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Understanding Mass Culture Dynamics to Maximize Production and Quality of Omega-3 Essential Fatty Acid Commercial Algal Cultivation

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Understanding Mass Culture Dynamics to Maximize Production and Quality of Omega-3 Essential Fatty Acid Commercial Algal Cultivation

by

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Abstract

Omega-3 fatty acid supplements derived from algal oil are a fast-growing nutraceutical product because they provide an alternative to fish-meal based oil; they are sustainable, economically viable, and appeal to a wider consumer market. There are many factors that have been shown to affect the fatty acid profiles of algae: environmental stressors, nutrient availability, and temporal cycles. This thesis explores the effects of temporal culture dynamics on the fatty acid profiles of *Cyclotella meneghiniana* in both small-scale and large-scale growth models. these results can be used to develop a strategic growth model for the optimization of Omega-3 product yield in a commercial scale algal growth facility. A market assessment of the quality and consistency of current algal omega-3 supplements was completed to identify possible challenges of a commercial algal based product. Maximizing the lipid content and optimizing the fatty acid profiles will have a significant impact on the quantity, quality, and profitability of these products.

Acknowledgments

The first time I heard the term “algae” in a positive light was in my junior year as an undergraduate in the BS biology program at East Carolina University. The professor of the global climate change class was outlining some potential bioremediation technologies and algae was featured as a possible carbon fixer/toxin remover for the environment. With a botany/ecology concentration, I had already discovered my love for growing things, but these tiny aquatic plants intrigued me, the more I learned about these organisms, the more fascinating they became. After my undergraduate education I decided that I wanted to start my own algal growth facility. To that end I began looking for a graduate level program that was able to offer a phycology focus, specifically on the growth and commercial use of algae. While researching the next step in producing algae commercially I came across an article about Dr. Julius and his phytoplankton laboratory at St Cloud State University, near my hometown of Big Lake MN. I reached out to Dr. Julius and in our first meeting expressed my interest and desire to learn more about algae and their potential for commercial growth. St Cloud State was able to offer me a flexible graduate program allowing me to pursue both of my passions, biology and business, simultaneously. My research combined work from the Herberger school of business, centering around entrepreneurship and new product development, with a foundation in the school of science and engineering curriculum. The purpose of this thesis is to highlight some of the research that was completed during my Master of Science degree studies. I am exceedingly grateful for all of Dr. Julius’ support in the completion of this research. This is one of the steps in my journey towards the launch of an innovative nutraceutical grade algal biomass facility. I will be using the knowledge gained from my time at St Cloud State University as a foundation

for the launch of a scientifically based, economically viable business in the algae industry. I will be refining my business plan, with the intentions to procure funding and execute a proof of concept commercial algal growth pilot facility outlined in the appendix. These core concepts will be the launching pad for my future career as a business owner and algal researcher.

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Chapter 1 : Diatom growth and yield factors, a review

Introduction

The term “diatom” refers to organisms in the family Bacillariophyceae, also termed “golden algae”. Diatoms are a group of oleaginous algae, characterized by their distinctive silica frustules. Diatoms are one of the most diverse group of organisms; with over 200 known genera and an estimated 200,000 species, of which only approximately 10% are currently described (Mann and Droop 1996). This group of organisms are environmentally significant because they provide an estimated 20% of available oxygen to the planet (Mann 1999). Diatoms have been of commercial interest for almost 40 years because of the high non-polar lipid content stored in their vacuoles (Chisti 1980).

The most popular commercial application for these lipids is biodiesel, which can be used as a direct replacement for petrol-based fuels. The lipid body in diatoms contains primarily triglycerides which can be reacted with methanol via transesterification to make fatty acid methyl esters (biodiesel). This process can be completed for a biodiesel yield of over 98% of the crude algal oil (Fukuda et al. 2001). It is estimated that if 2.5% of United States crop land is converted to production of algae-based biofuel, 50% of the US petrol consumption could be replaced with biodiesel (Chisti 2007).

Some species of diatoms produce long chain polyunsaturated fatty acids (omega-3 PUFA), Specifically docosahexanoic acid (DHA) and eicosapentaenoic acid (EPA). These fatty acids have been used in fish feed to increase the omega-3 fatty acids in their tissues (Stoneham et al. 2018). Direct supplementation of DHA and EPA has also shown to be beneficial for brain health. Showing neuroprotective properties against heavy metal poisoning

(Singh et al. 2019) as well as treatment of some mental ailments such as depression (Tayama 2018). Omega-3 fatty acids have also shown to prevent coronary heart disease and regular intake of DHA and EPA is recommended by the American Health Association (Ajith and Jayakumar 2019). Commercial production of algal oil, specifically from diatom species with high oil content, have the potential to reduce our dependence on fossil-based fuels and provide valuable health benefits both indirectly through animal feed additives and directly through nutraceutical supplements.

Lipid Production Strategies

Research on the lipid synthesis pathways in diatoms is relatively limited, current understanding suggests that their lipid biosynthesis

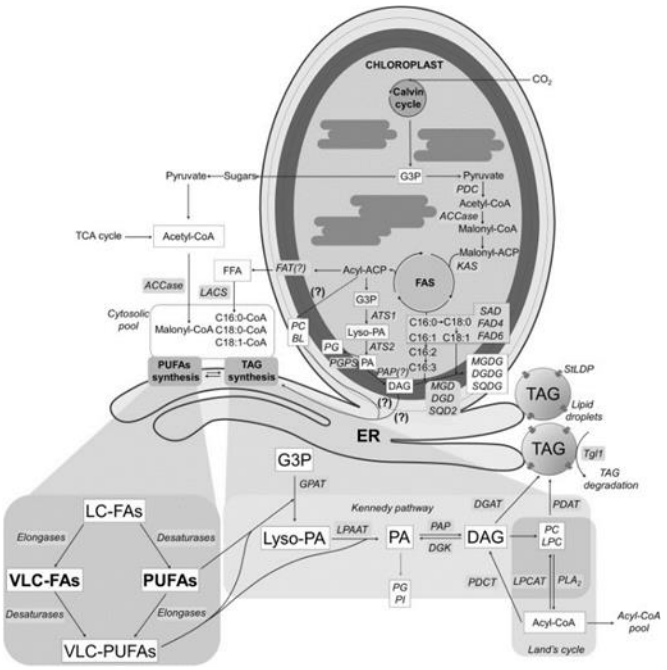

Figure 1.1 Diagram of two lipid metabolic pathways in diatoms: Saturated and monounsaturated medium chain fatty in the chloroplast and the endoplasmic reticulum to convert medium chain fatty acids to long chain PUFA (Zulu et al. 2018)

pathways contain elements from both prokaryotic (in the plastid envelope membranes) and eukaryotic (localized in the endoplasmic reticulum) pathways (Zulu et al. 2018). Fatty acid synthesis is thought to occur via prokaryotic pathways in the envelope membranes of the plastids similar to flowering plants (figure 1.1). This process produces a variety of medium chain saturated and monounsaturated fatty acids (MUFA), primarily 16:0, 18:0, 16:1, 18:1. The medium chain fatty acids produced in this manner can then be metabolized in the endoplasmic reticulum via the eukaryotic synthesis pathways.

