At the end of the exponential phase, the total protein concentration of *B. hooglandii* peaked at 556 ±11 mg g^{-1} dw (Figure 6). Zhu et al. (1997) noted a similar trend when examining the growth of the marine alga *Isochrysis galbana*, in which protein concentration increased during the lag and exponential phases, followed by a drop in concentration in the stationary phase. For *I. galbana,* this drop was attributed to nitrogen limitation, resulting in decreased synthesis of the proteins involved in photosystems I and II (Markou et al., 2012). A similar process may be occurring in *B. hooglandii,* as the concentration of total proteins in this study

followed a comparable trajectory- as the exponential phase came to an end and the stationary phase began, the cellular composition shifted away from protein production and towards lipid production. As the algal culture moves through the different growth phases, pigments, proteins, lipids and carbohydrates cellular content varies (Markou et al., 2012).

3.3.6 Algal carbohydrate content

In the present study, carbohydrate content peaked in *B. hooglandii* at 490 ± 116 mg g^{-1} during the final growth phase (death). Carbohydrate concentration steadily increased, from the end of lag phase (day 23) to the end of death phase (day 70), which is in agreement with previous studies demonstrating a positive correlation between the age of an algal culture and relative carbohydrate concentration (Fidalgo et al., 1998; Fernández-Reiriz et al., 1989). This positive correlation may be a result of phosphorous limitation in the death phase, inducing increased carbohydrate content. Markou et al. (2012) demonstrated the effects of phosphorous limitation on *C. vulgaris* and *Spirulina platensis,* resulting in the carbohydrate content increasing from 15% to 55% and 11% to 63%, respectively. Phosphorus limitation changes the composition of algal cells due to a reduction in protein synthesis and an accumulation of carbohydrates and lipids (Markou et al., 2012). In addition to phosphorous limitation, sulfur deprivation could be a potential cause of the doubling in carbohydrate concentration from day 56 to 61. Sulfur is necessary for the synthesis of proteins, thus sulfur limitation would result in the accumulation of other nutritional compounds, such as carbohydrates (Markou et al., 2012). Electron transport carriers require sulfur, and in its absence, cell division halts and carbohydrate content rapidly increases. The specific mechanism behind the dramatic spike in carbohydrate concentration due to sulfur is unknown (Markou et al., 2012). A 2011 study investigated the effects of sulfur limitation on carbohydrate content in *Chlorella* sp., and demonstrated that sulfur-limited cultures

achieved a starch concentration of 2 g L^{-1} compared to 0.4 g L^{-1} in untreated cultures (Brányiková et al., 2011). Tracking the growth-limiting nutrients during algal growth and understanding the correlations that exist among the different nutritional characteristics are key to cost-effective harvesting.

3.3.7 Nitrogen deficiency

Nitrogen deficiency has pervasive effects, influencing the cellular content of growth, pigments, fatty acids, and proteins. Nitrogen limitation has a negative effect on the production of proteins and pigments (such as chlorophyll and carotenoids) in photosystem I and II (Markou et al., 2012). For example, nitrogen deficiency results in phycobilisome degradation in photosystem II (Hu, 2004), due to decreased synthesis and utilization of phycobilisome proteins as an internal nitrogen source (Collier & Grossman, 1992). Under nitrogen limiting conditions protein synthesis decreases, due to the structural role of nitrogen in proteins, and the synthesis of lipids or carbohydrates increases (Markou et al., 2012). Nitrogen deficiency and decreased protein synthesis lead to reduced biomass accumulation and enhanced lipid or carbohydrate content in the cell (Markou et al., 2012). This correlation is clearly illustrated by the correlation (r-value) of 0.934 ($p=0.020$) between the protein content and the growth rate. Thus, growth rate can act as a predictor of protein concentration during the different growth phases, and could potentially be used by the aquaculture industry to identify a protein-rich culture.

3.3.8 Aquaculture implications

Cell size, digestibility, growth rate, pigment content, and nutrition composition are all characteristics that must be considered when selecting microalgae for aquaculture (Glaude & Maxey, 1994). A cell size of 6 µm (Microalgal Technology Research Group, 1986), as well as the absence of a cell wall, makes *B. hooglandii* easily digestible for larval mollusks and shrimp

(Glaude & Maxey, 1994; Mussgnug et al., 2010). The biomass productivity of this algal species (0.52 g L^{-1} day^{-1}) was relatively high in comparison with previously estimated growth rates of currently used aquaculture feeds, e.g. *I. galbana* (0.16 g L^{-1} day^{-1}), *Tetraselmis suecica* (0.27 g L^{-1} day^{-1}), and *Pavlova* sp. (0.28 g L^{-1} day^{-1}) (Patil et al., 2007). In addition, the high biomass productivity will result in increased productivity of the nutritional components. For example, in the late exponential phase, the PUFA content in *B. hooglandii* was relatively low (Table 14); however, when biomass productivity was taken into consideration, the PUFA productivity was 20.28 mg L^{-1} day^{-1} and 7.20 mg L^{-1} day^{-1} for *B. hooglandii* and *I. galbana*, respectively. These characteristics suggest that this species may be a suitable candidate for aquaculture feed.

Boekelovia hooglandii can be used as an aquaculture feed, and can be optimized to be rich in protein (max. 55.6%), fatty acid (max. 27.1%), or carbohydrate (max. 49.0%) content depending on growth and harvest conditions. Collecting algal biomass on day 35 creates an aquaculture feed rich in protein (55.6%), with moderate levels of carbohydrates (13.9%) and fatty acids (18.6%). This combination is ideal for enhancing the growth of bivalve larvae, which prefer a protein and carbohydrate composition between 30-60% and 5-30%, respectively (Renaud et al., 1999). Day 35 biomass was also rich in PUFAs (3.9%), which can enhance the nutritional value of the aquaculture feed (Brown et al., 1997). Collecting *B. hooglandii* on day 63 will create an aquaculture feed high in carbohydrates (49.0%) and moderate levels of PUFAs (1.6%). This nutritional profile is ideal for enhancing the growth of juvenile oysters and larval scallops (Brown et al., 1997). When protein, fatty acid, carbohydrate, and pigment content are taken into consideration, this species appears to be a viable aquaculture feed when compared to the commonly used microalgal feed *I. galbana*, as seen in Table 14. Nutrient requirements vary by aquaculture organism, so in addition to characterization of nutrients, feeding experiments

would be necessary before implementing *B. hooglandii* as a commercial feed.

This study proposes the halotolerant alga *B. hooglandii* as a species for potential use as aquaculture feed. This research also identifies the peak concentrations and correlations among the nutritional constituents (protein, fatty acid, pigments and carbohydrates) throughout the four growth phases. These findings can be used by the aquaculture industry for future decision-making concerning microalgal nutritional composition. For example, the ability of certain cellular characteristics to predict others could provide a high-throughput method for identifying potential microalgae for aquaculture. In conclusion, the small cell size, easy digestibility, and nutritional profile of *B. hooglandii* suggests that this microalga is a suitable candidate as aquaculture feed for bivalve larvae and juvenile oysters (Microalgal Technology Research Group, 1986; Glaude and Maxey, 1994; Mussgnug et al., 2010, Renaud et al., 1999; Brown et al., 1997).

Table 13. A comparison of B. hooglandii *and* I. galbana *as aquaculture feeds, using percentage of total biomass.*

Aquaculture Characteristic	*Boekelovia hooglandii* (Day 35; late exponential)	*Boekelovia hooglandii* (Day 63; mid-death)	*Isochrysis galbana* (Dörner et al., 2014) (late exponential phase)	*Isochrysis galbana* (Fidalgo et al., 1998) (early stationary phase)	*Isochrysis galbana* (Fidalgo et al., 1998) (late stationary phase)
Protein	55.6%	28.0%	12.4%	34.5%	28.0%
Carbohydrates	13.9%	49.0%	51.2%	10.6%	12.0%
Fatty acid	18.6%	21.4%	15.3%	34.0%	38.5%
PUFA	3.93%	1.55%	4.45%	7.15%	6.82%
Omega-3	3.08%	1.17%	1.07%	4.56%	3.44%
EPA + DHA	2.83%	1.03%	0.505%	2.69%	2.44%

Chapter 4: The effect of growth conditions on omega-3 fatty acid production in five microalgae

4.1 Introduction

Omega-3 fatty acids are essential to the healthy development of a wide range of organisms, including zooplankton, zoobenthos, fish, and humans (Kainz et al., 2004; Koussoroplis et al., 2011). Algae play a valuable role as a natural source of these essential lipids in both marine and freshwater food webs (Kainz et al., 2004). The ability of these organisms to synthesize essential fatty acids is due to the presence of desaturase Δ12 and Δ15 enzymes, which enables some algal groups to produce 18:2(n-6) and 18:3(n-3) fatty acids. The synthesis of these two fatty acids is an important step in the development of longer chain PUFAs including omega-3s (Wallis et al., 2002). Omega-3 fatty acids are synthesized directly in algal cells (e.g. Cardozo et al., 2007), are present in the lipid bilayers of the membranes, and contribute to the maintenance of membrane function under varying growth conditions (Cardozo et al., 2007; Zhang et al., 2011). The algal omega-3 content influences the food quality throughout aquatic trophic levels (Kainz et al., 2004), largely because consumers lack Δ12 and Δ15 enzymes and need to obtain essential fatty acids from dietary sources (Schmid and Ohlrogge, 2002; Wallis et al., 2002). Hence, access to omega-3 fatty acids through diet directly affects the health of consumers (Kainz et al., 2004), and is closely associated with omega-3 content within the algal cell.

The necessity for omega-3 fatty acids in the human diet has resulted in increased demand from the pharmaceutical and food industries for novel sources of omega-3 fatty acids, such as omega-3-rich algal species (Simopoulos 1999; Sidhu 2003; San Giovanni & Chew 2005; Lane et al., 2014). Omega-3 fatty acids have been linked to the prevention of a variety of neurodegenerative and cardiovascular disease (San Giovanni and Chew 2005; Simopoulos 1999). Docosahexaenoic acid [DHA; 22:6(n-3)] intake during pregnancy and early childhood is critical for eye and brain development (Lane et al. 2014). This fatty acid is also beneficial throughout life as it aids in the maintenance of vision and cognition over time (Lane et al. 2014). Eicosapentaenoic acid [EPA; 20:5(n-3)] is postulated to play a major role in reducing the effects of deep vein thrombosis (Lane et al. 2014). Furthermore, it can be used as a preventative measure against atherosclerosis, heart disease, and inflammatory disease (Dyerberg 1986; Ziboh 1991; Mata et al. 2010).

Some algae are a rich source of omega-3s, and growth conditions can be manipulated to further increase the cellular content of these fatty acids (Guschina & Harwood 2006). Although they are a component of all membranes, FAs in algae are located primarily in the thylakoid membranes (rich in PUFAs) or as storage lipids (rich in saturated and monounsaturated fatty acids) (Guschina & Harwood 2006). Under favourable nutrient and light conditions, fatty acids are used to a greater degree in the thylakoids for membrane synthesis, which can result in algae with higher PUFA content (Harwood & Guschina 2009). Under stressful growth conditions, such as nutrient limitation or high light intensity, FA content increases in storage lipids, resulting in algae rich in saturated and monounsaturated fatty acids (Harwood & Guschina 2009). Algae can also shift their membrane composition to maintain function and fluidity under different growth conditions (Harwood & Guschina 2009). For example, low growth temperatures can result in a

modified FA chain length as well as a differing level of unsaturation to create a higher PUFA content within an algal cell (Harwood & Guschina 2009).

The present study compared fatty acid production in five species of microalgae previously reported to have high levels of omega-3 fatty acids (Yongmanitchai, 1991; Sheehan et al., 1991; Lang et al., 2011). Experiments were conducted to identify patterns in omega-3 fatty acid production and growth conditions that increase total and omega-3 fatty acid content, and biomass productivity. Four different growth conditions were manipulated using a single factorial design, which tested the effects of light intensity, light cycle, temperature, and vitamin B_{12} content. The growth and fatty acid content from two lipid-rich freshwater microalgae (*Goniochloris sculpta* and *Chloridella simplex*) were compared to those of three marine microalgae (*Rhodella maculata, Boekelovia hooglandii* and *Phaeodactylum tricornutum*). This study identified algal species and growth conditions that produced both a high biomass productivity and omega-3 content. Omeag-3 rich cells are beneficial for consumer health, however the combined high productivity and omega-3 content is a highly desirable feature for industrial application as well (Kainz et al. 2004; Yongmanitchai, 1991)

4.2. Materials and Methods

4.2.1. Microalgal cultures

Cultures were purchased from the Canadian Phycological Culture Centre (CPCC) (Waterloo, ON, Canada; http://uwaterloo.ca/canadian-phycological-culture-centre/) and the Culture Collection of Algae at Göttingen University (SAG) (Göttingen, Germany; www.uni-goettingen.de/en/184982.html) for the following microalgal taxa: *B. hooglandii* Nicolai et Baas Becking CPCC 484 (Synurophyceae, Heterokontophyta), *P. tricornutum* Bohlin CPCC 162

(Bacillariophyceae, Heterokontophyta), *G. sculpta* Geitler SAG 29.96 (Xanthophyceae, Heterokontophyta), *R. maculata* L. V. Evans (SAG 45.85) (Rhodellophyceae, Rhodophyta) and *C. simplex* Pascher SAG 51.91 (Xanthophyceae, Heterokontophyta). Strains were supplied as non-axenic monospecific cultures.

4.2.2. Culture maintenance

The cultures were maintained in 250 mL Erlenmeyer flasks in 50 mL of medium, from which inoculate was aseptically transferred into new sterile flasks containing prepared media every two weeks. Cultures were incubated in a Model E-36HO (Percival Scientific, Inc., IA, United States) growth chamber with 55 W PlusRite Compact Fluorescent FTL55/2G11/841 lights (USA) under a 17 h:7h light:dark cycle, at 80 ± 5 µmoles m^{-2} s^{-1} light intensity, and 18 ± 1°C without shaking or aeration. Media was sterilised and prepared according to the recipes provided by the culture collections. SAG Bold's Basal Medium (SAG BBM) was used for *G. sculpta*, ESP for *C. simplex,* SWES for *R. maculata* and F/2 for both *P. tricornutum* and *B. hooglandii*. The above reference conditions were kept constant during experiments with the exception of changes to individual growth parameters under investigation.

4.2.3. Experimental protocol

Media was inoculated with stock cultures on day 0, with a starting biomass concentration of 1.27 ± 0.22 g L^{-1}, expressed as dry weight biomass per liter of medium. The concentration was estimated every two days using optical absorbance measured with a Spectronic 200 spectrophotometer (Thermo Fisher Scientific). To establish the linear equation required to convert absorbance values to weight, a calibration curve was made using dilution series of the algal cultures. These dilutions were then measured for absorbance and filtered using pre-weighed filter paper. The filter paper was then dried and re-weighed to determine the concentration (g L^{-1})

of the algae present. The algal concentration and corresponding absorbance were graphed and a line of best fit was calculated using Microsoft Excel (2007). The line of best fit (*B. hooglandii* R^2 = 0.93, *P. tricornutum* R^2 = 0.91, *G. sculpta* R^2 = 0.96, *R. maculata* R^2 = 0.92 and *C. simplex* R^2 = 0.91) was used to convert subsequent absorbance values to biomass concentrations. Absorbance at 600 nm was used for *P. tricornutum* and *B. hooglandii* due to a dominant chlorophyll c content (Fawley, 1989; Rabinowitch and Govindjee, 1969), using previously established methods by Yongmanitchai and Ward (1991). Absorbance at 680 nm was used for *G. sculpta, R. maculata,* and *C. simplex* due to a dominant chlorophyll a content (Jones et al., 2013).

Growth parameters were varied independently while all other conditions were maintained as described in section 2.2. All tests were performed in replicates of four. After eight days, samples were pelleted via centrifugation, the pellets were freeze dried and stored at -80°C for fatty acid analysis (Yongmanitchai and Ward, 1991). Lipid extraction and derivatization to fatty acid methyl esters was performed using a one-step direct transesterification followed by gas chromatography to quantify fatty acid content (Zuñiga et al., 2012). The direct transesterification method results in a greater recovery of total and PUFA content, compared to separate extraction and transesterification (Zuñiga et al., 2012). Boron trifluoride in methanol and hexane were added to freeze-dried algae to produce fatty acid methyl esters. The freeze-dried samples were hexane extracted to remove the fatty acid methyl esters. A Varian 3900 gas chromatograph equipped with a flame ionization detector was used to quantify the fatty acids. Derivatized fatty acids were identified through comparison of peak retention times with corresponding standards (Metherel et al., 2013).

The following growth conditions were tested: light cycle (at 24h:0h and 17h:7h light:dark), light intensity (45, 80 and 130 µmoles m^{-2} s^{-1}), and vitamin B_{12} supplementation (0,

10, and 100 ng L^{-1}). Each organism was also grown in the media recommended by the source culture collection; the culture collections used F/2 for *B. hooglandii*, F/2 for *P. tricornutum*, SAG BBM for *G. sculpta*, SWES for *R. maculata*, and ESP for *C. simplex*. The F/2 saltwater medium is supplemented with silica (Guillard 1975), SWES is a saltwater medium that contains soil extract, SAG BBM is a freshwater media, and ESP is a freshwater medium with soil extract (SAG 2014). All media types contain nitrate as the nitrogen source. While one growth condition was being manipulated, the remaining growth conditions followed the appropriate culture collection criteria. All tests were performed in replicates of four.

4.2.4. Statistics

The biomass productivity over 8 days was calculated using the algal biomass on day 8 and 0. Mean biomass productivity (g L^{-1} day^{-1} dry weight) was calculated using $(X_2-X_1)/(t_2-t_1)$, where X_2 was day 8 biomass concentration (g L^{-1}), X_1 was day 0 biomass concentration (g L^{-1}), and t was the sample day (t_2=8 and t_1=0) (Hempel et al., 2012).

SigmaPlot 10.0.1 (San Jose, CA, US) was used for all statistical analyses. T-tests were used to compare the two light cycle conditions, and one-way ANOVA was used for multi-factor comparison of fatty acid abundances, and biomass production under different growth conditions. If significant results were obtained using one-way ANOVA, the Holm-Sidak method was used to perform an all-by-all pairwise multiple comparison. The ANOVA data was additionally assessed for normality and for equal variances, and if data did not pass normality or equal variance tests, a Kruskal-Wallis One Way ANOVA on Ranks was used. The non-parametric Kruskal-Wallis test does not have to meet the assumptions of the parametric ANOVA, and can be used when these assumptions are not met by the data. If significant results were obtained using the Kruskal-Wallis One Way ANOVA on Ranks, the Student-Newman-Keuls method was used to perform a

pairwise comparison. Superscript letters were used in Tables 14 to 17 to indicate whether data points were significantly different (using the same letter; $p<0.05$) or not significantly different (using different letters, such as A, B, and C; $p<0.05$). Four repeats were used for each taxa under each growth condition, and the standard deviation for each mean is indicated in the data tables with plus-minus symbols (±).

4.3. Results and Discussion

4.3.1. Effects of light intensity on biomass productivity and fatty acid content

This study examined the effects of 45, 80 and 130 µmoles m^{-2} s^{-1} on biomass productivity and omega-3 content. This range is comparable to the surface water of natural aquatic habitats, (e.g. within the English Channel at ~25-356 µmoles m^{-2} s^{-1}) (Edwards et al., 2013). Within the present study, *B. hooglandii* ($p=0.002$), *P. tricornutum* ($p=0.004$), and *G. sculpta* ($p=0.0002$) exhibited a significant increase in biomass productivity with increasing light intensity (Table 14). *Chloridella simplex* exhibited an 8.8% increase in biomass productivity at 80 µmoles m^{-2} s^{-1} ($p=0.0005$), followed by a decline in biomass productivity of 7.5% at 130 µmoles m^{-2} s^{-1} ($p=0.0001$). The biomass productivity of *R. maculata* was not significantly impacted. The maximum biomass productivity for an algal strain occurs at the light saturation point and decreases in response to suboptimal light intensities (Wahidin et al., 2013). The marine synurophyte in the present study, *B. hooglandii*, exhibited its maximum biomass productivity in 130 µmoles m^{-2} s^{-1} at 18°C. A 1997 study of the synurophyceae *Synura petersenii* identified a saturation point at 20°C and 167 µmoles m^{-2} s^{-1} (Saxby-Rouen et al., 1997). The high growth of *S. petersenii* at 167 µmoles m^{-2} s^{-1} suggests that *B. hooglandii* had not reached the saturation point. On the other hand, *P. tricornutum* (Bacillariophyceae), exhibited increased biomass productivity up to a plateau at ~80 µmoles m^{-2} s^{-1}, and maintained this level at 130 µmoles m^{-2} s^{-}

^{1}. This agrees with the study by Beardall and Morris (1976), who identified a similar plateau in *P. tricornutum* above ~80 µmoles m^{-2} s^{-1}. The light saturation point for *P. tricornutum* was reached at 80-130 µmoles m^{-2} s^{-1}, as demonstrated by the plateau. A similar plateau was observed in the biomass productivity of *C. simplex;* however, growth began to decline at 130 µmoles m^{-2} s^{-1}. The high biomass productivity at 80 µmoles m^{-2} s^{-1} agrees with a study on the *Vaucheria dichotoma* (Xanthophyceae) in which growth peaked at 86 µmoles m^{-2} s^{-1} (Aberg & Fries, 1976). The subsequent drop in the biomass productivity of *C. simplex* at 130 µmoles m^{-2} s^{-1} would suggest that light intensity surpassed the saturation point for this particular species. Finally, Phooprong et al. (2007) studied the response of the macroalga *Gracilaria salicornia* to irradiance at 20°C and identified a light saturation point at 395 µmoles m^{-2} s^{-1}. If *R. maculata* has a similarly high saturation point, then the three intensities analyzed in the present study would all be relatively low (in comparison with 395 µmoles m^{-2} s^{-1}), which could explain this species' lack of a significant response. Light intensity and saturation point can influence microalgal biomass productivity; the change in biomass productivity depends on the physiological capabilities of the algal species and previous light and temperature adaption.

Of the microalgae examined in this study, only the diatom *P. tricornutum* exhibited significantly increased omega-3 content ($p=0.0006$) as light intensity decreased (Table 14). During light limitation, the algal cell will attempt to capture more light by increasing thylakoid synthesis, subsequently increasing the cellular content of PUFA-rich thylakoid membranes (Guschina and Harwood, 2009; Wacker et al., 2016). This mechanism would explain the high omega-3 content at the lowest light intensity. However, another mechanism for low light intensity has been demonstrated using *D. tertiolecta* (Wacker et al., 2016), which may explain the absence of a significant change in omega-3 content in the other four microalgae. Under

limited light availability, lower PUFA content reduces thylakoid fluidity, thereby decreasing proton leakage and preventing energy loss (Wacker et al., 2016). Thus, these two competing

Table 14. The effects of three light intensities, 45, 80 and 130 µmoles m^{-2} s^{-1} (± 5 µmoles m^{-2} s^{-1}) on the total and omega -3 fatty acid content of five algae growing under 17h:7h light:dark at 18 ± 1°C.