The eukaryotic fatty acid synthesis pathway may directly associate with the prokaryotic pathway but no conclusive research has been found (Zulu et al. 2018). The endoplasmic reticulum houses the synthesis of both the polyunsaturated fatty acids (PUFA) as well as the triacylglycerols (TAG) (Figure 1.1). The synthesis of MUFA, PUFA, and TAG in diatoms has been of interest to the scientific community due to its potential environmental and commercial applications. While the exact nature of these processes is not yet fully understood, there several triggers have been identified for stimulating the production of lipids in diatoms including: silica limitation, carbon dioxide infusion, photoperiods, and culture age.

Silica limitation

Silicon depletion to stimulate lipid production in Cyclotella cryptica was first documented over 30 years ago. Roessler published that 4 hours after the silicon in the media was depleted, the lipid composition of the cells increased from 27.6% to 54.1%. he believed that this accumulation of lipids was caused by two mechanisms: the creation of new lipids in the chloroplasts, and the conversion of other sugars and storage products into lipids for the protection of the cell against the environmental stress (Roessler 1988). The

primary challenge of the utilization of this strategy is biomass production, which is limited by the initial silicon concentration.

Ozkan and Rorrer aimed to develop a high-density diatom strategy using a multi-stage silicon and nitrogen feeding system to achieve high density culture in photobioreactors. After initial growth and nutrient depletion, a nitrogen and silicon mixture were infused into the bioreactor 11 times over the course of 10 days. Roessler's 1988 protocol was used as control for the silicon deplete condition. In the silicon replete treatment, Ozkn and Rorrer observed a staggering 10-fold increase in yield (5g/L biomass), but that was accompanied by a significant drop in lipid levels at 8% in comparison to the 39% lipids control in their control culture (2017b).

Jeffryes et al. developed a multi-stage silicon feed protocol where they aimed to increase biomass while maintaining a silicon starved culture with high lipids. This protocol involved a 48, 72, and 96 hour window during which they had a slow silicon feed infused into the culture after initial silicon depletion. This experiment was able to maintain the silica-starved state in the 48 and 72 hour treatments, and they were successful at increasing biomass and yield while maintaining a 45% lipid biomass (2013). Silica limitation appears to be an effective strategy for increasing lipid production in *Cyclotella* sp. and could be used in a commercial growth feeding strategy to maximize yields.

Carbon Dioxide Infusion

Available dissolved carbon dioxide is a primary challenge to commercially cultivated algae systems. Most photobioreactors (and some raceways) use air bubblers to increase carbon availability for the algae. To further increase yield, CO_2 can also be bubbled into the bioreactors, a 10%

carbon dioxide mixture was found to increase diatom yields by almost two-fold (Wang et al. 2014). In another study, Ozkan and Rorrer found that a 10% CO_2 infusion increased the lipid content of *Cyclotella* sp. while maintaining chitin production, thus increasing lipid yields without decreasing the valuable chitin co-product (2017a). Botte et al. found that while a 10% CO2 infusion increased lipid yields by two or three-fold in *Cyclotella cryptica* it did not significantly improve the creation of new biomass. They developed a growth strategy that alternated nutrient additions and 10% CO2 infusions when the nutrients reached depletion. They were able to achieve 1.25g/L dry biomass without seeing a decrease in lipid yield (Botte et al. 2018). Based on these studies, a 10% CO2 enrichment would be an ideal addition to a commercial *Cyclotella* sp. production strategy.

Photoperiods

Sicko-goad et al. identified cyclic trends in their *Cyclotella meneghiniana* cultures regarding lipid content and composition that related to each photoperiod. By combining the control data from 4 different experiments and separating the data into 3 different photoperiods (early light, late light, and dark) they were able to identify shifts in fatty acid composition and abundance. They found that while the major fatty acids (C16's) stayed relatively consistent, the unsaturated fatty acids (including EPA) were lowest in the early light period and highest in the dark period. Also the lipid content is highest in the dark period and lowest in the early light period, suggesting that the cell division events likely happen in the late dark or early light period (1989). Gaidarenko et al. found contrasting results when they evaluated these day/night cycles in their *Cyclotella cryptica* culture. They found that the lipid levels increased during the day and decreased at night, with a mid-day decrease in lipids while the cells

divided (2019). This difference in temporal reproduction rhythms are possibly due to taxonomic adaptations. *Cyclotella cryptica* is found in colder regions so they could have adapted their reproductive cycle to be during the warmest part of the day cycle. In contrast it would be more advantageous for the temperately located *Cyclotella meneghiniana*, who favor long light cycles (Sicko-goad and Andresen 1991) to divide when the temperature is at the coolest, the last part of the night, or first part of the day. These results suggest that identifying the optimal photoperiod cycles is essential when developing a commercial algal growth strategy.

Culture Age

Diatoms also have distinct growth phases that impact fatty acid composition and yield. While evaluating the ideal day/night scheme for lipid production, Sicko-goad and Andresen identified a specific period, after exponential growth when the lipid content of the biomass was the highest. For their culture of *Cyclotella meneghiniana* this period was on day 14, after which, lipid yield started to decrease (1991). Stepaneck et al, observed a similar trend when they compared *Cyclotella meneghiniana* and *Phaeodactylum tricornutum* DHA lipid yields based on when the culture was harvested (early/late exponential and early/late exponential). They found that very shortly after exponential growth phase has ended, lipids begin to decrease, specifically the long chain polyunsaturated fatty acids (DHA) (unpublished data). These trends suggest that careful monitoring and precise harvest timing can maximize product yield in commercial algal DHA production.

Commercial Cultivation Strategies

Maximizing lipid yield is not the only factor that can influence the successful commercial production of algal biomass. Bioreactor type has a large influence on the economic viability of the business. There are three

major bioreactor types that will be evaluated: fermentation bioreactors, photobioreactors, and raceway ponds. There are several environmental factors that will influence growth in all these environments including: carbon source, light saturation, and nutrient competition. These three factors will be contrasted for economic viability on a commercial scale.

Fermentation Bioreactors

Heterotrophic growth of diatoms was first identified in 1953 when Lewin tested 42 different strains for the ability to thrive in the absence of light. He was able to successfully cultivate 13 isolates in the dark with a glucose rich medium (Lewin 1953). Since that time, heterotrophic growth of algae has become one of the most popular cultivation methods for commercial algal production. Heterotrophic bioreactors are completely sealed, all environmental factors are controlled and measured, this allows for a very consistent and high-quality product (Barclay et al. 1994). The absence of light also allows for very high cell density because the diatoms are not dependent on light saturation for growth. Heterotrophically grown biomass and lipid yield have been published at more than 3 fold higher than traditional photobioreactor or raceway ponds (Morales-Sanchez et al. 2016; Barclay et al. 1994). Although these high yields are very appealing for commercial cultivation, they are accompanied by very high capital investment and operational costs (Morales-sanchez et al. 2016). Heterotrophic growth has several limitations including, high initial capital cost, high operational cost, and limited target isolates. However, if the target product has a very high value, these limitations can be overcome (Morales-Sanchez et al. 2016).

Photobioreactors

Photobioreactors are closed bioreactors made of either glass or clear plastic to allow for maximum light penetration, which can be supplied by either natural or artificial light. Tubular photobioreactors are the most common type and consist of large tubes that have continuously flowing culture medium in them (Slegers et al. 2013). Other types of photobioreactors include flat panel (Endres et al. 2016) and clear polyethylene bags (Harris et al. 2013). Photobioreactor systems typically have a higher initial capital investment and maintenance costs than raceway ponds but have less contamination or environmental hazards. Closed systems also have less water loss and can achieve higher biomass concentrations than their pond/raceway counterparts (Chisti 2007). Photobioreactors also make it possible to cultivate algae on a commercial scale in the northern climates by heating and supplementing with artificial light in the winter months (Pankratz 2017). Viability of a photobioreactor algae production facility is largely dependent on the value and importance of purity in the product.

Raceway Ponds

Raceways generally consist of a long, shallow pond that is dug into the ground, or in a frame above ground, and lined with either cement or plastic, they maintain a continuous flow with a large paddlewheel. Open systems are subject to significant water loss through evaporation and their yields are limited by light saturation because of shading, Raceway ponds typically have a maximum depth of approximately 12 inches (Chisti 2007). In addition to depth measurements, the correct pond length to height ratio is also very important to minimize dead zones and maintain homogeneity of nutrients to maximize CO2 utilization (Hadiyanto et al. 2013). Raceway ponds can either be outdoors or indoors with artificial light sources. Because outdoor systems

are exposed to the environmental conditions, location is very important. In a simulation using several environmental and operating conditions Rarrek et al. concluded that Les Cruz, New Mexico had optimal growing conditions for a raceway pond, specifically in the month of June (2016). Location of indoor systems are more flexible but will require a large amount of temperature-controlled space to function. Despite these challenges, raceways are very popular because of their low initial capital investment and maintenance costs.

Harvesting Strategies

In addition to taxa selection, Lipid accumulation strategy, and bioreactor type, harvest strategy will have a large impact on your overall commercial cultivation strategy. Most algal cultures follow a predictable growth pattern called the s-curve. When initially inoculated, the concentration of cells in the culture is usually very low, the growth experiences a lag phase where the algae are adjusting to the new environment. Once the concentration has reached the species dependent threshold, the population experiences rapid expansion, termed the "exponential" growth phase. In this growth stage, the cells are actively dividing and doubling every 24-36 hours. When the population reaches nutrient depletion, the cells start to transition from active growth to stationary phase. In this phase they will start converting all available energy into protective compounds (lipids in diatoms) for storage and protection. Commercial cultivation and harvest can be achieved through a few different strategies: Batch, continuous, and semi-continuous harvest. For a batch harvest, the algae are allowed to grow until they reach the stationary phase. Once maximum biomass is reached 90-95% of the cells are harvested, just leaving enough culture to inoculate the next batch, which will start again at the lag phase. This

results in a longer period between harvest times because each batch begins again at the lag phase.

The second type of commercial cultivation strategy is called continuous culturing. In a continuous harvest, the culture is grown until it nears the end of exponential growth phase. Once exponential growth reaches its peak, a constant small harvest stream begins, the lost volume is replaced with fresh media to prevent nutrient depletion. This cultivation strategy can be very effective for blue-green or green algae, however golden algae have the lowest lipid content during the exponential phase.

The third type of cultivation strategy is called semi-continuous culturing. In a semi-continuous harvest the culture is grown until it reached the stationary phase, then 40-50% of the culture is harvested and the lost volume is replaced with fresh media to begin the culture again at exponential phase, the cell concentration never falls low enough to send it back to lag phase. Depending on the taxa, you can either harvest at the end of exponential phase or beginning of stationary phase, the harvest intervals will be the approximate doubling time of the taxa (24 – 36 hours). With an oil producing taxa, the harvest frequency will be the doubling time plus the transition time to the stationary phase (48-72 hours). This hybrid harvest method effectively reduces time between harvests when compared to batch phase and does not require constant flow harvest or monitoring. There are many different growth strategies for commercial algal cultivation, strategy selection largely relies on the target taxa and potential product value.

Economic Modeling

Economic analysis of commercial algal production is complex depending on productivity, bioreactor type, and product value. Below are three different cost models based on single product portfolios published over the last 10 years (Table 1.1). There are several major differences between these estimates, mostly centering around advancing techniques and taxa. In all cases, only biomass production was evaluated, oil content and processing cost

Table 1.2 Economic model comparison from three different studies: Model 1 and Model 2 were designed for biofuel production; Model 3 centered around dietary supplement production.

	Photobioreactor Biomass		Raceway Pond Biomass		References
	Product-ivity	Production Cost	Product-ivity	Production Cost	
Model 1	72 g/m2/d	$2.95 /kg	35 g/m2/d	$3.80 /kg	(Chisti 2007)
Model 2	20 g/m2/d	$11.70 /kg	10 g/m2/d	$2.26 /kg	(Slade and Bauen 2013)
Model 3	30 g/m2/d	$2.80 /kg	12 g/m2/d	$1.10 /kg	(Xiang et al. 2017)

vary widely depending on taxa. Model 1 was centered around biofuel production (Chisti 2007), target taxon was not specified but from biomass productivity claims this model was probably designed around a green algae, probably *Chlorella* sp. Economic model 1 did not include capital depreciation cost in their calculations, resulting in a much lower photobioreactor production cost than model 2. The second model used a conservative biomass productivity, and a high capital depreciation for photobioreactors (60% of total costs) compared to raceway ponds (20%) (Slade and Bauen 2013), resulting in a much

higher photobioreactor production cost than the other two models. The third model estimated the photobioreactor cost to be only 3 times higher than raceway cost, with a higher operating cost resulting in smaller differences between the two production costs (Xiang et al. 2017). Based on these three independent economic models, commercial cultivation of algal oil is technically feasible depending on bioreactor type and potential product value. To create an economically viable business, a strategic approach toward strain selection, product development, process design and especially lipid content optimization are vital.