	Light Int-ensity	Biomass productivity (g L^{-1} day^{-1} dw)	Omega-3 Fatty acid Production (mg g^{-1} dw)							Total Omega-3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
			16:4n3	18:3n3	18:4n3	20:3n3	20:4n3	20:5n3	22:5n3	22:6n3		
Boekelovia hooglandii	130	0.105 ±0.016^{Aa}	0.082 ±0.022^{C}	0.854± 0.069^{A}	11.245± 0.632^{B}	0.056 ±0.016^{A}	0.049± 0.019	36.19 ±2.17	0.648± 0.0411^{A}	12.969 ±0.872^{A}	62.6 ±3.79	241± 9.84^{B}
	80	0.100 ±0.018^{AB}	0.509 ±0.038^{A}	0.635± 0.075^{B}	11.0 ±1.21^{B}	0.0219± 0.0041^{B}	0.039± 0.013	32.5± 2.8	0.289± 0.019^{C}	8.94 ±0.72^{B}	54.0 ±4.8	285± 15.5^{A}
	45	0.045 ± 0.015^{B}	0.19± 0.0149^{B}	1.16± 0.37	13.9± 0.763^{A}	0.056± 0.026^{A}	0.062± 0.031	47± 17	0.63± 0.20^{B}	15.6 ±4.3^{A}	78.6±28	323± 57^{A}
Phaeodactylum tricornutum	130	0.172± 0.0048^{A}	0.0503 ±0.0149	0.32 ±0.17	2.1 ±1.3	0.0176 ±0.0046	0.77 ±0.48	22± 14	0.169 ±0.0151	1.57 ±0.91^{B}	27.1 ±4.7^{A}	287± 58
	80	0.17±0.01^{AB}	0.26 ±0.13	0.57± 0.26	3.7±1.7	0.025± 0.021	1.28 ±0.56	41±18	0.25± 0.10	3.2 ±1.2^{AB}	50.0 ±21.9^{AB}	402± 182
	45	0.1544± 0.0061^{B}	0.291 ±0.012	0.444 ±0.026	2.13 ±0.14	0.0182 ±0.0025	0.82 ±0.11	36.4 ±1.4	0.1710 ±0.0086	2.942± 0.063^{A}	43.3 ±1.5^{B}	308± 11
Goniochloris sculpta	130	0.041± 0.0087^{A}	0.16± 0.11	0.86 ±0.37	0.273± 0.091	0.0168± 0.0047	0.190± 0.091	20±11	0.0194± 0.0015	0.047± 0.015^{A}	22±11	61± 20
	80	0.033± 0.0069^{A}	0.308± 0.072	0.91 ±0.16	0.256± 0.029	0.0156± 0.0063	0.13 ±0.11	21.5 ±3.1	0.0163± 0.0075	0.0147 ± 0.0074	23.2 ±3.4	70± 12
	45	0.00073± 0.0047^{B}	0.347± 0.067	0.98 ±0.11	0.265± 0.043	0.0151± 0.0079	0.204± 0.039	22.2 ±2.5	0.032± 0.012	0.0213 ± 0.0095	24.1 ±2.7	71.1± 9.7
Rhodella maculata	130	0.156 ±0.0074	0.0047 ±0.0017	0.0077 ± 0.0027	0.020± 0.017	0.0109± 0.0035^{A}	0.013± 0.012	4.4± 3.9^{AB}	0.012± 0.012	0.048± 0.027^{B}	4.5±3.8	23.1± 5.6

	Light Int-ensity	Biomass productivity (g L^{-1} day^{-1} dw)	Omega-3 Fatty acid Production (mg g^{-1} dw)							Total Omega-3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
			16:4n3	18:3n3	18:4n3	20:3n3	20:4n3	20:5n3	22:5n3	22:6n3		
Chloridella simplex	80	0.147± 0.018	0.0040 ±0.0064	0.0075 ± 0.0047	0.0073± 0.0023	0.0035± 0.0018[B]	0.0040 ± 0.0018	5.98± 0.80[B]	0.0110± 0.0037	0.0065 ± 0.0026	6.06± 0.81	21.5± 2.9
	45	0.1563± 0.0057	0.067 ±0.017	0.0066 ± 0.0019	0.0061 ±0.0015	0.0054± 0.0014[B]	0.0070 ± 0.0015	8.01± 0.50[A]	0.0140± 0.0038	0.0059 0±0.00 094[A]	8.12 ±0.52	28.8± 2.6
	130	0.148 ±0.001[B]	0.281 ±0.043 [B]	2.33± 0.29[A]	0.259 ±0.036	0.063± 0.011	0.763± 0.089[A]	53.7 ±5.6	0.0272± 0.0169	0.023± 0.014	57.5± 6.0	139± 15.4
	80	0.160 ±0.002[A]	0.28 ±0.13 [AB]	2.33 ±0.14[A]	0.284 ±0.017	0.069 ±0.013	0.870 ±0.060	54.1 ±4.6	0.033 ±0.019	0.0292 ±0.016	58.1± 5.0	150.0± 8.0
	45	0.147 ±0.0032[B]	0.475±0 .043[A]	1.810± 0.032[B]	0.248 ±0.0016	0.068 ±0.013	0.598± 0.024[B]	49.4 ±2.5	0.0244 ±0.0066	0.022 ±0.015	52.6± 2.7	140.0± 3.7

[a] Superscripts A, B, and C indicate statistically significant differences due to light intensity within a given species (refer to Materials and Methods).

mechanisms may be used by the algal cell to maintain functioning during low light intensity.

4.3.2. Effects of light cycle on biomass productivity and fatty acid content

Light cycle (17h light:7h dark) and continuous illumination (24h:0h) greatly influenced three of the five cultures studied (Table 15). Under continuous light, *B. hooglandii* and *G. sculpta* experienced a significant increase in EPA, total omega-3, and biomass productivity ($p = 0.003$, 0.002, and 0.0001 for *B. hooglandii* and $p = 0.005$, 0.007, and 0.0001 for *G. sculpta*). Brown et al. (1996) identified a relationship between a culture approaching light saturation and a resulting increase in biomass productivity and omega-3 content. Based on this relationship, the high biomass productivity and omega-3 content of *B. hooglandii* and *G. sculpta* under continuous light suggests that these species were approaching the light saturation point, which was not reached at 130 μmoles m^{-2} s^{-1}under a 17h:7h light:dark cycle. Within this study, *P. tricornutum* and *C. simplex* reached light saturation under 17h:7h from 80-130 and 80 μmoles m^{-2} s^{-1}, respectively. There was no significant difference in biomass productivity between 24h:0h and 17h:7h in *P. tricornutum* and *C. simplex*, which may be due to cultures undergoing photoacclimation to maintain growth with increasing irradiance. These photoprotective mechanisms help prevent damage to the cell due to the wide range of irradiances experienced in nature (Torzillo et al., 2012). As members of the Bacillariophyceae and Xanthophyceae respectively, *P. tricornutum* and *C. simplex* utilize the diadinoxanthin cycle, which removes excess energy in photosystem II by converting diadinoxanthin to diatoxanthin. This cycle prevents damage to the photosynthetic apparatus (Jahns et al., 2009) and may have contributed to the lack of a significant change in biomass productivity for these two species. The Rhodophyta, *R. maculata*, was also significantly affected by light:dark cycle. Both the biomass productivity and total fatty acid content significantly decreased ($p=0.004$ and $p=0.012$, respectively) under

continuous light. However, it is unclear which mechanisms contributed to this response. Algal omega-3 content and biomass productivity are physiologically determined by algal species and their associated light saturation point. The impact of light cycle and continuous light on omega-3 content and biomass productivity was dependent on algal species and the associated light saturation point, resulting in a significant response in three of the five algae studied.

4.3.3. Effects of temperature on biomass productivity and fatty acid content

Within the present study, the effect of varying temperature (15, 20 and 25°C) on biomass productivity and fatty acid content was examined. This is within the natural temperature range of temperate climates (15-40°C varying with seasonal and regional changes) (Juneja et al., 2013). Three microalgae *B. hooglandii, P. tricornutum* and *G. sculpta* experienced significantly greater biomass productivities at 25°C [*B. hooglandii* (p=0.0001), *P. tricornutum* (p=0.0002) and *G. sculpta* (p=0.0001)]. The high biomass productivity of *B. hooglandii* under 25°C agrees with the results for other species within the Synurophyceae, such as *S. petersenii* which had optimal growth at 25°C (Saxby-Rouen et al., 1997). A study by Yongmanitchai and Ward (1991) supports the high biomass productivity of *P. tricornutum* at 25°C, establishing that this species had optimal growth at a temperature of 23°C. With respect to *G. sculpta*, it was previously determined (Iliev et al., 2008) that the optimal temperature for *Trachydiscus lenticularis* (Pleurochloridaceae) was 26°C, which is consistent with the high biomass productivity of the Pleurochloridaceae *G. sculpta* at 25°C within this study. Of the five species analyzed in the present study, *B. hooglandii, P. tricornutum* and *G. sculpta* exhibited significantly lower omega-3 content at 20°C, compared to 15°C and 25°C [*B. hooglandii* (p=0.0001 and p=0.029, respectively), *P. tricornutum* (p=0.0001 and p=0.0001 respectively) and *G. sculpta* (p=0.020 and p=0.006, respectively)] (Table 16). Two different mechanisms may have resulted in the higher

Table 15. The effects of two light cycles, 24h:0h and 17h:7h (light:dark), on the omega-3 fatty acid and total fatty acid content of the five test algae growing under 80 ± 5 µmoles m^{-2} s^{-1} light intensity at 18 ± 1°C.

	Light Cycle	Biomass productivity (g L^{-1} day^{-1} dw)	Omega-3 Fatty acid Production (mg g^{-1} dw)								Total Omega-3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
			16:4n3	18:3n3	18:4n3	20:3n3	20:4n3	20:5n3	22:5n3	22:6n3		
Boekelovia hooglandii	24h:0h	0.255± 0.025^{Aa}	0.238± 0.068^{A}	0.357± 0.058^{A}	2.70± 0.34^{A}	0.0369± 0.0081	0.0304± 0.0037	6.1± 1.4^{A}	0.052± 0.015^{A}	1.36± 0.21^{A}	10.9± 1.9^{A}	126±11^{A}
	17:7h	0.108± 0.016^{B}	0.083± 0.014^{B}	0.159± 0.074^{B}	1.08± 0.50^{B}	0.039± 0.019	0.035± 0.022	2.1± 1.1^{B}	0.0201± 0.0053^{B}	0.48± 0.19^{B}	4.0± 1.8^{B}	88± 11^{B}
Phaeodactylum tricornutum	24h:0h	0.2098± 0.0076	0.52 ±0.12	0.294± 0.031^{A}	0.439± 0.062	0.0494± 0.0044	0.203± 0.034	6.77± 0.84^{B}	0.058± 0.022	0.532± 0.035	8.86± 0.74^{B}	153±13^{A}
	17h:7h	0.1943± 0.0082	0.048± 0.022	0.215± 0.019^{B}	0.340± 0.071	0.034± 0.013	0.199± 0.035	11.7± 2.4^{A}	0.0404± 0.0046	0.521± 0.083	13.1± 2.6^{A}	91.2± 8.9^{B}
Goniochloris sculpta	24h:0h	0.158± 0.016^{A}	0.067± 0.032	1.06± 0.25^{A}	0.323± 0.039^{A}	0.019± 0.013	0.085± 0.020	13.3± 3.2^{A}	0.0168± 0.0077	0.295± 0.027^{A}	15.1± 3.8^{A}	52± 12
	17h:7h	0.065± 0.039^{B}	0.029± 0.018	0.217± 0.042^{B}	0.182± 0.026^{B}	0.093± 0.030	0.046± 0.023	3.07± 0.81^{B}	0.0168± 0.0034	0.171± 0.034^{B}	3.83± 0.94^{B}	45.8± 9.6
Rhodella maculata	24h:0h	0.103± 0.012^{B}	0.063± 0.016	0.0078± 0.0019	0.0049 ±0.004	0.0105± 0.0015	0.0058± 0.0017	7.33± 0.67	0.0134± 0.0019	0.0154± 0.0013	7.45± 0.69	29.5 ±3.2^{B}
	17h:7h	0.1336± 0.0061^{A}	0.034± 0.042	0.039± 0.017	0.012± 0.015	0.029± 0.013	0.018± 0.012	5.2± 1.2	0.0201± 0.0078	0.036± 0.047	5.4± 1.1	50± 11^{A}
Chloridella simplex	24h:0h	0.0966± 0.0050	0.505± 0.036	1.65± 0.12^{B}	0.142± 0.006^{B}	0.0706± 0.0036	0.187± 0.015^{B}	38.2± 1.2^{A}	0.0190± 0.008	0.0260± 0.006	40.8± 1.3	135± 4.7
	17h:7h	0.100± 0.0060	0.063± 0.018	2.99± 0.29^{A}	0.215± 0.012^{A}	0.096± 0.025	0.288± 0.012^{A}	33.6±2 .9^{B}	0.0076± 0.0064	0.0438± 0.0093	37.3± 3.2	138± 11

[a]A, B and C indicate statistical significance ($p<0.05$)

omega-3 responses exhibited by these three microalgae at 15°C and 25°C. At 25°C, the significantly higher biomass productivities have been documented to result in increased thylakoid synthesis in preparation for cell division, resulting in a significantly higher omega-3 content at 25°C, compared to 20°C (Sukenik & Carmeli, 1990). A different mechanism is likely to occur under the 15°C growth condition; during low temperature growth conditions the algal cell will increase omega-3 content to maintain membrane fluidity (Juneja et al., 2013). *Rhodella maculata* exhibited significantly increased biomass productivity of 0.122 ± 0.018 g L^{-1} day^{-1} ($p<0.0002$) at the lowest tested temperature. The high biomass productivity exhibited by this algal species may be due to the adaptive mechanisms within the Rhodophyta, which allow this phylum to grow in deeper waters with reduced temperatures (Graham & Wilcox, 2000). The biomass productivity and omega-3 fatty acid content of *C. simplex* was not significantly influenced by the temperature range studied. Previously, Gigova et al. (2011) examined the impact of a 15-40°C temperature range on the cellular growth of another Xanthophyceae, *T. minutus*. From this study they determined that *T. minutus* could grow under a wide range of temperatures, only exhibiting growth inhibition at 15°C and 40°C (Gigova et al., 2011). A broad temperature tolerance, similar to *T. minutus,* may explain the lack of a significant response due to 15, 20 and 25°C in *C. simplex.* The results in the present study are noteworthy as they suggest that certain tested microalgae, notably *G. sculpta*, *P. tricornutum* and *B. hooglandii,* have high omega-3 content and biomass production under similar growth conditions, and thus may have potential industrial application.

4.3.4. Effects of vitamin B_{12} (cobalamin) on biomass productivity and fatty acid content

Typically free vitamin B_{12} is present within nature at a concentration of 2-6 ng L^{-1} in freshwater and approximately 3 ng L^{-1} in marine environments, however this does not meet the minimum

typically required (>10 ng L^{-1}) for algal growth (Croft et al., 2005). Research has shown that some algae can synthesize cobalamin de novo, however many species are cobalamin auxotrophs and exist in a symbiotic relationship with bacteria that are able to synthesize this vitamin (Croft et al., 2005). There does not appear to be a clear taxonomic relationship to indicate whether an algal species requires vitamin B_{12} or the source of this vitamin, via de novo synthesis or symbiotic relationships (Carlucci & Bowes, 1970; Croft et al., 2005). This is due to vitamin B_{12} auxotrophy independently evolving multiple times (Croft et al., 2005; Croft et al., 2006). The present study examined the effect of added vitamin B_{12} (0, 10 and 100 ng L^{-1}), as shown in Table 17, on omega-3 content and biomass productivity. The presence of vitamin B_{12}, as 10 or 100 ng L^{-1}, significantly increased biomass productivity by 20.5% (p=0.010) and 33.8% (p=0.002) in *B. hooglandii*, suggesting that this species clearly benefits from vitamin B_{12} supplementation. The biomass productivity of two of the five cultures, *G. sculpta* and *R. maculata* did not significantly increase with increased vitamin B_{12}, indicating that these species may not require vitamin B_{12} supplementation. This may be due to these species having the mechanisms necessary to synthesize vitamin B_{12} themselves or being part of symbiotic relationships with bacteria (Carlucci & Bowes, 1970). Previous research on *P. tricornutum* determined that this species is able to synthesize vitamin B_{12} (Carlucci et al., 1974), which may explain the significantly increasing biomass productivity from 10 to 100 ng L^{-1} (p=0.002), but neither were significantly different from 0 ng L^{-1}. The lack of a significant difference between 100 and 0 ng L^{-1}, and 10 and 0 ng L^{-1} suggests that under growth conditions lacking in vitamin B_{12} the algal cell may compensate by synthesizing vitamins. One of the five cultures analyzed, *C. simplex*, preferred no supplementation with 10 ng L^{-1} and 100 ng L^{-1} resulting in a significant reduction in biomass productivity by 10.5% as vitamin content increased (p=0.005 and p=0.013, respectively). It is

Table 16. The effects of different temperatures, 15°C, 20°C and 25°C, on the total and omega-3 fatty acid content of five test algae growing under 17h:7h, and 80 ± 5 µmoles m^{-2} s^{-1} light intensity.

	Temp-erature	Biomass prod-uctivity (g L^{-1} day^{-1}dw)	Omega-3 Fatty acid Production (mg g^{-1} dw)								Total Omega-3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
			16:4n3	18:3n3	18:4n3	20:3n3	20:4n3	20:5n3	22:5n3	22:6n3		
Boekelovia hooglandii	25°C	0.1847± 0.0018^{Aa}	0.428± 0.083^{A}	1.55 ±0.20^{A}	9.4 ±1.4^{A}	0.0290± 0.0090	0.061± 0.017	25.7± 3.1^{A}	0.082± 0.014^{A}	5.30 ±0.75^{A}	42.5± 5.5^{A}	158 ±26.7^{A}
	20°C	0.108± 0.016^{B}	0.083± 0.014^{B}	0.159± 0.074^{B}	1.08± 0.50^{B}	0.039± 0.019	0.035± 0.022	2.1 ±1.1^{B}	0.0201± 0.0053^{B}	0.48 ±0.19^{B}	4.0± 1.8^{C}	87.7 ±10.7^{B}
	15°C	0.0412± 0.0034^{C}	0.30± 0.20^{AB}	0.32 ±0.34^{B}	2.6 ±2.8^{B}	0.15 ±0.15	0.065± 0.069	7.5 ±9.6^{B}	0.064± 0.068^{A}	2.2 ±3.2^{AB}	13.2 ±6.2^{B}	207± 103^{A}
Phaeodactylum tricornutum	25°C	0.108± 0.015^{B}	0.18± 0.029^{B}	0.268± 0.035	0.560± 0.060^{B}	0.0132± 0.0039^{A}	0.695± 0.068^{A}	24.8± 1.6^{A}	0.125± 0.015^{A}	1.70 ±0.12	28.3 ±1.9^{A}	206± 17.4^{B}
	20°C	0.1943± 0.0082^{A}	0.051± 0.022^{C}	0.215 ±0.019	0.340± 0.071^{C}	0.034± 0.013^{B}	0.199± 0.035^{B}	11.7± 2.4^{B}	0.0404± 0.0046^{B}	0.52 ±8.3	13.1 ±2.6^{B}	91.2± 8.9^{C}
	15°C	0.0477± 0.0043^{C}	0.54± 0.17^{A}	0.45 ±0.18	1.97± 0.73^{A}	0.0355± 0.0084^{B}	0.271± 0.098^{B}	17.9± 8.9^{B}	0.103± 0.061^{AB}	1.45 ±0.82	28.0 ±1.05^{A}	411± 83^{A}
Goniochloris sculpta	25°C	0.166± 0.027^{A}	0.442± 0.092^{A}	3.1 ±1.5^{A}	0.86± 0.32^{A}	0.178± 0.089	0.38± 0.16^{A}	55 ±17^{A}	0.092± 0.046^{A}	0.0451 ±0.0030^{B}	60.2 ±19.1^{A}	170 ±52^{A}
	20°C	0.065± 0.040^{B}	0.029± 0.018^{B}	0.217± 0.042^{C}	0.182± 0.026 ^{C}	0.093± 0.030	0.046± 0.023^{B}	3.07± 0.81 ^{C}	0.0168± 0.0034^{B}	0.171 ±0.034^{A}	3.83 ±0.94^{C}	45.8 ±9.6^{B}
	15°C	0.00207± 0.00075^{C}	0.09± 0.12^{B}	0.96 ±0.12^{B}	0.278± 0.044^{B}	0.060± 0.028	0.120± 0.024^{B}	14.7± 2.0^{B}	0.014± 0.013^{B}	0.060 ±0.030^{B}	16.2 ±2.1^{B}	52.7 ±2.6^{B}
Rhodella maculata	25°C	0.0152± 0.0061^{C}	0.092± 0.050	0.0286± 0.0070	0.0116± 0.0057	0.0165± 0.0040	0.0159± 0.0043	13.7± 2.5^{A}	0.037± 0.012	0.043± 0.015	13.9±2.5^{A}	53±11
	20°C	0.1336± 0.0061^{B}	0.034± 0.042	0.039 ±0.017	0.012± 0.015	0.029± 0.013	0.018± 0.012	5.2± 1.2^{B}	0.0201± 0.0078	0.036 ±0.047	5.4±1.1^{B}	50±11

	Temp-erature	Biomass prod-uctivity (g L^{-1} day^{-1}dw)	Omega-3 Fatty acid Production (mg g^{-1} dw)								Total Omega-3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
			16:4n3	18:3n3	18:4n3	20:3n3	20:4n3	20:5n3	22:5n3	22:6n3		
	15°C	0.1762± 0.0086[A]	0.0147 ±0.008 8	0.039± 0.015	0.0070± 0.0058	0.034± 0.028	0.0077± 0.0069	7.1 ±1.7[B]	0.019± 0.011	0.036 ±0.026	7.2 ±1.7[B]	43 ±13
Chloridella simplex	25°C	0.0916± 0.0086	0.058 ±0.029	1.03 ± 0.12 [C]	0.170 ± 0.038	0.064 ± 0.021	0.321 ±0.053	26.7 ±2.7[AB]	0.018 ±0.012	0.039 ±0.013	28.4 ±2.9	114 ± 11
	20°C	0.1047± 0.0034	0.063 ±0.018	2.99 ± 0.29 [A]	0.215 ±0.012	0.096 ± 0.025	0.288 ±0.012	33.6 ±2.9[A]	0.0076 ±0.0064	0.0438± 0.0093	37.3± 3.2	138±11
	15°C	0.122 ± 0.018	0.064 ±0.022	2.14 ± 0.68 [B]	0.192 ±0.064	0.061 ±0.021	0.238 ±0.091	20.4 ±6.7[B]	0.0067 ±0.0075	0.029 ±0.011	23.2 ±7.5	127 ±45

[a] A, B and C indicate statistical significance ($p<0.05$)

unclear why this has occurred, as previous research has illustrated a plateau, not a decrease, in biomass productivity as vitamin B_{12} supplementation increased (Ford, 1958; Swift & Taylor, 1974). With respect to the response of omega-3 fatty acids to vitamin B_{12}, the omega-3 content of *B. hooglandii* ($p=0.0001$), *P. tricornutum* ($p<0.0005$) and *G. sculpta* ($p<0.004$) significantly increased during supplementation with vitamin B_{12}. It was unclear what mechanisms were resulting in the omega-3 response to vitamins, as vitamin B_{12} typically plays a role in the synthesis of deoxyriboses and amino acids within the algal cell (Tang et al., 2010). Thus, further research exploring the potential role of vitamin B_{12} within the algal cell would be necessary before any relationships between vitamin B_{12} and omega-3 content can be identified. From an industrial perspective, the findings from this study suggest that the requirement for vitamin B_{12} must be established on a species-by-species basis, however some microalgal cultures should be supplemented with vitamin B_{12} to increase their omega-3 content and biomass productivity.

4.3.5. Abiotic influences on omega-3 production and its industrial implications

Omega-3 fatty acids, notably EPA and DHA, are of primary importance due to their role in early development, reproduction and growth in consumers within the food chain (Kainz et al., 2004). Of the omega-3 fatty acids analyzed, EPA consistently represented over 50% of the total omega-3 content in all microalgae under all growth conditions tested. Across all tested growth conditions, the greatest concentration of omega-3 fatty acids was produced by *G. sculpta* at 25°C, making up 61% (60.2 ± 19.1 mg g^{-1}) of the cells total fatty acid content. Due to their rich EPA content, all five algal species could provide essential omega-3 fatty acids to consumers at higher trophic levels to maintain healthy development (Koussoroplis et al., 2011; Brett et al., 2009).

Table 17. The effects of vitamin B12, 100 ng L^{-1}, 10 ng L^{-1} and 0 ng L^{-1}, on the total and omega-3 fatty acid content of the five algae growing under 17h:7h, 80 ± 5 µmoles m^{-2} s^{-1} light intensity, and 18 ± 1°C.

	Vitamin B_{12}	Biomass productivity (g L^{-1} day^{-1}dw)	Omega-3 Fatty acid Production (mg g^{-1} dw)								Total Omega-3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
			16:4n3	18:3n3	18:4n3	20:3n3	20:4n3	20:5n3	22:5n3	22:6n3		
Boekelovia hooglandii	100	0.1498± 0.0091^{Aa}	0.235 ±0.038^{A}	0.611 ± 0.092^{A}	11.4 ±1.6^{B}	0.0303 ±0.0073^{B}	0.0539 ±0.0031^{A}	27.7 ±4.0^{B}	0.296 ±0.040^{A}	9.1± 1.48^{B}	49.5± 7.0^{B}	179± 16^{B}
	10	0.135± 0.012^{A}	0.225 ±0.039^{A}	0.718 ±0.060^{A}	14.1 ±1.0^{A}	0.0547 ±0.0079^{A}	0.068± 0.017^{A}	35.9± 2.6^{A}	0.331± 0.039^{A}	11.72±0 .82^{A}	63.1± 4.6^{A}	214 ±16^{A}
	0	0.112± 0.0028^{B}	0.146± 0.035^{B}	0.224 ±0.085^{B}	2.5 ±1.0^{C}	0.0355 ±0.0096^{B}	0.034 ±0.013^{B}	5.0 ±2.5^{C}	0.096± 0.038^{B}	1.39± 0.70 ^{C}	9.5± 4.3^{C}	141± 22^{C}
Phaeodactylum tricornutum	100	0.180± 0.011 ^{A}	0.211± 0.031^{A}	0.413 ±0.029^{A}	0.694± 0.091^{A}	0.0199 ±0.0046	0.538± 0.050^{A}	25.9± 2.1^{A}	0.0957 ±0.0091	2.01± 0.22 ^{A}	29.9± 2.5^{A}	140± 11^{A}
	10	0.1473± 0.0061 ^{B}	0.178± 0.043^{A}	0.41 ±0.12^{A}	0.276± 0.072^{B}	0.0206 ±0.0090	0.27± 0.12^{B}	19.8 ±6.5^{B}	0.098 ± 0.042^{A}	2.08 ±0.63^{A}	23.1± 7.5^{A}	100± 27^{AB}
	0	0.16± 0.018 ^{AB}	0.068± 0.029^{B}	0.196 ±0.050^{B}	0.29 ±0.11^{B}	0.038 ±0.026	0.212± 0.064^{B}	8.1 ±2.4^{C}	0.0504 ±0.0066 ^{B}	0.67 ±0.20^{B}	9.6± 2.9^{B}	89± 23^{B}
Goniochloris sculpta	100	0.1531± 0.0064	0.79± 0.16^{A}	4.3 ±1.3^{A}	1.23± 0.45^{A}	0.41 ±0.18	0.88± 0.29^{A}	81 ±23^{A}	0.50 ± 0.20^{A}	0.26 ±0.16	89± 26^{A}	254 ±78^{A}
	10	0.1350± 0.0097	0.591± 0.111^{A}	0.37 ±0.62^{A}	1.04± 0.29^{A}	0.25 ±0.16	0.65± 0.12^{A}	74±14 ^{A}	0.46 ±0.11^{A}	0.173 ±0.093	81± 16^{A}	207± 26^{B}
	0	0.173± 0.031	0.0200 ±0.0098 ^{B}	0.577 ±0.092^{B}	0.245± 0.057^{B}	0.22 ±0.29	0.091 ±0.012^{B}	8.79 ±0.90^{B}	0.057 ± 0.061 ^{B}	0.19 ±0.16	10.22 ±0.81^{B}	63± 19^{C}
Rhodella maculata	100	0.155± 0.018	0.0119 ±0.0056	0.0225 ±0.0047	0.0335 ± 0.0098	0.028± 0.023	0.0084± 0.0041	4.8 ±3.1	0.021 ±0.022	0.017 ±0.016	4.9 ±3.1	30.9± 6.6

	Vitamin B_{12}	Biomass productivity (g L^{-1} day^{-1}dw)	Omega-3 Fatty acid Production (mg g^{-1} dw)								Total Omega-3 (mg g^{-1} dw)	Total FA (mg g^{-1} dw)
			16:4n3	18:3n3	18:4n3	20:3n3	20:4n3	20:5n3	22:5n3	22:6n3		
	10	0.144± 0.026	0.0143 ±0.0081	0.0171 ±0.0049	0.051 ±0.021	0.037 ±0.019	0.026 ±0.025	1.86± 0.92	0.0127 ±0.0069	0.020 ±0.021	2.04± 0.92	22.6±2.2
	0	0.1589± 0.0096	0.0154 ±0.0077	0.036 ±0.036	0.043± 0.032	0.051± 0.059	0.037± 0.061	7±10	0.034 ±0.054	0.090± 0.075	7±11	49±60
Chloridella simplex	100	0.1375± 0.0081^{B}	0.23 ±0.13	2.04 ±0.10	0.323± 0.025	0.061± 0.010^{A}	0.648± 0.050	39.9 ±1.5	0.0170 ±0.0096	0.067±0.038	43.3± 1.5	144.0±4.8
	10	0.1362± 0.0058^{AB}	0.17 ±0.15	1.85 ±0.23	0.304± 0.025	0.0530 $\pm0.0097^{A}$	0.581± 0.072	36.9 ±4.4	0.0323^{B} ±0.0045	0.100±0.039	40.0± 4.6	128± 13
	0	0.1231± 0.0017^{A}	0.146 ±0.050	2.05 ±0.29	0.326± 0.054	0.00412±0.0048^{B}	0.67± 0.12	41.0 ±5.4	0.0161^{AB} ± 0.0086	0.053 ±0.024	44.3± 5.8	144± 18

[a]A, B and C indicate statistical significance ($p<0.05$)

Docosahexaenoic acid [DHA; 22:6(n-3)] makes up over 60% of the total lipid content in retinal tissue and is required for healthy visual development within infants (Giusto et al., 2000). Of the five species and four growth conditions studied within this chapter, DHA was present in the greatest concentrations within *B. hooglandii* at 20.8% of total omega-3 content and 5.38% of total fatty acid content at 130 µmoles m^{-2} s^{-1}. Under these same growth conditions, the greatest DHA productivity was from *B. hooglandii* at 1.36 mg L^{-1} day^{-1}.This productivity was greater than the DHA productivity of the other four species studies in this project (≤ 0.544 mg L^{-1} day^{-1}), however it was lower than the algal DHA producers used within industry. For example, the marine heterotroph *Crypthecodinium cohnii* has produced 3.33 g L^{-1} day^{-1} (Yoguchi et al., 1997) and 2.73 g L^{-1} day^{-1} (Fan et al., 2001). As a consequence of the significantly greater DHA productivity present within the literature, a high DHA producer for industrial applications was not established within this study. Future studies can continue to manipulate growth conditions, using trends identified within this study, to further increase DHA productivity.

This study compared two freshwater and three marine microalgae under different light cycles, intensities, temperatures and vitamin B_{12} concentrations to identify key patterns in omega-3 content and biomass productivity. Overall, a few general patterns in fatty acid production and growth were identified. High light intensity resulted in increased biomass productivity and no significant change in omega-3 fatty acid. Continuous light resulted in both increased growth and higher cellular omega-3 content. The presence of vitamin B_{12} was positively correlated with omega-3 content, while the impact on biomass productivity was more variable. Finally, increased temperature resulted in increased omega-3 content. All algal strains studied were rich in EPA content (>50% of total fatty acid content). The effects of growth conditions on lipid content and biomass productivity were also examined on the species level.

The highest biomass productivity was noted in *B. hooglandii* (0.255 ± 0.025 g L^{-1} day^{-1}). However, *P. tricornutum* exhibited the highest total fatty acid content at 411 ± 83 mg g^{-1} with 22.7 ± 10.6 mg g^{-1} omega-3 fatty acid (79% EPA). Overall, *G. sculpta* had the greatest omega-3 productivity at 100 ng L^{-1} due to a 0.153 ± 0.006 g L^{-1} day^{-1} biomass productivity and 89.1 ± 26.3 mg g^{-1} dw omega-3 content. Of the five microalgae and four growth conditions studied, *G. sculpta* resulted in a high biomass productivity and omega-3 content under the same growth conditions. A strain that is capable of producing high levels of omega-3 fatty acids can greatly influence consumer health throughout the trophic levels, while maintaining a high biomass productivity is very valuable from a commercial point of view (Yongmanitchai & Ward 1991).

Chapter 5: Conclusion

The objective of this research was to examine industrially relevant characteristics of algal lipids, in addition to the broader implications within the food web. Algae can naturally synthesize a variety of fatty acids (Yaakob et al., 2014), which play a valuable role within industry and the food web. Chapters 2 through 4 explore these potential roles within pharmaceuticals, aquaculture, and consumer health. These 3 chapters highlight the significant influence of growth conditions, algal species, and lipid structure on algal fatty acids for industry and food webs.

Evolutionary adaptations utilized by an algal cell are closely tied to the surrounding growth conditions (Guschina & Harwood, 2013). Each alga has adapted to a certain range of environmental conditions. Once an organism is placed under non-optimal conditions the cell triggers a physiological response in order to maintain cellular functioning (Guschina & Harwood, 2013). These adaptive mechanisms can be used to manipulate the fatty acid profile of a species in order to establish growth conditions that enhance fatty acid content, or highlight the impact of certain fatty acids on higher trophic level consumers (Guschina & Harwood, 2013; Koussoroplis et al., 2011). This project identified different growth conditions required to produce antibacterial and omega-3 fatty acids. For example, this study determined that longer photoperiod increased antibacterial and omega-3 fatty acid content, while lower light intensities increased total, but not omega-3, fatty acid content. In addition, higher PUFA productivity occurred in media analogous with the species natural habitat (ie. freshwater media induced higher PUFA productivity in freshwater algae). Consumers, such as zooplankton and fish, that

supplement their diet with essential fatty acids, would have greater reproductive success and survival in low intensity-high photoperiod light conditions due to the higher availability of omega-3s (Dawidowicz et al., 2012; Kainz et al., 2004). Thus, identifying the optimal growth conditions of a specific algal species are key to better understanding an algal cell's fatty acid response, and the impacts these shifts may have on the consumers.

Among algal species there is huge variability in the fatty acid profiles, however fatty acid trends exist within classes (Lang et al., 2011). These trends in fatty acid content can be used to assist in the selection of species with high concentrations of specific fatty acids. A 2011 study analyzed the fatty acid profile of over 2000 algal strains, and provided valuable information for selecting lipid rich algal species (Lang et al., 2011). All five microalgae studied in this project, Synurophyceae (*B. hooglandii),* Bacillariophyta *(P. tricornutum),* Xanthophyceae (*G. sculpta* and *C. simplex),* and Rhodellophyceae (*R. maculata),* were selected because of the expected high EPA and DHA content (Lang et al., 2011). For example, of the 81 strains of Xanthophyceae studied 75.3% contained EPA, and of the eighteen strains of Bacillariophyceae studied 22.2% contained DHA (Lang et al., 2011). Within these classes, a review of the literature identified the above genus and species as high lipid producers (Lang et al., 2011; Sheehan et al., 1998; Yongmanitchai, 1991; Desbois et al., 2009). For example, within Chapter 4 the Heterokontophyta consistently had high EPA content (>50%). However, considerable variation in fatty acid content was present, for instance the EPA content of the freshwater *G. sculpta* and *C. simplex* responded differently to light cycle, with one (*G. sculpta*) of two significantly increasing. Thus, algal taxonomy can assist in the search for an organism rich in a desired lipid, however it can only provide an initial selection of species that may be appropriate.

Lipid components within the algal cell can vary in carbon chain length, number of double bonds, and positioning of double bonds on the chain (William, 1998). These three components are significant factors in determining the characteristics of the fatty acid. Depending on the combination of these three structural features a lipid can be an essential fatty acid, have antibacterial properties, or have a significant role in the food web. For example, 18:3(n-6), 20:4(n-6) and 22:2(n-6) have antibacterial properties (Ruffell et al., 2015). However by slightly shifting the structure of the carbon chain (i.e. changing the positioning of a double bond, or adding/removing a carbon) omega-3 fatty acids are formed which are essential fatty acids (Kainz et al., 2004). In addition, growth conditions and growth phase can greatly influence what fatty acids are dominant within the algal cell. For example, Chapter 2 and 4 examined the influence of different growth conditions on fatty acid content, while Chapter 3 focused on the change in these fatty acids over different growth phases. Structure greatly influences the potential applications and responses of algal fatty acids to external pressures, and as a consequence molecular structure must be taken into consideration when studying fatty acids within the cell.

The purpose of this project was to identify the potential role of algal fatty acids within industry and the food web. Through this research, industrial applications were identified within two business sectors. In addition, the impact of light and omega-3 content on consumer selective grazing was discussed. Overall, growth conditions, algal species and lipid structure were critical components influencing the fatty acid profile in the algal cell. These three factors are critical to the identification of industrial applications and food web implications of fatty acids.

5.1. Validation of the Hypotheses

Of the twenty-nine fatty acids examined, the number of inhibitory fatty acids increased as the number of carbons and double bonds in the chain increased. There were exceptions to this trend, such as the inhibitory saturated fatty acids 10:0 and 12:0. After twenty-four hours, both fatty acids were inhibitory towards *S. aureus* at 800 and 2000 µg. Previous literature has demonstrated that fatty acids can be inhibitory towards bacteria (Desbois et al., 2009; Ohta et al., 1994; Sun et al., 2003), however this study examined a larger number of fatty acids and was the first study to quantify the inhibitory effects of the four clinically applicable doses (25, 250, 800, and 2000 µg). This study does have some limitations, due to the small number of doses tested and the limited number of bacterial species selected. As a consequence of four doses being tested and the large gaps between concentrations, it is not possible to identify the minimum inhibitory concentration and only broad statements can be made regarding the inhibitory nature of the fatty acid. This element of the experimental design was intentional, as this study was a preliminary investigation into the inhibitory nature of these twenty-nine fatty acids. Thus, future research would be required to verify whether the trends observed can be applied to a broader set of fatty acids and bacterial species. In conclusion, Chapter 2 provides insufficient evidence to reject the hypothesis that polyunsaturated fatty acids are more inhibitory towards bacteria, compared to monounsaturated and fatty acids.

Nutritional composition within the algal cell will shift with growth conditions and growth phase. This chapter tracked the composition and growth of the culture, and identified the key sampling period on day 35 when the composition of the cell (55.6% protein, 13.9% carbohydrates, and 18.6% fatty acids) was ideal as bivalve larvae feed. This was the first study to analyze the pigments, protein, fatty acids, and carbohydrate content of *B. hooglandii* over a 70

day time period. In addition to tracking the nutritional composition, this chapter provides the most complete growth curve, including lag, exponential, stationary and death phases, for this algal species. From this collected data, unique correlations were identified between the nutritional components. For example, the omega-3 fatty acid 22:5(n-3) was strongly positively correlated with β-carotene ($r=0.930$, $p=0.022$). There are some limitations to this study, due to the small scale of the experiments and batch culture approach. This experiment has potential industrial applications, therefore a larger batch size would have produced results more closely aligned to industrial conditions. For instance, larger batch size has a variety of challenges associated with it, such as light penetration, which would have been more accurately represented with a larger batch volume (Priyadarshani & Rath, 2012). The use of a batch culture approach was another limitation, as many industrial facilities use continuous cultures to reduce the periods of time with slower rates of algal growth (Priyadarshani, & Rath, 2012). Some implications from these limitations include potential variation in the results as culture size increases and culturing technique changes. This may change the nutritional composition and growth rate, possibly shifting the ideal day for harvesting biomass for bivalve larvae feed. Even with these limitations, this chapter identified key trends in nutritional composition and highlighted *B. hooglandii* as a potential aquaculture feed for further study. In conclusion, Chapter 3 provides insufficient evidence to reject the hypothesis that the microalga *B. hooglandii* has a high polyunsaturated fatty acid and protein content during late exponential phase, which would make them an ideal aquaculture feed for bivalve larvae.

During the light intensity experiments biomass productivity and omega-3 fatty acid content varied between algae, and were influenced by the light saturation point of the algal strain. For example, Beardall and Morris (1976) identified a saturation point for *P. tricornutum*

at 80 µmoles m^{-2} s^{-1}, which was exemplified by the plateau in biomass productivity in this study at 80 µmoles m^{-2} s^{-1}. Within the vitamin experiments, the lack of a relationship between taxonomy and cobalamin requirements made it difficult to identify if an algal strain was an auxotroph, synthesized or did not require cobalamin. However, supplementation of growth medium with cobalamin significantly increased omega-3 productivity (p=0.010) for *B. hooglandii,* and did not have a significant influence on *G. sculpta* and *R. maculata,* suggesting that some species should be supplemented with cobalamin to enhance biomass productivity. The final hypothesis was tested using temperatures 15, 20 and 25°C, and as growth condition temperatures decreased relative to stock culture conditions (20°C), omega-3 fatty acid content increased in order to maintain membrane fluidity. For example, *B. hooglandii* had a significantly higher omega-3 content at 15°C (p=0.0001). Chapter 4 was the first study to identify the industrial potential of the algal species, *G. sculpta* and *C. simplex,* that are rich in omega-3 fatty acids and DHA, respectively. Overall, chapter 4 did have some limitations, such as the small number of species tested and the single factorial design. As a result of the five species chosen, an analysis of taxonomic trends under the different growth conditions was not feasible. Thus, further research would be required to determine if the trends observed in this chapter apply to other individuals within these taxonomic groups. The second limitation, single factorial design, allows for the identification of the impact of a single growth condition on biomass productivity and omega-3 content. A multifactorial design would be necessary in order to optimize the biomass productivity and total omega-3 content of these algal strains. This design would accommodate the competing and complementary influences of the different growth conditions on each other. The small number of species and single factorial design limited the discussion of trends in algal taxonomy or broader trends related to the impact of growth conditions on the algal

cell. Nonetheless, both limitations were included within the design to create a manageable study that would provide preliminary information on the effects of these growth conditions on five algal species. In conclusion, chapter 4 provides insufficient evidence to reject hypothesize A, B, and C.

5.2. Commercial implications

5.2.1. Pharmaceutical industry

Fatty acids have a variety of different antibacterial applications and are becoming increasingly important in medicine, agriculture, and food processing (Desbois & Smith, 2010). This project highlighted multiple algal fatty acids with significant antibacterial properties against *E. coli* and *S. aureus* (Ruffell et al., 2015). In addition, this study identified growth conditions that increase the antibacterial fatty acid content within the cell (Ruffell et al., 2015). Studies focusing on antibiotic resistance have observed that rates of bacterial resistance towards fatty acids are significantly lower than conventional antibiotics (Desbois et al., 2009; Desbois & Smith, 2010; Petschow et al., 1996). In addition, MRSA has become a considerable concern due to increasing resistance of this bacteria (and others) to traditional antibiotics (Desbois, 2009). Fatty acids may have a competitive advantage as topical antibiotics that can be used as a treatment against resistant bacteria. Furthermore, various studies have demonstrated a variety of other antibacterial applications, which include cavity prevention, enriching milk to reduce infant gastrointestinal infections and inhibition of *Propionibacterium acnes*, the primary cause of acne (Desbois & Smith, 2010; Isaacs et al., 1995; Yang et al., 2009). Thus, there may be an important role for algae as a topical antibacterial agent, due to these naturally synthesized antibacterial fatty acids.

5.2.2. Aquaculture industry

This study identified key sampling periods in the growth curve of *B. hooglandii* that provide algal biomass rich in protein, fatty acids, carbohydrates or pigments, as well as identifying correlations between different nutritional characteristics relevant to the aquaculture industry. Identifying shifts in the nutritional profile and correlations present throughout the growth phases allows for more informed decisions to be made during culturing and biomass collection. For example, growth conditions that produce high growth rates may not be the same that result in the high omega-3 fatty acid content or productivity (Thompson et al., 1990). Thus, selection of a species and appropriate growth conditions that maintain both a high growth rate and a high lipid content under the same growth conditions is highly desirable for industrial applications (Yongmanitchai & Ward, 1991).

5.2.3. Nutritional supplement industry

Currently, fish oils are the most common source of omega-3 fatty acids on the market. However, methyl mercury and carcinogen contamination in fish lipids is a concern (Sidhu, 2003), as is the sustainability of fish oil production (Yongmanitchai & Ward, 1991). Flaxseeds and walnuts are also good sources of omega-3 fatty acids, but primarily in the form of alpha-linolenic acid [ALA; 18:3(n-3)] (Lane et al., 2014). This fatty acid is the most prevalent omega-3 fatty acid in vegetarian diets, although very little is metabolised to the more beneficial DHA and EPA (Lane et al., 2014). Algae, on the other hand, are an excellent source of DHA and EPA, and can provide a safe, contaminant-free, animal-free source of omega-3 FAs that can be extracted or ingested whole (Lane et al., 2014). In addition, algae are a sustainable source of omega-3 fatty acids and can grow in a range of environmental conditions, directly assimilating carbon dioxide (Mata et al., 2010; Gosch et al., 2012). This study identified adaptive mechanisms used by algal

cells, which could be used to enhance cellular content of omega-3 fatty acids. From an industrial perspective, algae are a viable source of omega-3 fatty acids and growth conditions resulting in increased production would assist in the scale up of these valuable fatty acids.

5.3. Future Research

From this research a variety of different avenues can be taken.

- Further antibacterial experiments, similar to Chapter 2, could be performed on a larger range of gram-positive and gram-negative bacteria, most notably a biosafety level 2 bacterium due to the greater resistance of these strains to antibiotics.

- A study should be developed that tests the use of the antibacterial fatty acids on animals and humans. Some of these fatty acids were stable over 48 h, and could potentially be used as a topical antibacterial agent.

- A study could be performed that uses *B. hooglandii* as an aquaculture feed to determine if the nutritional properties identified are translated into nutrition for the aquaculture organism.

- A cost analysis could be performed on the use of *B. hooglandii* as an aquaculture feed.

- Future research should focus on continued analysis of optimal growth conditions, in order to further increase growth rates and lipid productivity. Some suggestions include a detailed examination of salt content, pH, density, or soil medium.