Conclusion

Commercial production of high value lipids and co-products from *Cyclotella meneghiniana* can be economically feasible if a strategic approach to the bioreactor design, location, and product marketing is applied. Media composition, feeding profiles, carbon dioxide addition, photoperiod, and harvest timing will all have significant impacts on the lipid composition and yield. Additionally, expanding the product portfolio to utilize more of the biomass would increase profits and decrease waste streams. Developing these key growth strategies and new technologies will have long term economic benefits including but not limited to the biodiesel, nutraceuticals and animal feed industries. Algal cultivation continues to further our progress towards the economic viability of a complete displacement of fossil-based fuels, human and animal nutraceutical supplementation and sustainable agriculture.

Chapter 2 : Bioreactor scale-up for commercial cultivation

Introduction

The potential health benefits from dietary omega-3 oils is well known (Swanson et al. 2012). The majority of the Omega-3 supplement market is made using fish-derived oils, but fish do not make these oils, they acquire them from their diets (Jenkins and Josse 2008); algae are the primary producers of these essential fatty acids. Omega-3 supplements derived from algal oil are fast growing in popularity on the nutraceutical market (Finco et al. 2016). This trend stems from two main factors: sustainability and market demand. Demand for fish meal and oil has grown significantly over the last 50 years (Shepherd & Jackson 2013), this rapid growth of the aquaculture industry has been limited by space and resource availability (Shepherd & Jackson 2013) and has been shown to contribute to declining fish stocks (Adarme-Vega et al. 2014) and environmental degradation (Cole et al. 2009). Commercial cultivation of algal for omega-3 supplements provide a sustainable alternative to fish-meal based oil (Lenihan-Geels et al. 2013). Supplements derived from fish meal can contain marine pollutants or toxins (Finco et al. 2016) and cannot be used by consumers with dietary restrictions stemming from health (eg. shellfish allergy) or lifestyle choices (eg. Veganism). Algal derived omega-3 fatty acid supplements are vegan, toxin, and shellfish free, providing bioavailable omega-3 fatty acids without the consumption of fish (Lane et al. 2013, Doughman et al. 2007). These limitations on the production and usability of fish-based omega-3 supplements created a prime market space for alternative sources of omega-3s fatty acids, specifically from algal feedstocks. Algae oil based omega-3

supplements stand out against their fish oil counterparts because they are sustainable, economically viable, and appeal to a wider consumer market.

Omega-3 Producing Algal Species

Several Species of algae are currently being investigated for commercial cultivation of long chain polyunsaturated fatty acids (Omega-3's specifically), those include *Phaeodactylum tricornutum* (Yongmanitchai and Ward 1991), *Schizochytrium* sp. (Sahin et al. 2018), *Skeletonema menzeli*(Jiang et al. 2016), and *Nannochloropsis salina*(Hoffmann et al. 2010) and *Cyclotella meneghiniana* (Phytolab Unpublished). Total fatty acids and EPA yield comparison are summarized on table 2.1. In a broad strain review comparing 21 different algae species as potential biofuel feedstocks *Cyclotella cryprica*

Table 2.1 Comparison of Omega-3 producing diatoms total lipids production and omega-3 content used to calculate total omega-3 oil yield per kg biomass.

	Total lipids (% biomass)	Omega-3 content (% lipids)	**Omega-3 yield/ kg biomass**	
Schizochytrium sp.	29.4%	31.5%	**9.26g**	(Sahin et al. 2018)
Phaeodactylum tricornutum	10.7%	27.9%	**2.99g**	(Tongmanitchai et al.1991)
Skeletonema menzeli	17.9%	17.2%	**3.08g**	(Jiang et al. 2016)
Nannochloropsis salina	38%	8.4%	**3.19g**	(Hoffmann et al. 2010)
Cyclotella meneghiniana	43%*	18.2%**	**7.83g**	*(d'Ippolito et al. 2015) **(Phytolab Unpublished)

was identified as one of the most promising taxa with lipids accounting for 42% of biomass and TAG percentage (storage lipids) comprising 55% of total lipids (d'lppolita et al. 2015). These characteristics that Cyclotella appealing for use in the industrial market also translates to high yields in the dietary lipids space.

Cyclotella meneghiniana has been shown to include omega-3 fatty acids in its fatty acid profile (Sicko-Goad et al. 1989, Phytolab Unpublished data). Although *Cyclotella meneghiniana* has a comparatively lower concentration of Omega-3 fatty acids in the lipid bodies than the other currently targeted taxa, the yield of Omega-3 PUFA per kg of biomass of *Cyclotella meneghiniana* (7.83 g/kg) is only 15% lower than the current commercial strain *Schizochytrium* sp.(9.26 g/kg) (ref. table 2). High total lipids and the capacity to produce omega-3 fatty acids, Cyclotella is a promising potential feedstock for the omega-3 lipid nutraceutical market.

Cyclotella Product portfolio

Cyclotella sp. is attractive for commercial cultivation not only because if its high lipid content and capability of producing omega-3 fatty acids, but also because it produces a marketable co-product, chitin (N-acetyl glucosamine). This biopolymer nanofiber extrudes from the frustule pores (Herth and Zugenmaier 1977) and can be marketed as a joint supplement (Ozkan and Rorrer 2017a). Algal sourced chitin is a direct replacement for fish-based chitin feedstocks, making it a complementary product for the Cyclotella portfolio. Although chitin is not economically feasible as a sole product from *Cyclotella* sp. the ability to market a secondary vegan, sustainable, nutraceutical supplement could enhance the business portfolio of a *Cyclotella* photobioreactor plant. Tipping cultures of Cyclotella to either favor

production of oils or chitin can be manipulated by altering growth strategies (Ozkan and Rorrer 2017b), providing the agility to adapt to market demands.

In addition to these two products Cyclotella is also being researched for osteopathic bone therapies using the cleaned glass frustules in a bone paste that acts as a scaffolding to encourage faster bone growth (Walsh et al. 2018), this pharmaceutical application is currently in human research

Table 2.2 Percentage of Cyclotella meneghiniana biomass that contains potentially marketable products with target product type.