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Appendices

A.1. Chapter 3 supplementary data

*Table A1. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for biomass and cell size from Chapter 3 data***.*

Characteristic	Biomass (g L^{-1})	Cell size (µm)
Biomass (g L^{-1})		
Cell size (µm)		
C10:0		
C12:0		
C14:0	0.749 (0.02)*	
C16:0	0.742(0.022)	
C17:0		
C18:0		
C20:0		
C22:0		
C23:0		
C24:0		
SFAs	0.783(0.013)	
C12:1		
C14:1		
C16:1		
C18:1(n-7)	0.752(0.019)	0.743(0.022)
C18:1(n-9)		
C20:1(n-9)	**-0.901 (0.001)****	0.73(0.026)
C22:1(n-9)		
C24:1(n-9)	0.745(0.021)	0.821(0.007)
MUFAs		
C18:2(n-6)		
C18:3(n-6)		
C20:2(n-6)	**-0.796(0.01)**	0.736(0.024)
C20:3(n-6)		
C20:4(n-6)		
C22:2(n-6)		
C22:4(n-6)		
C22:5(n-6)		
N-6		
C16:4(n-3)		
C18:3(n-3)		
C18:4(n-3)	0.825(0.006)	

Characteristic	Biomass (g L^{-1})	Cell size (µm)
C20:3(n-3)		
C20:4(n-3)	0.855(0.003)	
C20:5(n-3)		
C22:5(n-3)		
C22:6(n-3)		
N-3		
PUFAs		
EPA+ DHA		
Total	0.708(0.033)	
Protein (mg g^{-1})		
Carbohydrates (mg g^{-1})		
Growth rate		
Fucoxanthin w/ derivatives		
Diatoxanthin w/ derivatives		
Diadinoxanthin w/ derivatives		
Zeaxanthin w/ derivatives		
Chl_c w/ derivatives		
Chl_a w/ derivatives		
B- carotene w/ derivatives		
Total Pigment (w/ derivatives)		

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A2. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for saturated FAs 10:0 to 17:0 (mg g^{-1} dw) from Chapter 3 data***.*

Characteristic	C10:0	C12:0	C14:0	C16:0	C17:0
Biomass (g L^{-1})			0.562	0.55	
Cell size (μm)					
C10:0			0.652		0.635
C12:0					
C14:0	0.808(0.008)			0.567	0.503
C16:0			0.753(0.019)		
C17:0	0.797(0.010)		0.709(0.032)		
C18:0			0.782(0.013)	0.949(0.000)	
C20:0		0.854(0.003)			
C22:0		0.812(0.008)			
C23:0		0.903(0.001)			
C24:0	0.784(0.012)	0.767(0.016)	0.817(0.007)		0.744(0.022)
SFAs			0.857(0.003)	0.98(0.000)	
C12:1		0.912(0.001)			
C14:1					
C16:1					
C18:1(n-7)			**-0.773 (0.015)**		**-0.716 (0.03)**
C18:1(n-9)		**-0.833 (0.005)**			
C20:1(n-9)			**-0.772 (0.015)**	**-0.846 (0.004)**	
C22:1(n-9)		0.868(0.002)			
C24:1(n-9)			**-0.804 (0.009)**		
MUFAs					
C18:2(n-6)	0.810(0.008)		0.735(0.024)		0.917(0.001)
C18:3(n-6)					0.829(0.006)
C20:2(n-6)			**-0.829 (0.006)**	**-0.793 (0.011)**	
C20:3(n-6)					0.819(0.007)
C20:4(n-6)					0.884(0.002)
C22:2(n-6)					
C22:4(n-6)					
C22:5(n-6)					
N-6					0.968(0.000)
C16:4(n-3)		0.929(0.000)			
C18:3(n-3)	0.713(0.031)				0.917(0.000)

Characteristic	C10:0	C12:0	C14:0	C16:0	C17:0
C18:4(n-3)			0.79(0.011)	**-0.748(0.02)**	0.732(0.025)
C20:3(n-3)		0.743(0.022)			
C20:4(n-3)			0.817(0.007)		0.916(0.001)
C20:5(n-3)					0.806(0.009)
C22:5(n-3)					
C22:6(n-3)					0.878(0.002)
N-3					0.851(0.004)
PUFAs					0.888(0.001)
EPA+ DHA					0.823(0.006)
Total				**-0.742 (0.022)**	
Protein (mg g^{-1})					0.806(0.009)
Carbohydrates (mg g^{-1})					
Growth rate					
Fucoxanthin w/ derivatives					
Diatoxanthin w/ derivatives					
Diadinoxanthin w/ derivatives					
Zeaxanthin w/ derivatives					
Chl_c w/ derivatives					
Chl_a w/ derivatives					
B- carotene w/ derivatives					
Total Pigment (w/ derivatives)					

*Table A3. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for saturated FAs 20:0 to 24:0 (mg g^{-1} dw) from Chapter 3 data***.*

Characteristic	C20:0	C22:0	C23:0	C24:0	SFAs
Biomass (g L^{-1})					0.613
Cell size (µm)					
C10:0				0.615	
C12:0	0.729	0.66	0.816	0.589	
C14:0				0.668	0.735
C16:0					0.961
C17:0				0.554	
C18:0					0.911
C20:0			0.705		
C22:0					
C23:0	0.84(0.005)				
C24:0					
SFAs					
C12:1	0.929(0.000)				
C14:1					
C16:1			**-0.713 (0.031)**		
C18:1(n-7)				**-0.75(0.02)**	
C18:1(n-9)		**-0.743 (0.022)**			
C20:1(n-9)					**-0.867 (0.002)**
C22:1(n-9)	0.832(0.005)	0.944(0.000)			
C24:1(n-9)					**-0.75(0.02)**
MUFAs			0.774(0.014)		
C18:2(n-6)				0.91(0.001)	
C18:3(n-6)					
C20:2(n-6)					**-0.836 (0.005)**
C20:3(n-6)					
C20:4(n-6)					
C22:2(n-6)	0.812(0.008)		0.815(0.007)		
C22:4(n-6)					
C22:5(n-6)			0.769(0.015)		
N-6				0.734(0.024)	
C16:4(n-3)	0.857(0.003)	0.851(0.004)	0.794(0.011)		
C18:3(n-3)				0.846(0.004)	
C18:4(n-3)					**-0.801**

Characteristic	C20:0	C22:0	C23:0	C24:0	SFAs
					(0.009)
C20:3(n-3)	0.833(0.005)		0.802(0.009)		
C20:4(n-3)					
C20:5(n-3)					
C22:5(n-3)					
C22:6(n-3)					
N-3					
PUFAs					
EPA+ DHA					
Total					0.729(0.026)
Protein (mg g^{-1})				0.763(0.017)	
Carbohydrates (mg g^{-1})			**-0.753(0.019)**		
Growth rate			0.987(0.002)	0.915(0.029)	
Fucoxanthin w/ derivatives					
Diatoxanthin w/ derivatives					
Diadinoxanthin w/ derivatives					
Zeaxanthin w/ derivatives					
Chl_c w/ derivatives					
Chl_a w/ derivatives					
B- carotene w/ derivatives					
Total Pigment (w/ derivatives)					

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A4. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for unsaturated FAs 12:1 to 18:1(mg g^{-1} dw) from Chapter 3 data***.*

Characteristic	C12:1	C14:1	C16:1	C18:1(n-7)	C18:1(n-9)
Biomass (g L^{-1})				0.565	
Cell size (µm)				0.552	
C10:0					
C12:0	0.832				0.695
C14:0				0.598	
C16:0					
C17:0				0.513	
C18:0					
C20:0	0.863				
C22:0					0.552
C23:0			0.509		
C24:0				0.562	
SFAs					
C12:1			0.599		0.706
C14:1					
C16:1	**-0.774 (0.014)**				0.587
C18:1(n-7)					
C18:1(n-9)	**-0.840 (0.005)**		**-0.766 (0.016)**		
C20:1(n-9)					
C22:1(n-9)	0.853(0.003)				**-0.801 (0.010)**
C24:1(n-9)				0.793(0.011)	
MUFAs	0.837(0.005)		0.983(0.000)		0.862(0.003)
C18:2(n-6)				**-0.82(0.007)**	
C18:3(n-6)					
C20:2(n-6)				0.715(0.030)	
C20:3(n-6)					
C20:4(n-6)					
C22:2(n-6)	0.856(0.003)		**-0.751 (0.020)**		
C22:4(n-6)					
C22:5(n-6)					
N-6					
C16:4(n-3)	0.88(0.002)				0.804(0.009)
C18:3(n-3)					
C18:4(n-3)					

Characteristic	C12:1	C14:1	C16:1	C18:1(n-7)	C18:1(n-9)
C20:3(n-3)	0.844(0.004)				
C20:4(n-3)				**-0.805 (0.009)**	
C20:5(n-3)					
C22:5(n-3)					
C22:6(n-3)					
N-3					
PUFAs					
EPA+ DHA					
Total			0.941(0.000)		
Protein (mg g^{-1})				0.88(0.002)	
Carbohydrates (mg g^{-1})					
Growth rate					
Fucoxanthin w/ derivatives		**-0.927 (0.023)**			
Diatoxanthin w/ derivatives					
Diadinoxanthin w/ derivatives					
Zeaxanthin w/ derivatives					
Chl_c w/ derivatives					
Chl_a w/ derivatives					
B- carotene w/ derivatives					
Total Pigment (w/ derivatives)					

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A5. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for unsaturated FAs 20:1 to 24:1 (mg g^{-1} day) from Chapter 3 data***.*

Characteristic	C20:1(n-9)	C22:1(n-9)	C24:1(n-9)	MUFAs
Biomass (g L^{-1})	0.812		0.555	
Cell size (µm)	0.533		0.674	
C10:0				
C12:0		0.753		
C14:0	0.597		0.646	
C16:0	0.715			
C17:0				
C18:0	0.557			
C20:0		0.691		
C22:0		0.891		
C23:0				0.599
C24:0				
SFAs	0.752		0.562	
C12:1		0.727		0.701
C14:1				
C16:1				0.966
C18:1(n-7)			0.629	
C18:1(n-9)		0.641		0.743
C20:1(n-9)			0.813	
C22:1(n-9)			0.537	
C24:1(n-9)	0.902(0.001)	**-0.733(0.025)**		
MUFAs				
C18:2(n-6)				
C18:3(n-6)				
C20:2(n-6)	0.95(0.000)		0.971(0.000)	
C20:3(n-6)				
C20:4(n-6)				
C22:2(n-6)				**-0.751 (0.020)**
C22:4(n-6)				
C22:5(n-6)				
N-6				
C16:4(n-3)		0.893(0.001)		
C18:3(n-3)				
C18:4(n-3)				
C20:3(n-3)				
C20:4(n-3)				

Characteristic	C20:1(n-9)	C22:1(n-9)	C24:1(n-9)	MUFAs
C20:5(n-3)				
C22:5(n-3)				
C22:6(n-3)				
N-3				
PUFAs				
EPA+ DHA				
Total				0.888(0.001)
Protein (mg g^{-1})				
Carbohydrates (mg g^{-1})				
Growth rate				
Fucoxanthin w/ derivatives				
Diatoxanthin w/ derivatives				
Diadinoxanthin w/ derivatives				
Zeaxanthin w/ derivatives				
Chl_c w/ derivatives				
Chl_a w/ derivatives				
B- carotene w/ derivatives				
Total Pigment (w/ derivatives)				

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A6. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for omega-6 FAs 18:2 to 20:4 (mg g^{-1} day) from Chapter 3 data***.*

Characteristic	C18:2(n-6)	C18:3(n-6)	C20:2(n6)	C20:3(n-6)	C20:4(n-6)
Biomass (g L^{-1})			0.633		
Cell size (µm)			0.542		
C10:0	0.656				
C12:0					
C14:0	0.54		0.688		
C16:0			0.628		
C17:0	0.841	0.687		0.671	0.782
C18:0			0.538		
C20:0					
C22:0					
C23:0					
C24:0	0.828				
SFAs			0.699		
C12:1					
C14:1					
C16:1					
C18:1(n-7)	0.672		0.511		
C18:1(n-9)					
C20:1(n-9)			0.92		
C22:1(n-9)					
C24:1(n-9)			0.943		
MUFAs					
C18:2(n-6)				0.535	0.62
C18:3(n-6)				0.713	0.652
C20:2(n-6)					
C20:3(n-6)	0.731(0.025)	0.844(0.004)			0.666
C20:4(n-6)	0.787(0.012)	0.808(0.008)		0.816(0.007)	
C22:2(n-6)					
C22:4(n-6)					
C22:5(n-6)					
N-6	0.917(0.001)	0.807(0.009)		0.861(0.003)	0.957(0.000)
C16:4(n-3)					
C18:3(n-3)	0.963(0.000)			0.812(0.008)	0.889(0001)
C18:4(n-3)		0.806(0.009)			
C20:3(n-3)					
C20:4(n-3)	0.820(0.007)	0.791(0.011)		0.771(0.015)	
C20:5(n-3)	0.801(0.009)				0.897(0.001)

Characteristic	C18:2(n-6)	C18:3(n-6)	C20:2(n6)	C20:3(n-6)	C20:4(n-6)
C22:5(n-3)					0.879(0.002)
C22:6(n-3)	0.786(0.012)	0.822(0.007)		0.824(0.006)	0.997(0.000)
N-3	0.837(0.005)			0.725(0.027)	0.925(0.000)
PUFAs	0.865(0.003)	0.721(0.028)		0.764(0.016)	0.944(0.000)
EPA+ DHA	0.808(0.008)				0.918(0.000)
Total Protein (mg g^{-1})	0.894(0.001)			0.737(0.023)	
Carbohydrates (mg g^{-1})					
Growth rate	0.932(0.021)				
Fucoxanthin w/ derivatives					
Diatoxanthin w/ derivatives					
Diadinoxanthin w/ derivatives					
Zeaxanthin w/ derivatives					
Chl_c w/ derivatives					
Chl_a w/ derivatives					
B- carotene w/ derivatives					
Total Pigment (w/ derivatives)					

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A7. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for omega-6 FAs 22:2 to 22:5 (mg g^{-1} day) from Chapter 3 data***.*

Characteristic	C22:2(n-6)	C22:4(n-6)	C22:5(n-6)	N-6
Biomass (g L^{-1})				
Cell size (µm)				
C10:0				
C12:0				
C14:0				
C16:0				
C17:0				0.938
C18:0				
C20:0	0.66			
C22:0				
C23:0	0.665		0.592	
C24:0				0.538
SFAs				
C12:1	0.732			
C14:1				
C16:1	0.564			
C18:1(n-7)				
C18:1(n-9)				
C20:1(n-9)				
C22:1(n-9)				
C24:1(n-9)				
MUFAs	0.563			
C18:2(n-6)				0.841
C18:3(n-6)				0.652
C20:2(n-6)				
C20:3(n-6)				0.742
C20:4(n-6)				0.917
C22:2(n-6)			0.806	
C22:4(n-6)				
C22:5(n-6)	0.898(0.001)			
N-6				
C16:4(n-3)				
C18:3(n-3)				0.964(0.000)
C18:4(n-3)				
C20:3(n-3)	0.835(0.005)		0.73(0.025)	
C20:4(n-3)		0.726(0.027)		0.816(0.007)
C20:5(n-3)				0.896(0.001)

Characteristic	C22:2(n-6)	C22:4(n-6)	C22:5(n-6)	N-6
C22:5(n-3)				0.824(0.006)
C22:6(n-3)				0.954(0.000)
N-3				0.931(0.000)
PUFAs				0.958(0.000)
EPA+ DHA				0.912(0.001)
Total				
Protein (mg g^{-1})				0.813(0.008)
Carbohydrates (mg g^{-1})				
Growth rate				
Fucoxanthin w/ derivatives			0.893(0.041)	
Diatoxanthin w/ derivatives				
Diadinoxanthin w/ derivatives			0.931(0.022)	
Zeaxanthin w/ derivatives				
Chl_c w/ derivatives				
Chl_a w/ derivatives				
B- carotene w/ derivatives				
Total Pigment (w/ derivatives)			0.901(0.037)	

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A8. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for omega-3 FAs 16:4 to 20:4 (mg g^{-1} dw) from Chapter 3 data***.*

Characteristic	C16:4 (n-3)	C18:3 (n-3)	C18:4 (n-3)	C20:3 (n-3)	C20:4 (n-3)
Biomass (g L^{-1})			0.681		0.731
Cell size (µm)					
C10:0		0.508			
C12:0	0.863			0.552	
C14:0			0.624		0.668
C16:0			0.559		
C17:0		0.841	0.537		0.839
C18:0			0.553		
C20:0	0.735			0.694	
C22:0	0.724				
C23:0	0.631			0.643	
C24:0		0.716			
SFAs			0.642		
C12:1	0.774			0.712	
C14:1					
C16:1					
C18:1(n-7)					0.648
C18:1(n-9)	0.647				
C20:1(n-9)					
C22:1(n-9)	0.797				
C24:1(n-9)					
MUFAs					
C18:2(n-6)		0.928			0.672
C18:3(n-6)			0.65		0.626
C20:2(n-6)					
C20:3(n-6)		0.66			0.594
C20:4(n-6)		0.791			
C22:2(n-6)				0.698	
C22:4(n-6)					0.527
C22:5(n-6)				0.533	
N-6		0.929			0.671
C16:4(n-3)					
C18:3(n-3)					0.614
C18:4(n-3)					0.78
C20:3(n-3)					
C20:4(n-3)		0.784(0.012)	0.883(0.002)		

Characteristic	C16:4 (n-3)	C18:3 (n-3)	C18:4 (n-3)	C20:3 (n-3)	C20:4 (n-3)
C20:5(n-3)		0.871(0.002)			
C22:5(n-3)		0.884(0.004)			
C22:6(n-3)		0.891(0.001)			
N-3		0.904(0.001)			
PUFAs		0.928(0.000)			
EPA+ DHA		0.882(0.002)			
Total			0.882(0.002)		
Protein (mg g^{-1})		0.881(0.002)			0.828(0.006)
Carbohydrates (mg g^{-1})					
Growth rate		0.93(0.022)			
Fucoxanthin w/ derivatives					
Diatoxanthin w/ derivatives					
Diadinoxanthin w/ derivatives					
Zeaxanthin w/ derivatives					
Chl_c w/ derivatives					
Chl_a w/ derivatives					
B- carotene w/ derivatives					
Total Pigment (w/ derivatives)					

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A9. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for omega-3 FAs 20:5 to 22:6 (mg g^{-1} dw) from Chapter 3 data***.*

Characteristic	C20:5(n-3)	C22:5(n-3)	C22:6(n-3)	N-3
Biomass (g L^{-1})				
Cell size (µm)				
C10:0				
C12:0				
C14:0				
C16:0				
C17:0	0.009		0.77	0.724
C18:0				
C20:0				
C22:0				
C23:0				
C24:0				
SFAs				
C12:1				
C14:1				
C16:1				
C18:1(n-7)				
C18:1(n-9)				
C20:1(n-9)				
C22:1(n-9)				
C24:1(n-9)				
MUFAs				
C18:2(n-6)	0.642		0.618	0.701
C18:3(n-6)			0.676	
C20:2(n-6)				
C20:3(n-6)			0.679	0.526
C20:4(n-6)	0.804	0.772	0.994	0.856
C22:2(n-6)				
C22:4(n-6)				
C22:5(n-6)				
N-6	0.802	0.679	0.91	0.866
C16:4(n-3)				
C18:3(n-3)	0.758	0.712	0.764	0.817
C18:4(n-3)				
C20:3(n-3)				
C20:4(n-3)				
C20:5(n-3)		0.829	0.812	0.992

Characteristic	C20:5(n-3)	C22:5(n-3)	C22:6(n-3)	N-3	
C22:5(n-3)	0.911(0.001)		0.801		0.837
C22:6(n-3)	0.901(0.001)	0.895(0.001)			0.864
N-3	0.996(0.000)	0.915(0.001)	0.929(0.000)		
PUFAs	0.986(0.000)	0.906(0.001)	0.946(0.000)		
EPA+ DHA	0.999(0.000)	0.918(0.000)	0.922(0.000)		
Total					
Protein (mg g^{-1})					
Carbohydrates (mg g^{-1})	**-0.725 (0.027)**				
Growth rate					
Fucoxanthin w/ derivatives					
Diatoxanthin w/ derivatives					
Diadinoxanthin w/ derivatives					
Zeaxanthin w/ derivatives					
Chl_c w/ derivatives					
Chl_a w/ derivatives					
B- carotene w/ derivatives	0.939(0.018)	0.93(0.022)		0.933(0.021)	
Total Pigment (w/ derivatives)					

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A10. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for total FA, protein, carbohydrates and growth rate from Chapter 3 data***.*

Characteristic	Total FA	Protein (mg/g)	Carbohydrates (mg/g)	Growth rate
Biomass (g L^{-1})	0.502			
Cell size (µm)				
C10:0				
C12:0				
C14:0				
C16:0	0.551			
C17:0		0.65		
C18:0				
C20:0				
C22:0				
C23:0			0.567	0.974
C24:0		0.582		0.837
SFAs	0.531			
C12:1				
C14:1				
C16:1	0.885			
C18:1(n-7)		0.775		
C18:1(n-9)				
C20:1(n-9)				
C22:1(n-9)				
C24:1(n-9)				
MUFAs	0.788			
C18:2(n-6)		0.8		0.869
C18:3(n-6)				
C20:2(n-6)				
C20:3(n-6)		0.543		
C20:4(n-6)				
C22:2(n-6)				
C22:4(n-6)				
C22:5(n-6)				
N-6		0.661		
C16:4(n-3)				
C18:3(n-3)		0.775		0.866
C18:4(n-3)	0.779			
C20:3(n-3)				

Characteristic	Total FA	Protein (mg/g)	Carbohydrates (mg/g)	Growth rate
C20:4(n-3)		0.685		
C20:5(n-3)			0.526	
C22:5(n-3)				
C22:6(n-3)				
N-3				
PUFAs				
EPA+ DHA				
Total				
Protein (mg g^{-1})				0.872
Carbohydrates (mg g^{-1})				
Growth rate		0.934(0.020)		
Fucoxanthin w/ derivatives				
Diatoxanthin w/ derivatives				
Diadinoxanthin w/ derivatives				
Zeaxanthin w/ derivatives				
Chl_c w/ derivatives				
Chl_a w/ derivatives				
B- carotene w/ derivatives				
Total Pigment (w/ derivatives)				

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A11. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for pigments fucoxanthin, diatoxanthin, diadinoxanthin and zeaxanthin (µg g^{-1} dw) from Chapter 3 data***.*

Characteristic	Fucoxanthin w/ derivatives	Diatoxanthin w/ derivatives	Diadinoxanthin w/ derivatives	Zeaxanthin w/ derivatives
Biomass (g L^{-1})				
Cell size (µm)				
C10:0				
C12:0				
C14:0				
C16:0				
C17:0				
C18:0				
C20:0				
C22:0				
C23:0				
C24:0				
SFAs				
C12:1				
C14:1	0.86			
C16:1				
C18:1(n-7)				
C18:1(n-9)				
C20:1(n-9)				
C22:1(n-9)				
C24:1(n-9)				
MUFAs				
C18:2(n-6)				
C18:3(n-6)				
C20:2(n-6)				
C20:3(n-6)				
C20:4(n-6)				
C22:2(n-6)				
C22:4(n-6)				
C22:5(n-6)	0.798		0.866	
N-6				
C16:4(n-3)				
C18:3(n-3)				
C18:4(n-3)				

Characteristic	Fucoxanthin w/ derivatives	Diatoxanthin w/ derivatives	Diadinoxanthin w/ derivatives	Zeaxanthin w/ derivatives
C20:3(n-3)				
C20:4(n-3)				
C20:5(n-3)				
C22:5(n-3)				
C22:6(n-3)				
N-3				
PUFAs				
EPA+ DHA				
Total				
Protein (mg g^{-1})				
Carbohydrates (mg g^{-1})				
Growth rate				
Fucoxanthin w/ derivatives				
Diatoxanthin w/ derivatives				
Diadinoxanthin w/ derivatives				
Zeaxanthin w/ derivatives				
Chl_c w/ derivatives				
Chl_a w/ derivatives				
B- carotene w/ derivatives				0.927(0.023)
Total Pigment (w/ derivatives)	0.897(0.039)	0.890(0.043)	0.924(0.025)	

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

*Table A12. Summary of the statistically significant correlations (r; white area) and r-squared values (r^2; grey area) for pigments chlorophyll c, a and β-carotene (μg g^{-1} dw) from Chapter 3 data***.*

Characteristic	Chl_c w/ derivatives	Chl_a w/ derivatives	B- carotene w/ derivatives	Total Pigment (mg/g; includes pigment w/ derivatives)
Biomass (g L^{-1})				
Cell size (μm)				
C10:0				
C12:0				
C14:0				
C16:0				
C17:0				
C18:0				
C20:0				
C22:0				
C23:0				
C24:0				
SFAs				
C12:1				
C14:1				
C16:1				
C18:1(n-7)				
C18:1(n-9)				
C20:1(n-9)				
C22:1(n-9)				
C24:1(n-9)				
MUFAs				
C18:2(n-6)				
C18:3(n-6)				
C20:2(n-6)				
C20:3(n-6)				
C20:4(n-6)				
C22:2(n-6)				
C22:4(n-6)				
C22:5(n-6)				0.812
N-6				
C16:4(n-3)				
C18:3(n-3)				
C18:4(n-3)				

Characteristic	Chl_c w/ derivatives	Chl_a w/ derivatives	B- carotene w/ derivatives	Total Pigment (mg/g; includes pigment w/ derivatives)
C20:3(n-3)				
C20:4(n-3)				
C20:5(n-3)			0.882	
C22:5(n-3)			0.865	
C22:6(n-3)				
N-3			0.87	
PUFAs			0.85	
EPA+ DHA			0.877	
Total				
Protein (mg g^{-1})				
Carbohydrates (mg g^{-1})				
Growth rate				
Fucoxanthin w/ derivatives				0.805
Diatoxanthin w/ derivatives				0.791
Diadinoxanthin w/ derivatives				0.854
Zeaxanthin w/ derivatives			0.86	
Chl_c w/ derivatives				
Chl_a w/ derivatives				0.926
B- carotene w/ derivatives				
Total Pigment (w/ derivatives)		0.962(0.009)		

* p-value in the brackets; ** bolded numbers are negative correlations; ***The white section has r values with the corresponding p-value and the grey section has r-squared values (note: only p-value < 0.05, r > 0.5 or < -0.5 and r^2 > 0.5 were included)

A.2. Sequencing of the five microalgae studied

A.2.1. DNA extraction and amplification

DNA was extracted from the sample using liquid nitrogen and a mortar and pestle to break open the cells and a DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) to extract the DNA. The 18S ribosomal RNA gene of *P. tricornutum, B. hooglandii* and *C. simplex* were amplified in a solution of 72 µl water, 10 µl ThermoPol Reaction Buffer (New England BioLabs Inc., Ipswich, MA, USA), 8 µl $MgCl_2$, 2 µl dNTP, 4 µl primer 1, 4 µl primer 2, 0.4 µl Taq and 2 µl DNA. An Eppendorf Mastercycle gradient thermocycler (Eppendorf Canada Ltd., Mississauga, ON, Canada) was used for amplification, with 34 cycles of 93°C for 1 min, 47°C for 1 min and 72°C for 4 min, a pre-denaturation step of 95°C for 2 min and a final extension of 72°C for 6 min. The same forward and reverse primers were used to amplify the 18S region of these three microalgae, SSU1 5' TGG-TTG-ATC-CTG-CCA-GTA-G 3' and SSU2 5' TGA-TCC-TTC-CGC-AGG-TTC-AC 3' (Lewis et al., 1992). The PCR product was cleaned using a QiaQuick PCR Purification Kit (Qiagen Inc., Mississauga, ON, Canada) and Sanger sequenced with an ABI 3730XL sequencer (Applied Biosystems Canada, Streetsville, ON, Canada).

The same protocol was followed for *R. maculata* and *G. sculpta,* however a different PCR protocol and primers were used. The method used is based on a 2001 study by Katana et al. examining Chlorophyta. Amplification of 18S was performed using a pre-denaturation step of 95°C for 5 min, with 25 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. The same forward and reverse primers were used to amplify the 18S of *R. maculata* and *G. sculpta,* F 5' AAC-CTG-GTT-GAT-CCT-GCC-AGT 3' and R 5' TGA-TCC-TTC-TGC-AGG-TTC-ACC-TAC-G 3' (Katana et al., 2001).

A.2.2. Phylogenetic tree assembly

Sequences were aligned using MEGA (Tamura et al., 2007). The consensus sequences were created by combining a forward and reverse sequence, and regions of uncertain alignment, near the ends, were removed. The program MEGA was used to create a maximum likelihood tree with bootstrap values. Outgroups were included in the tree in accordance with the literature for the Heterokontophyta (Daugbjerg and Guillou, 2001) and Rhodophyta (Yoon et al., 2006). The outgroups were used to root the tree, and bootstrap values below 65% were removed from the phylogenetic tree.. The phylogenetic trees constructed are displayed below. All five microalgae sequenced were closely related to algae with the same species identification provided by the culture collection. Notably, an 18S sequence for *B. hooglandii* could not be found within the literature, thus the closest match was AJ269501 *P. tricornutum* strain UTEX 640. Microscopy was used to verify that the species received from the CPCC had the morphological features of *B. hooglandii* documented within the literature (Throndsen, 1997).

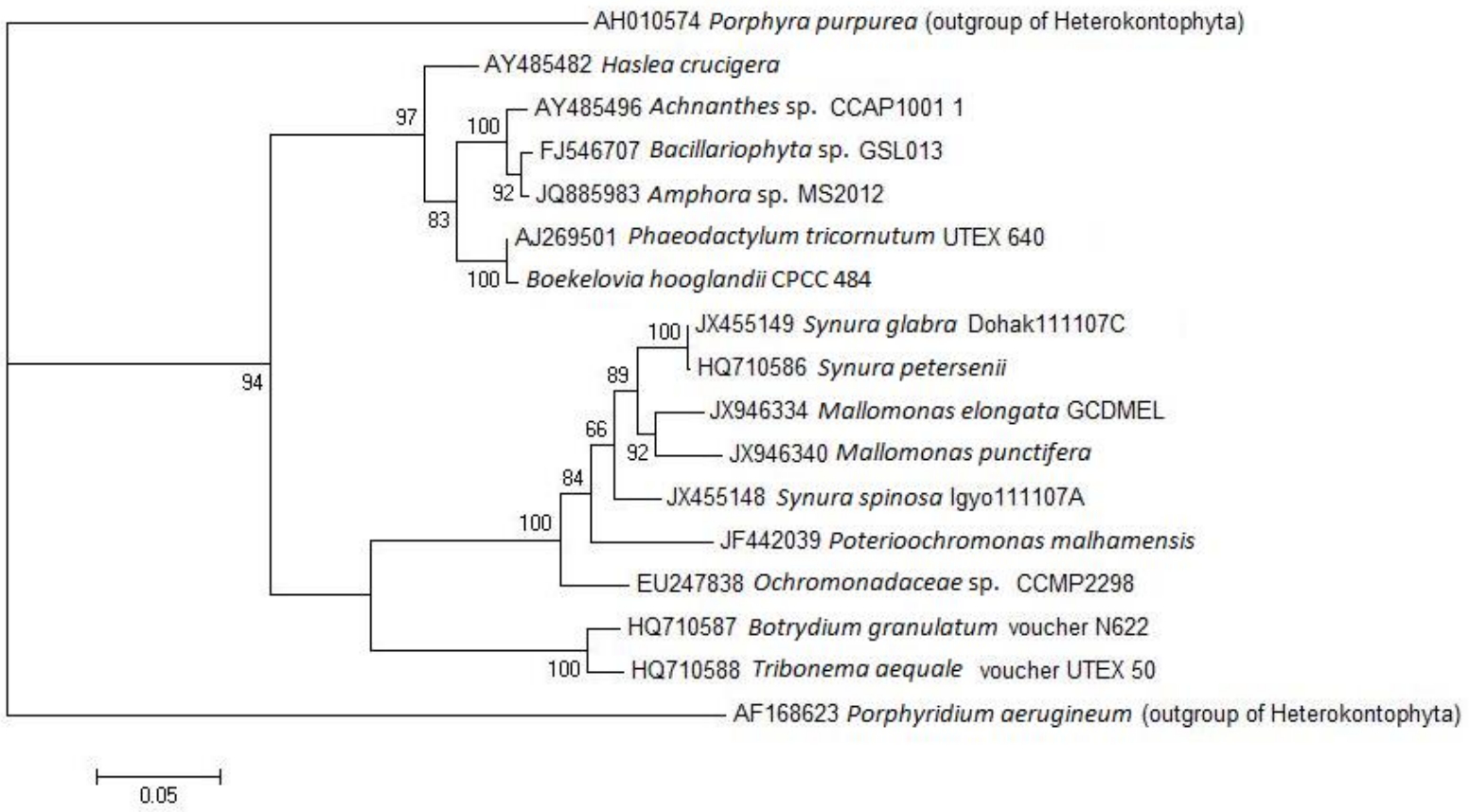


Figure 8. Phylogenetic tree for Boekelovia hooglandii.

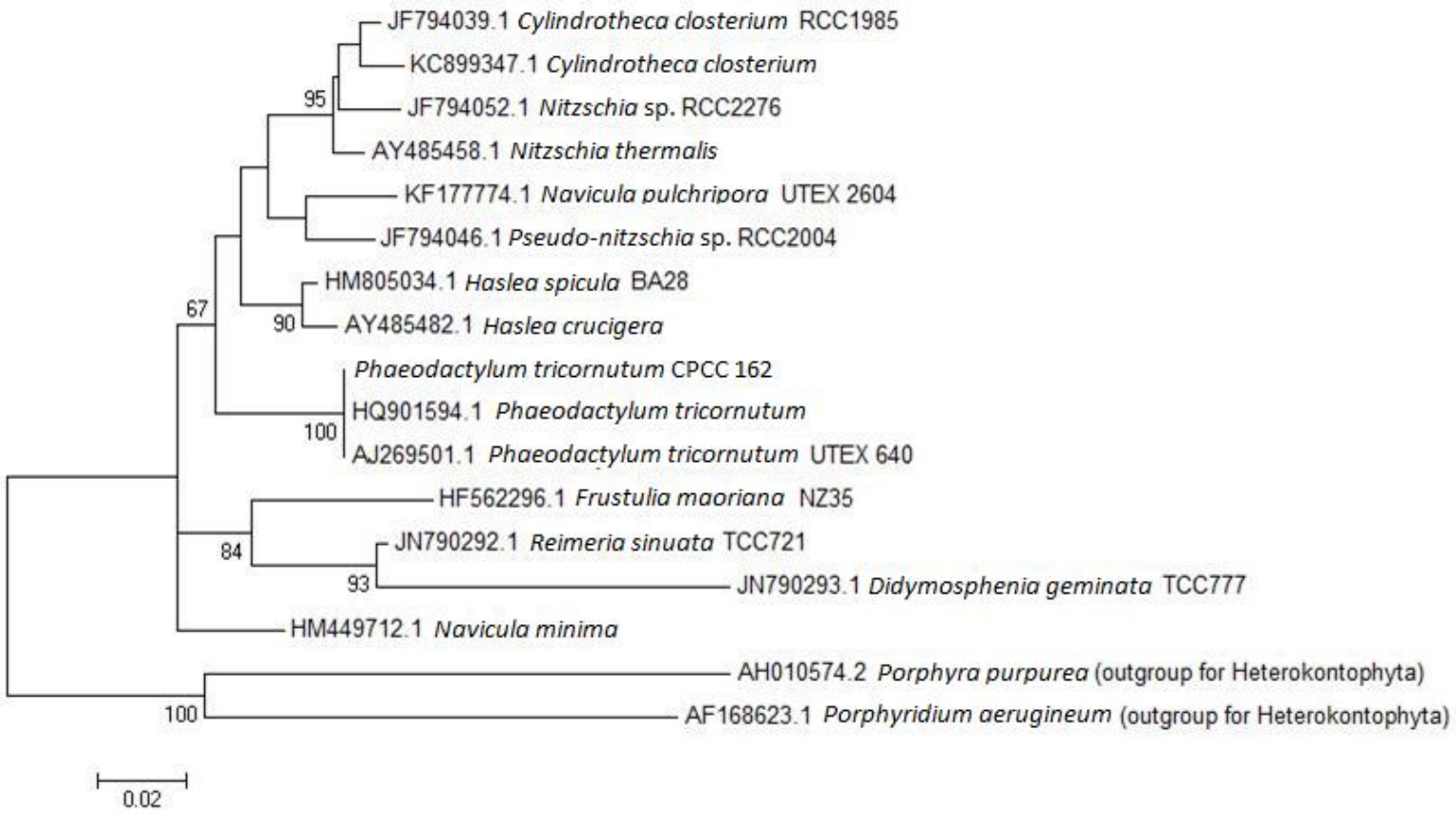


Figure 9. Phylogenetic tree for Phaeodactylum tricornutum.

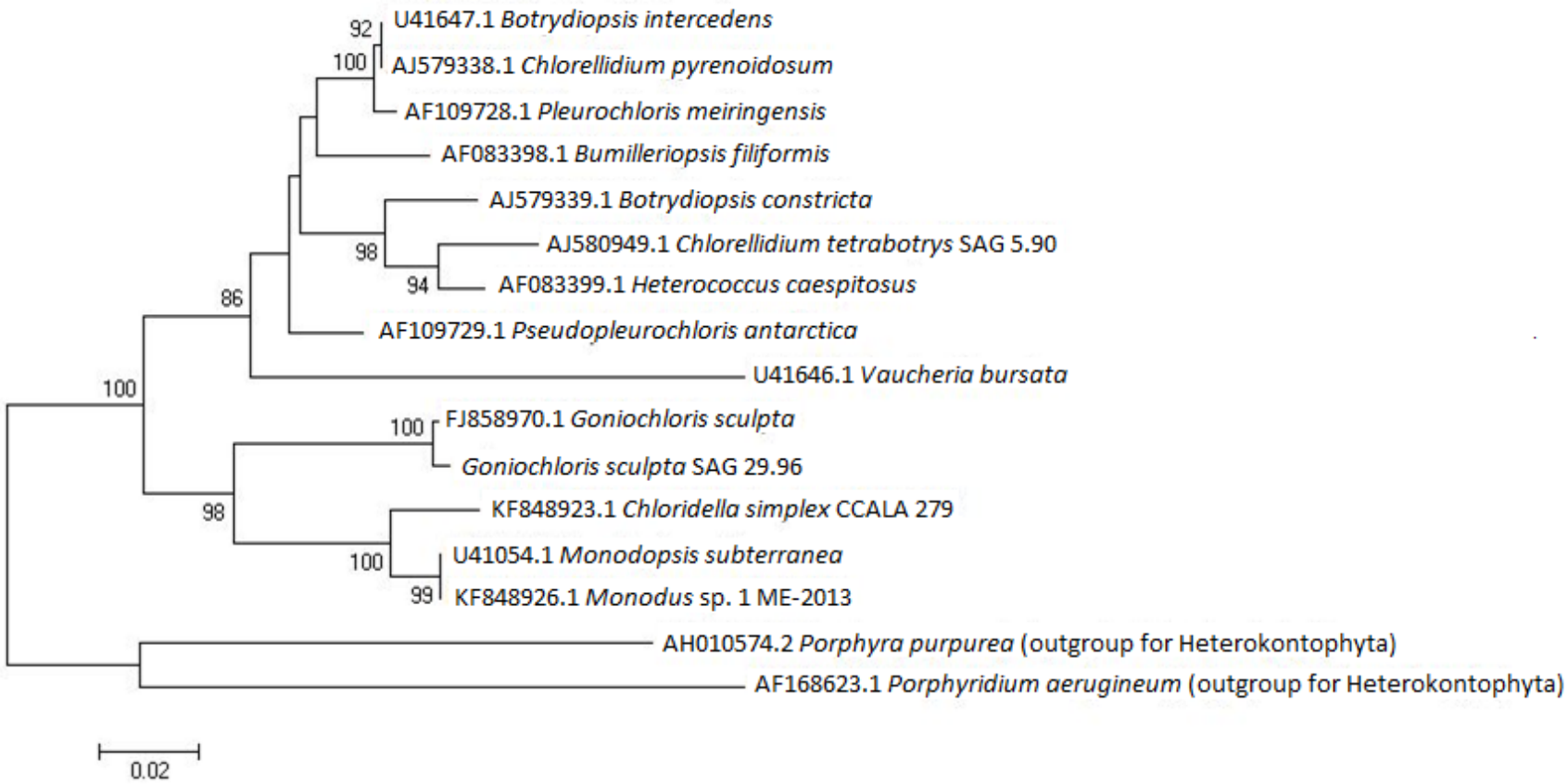


Figure 10. Phylogenetic tree for Goniochloris sculpta.

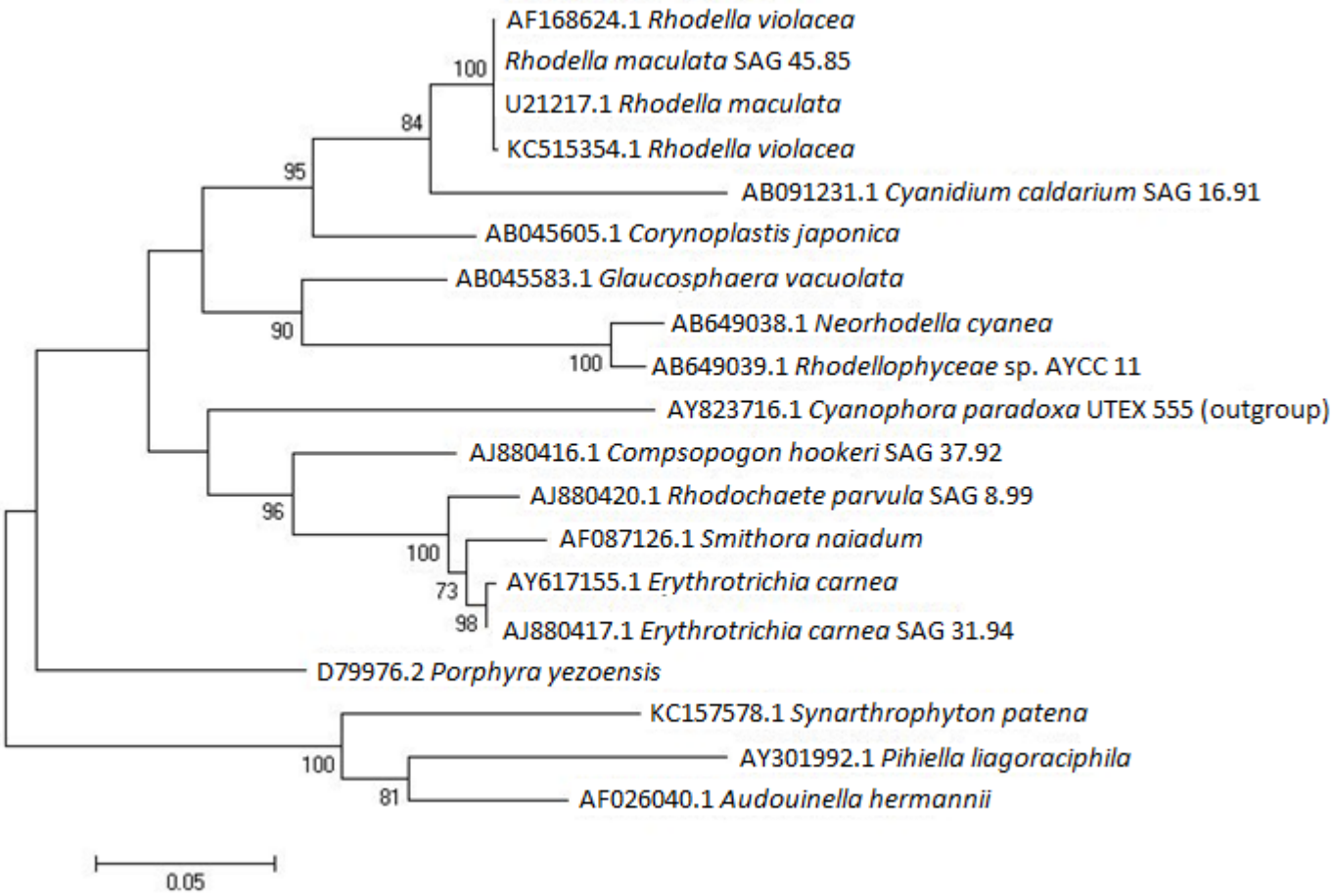


Figure 11. Phylogenetic tree for Rhodella maculata.

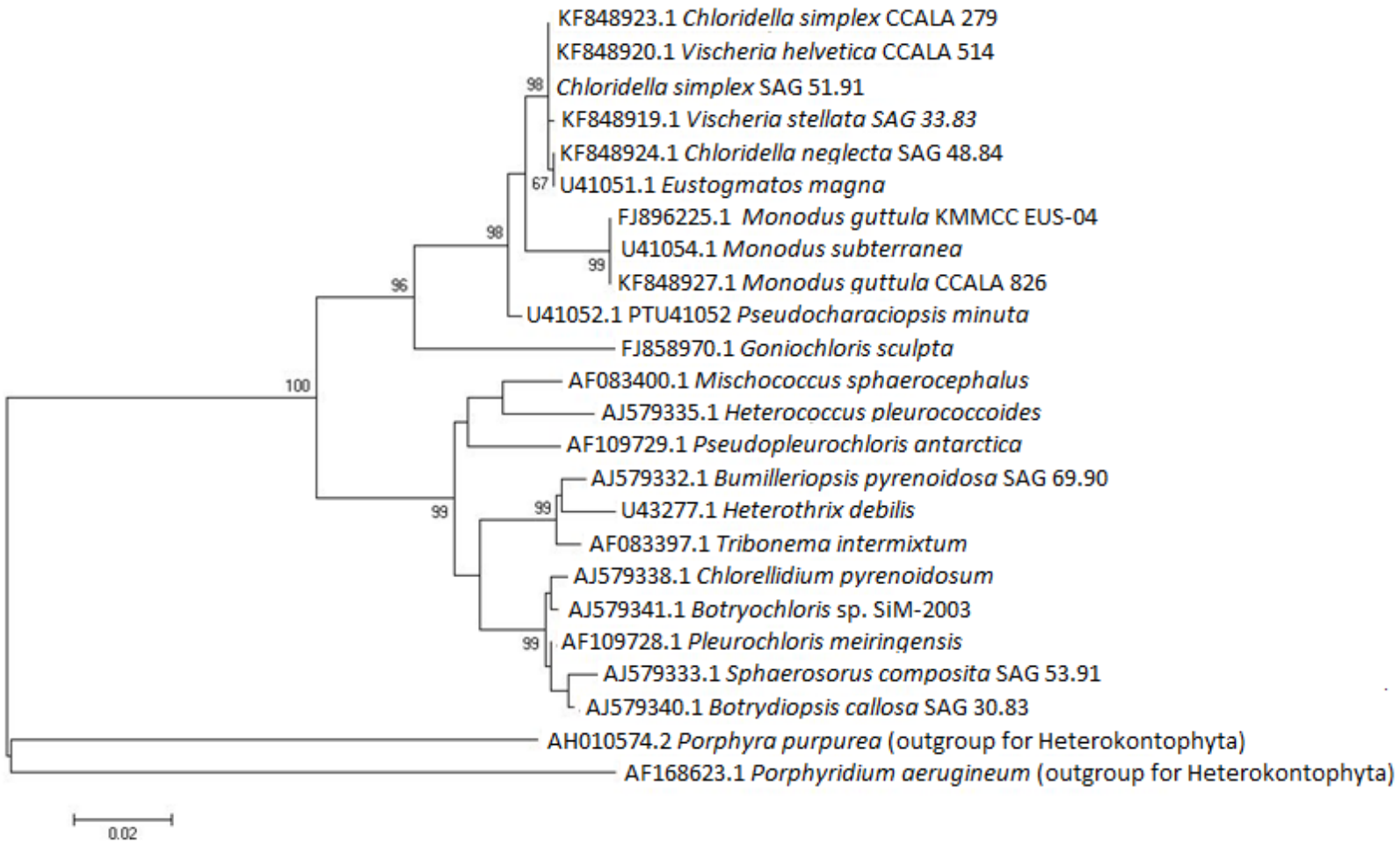


Figure 12. Phylogenetic tree for Chloridella simplex.

A.3. Culture growth curves

Note: Culture conditions followed culture collection conditions (section 2.2.1), unless otherwise specified in the figure title.