Product	Target Market	Composition (% Biomass)	Source
Lipids	Nutraceuticals	42%	(d'lppolitia et al. 2015)
Frustules	Agriculture, Pharmaceuticals	12%	(Sicko-goad et al. 1989)
Chitin	Nutraceuticals	20%	(Xiang et al. 2017)
Marketable		**74%**	

trials and are anticipated to complete in 5-7 years. These three potential products: dietary lipids, chitin filaments, and pharmaceutical treatment components make up approximately 74% of Cyclotella biomass (Table 2.2) making it a promising taxon for the development of a commercial growth strategy. The purpose of this study is to successfully scale up *Cyclotella meneghiniana* cultivation processes in a raceway pond photobioreactor resulting in comparable fatty acid profiles to established laboratory scale processes, specifically reflecting the peak in Omega-3 fatty acids in the early stationary stage of growth.

Methods

Cyclotella meneghiniana strains used in this study originated from the SCSU cell bank. This strain was isolated from local Mississippi river samples via single cell isolation in 2009 and typed by Dr. Matthew Julius. Raceway experiments were completed during the summer semester, 2018. Set-up, data collection, and tear down for the raceway runs were assisted by the Phytoplankton Laboratory staff at St. Cloud State University. The growth strategies that were used involve specific macro nutrient profiles, temperature, light wavelength, light intensity, and agitation that differ from established small-scale growth (20L) protocols. The biomass from the small-scale cultivations were the source of the initial fatty acid profiles collected by the Phytolab team in 2016 and provide the foundation for measuring effectiveness of experimental large-scale protocols. The large-scale processes were designed using a combination of literature research (chapter 1) and lab members personal expertise and experiences. A batch cultivation strategy was adopted (see chapter 1) was used for ease of input and output quantification as well as mitigating high contamination risk stemming from traffic and undergraduate student involvement.

Small-Scale

W.C medium was prepared according to published protocol (REF) in 20L Nalgene polypropylene carboy sterilized in an autoclave on a liquid cycle with a 60-minute sterilization duration. Sterile 20L glass carboys were filled with approximately 15L sterile W.C. Medium and inoculated with live *Cyclotella meneghiniana* culture from the phytoplankton laboratory cell bank. Carboys were fitted with a sterile vented cork fitted with a glass tube used to bubble filtered air through culture from a gas dispersion manifold and flow valves to adjust pressure. These air tubes were used to prevent carbon

limitation and settling. Filled and corked carboys were placed on wire shelving with horizontally mounted cool tone full spectrum grow bulbs on a 16:8 light/dark cycle at 200$\mu Em^{-1}s^{-1}$.

Large-Scale

The commercial scale growth experiments were completed with a Microbio engineering's RW29 model raceway pond with paddle. The raceway was filled with softened well water, Proline f/2 concentrated algae food by Pentair Aquatic Ecosystems, and sodium metasilicate dissolved in sterile water. Custom scaffolding was designed to cover the center portion of the raceway, mounted with 32 Kessil grow lights connected via synchronized controllers. Diatoms prefer the lower wavelength spectrum (Aidar et al. 2003) so a cooler lighting profile was utilized. A 16h:8h day:night cycle was adopted for this growth experiments as *Cyclotella meneghiniana* has shown a preference to long day cycles (Sicko-goad and Andresen 1991). Light intensity was adjusted dependent on culture density to achieve ideal light saturation (Ozkan and Rorrer 2017c) to minimize stress on the cells from over-exposure. Light saturation was measured using a Biospherical instruments QSL-2100 Scalar Irradiance sensor, target irradiance levels were 120-150 at the bottom of the raceway directly beneath the light bank. Temperature and pH were collected using a Neptune Apex controller with both pH and temperature probe mounted on the side of the pond for the entirety of the experiment. All raceway experiments were completed in the SCSU Phytoplankton laboratory algal growth facilities

Inoculation of the raceway pond was performed in a three-step process (figure 2.1). Four small scale carboys were allowed to grow for approximately 5 days until they were in their exponential phase. The densely growing carboys were transferred into a 1000L square tote filled with W.C medium. The top of the tote was modified to accommodate four Kessil brand cool tone

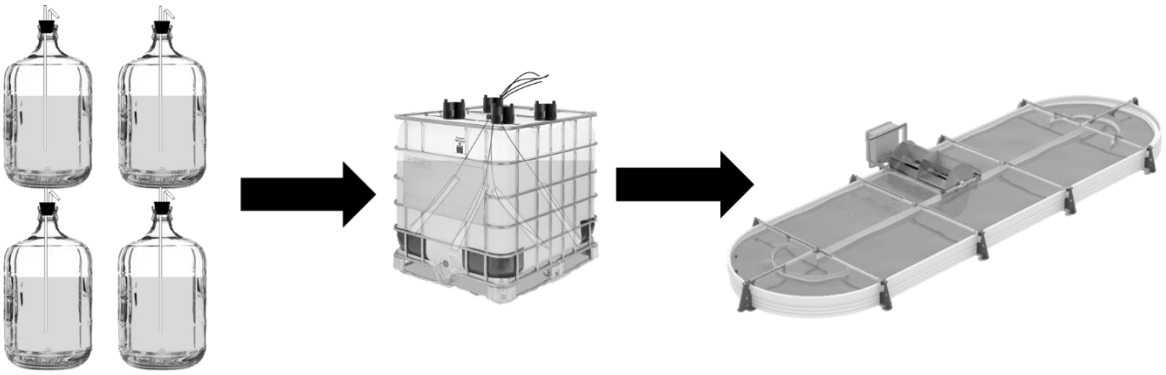

Figure 2.1 The three-step inoculation of a commercial scale raceway includes multiple 20L small scale reactors into an intermediary 1,000L growth tote, that then inoculates the 10,000L raceway.

growth lights and an access port for air lifters. Air lifters were designed using PVC pipe and air compressors fitted with a valve manifold. Tote culture was cultivated until a chlorophyll α level above 30 μg/L was reached, indicating that the population reached exponential growth phase. A single tote was used to inoculate the raceway pond for the pilot growth study. Fill height of the raceway after inoculation was 32 cm, resulting in a final volume of approximately 10,000 liters.

Data Collection

Data for these conclusions originated from two separate experiments. small-scale data were obtained from SCSU phytoplankton laboratory staff, generated in the summer semester of 2016. The Phytolab staff conducted a time wise study comparing fatty acid profiles of multiple omega-3 PUFA producing

algae, they identified the ideal harvest window for maximum omega-3 fatty acid content was 1-3 days after stationary phase begins. large scale data were collected from this set of large-scale raceway experiments conducted during the summer/fall semester 2018.