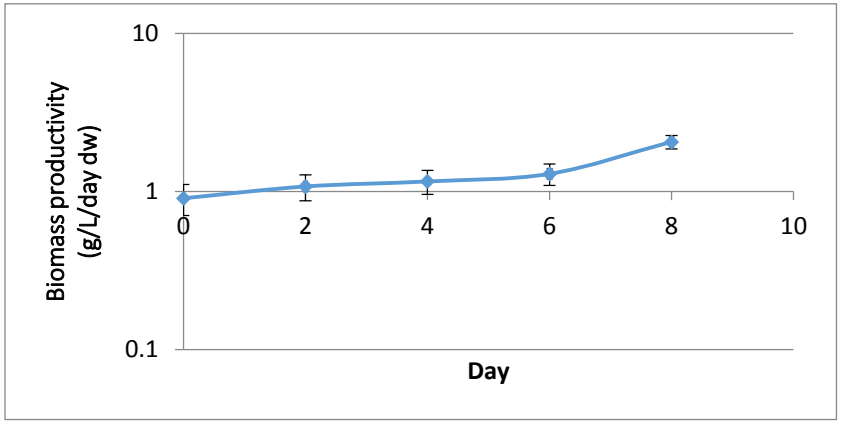


Figure A6. Growth curve of B. hooglandii *in F/2 medium.*

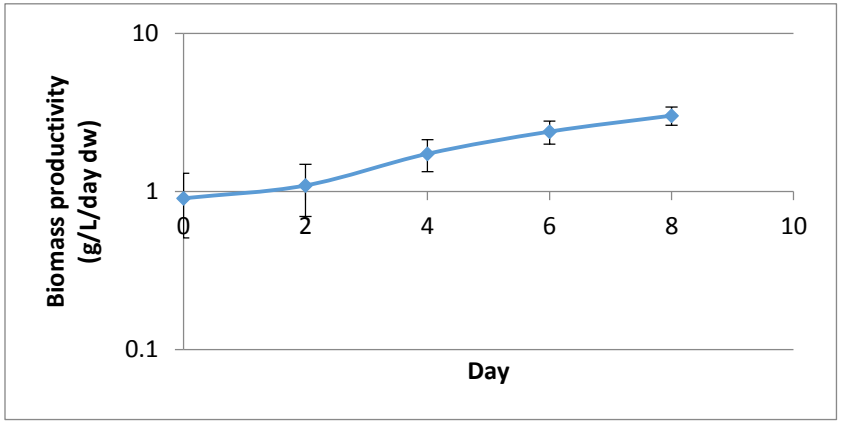


Figure A713. Growth curve of B. hooglandii *in CPCC BBM medium.*

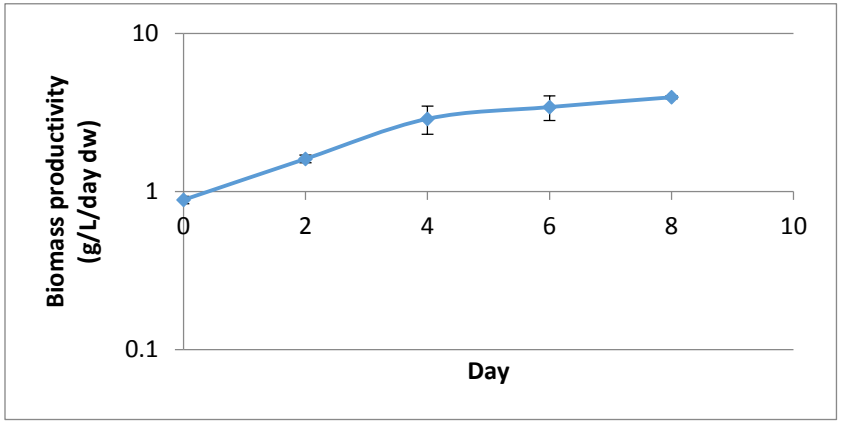


Figure A8. Growth curve of B. hooglandii *in ESP medium.*

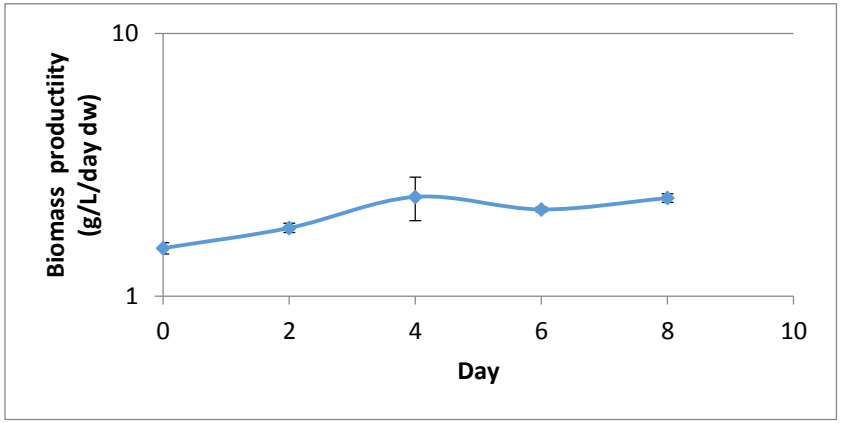


Figure A9. Growth curve of B. hooglandii *at 130 µmoles* m^{-2} s^{-1}.

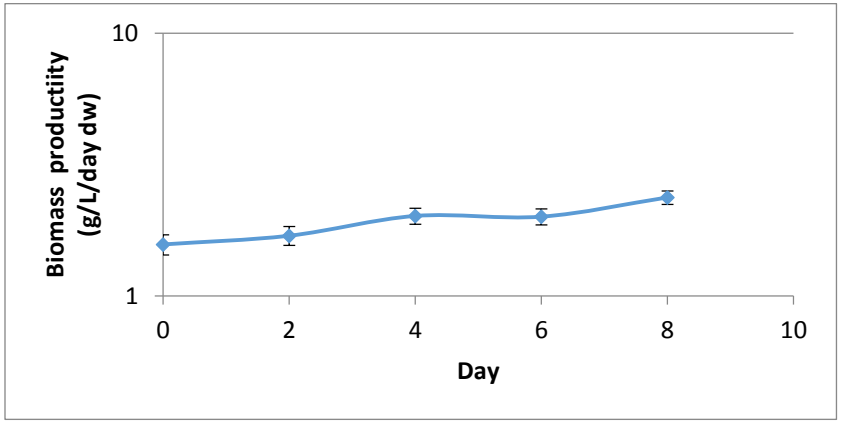


Figure A10. Growth curve of B. hooglandii *in 80 µmoles* m^{-2} s^{-1}.

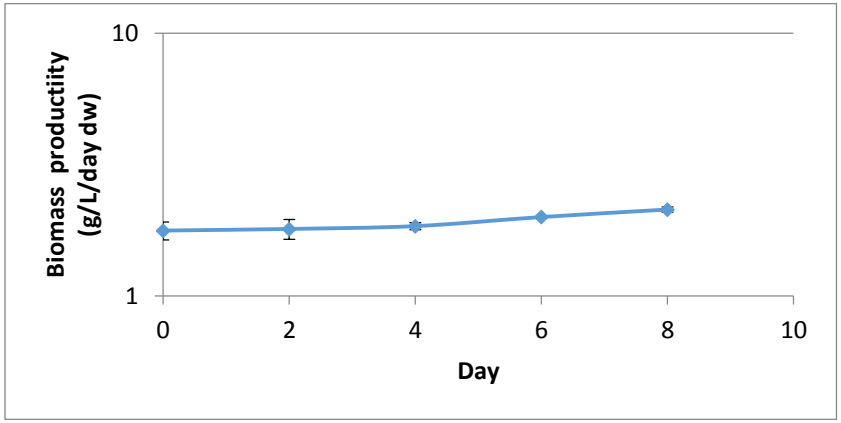


Figure A1114. Growth curve of B. hooglandii *in 45 µmoles* m^{-2} s^{-1}.

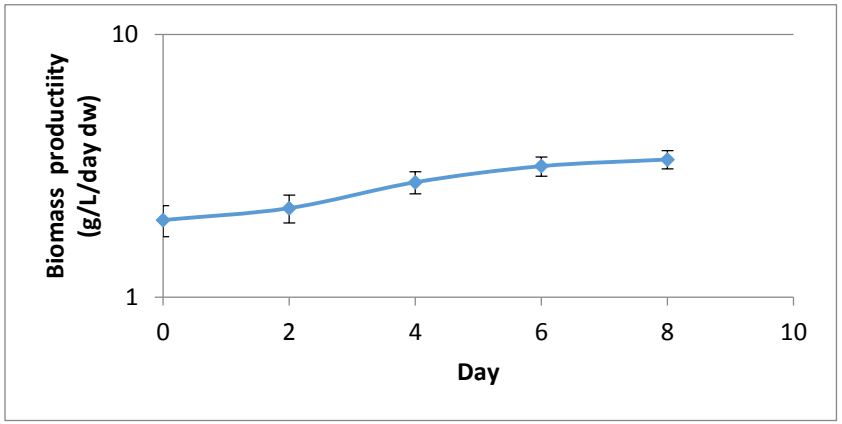


Figure A1215. Growth curve of P. tricornutum *in 130 µmoles* m^{-2} s^{-1}.

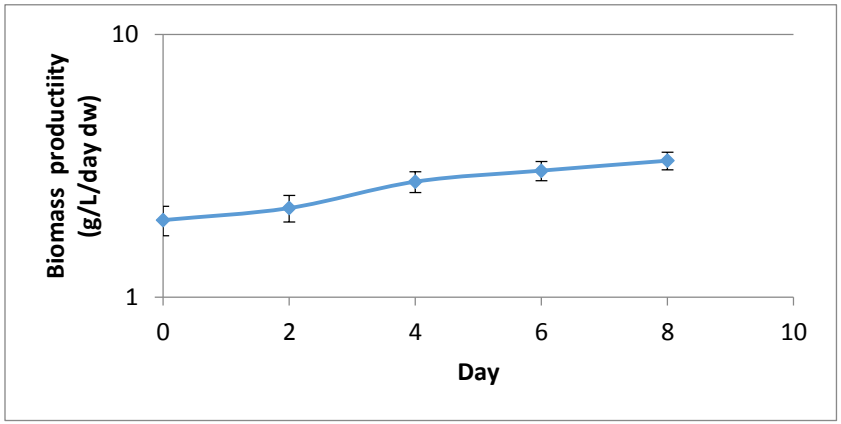


Figure A13. Growth curve of P. tricornutum *in 80 µmoles* m^{-2} s^{-1}.

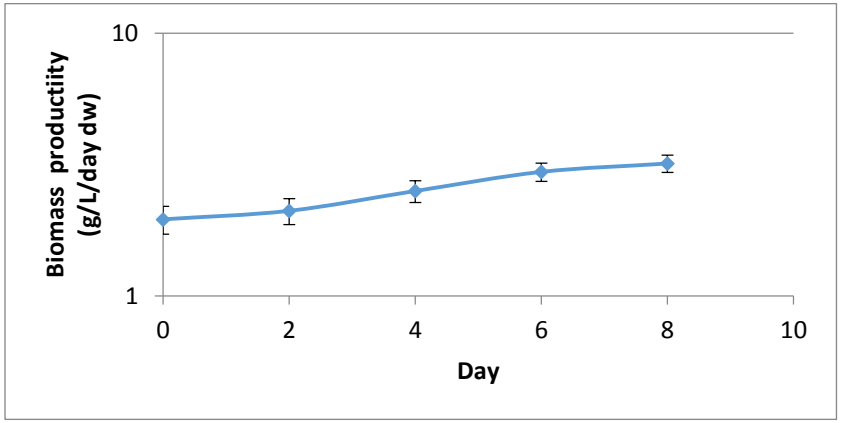


Figure A14. Growth curve of P. tricornutum *in 45 µmoles* m^{-2} s^{-1}.

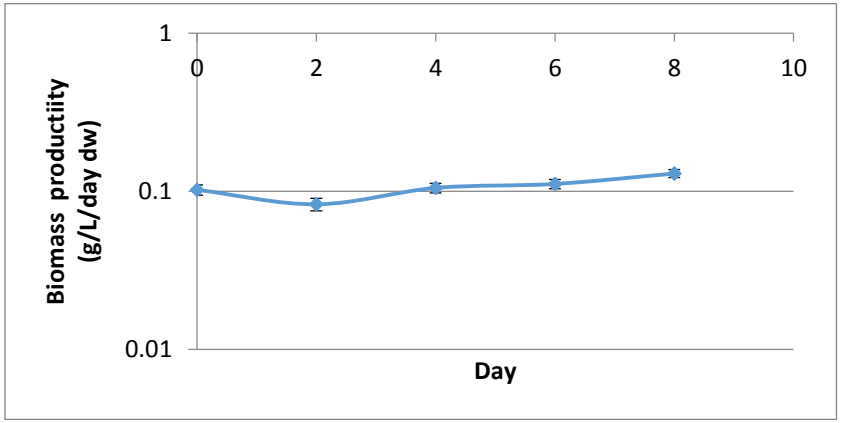


Figure A1516. Growth curve of G. sculpta *in 130 µmoles* m^{-2} s^{-1}.

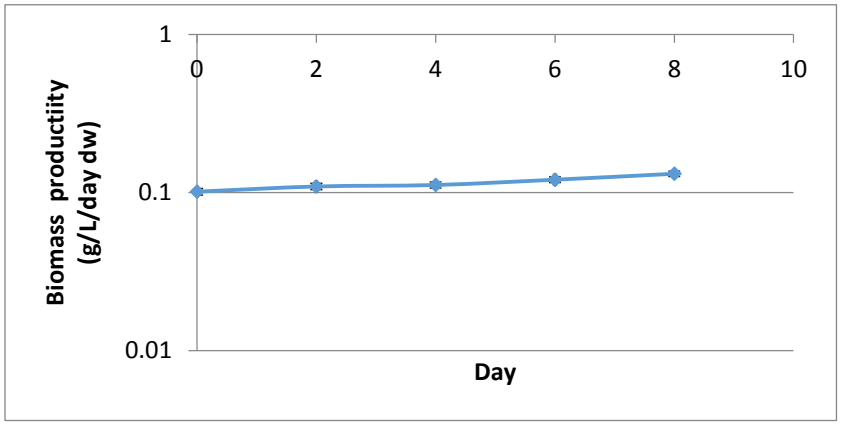


Figure A16. Growth curve of G. sculpta *in 80 µmoles* m^{-2} s^{-1}.

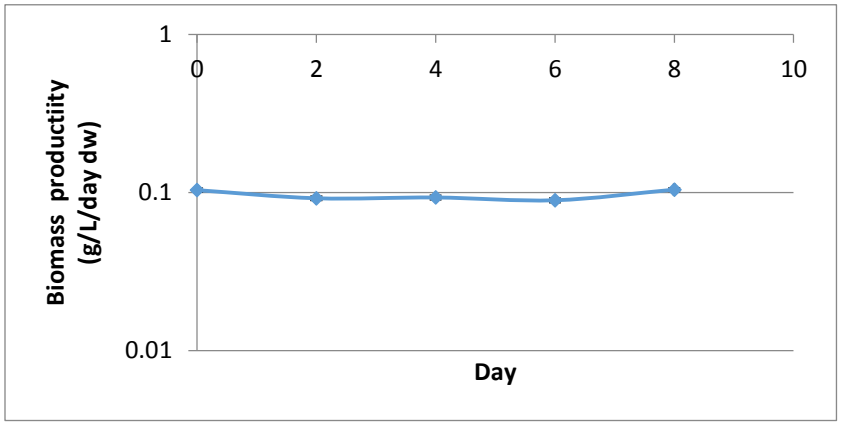


Figure A17. Growth curve of G. sculpta *in 45 µmoles* m^{-2} s^{-1}.

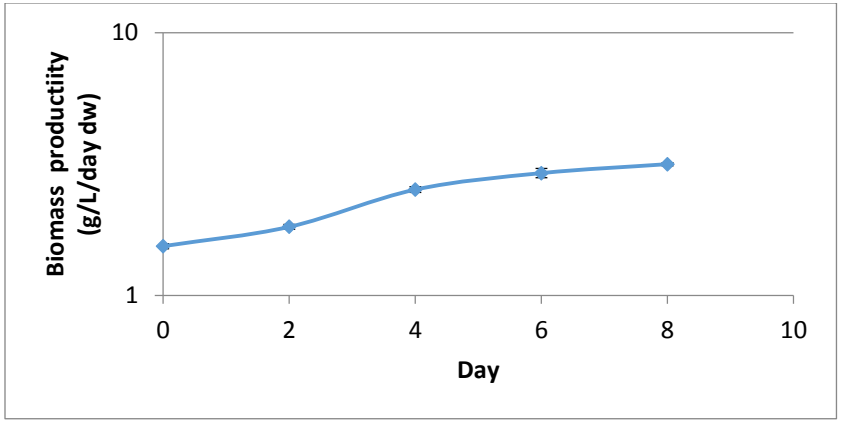


Figure A18. Growth curve of R. maculata *in 130 µmoles* m^{-2} s^{-1}.

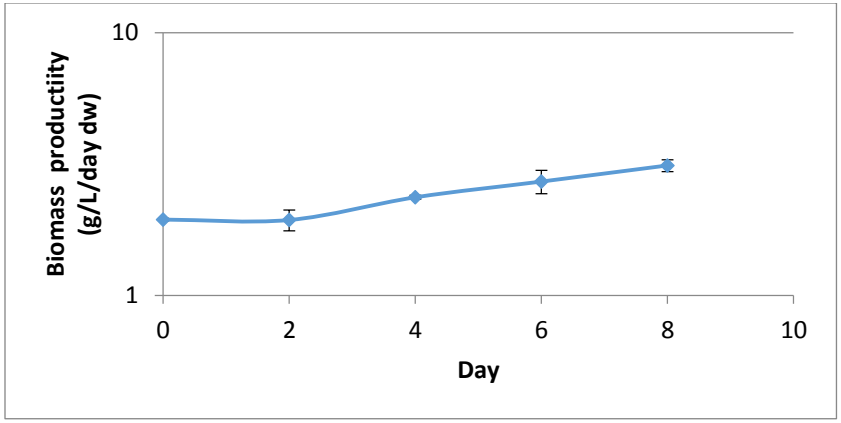


Figure A1917. Growth curve of R. maculata *in 80 µmoles* m^{-2} s^{-1}.

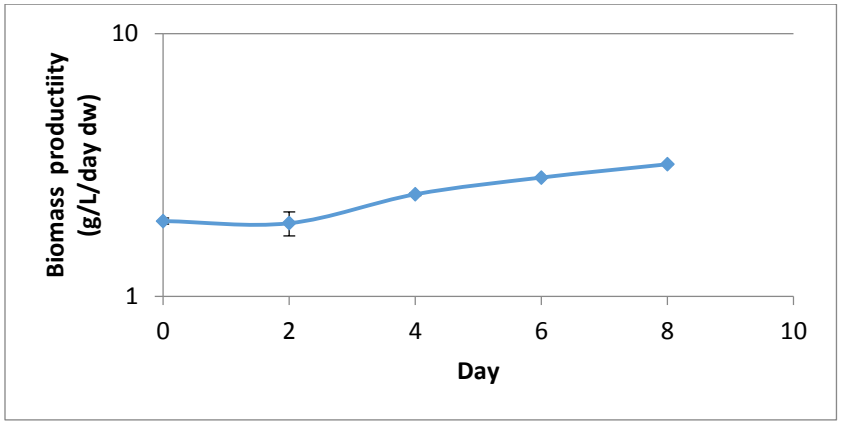


Figure A2018. Growth curve of R. maculata *in 45 µmoles* m^{-2} s^{-1}.

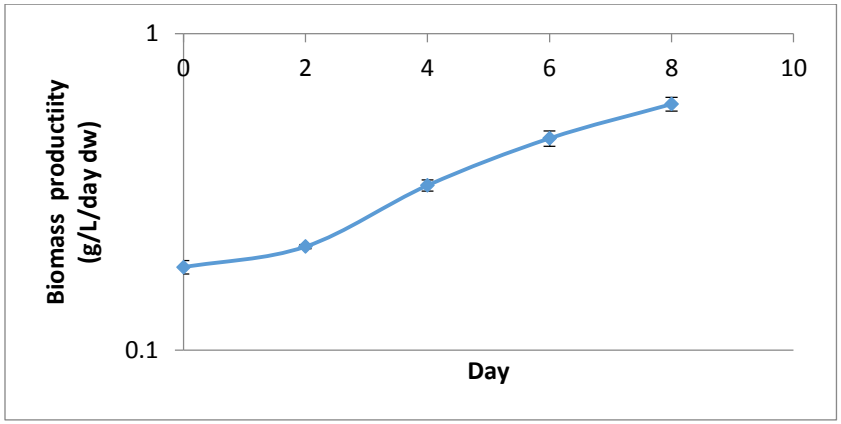


Figure A21. Growth curve of C. simplex *in 130 µmoles* m^{-2} s^{-1}.

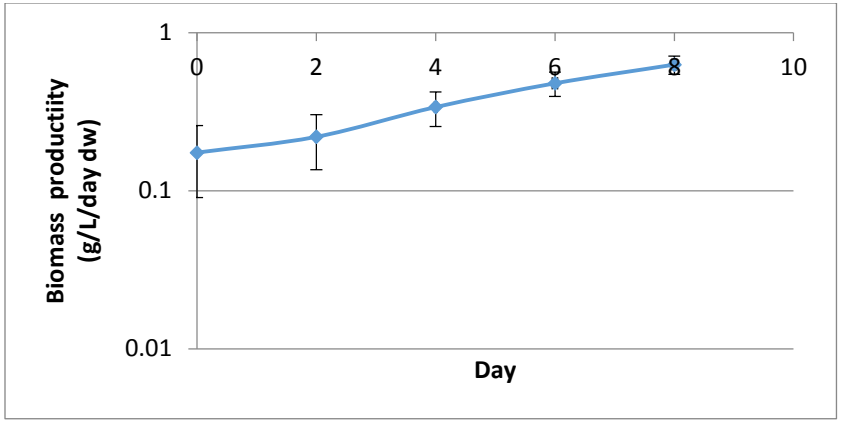


Figure A22. Growth curve of C. simplex *in 80 µmoles* m^{-2} s^{-1}.

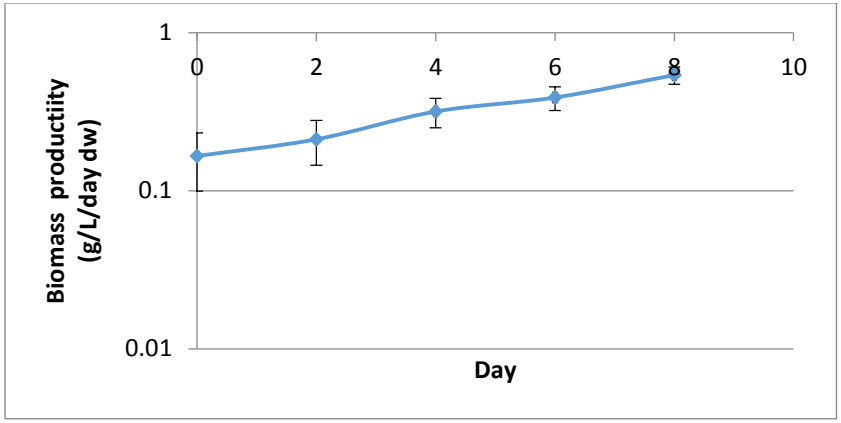


Figure A2319. Growth curve of C. simplex *in 45 µmoles* m^{-2} s^{-1}.

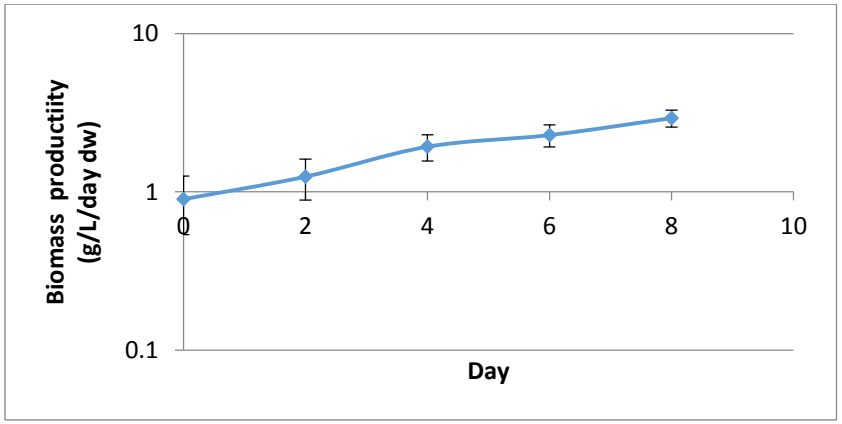
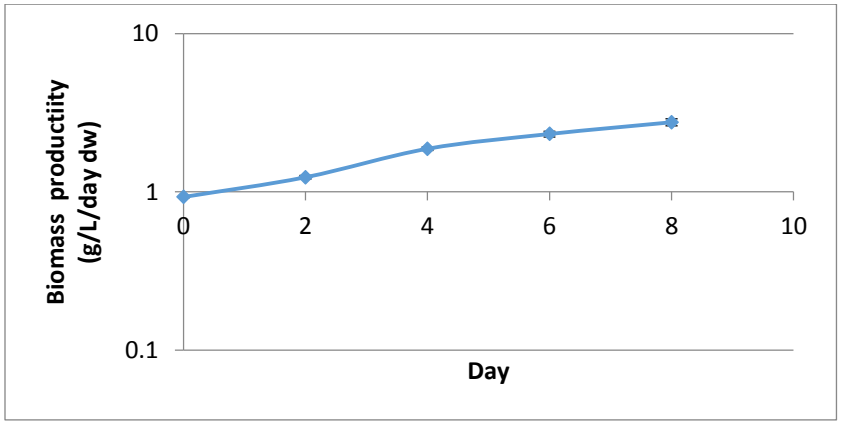