Cell Dry Weight

Samples for cell dry weights were filtered using a Pall magnetic filter funnel on a side-arm flask attached to house vacuum. Pre dried and weighed Gelman Sciences type A/E glass filters were placed into the apparatus and samples were places on pre-wetted filters; volume of sample used was dependent on call density. Filters with collected biomass were returned to 60˚C drying oven for 24 hours before final weight was recorded. Titer was determined using the change in filter weight, standardized for volume of sample used.

Chlorophyll α

The Chlorophyll α measurements were taken in duplicate using an AquaFluor Handheld Fluorometer by Turner Designs that collects in vivo chlorophyll α estimations using pulse florescence. All samples were collected approximately one meter beyond the paddlewheel 5 cm below the surface with sterile serological pipettes. All timepoints were collected at 24-hour intervals midway through the light cycle to standardize day/night variances.

Oil Extraction

Samples for small-scale fatty acid profiles were collected at exponential phase, first day of stationary phase (early) and Seventh day of stationary phase (late). Samples from large-scale experiment were collected on third day of stationary phase. Samples were dewatered using centrifugation followed by lyophilizing to remove remaining water from pellet. Oil was

extracted from dry biomass using an HCL direct derivatization method (Boulom et al. 2014). Dried biomass was combined with toluene, hydrochloric acid, methanol, pentadecanoic acid (internal standard), and sealed with a nitrogen infused headspace. After heating in water bath for approximately 2 hours, potassium carbonate and toluene were added to the sample and centrifuged. Oil layer was removed and used for fatty acid analysis.

Fatty Acid Profiles

The fatty acid methyl ester (FAME) composition of each sample was obtained with a Agilent 6890N gas chromatograph with a flame ionization detector, equipped with a phenomenex ZB-WAX capillary column. The sample inlet temperature was set to 250°C and the split ratio was 1:50. The internal chamber temperature was set to 70°C for 1 minute, then increased to 200°C with a ramp rate of 35°C/min. Once the chamber reached 200°C, an additional ramp of 5°C/min to 260°C. Peaks were mapped by comparing retention times to a Supelco 37 FAME mix (Sigma), along with a stearidonic acid methyl ester standard (Cayman Chemical). Fatty acids were quantified using the following equation:

$$FA\ content\ \left(\frac{mg}{g}\right) = IS\ added\left(\frac{mg}{sample}\right) x \frac{\frac{Area\ of\ Unknown\ Fame}{Area\ of\ IS\ x\ Response\ Factor\ x\ Derivitisation\ Rate}}{Mass\ of\ Algae\ (g)}$$

This has been modified to include the derivation rate which was calculated by comparing the peak area of pentadecanoic acid (internal standard) against the methyl pentadecanoate (external standard). Response factor was calculated using the areas of the FAME standard peaks and concentration from corresponding data sheet. Total omega-3 fatty acid content was calculated by summing the three different omega-3 PUFA types:

eicosatetraenoic acid (20:4ω3), eicosapentaenoic acid (20:5ω3), and docosahexaenoic acid (22:6ω3).

Results

Chlorophyll α measurements were used to construct growth curves for the large-scale experiments (Figure 2.2). Growth curves were standardized at the beginning of exponential phase, defined by a starting chlorophyll α measurement over 4 μg/L. Growth data were tested for differences using a linear regression analysis of covariance (ANCOVA). The growth curves from the two duplicate raceway experiments had no significant difference (p= 0.38).

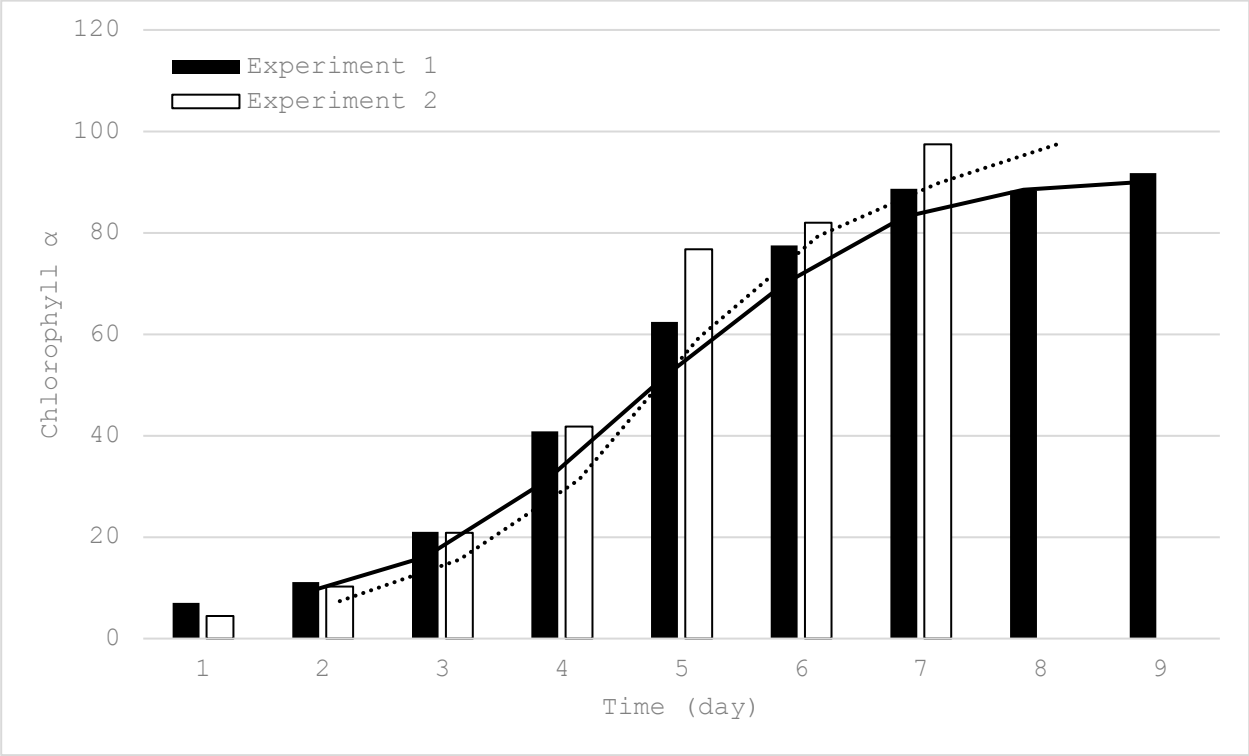

Figure 2.2. Growth curve comparison in duplicate large-scale raceway experiments. Experiment 2 was terminated on day 7 for fatty acid analysis. Linear regression analysis of covariance (ANCOVA) analysis determined no statistically significant difference between the two growth curves (p=0.38).

Small Scale Profiles

Fatty acid profiles obtained from small-scale experiments (Table 2.3) previously obtained from the Phytolab team show that the means of the omega-3 PUFA vary significantly dependent on growth stage (Figure 2.3). These means of the experimental values were tested for significant differences using student's t-test. There was a statistical significance between the omega-3

Table 2.3. Fatty acid profiles from small-scale and large-scale experiments performed in the Phytoplankton laboratory delineated by growth stage: exponential (Exp), early stationary (Early Stat) and late stationary (Late Stat). All values are percentage of total fatty acids.

	Large-scale	Small-scale		
	Early Stat	Exp	Early Stat	Late Stat
14:0	12.9	19.86	20.045	18.585
15:0	0	0.68	0.835	1.02
16:0	30.2	24.3	16.6	23.8
16:1	22.0	24.0	17.3	21.4
16:2	4.1	4.1	3.0	0
16:3	5.0	10.7	13.3	6.8
16:4	2.0	1.8	4.7	3.0
18:0	0	0	0.6	1.2
18:1	0.7	0	1.0	3.2
18:2	2.4	0	0	0.6
18:4	6.4	8.1	4.3	3.1
20:4ω3	0	0	0	0
20:5ω3	12.3	6.4	16.9	14.6
22:6ω3	1.5	0	1.7	2.4

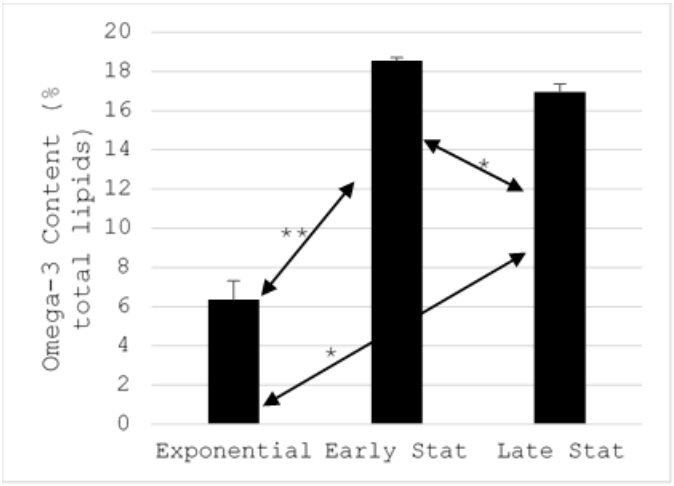

*Figure 2.3 Omega-3 polyunsaturated fatty acid (PUFA) content of small-scale experiments measured in each growth stage: exponential, early stationary (early stat) and late stationary stage (late stat). *significant difference (p<0.05) **very significant difference (p<0.01)*

fatty acid content at the 99% confidence level between the exponential and early stationary growth phase (p=0.008). There was also significant difference between the late stationary phase and exponential as well as early stationary phase at the 95% confidence level, p=0.014 and p=0.02 respectively.

Large-Scale Profiles

Large-scale experiments resulted in one set of duplicate fatty acid profiles collected on third day of stationary phase. These profiles were compared to the small-scale late stationary profiles for rank differences across the fatty acid fractions (Table 2.3). These profiles were tested for significant rank difference using the Wilcoxon signed rank test, no significant difference in fatty acid ranking between the large-scale and small-scale fatty acid profiles was found(p=0.916). The means of the Omega-3 PUFA were compared between the small-scale and large-scale experiments at the early stationary growth stage (Figure 2.4), there was not a significant

difference between small-scale early stationary and the large-scale Omega-3 PUFA fractions at the 95% confidence level (p=0.18).

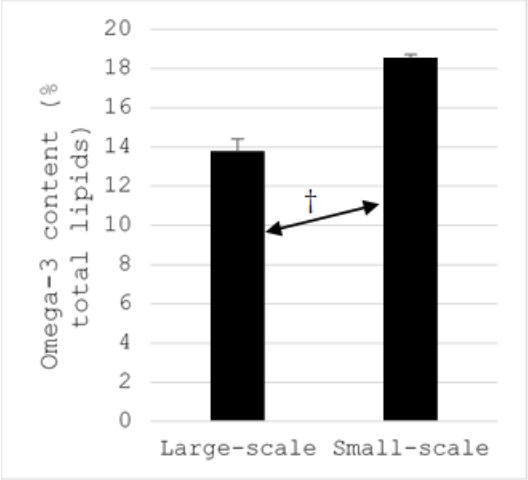

Figure 2.4 Comparison of the mean omega-3 fatty acid content of large-scale and small-scale experiments during the early exponential growth phase. † indicates no statistically significant difference (p>0.05).

Discussion

There were two critical comparisons evaluated in these experiments: consistency across the duplicate large-scale raceway experiments and fatty acid profile consistency between the raceway experiments and the previous small-scale experiments.

Large-Scale Consistency

The first comparison determined the repeatability of the scale-up efforts, verifying that the production strategy was able to be implemented in a repeatable fashion with consistent results. Although there were some individual time points that showed a significant difference with the t-tests, the overall slope of the growth curves was not statistically significant when compared with the ANCOVA analysis of co-variance. These differences of the individual time points may be attributed to a few different factors including temperature, nutrient concentrations, or Illumination variability. The first

large scale experiment was conducted in the spring of 2018, the initial water temperature was 65 Fahrenheit. The second run was completed in summer 2018, the initial water temperature was three degrees higher, and may have caused a decrease in growth. The water temperature of both experiments normalized by the third day of growth. The second possible reason for the discrepancy in max growth could be the nutrient concentration. The nutrient components used in the experiments were measured accurately, however, we were not able to measure the total water volume in the raceway accurately. The volumes are estimates calculated using the height of the water and total surface area of the raceway. If the second raceway experiment had less total volume with the same amount of media components, the yield per liter would predictably be higher. The third potential reason for the variability would be illumination differences, a responsive lighting profile was used to increase the illumination intensity as the biomass concentration got higher. On day three of the second experiment the lighting profiles were manipulated to provide maximum illumination instead of the 20% that was targeted by the lighting strategy. This deviation from the process was in place for 2-6 hours before returning to the correct settings, this difference may have impacted the growth curve data. Even with some individual time point differences in chlorophyll α and titer, the differences in growth curves were not statistically significant indicating that this large-scale growth strategy is consistent and repeatable for cultivation of *Cyclotella meneghiniana*.