Figure A2420.Growth curve of B. hooglandii *in 100* ngL^{-1} *cobalamin.*



FigureA25.Growth curve of B. hooglandii *in 10* ngL^{-1} *cobalamin.*

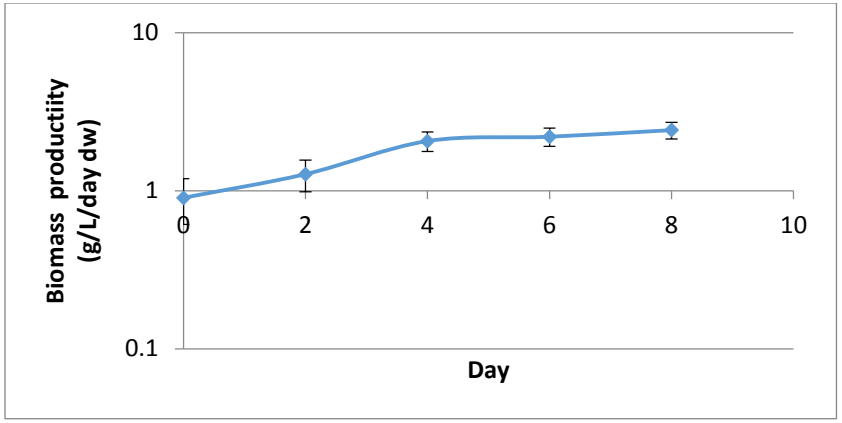


Figure A26.Growth curve of B. hooglandii *in 0 ngL^{-1} cobalamin.*

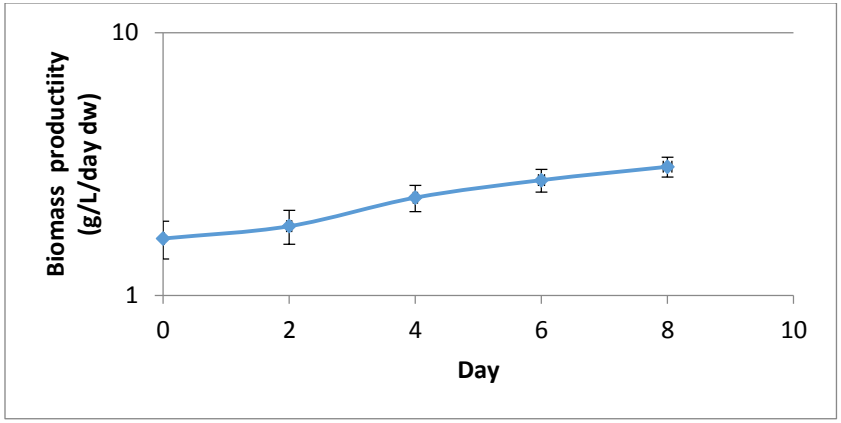


Figure A27.Growth curve of P. tricornutum *in 100 ngL^{-1} cobalamin.*

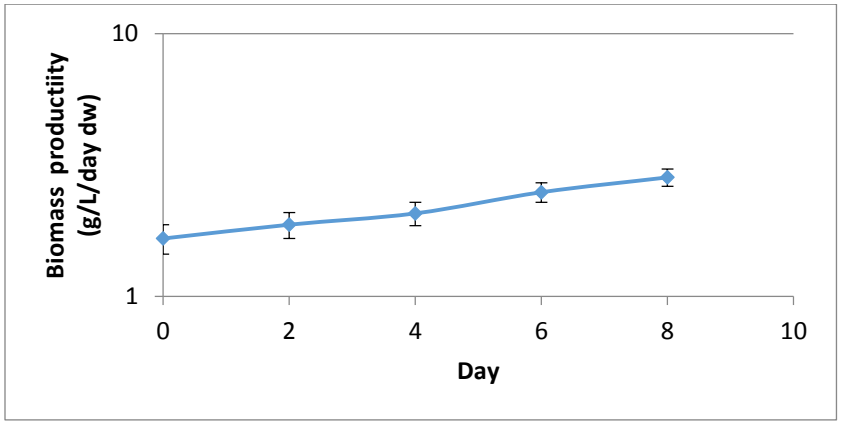


Figure A28. Growth curve of P. tricornutum *in 10 ng L^{-1} cobalamin.*

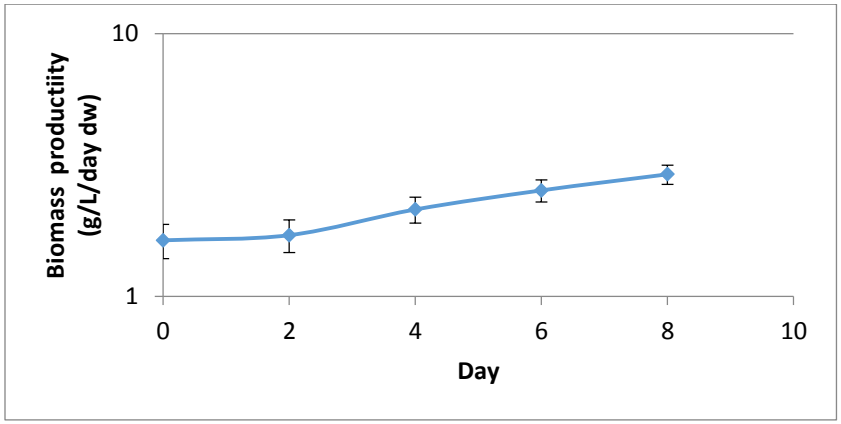


Figure A29. Growth curve of P. tricornutum *in 0 ng L^{-1} cobalamin.*

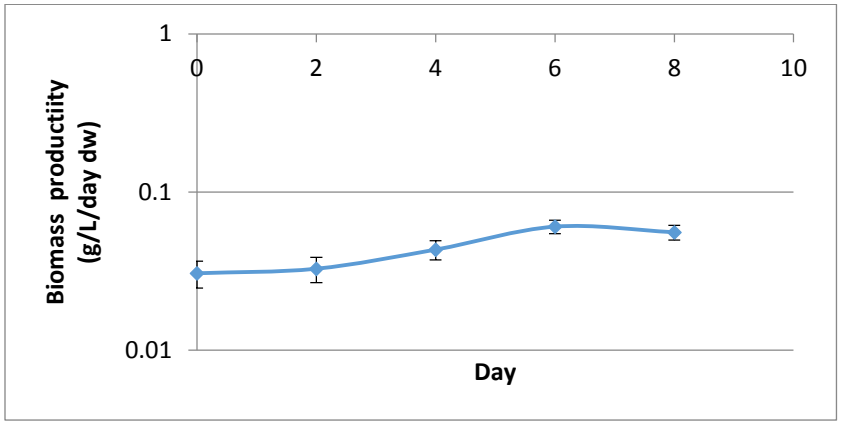


Figure A30. Growth curve of G. sculpta *in 100 ng L^{-1} cobalamin.*

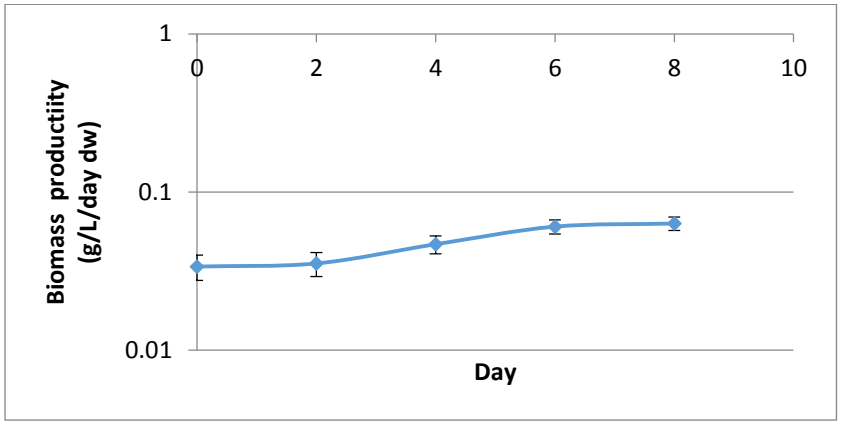


Figure A31. Growth curve of G. sculpta *in 10 ng L^{-1} cobalamin.*

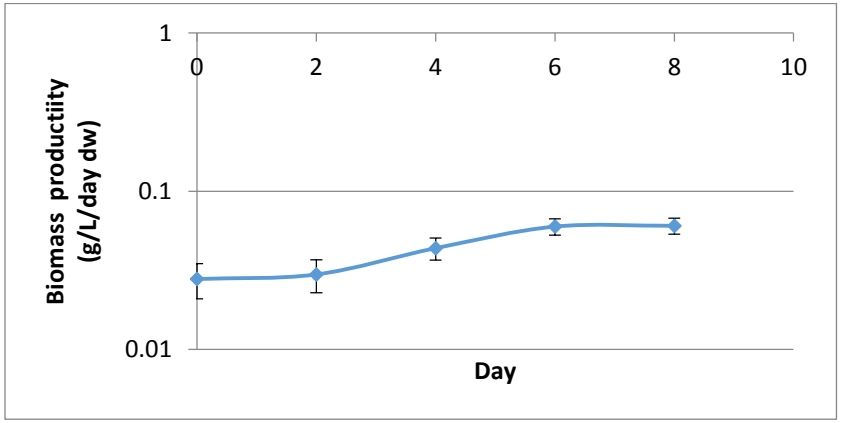


Figure A32. Growth curve of G. sculpta *in 0 ng L^{-1} cobalamin. cobalamin.*

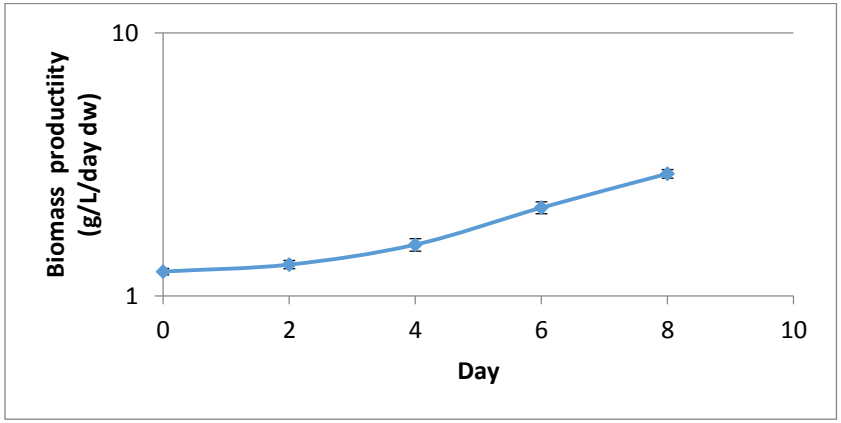


Figure A3321. Growth curve of R. maculata *in 100 ng L^{-1}*

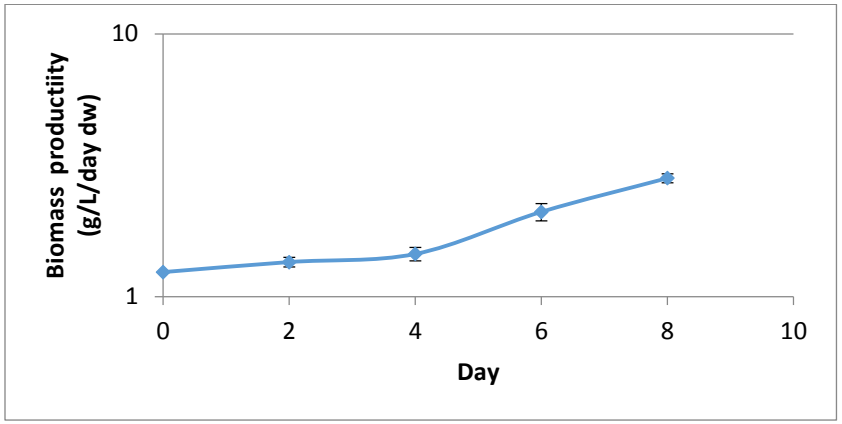


Figure A34. Growth curve of R. maculata *in 10 ng L^{-1} cobalamin.*

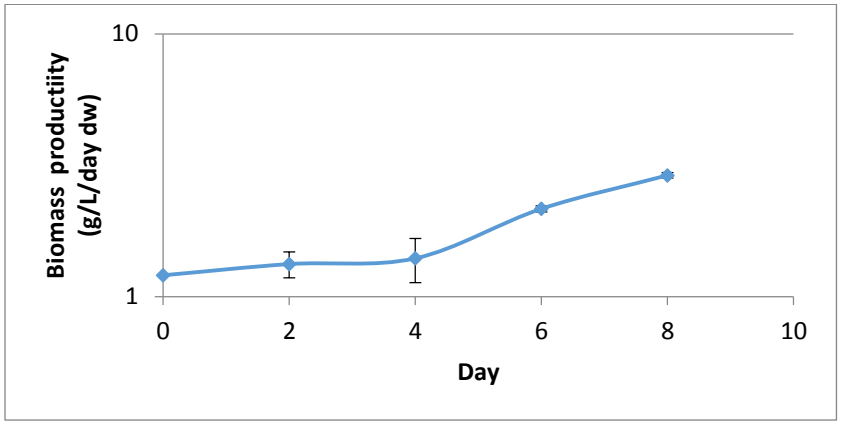


Figure A35. Growth curve of R. maculata *in 0 ng L^{-1} cobalamin.*

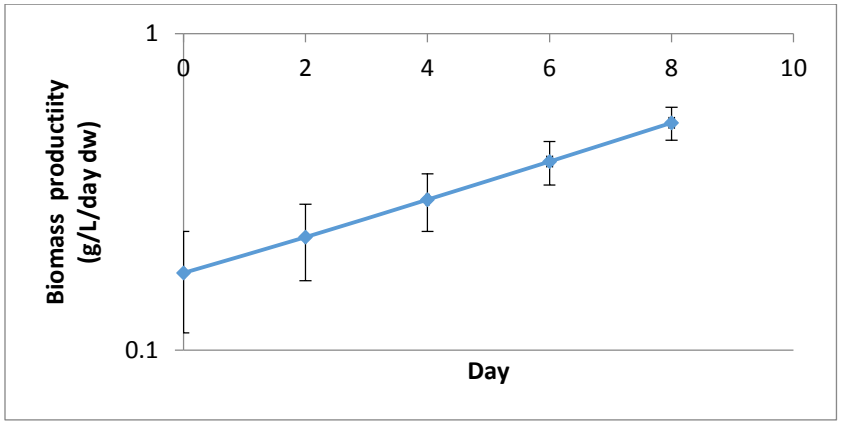


Figure A36. Growth curve of C. simplex *in 100 ng L^{-1} cobalamin.*

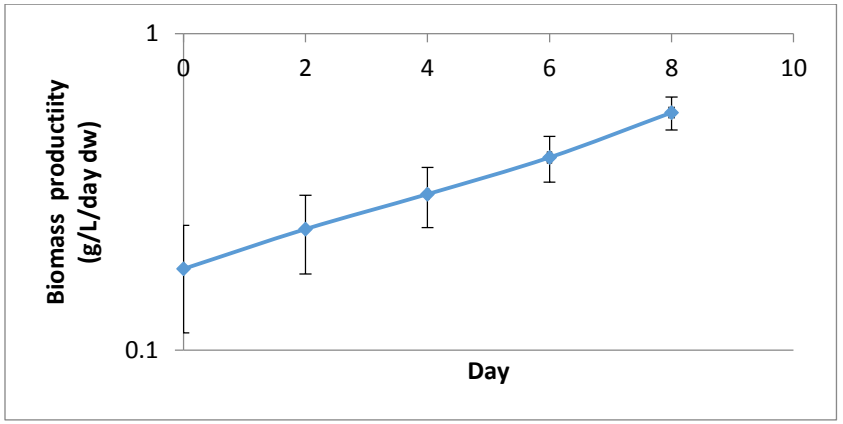


Figure A37. Growth curve of C. simplex *in 10 ng L^{-1} cobalamin.*

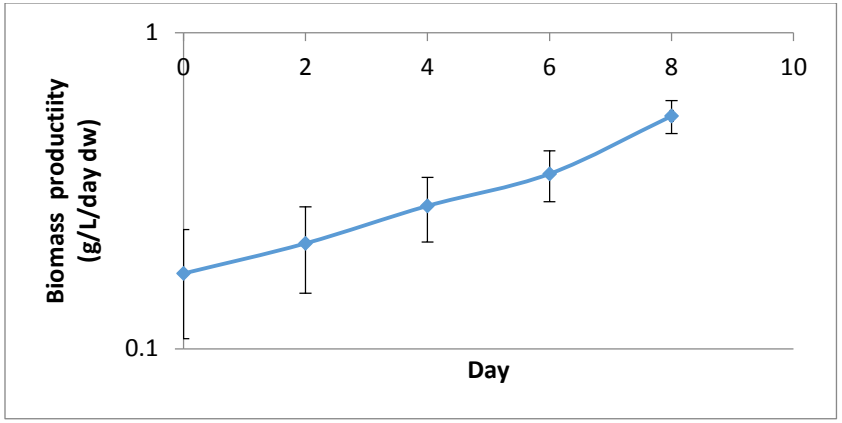


Figure A38. Growth curve of C. simplex *in 0 ng L^{-1} cobalamin.*

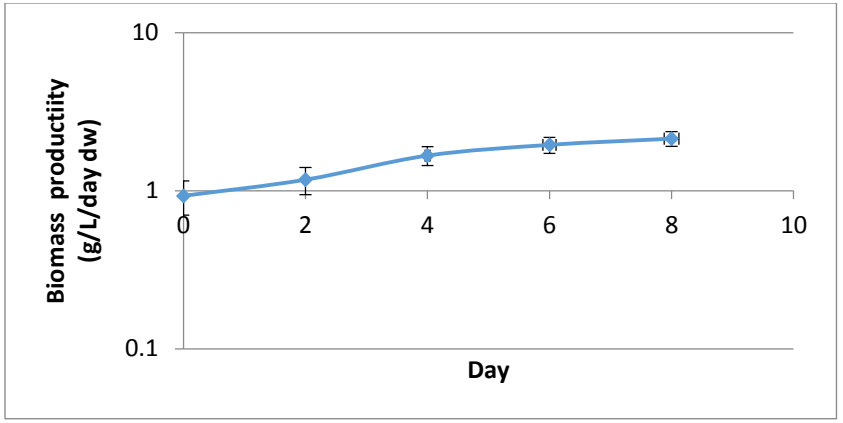


Figure A39. Growth curve of B. hooglandii *in 24:0 light:dark.*

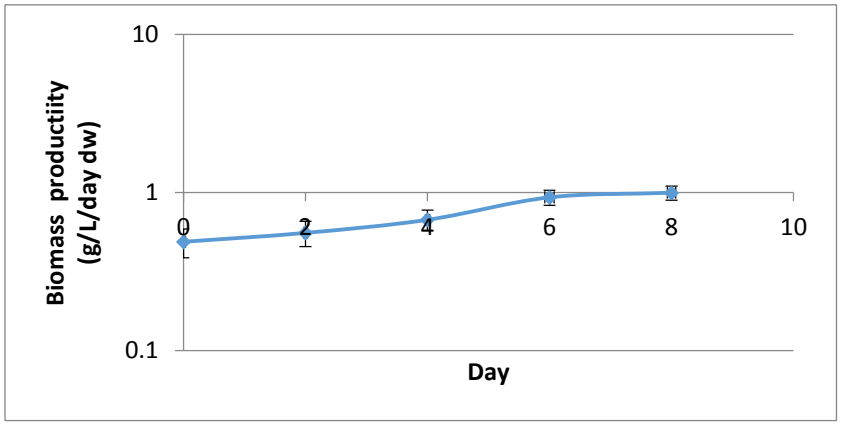


Figure A40. Growth curve of B. hooglandii *in 17:7 light:dark.*

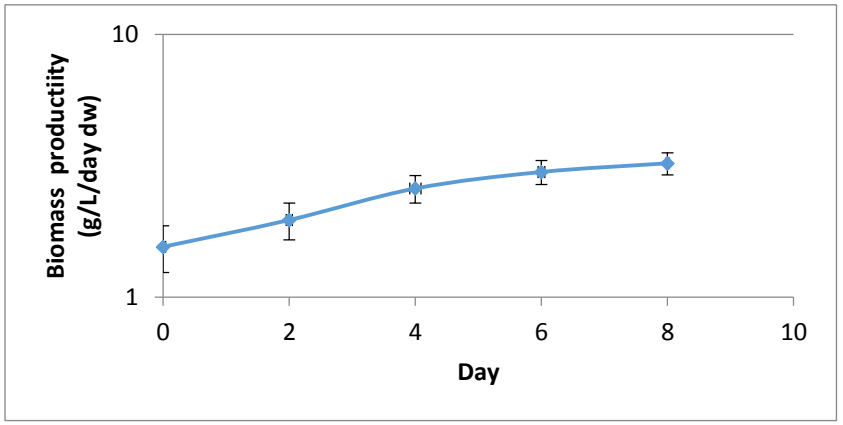


Figure A41. Growth curve of P. tricornutum *in 24:0 light:dark.*

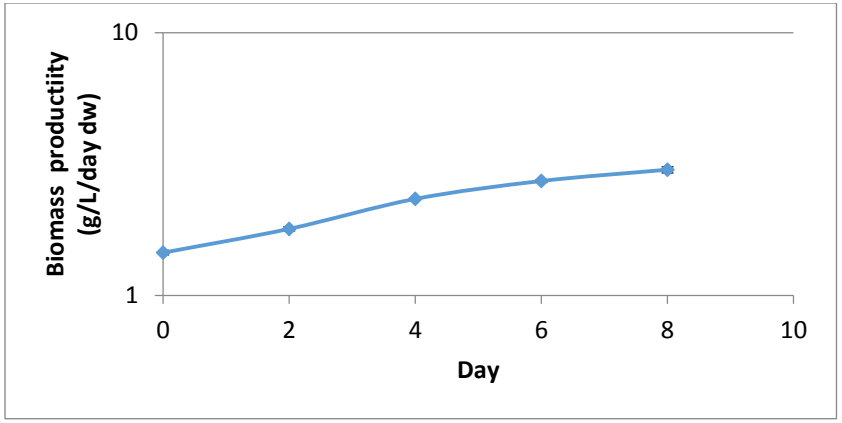


Figure A42. Growth curve of P. tricornutum *in 17:7 light:dark.*

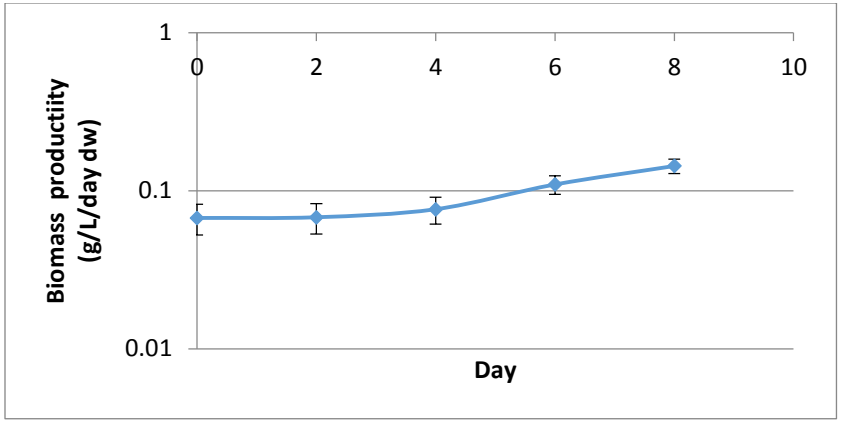


Figure A43. Growth curve of G. sculpta *in 24:0 light:dark.*

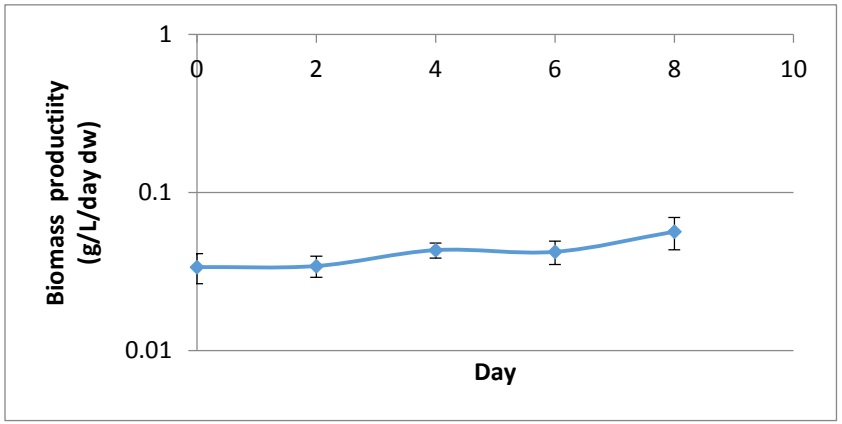


Figure A44. Growth curve of G. sculpta *in 17:7 light:dark.*

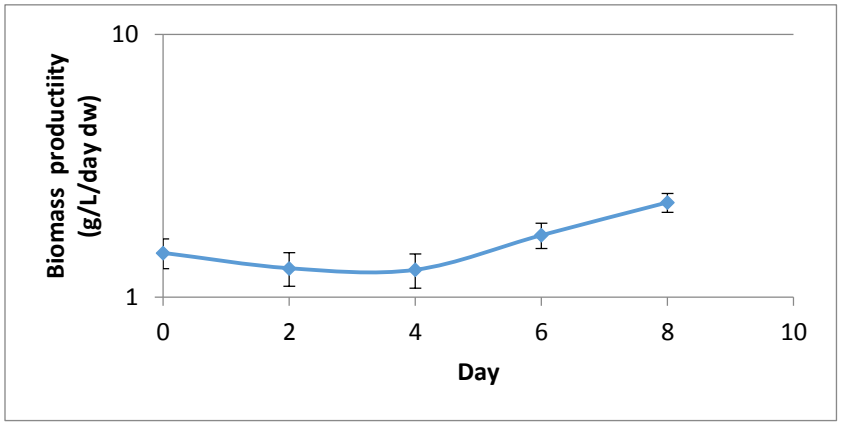


Figure A45. Growth curve of R. maculata *in 24:0 light:dark.*

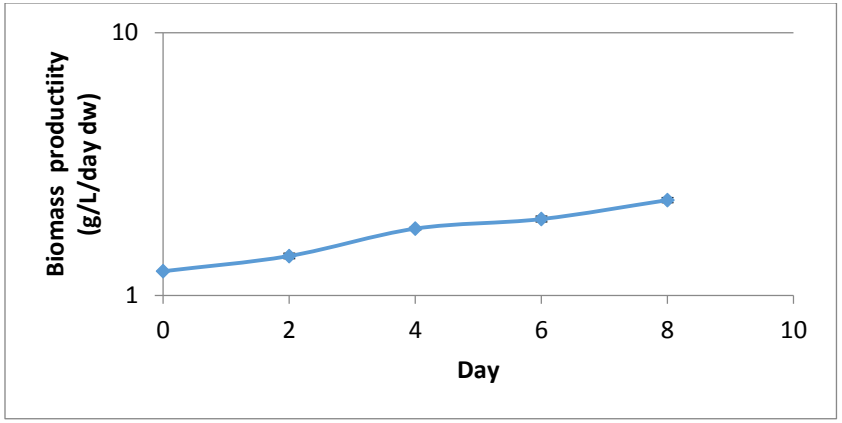


Figure A46. Growth curve of R. maculata *in 17:7 light:dark.*

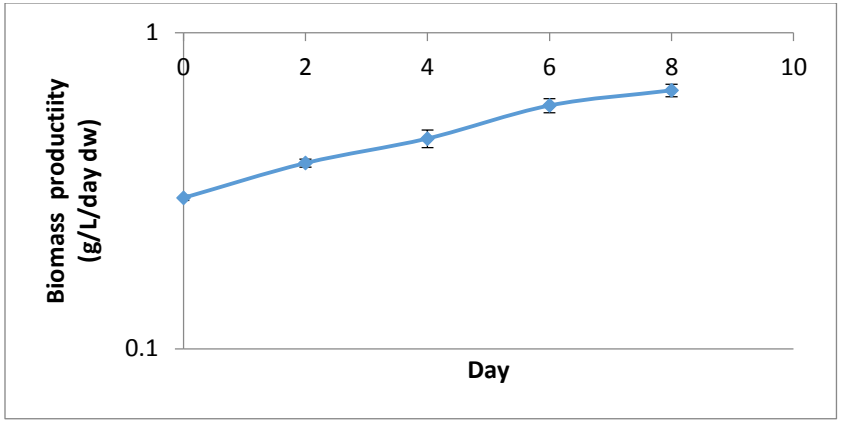


Figure A47. Growth curve of C. simplex *in 24:0 light:dark.*

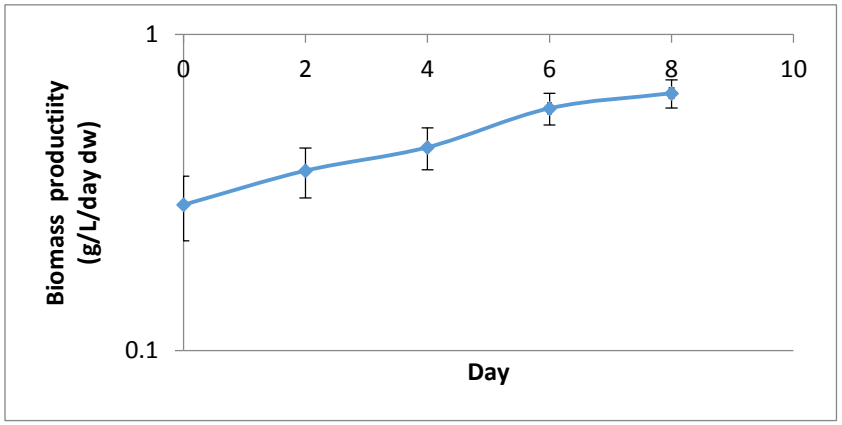


Figure A48. Growth curve of C. simplex *in 17:7 light:dark.*

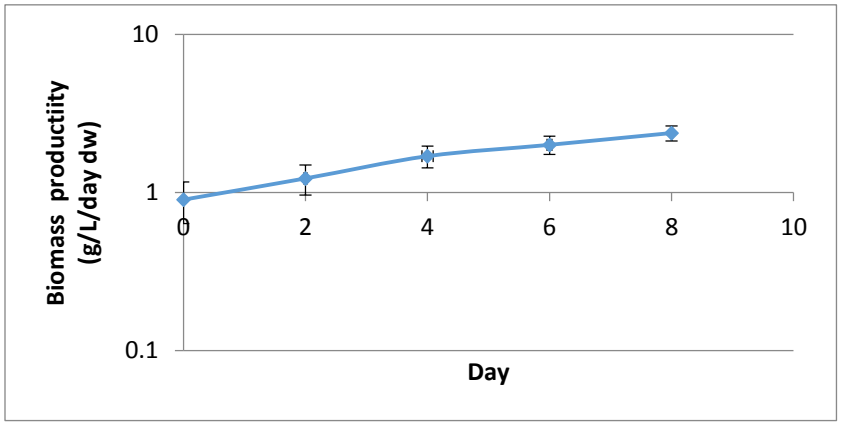


Figure A49. Growth curve of B. hooglandii *in 25°C.*

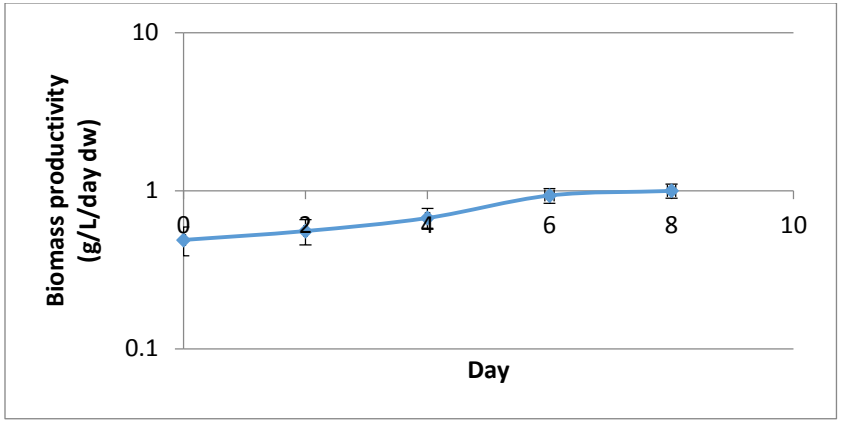


Figure A50. Growth curve of B. hooglandii *in 20°C.*

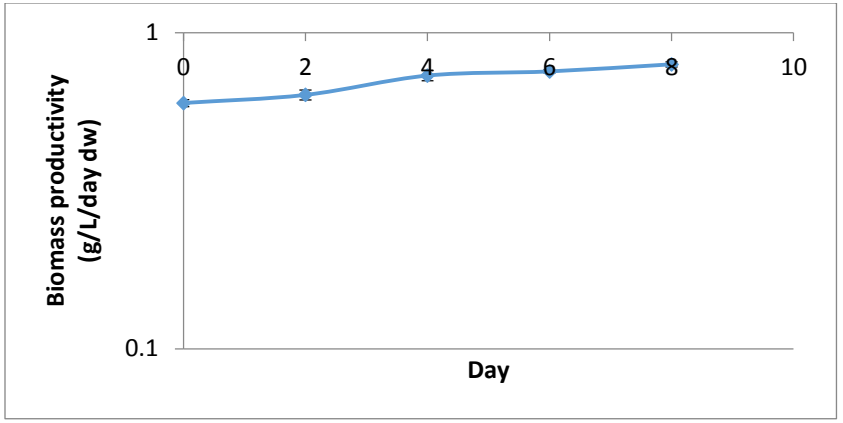


Figure A51. Growth curve of B. hooglandii *in 15°C.*

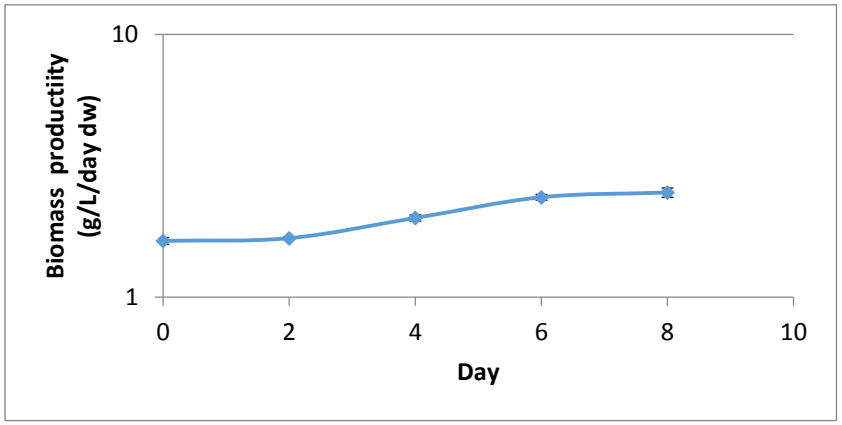


Figure A52. Growth curve of P. tricornutum *in 25°C.*

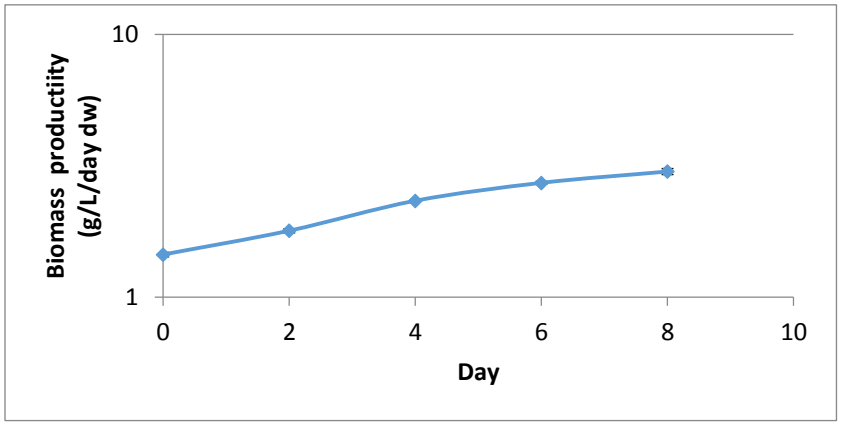


Figure A53. Growth curve of P. tricornutum *in 20°C.*

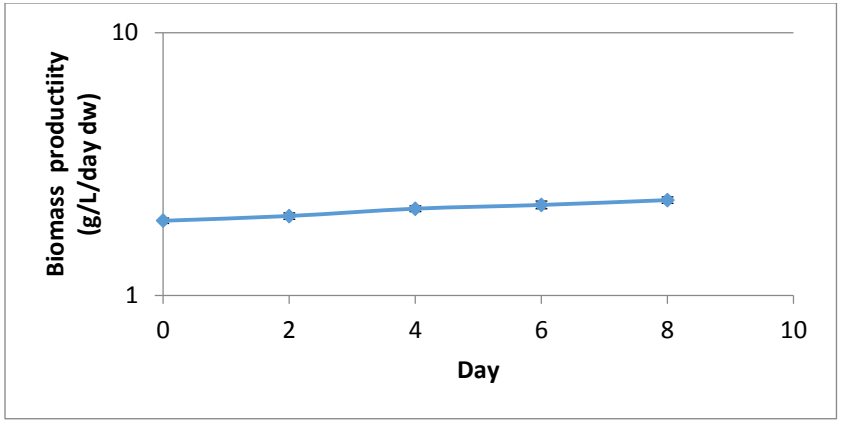


Figure A54. Growth curve of P. tricornutum *in 15°C.*

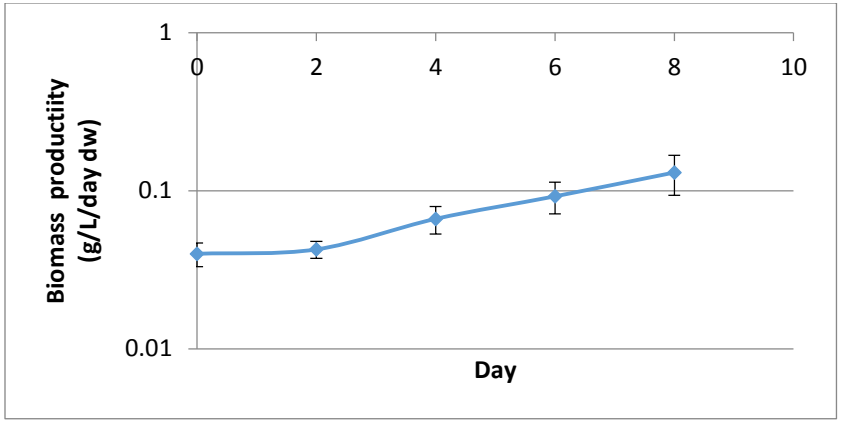


Figure A55. Growth curve of G. scuplta *in 25°C.*

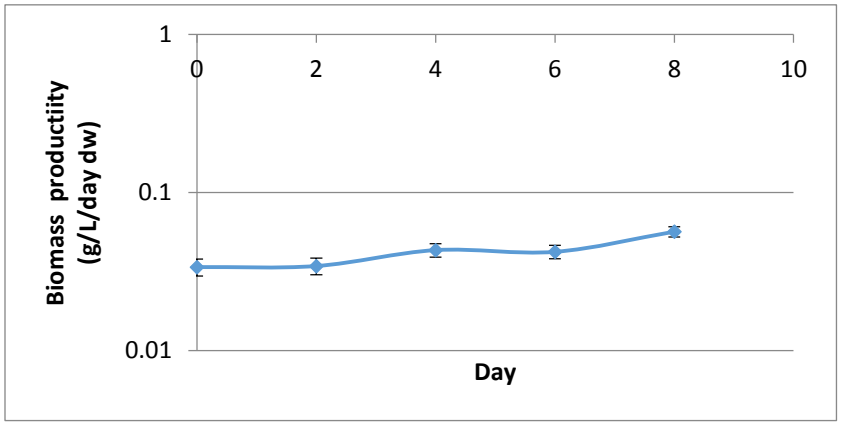


Figure A56. Growth curve of G. sculpta *in 20°C.*

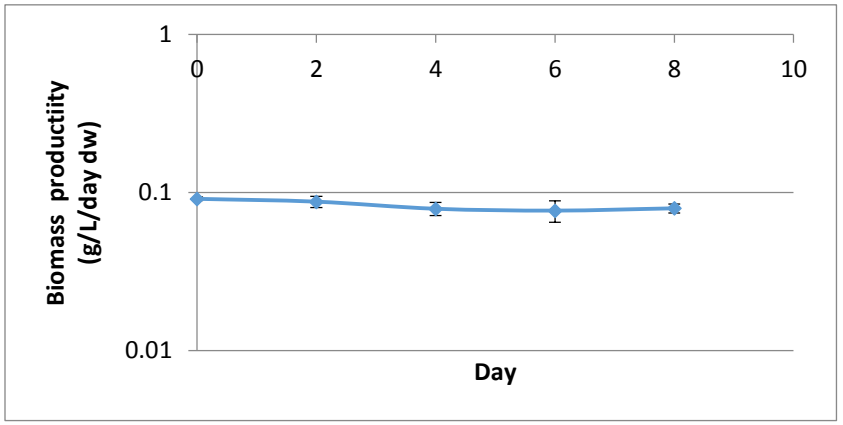


Figure A57. Growth curve of G. sculpta *in 15°C.*

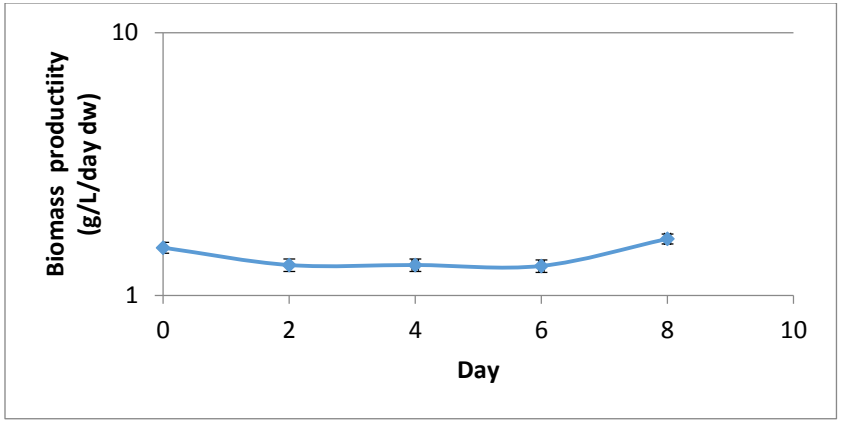


Figure A58. Growth curve of R. maculata *in 25°C.*

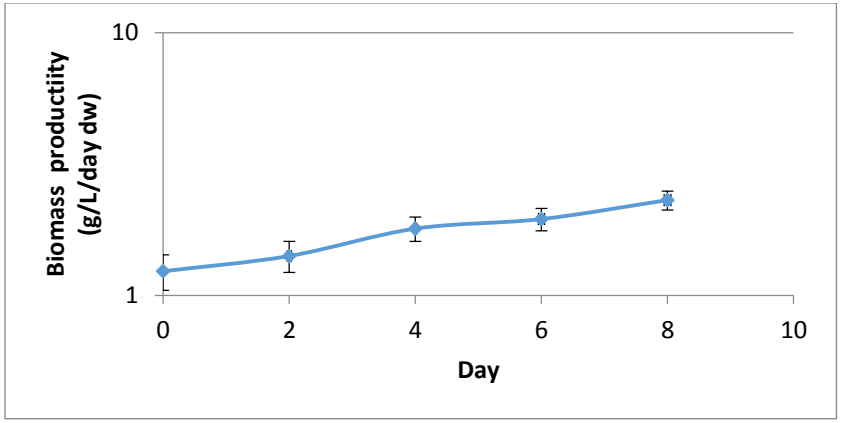


Figure A59. Growth curve of R. maculata *in 20°C.*

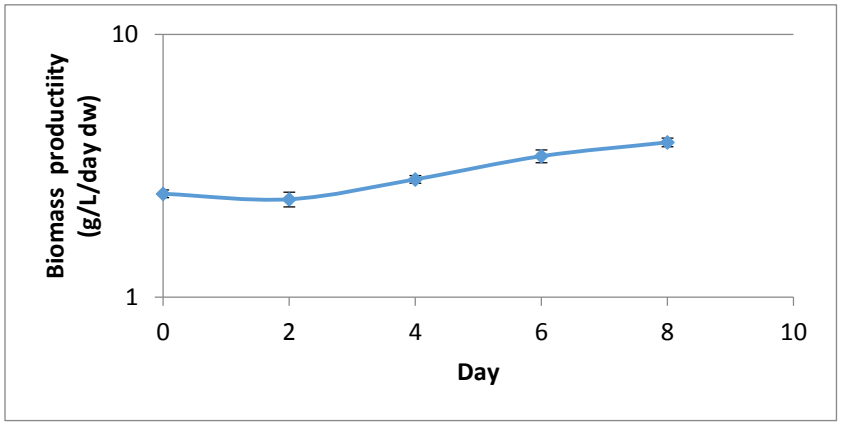


Figure A60. Growth curve of R. maculata *in 15°C.*

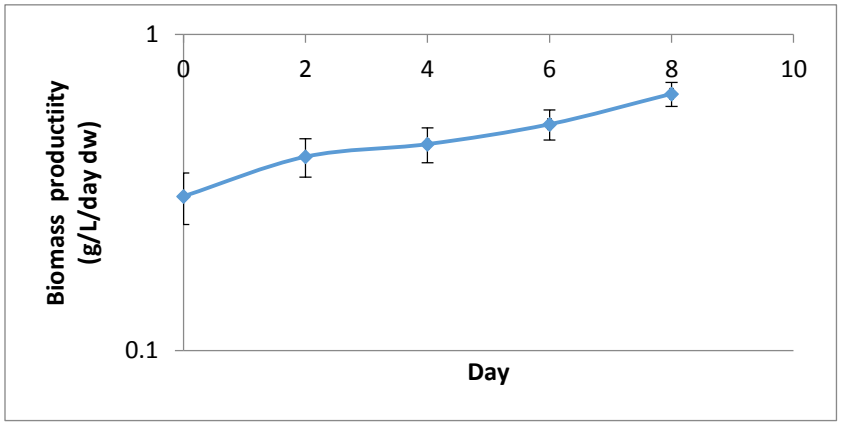


Figure A61. Growth curve of R. maculata *in 25°C.*

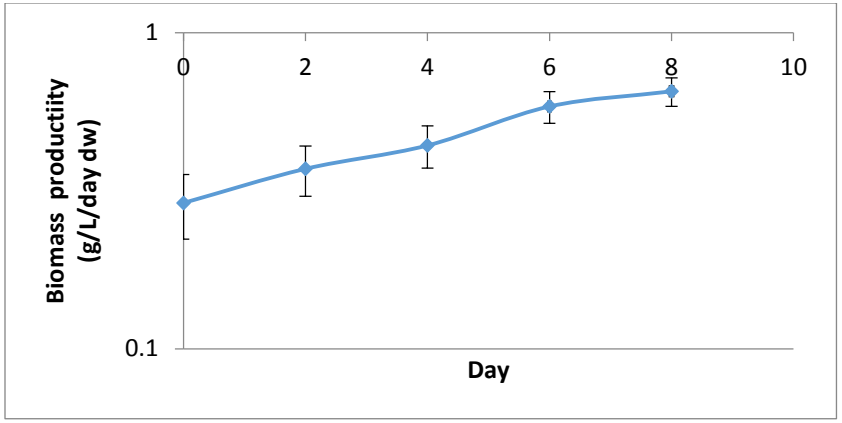


Figure A62. Growth curve of R. maculata *in 20°C.*

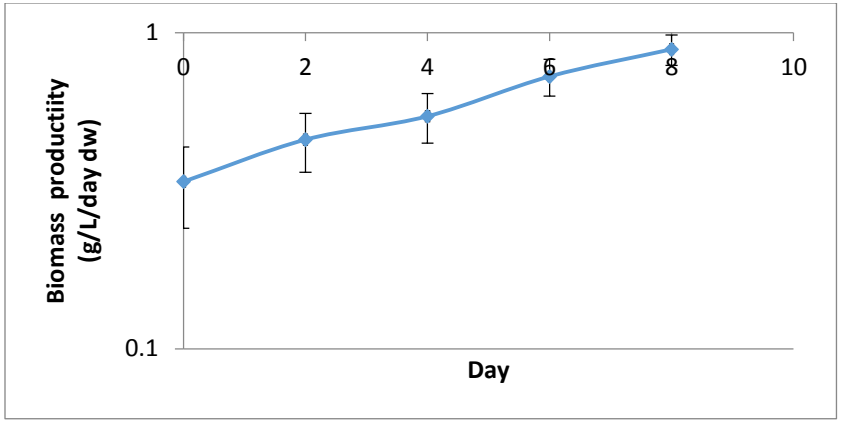


Figure A63. Growth curve of R. maculata *in 15°C.*