Small-scale vs. Large-scale Profiles

The small to large scale fatty acid comparison is to determine if the omega-3 content and oil quality was preserved in the scale-up effort. The fatty acid profiles collected by the SCSU Phytoplankton laboratory team identified a distinctive shift in polyunsaturated fatty acid content

dependent of the growth stage that the culture was in. While the cells were in exponential growth the EPA content was very low, this could be due to the growth and cell division that happens during this growth phase. The fatty acid profile from the early stationary stage show a large shift from the saturated and monounsaturated fatty acids to the long chain polyunsaturated fatty acids (EPA), when the cells reach nutrient depletion, cell division slows, and all excess energy is converted to lipids for storage. When the culture proceeds into late stationary phase, there is a significant drop in EPA concentration, the cell begins using the long chain fatty acids to fuel their metabolic processes in response to the unfavorable environmental conditions and the omega-3 fatty acid concentration begins to fall.

The large-scale experiment was harvested during early stationary phase, so the fatty acid profiles were compared to the early stationary profiles. The Wilcoxon rank sum tests confirmed the quality of the profiles, the two fatty acid profiles were similar in structure and ranking. The mean EPA content of the two experiments were also not significantly different with a 95% confidence interval. Potential sources of variability include: Growth matrix, light quality, and harvest timing. The large scale and small-scale experiments use different growth media. The small-scale media has a more complex macronutrient profile than the large-scale experiments, which could have impacted lipid profiles. The small-scale experiments use full spectrum florescent lighting as opposed to the targeted spectrum Kessil lights used on the large-scale experiments, this difference could have impacted the growth and lipid profiles. The small-scale samples for fatty acid analysis were collected on the first day of stationary period, the large-scale experiment was harvested of the third day of stationary, this likely introduced variability into the fatty acid profile data. The comparison between the

small-scale and large-scale fatty acid profiles and omega-3 content was consistent across experiments, there was no statistically significant differences resulting from the scale-up process.

Conclusion

The results of this study confirm the feasibility of scaling up *Cyclotella meneghiniana* for commercial production of Omega-3 fatty acids. With further process optimization, the product yield and quality from Cyclotella may exceed all other currently researched and grown omega-3 producing taxa. This oil data, in combination with the other two potential co-products (chitin and pharmaceutical additives) makes *Cyclotella meneghiniana* a versatile and marketable taxon for commercial algal cultivation. Algal based omega-3 fatty acid supplement market is growing in response to an increased consumer health-awareness and environmental responsibility. The commercial cultivation of algae satisfies the market demand for pure and sustainable nutraceutical products and *Cyclotella meneghiniana* is a promising addition to the commercial algae product portfolio.

Chapter 3 : Consistency and quality of dietary supplements

Introduction

Fatty acids are vital for the proper functioning of all living things, they make up the membrane around our cells, keeping the contents of the cell protected from the environment while maintaining permeability and nutrient uptake into the cell. The structure of these membranes consists of a double layer of phospholipids that differ dependent on the fatty acid composition. Fatty acids come in two types: saturated and unsaturated. The difference between these two types is the presence or absence of double bonds along the carbon chain. Saturated fatty acids have no double bonds, they are saturated with hydrogen. Saturated fatty acids are completely straight, and pack together tightly making them solid at room temperature. These fatty acids are used in the cell membrane for strength and stability. Unsaturated fatty acids lose hydrogen ions, forcing them to form double bonds along their carbon chain. The double bonds are shorter and less flexible thank single bonds making a kink in the chain, these types of fatty acids do not fit together as tightly, unsaturated fats are generally liquid at room temperature because of this. Unsaturated fats are vital because they make the cell membrane flexible and permeable. Unsaturated fats can be further delineated into two groups, monounsaturated and polyunsaturated fatty acids. Monounsaturated fatty acids (MUFA) have one double bond and have one kink in their chain. Polyunsaturated fatty acids (PUFA) have more than one double bond in each chain, giving them a more twisted or kinky structure. Both saturated and unsaturated fatty acids are needed for a healthy cell membrane.

Essential Fatty Acids

There are two types of polyunsaturated fatty acids (PUFA) have been termed “essential fatty acids” (EFA) because humans (and most animals) cannot

synthesize them, they must be sourced through diet. The two types of EFA's are the Omega-6 and Omega-3 PUFA. The numerical designation on these classes refer to the location of the first double bond along the carbon tail. On an omega-6 fatty acid, there are six single bonds from the end of the tail before the first double bond is found, similarly, there are three single bonds before the first double bond on omega-3 fatty acid chains. Linolenic acid (LA) is the shortest omega-6 fatty acid, it can be found many different common dietary oils like corn, canola, peanut, and soybean. LA can then converted into longer chain omega-6 fatty acids like gamma-linoleic acid (GLA) and Arachidonic (AA) that are vital for cell health. Alpha-linolenic acid (ALA) the smallest omega-3 fatty acid, is found in Chia and flax seed oils. ALA can be metabolized into longer chain omega-3 fatty acids like eicosatetraenoic acid (DPA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These essential fatty acids are integrated into the cellular membrane of the tissues from many difference systems including nervous, immune, cardiovascular, and optical (Surette 2008). These membrane lipids primary role is the uptake and regulation of metabolic pathways, making them a vital component in the function of the cell (McArthur 1999).

Before the agricultural revolution, the human diet consisted of an Omega-6:Omega-3 ratio of around 1 – 2:1, modeled by remote Greenland eskimos (Simopoulos 1999). The modern American diet consists of mostly corn, peanut and canola oil with very low flax, chia, and fish consumption, resulting in an Omega ratio as high as 25:1 (Simopoulus 1991). This over consumption of omega-6 oils and lack of omega-3 oils are suspected to contribute to chronic inflammatory and autoimmune diseases, cardiovascular disease, neurodegenerative diseases and some developmental disorders (Simopoulus 1999). Omega-3 fatty acids have shown to exhibit neuroprotective properties

in the retina and improve overall eye health (SanGiovanni & Chew 2005). Long chain omega-3 fatty acids have been shown to decrease cardiovascular disease and infarction risk (Psota et al. 2006). Omega-3 PUFAs contribute to healthy neurodevelopment in neonatal and adolescents (Kitajka et al. 2004). They have also been shown to mitigate deleterious neurological processes in adults like: stress responses, mood regulation and depression as well as the prevention of dementia and Alzheimer's disease (Bourre 2005). In addition to the optical, neurological and cardiovascular benefits, Omega-3 oils also have anti-inflammatory properties and could assist in the management of chronic inflammation and autoimmune diseases like Chron's disease, Lupus, and rheumatoid arthritis (Simopoulus 2002). Although the human body can convert the short chain EFA's into longer chains, the efficiency of this process is less than 6% conversion to EPA and less that 3.8% to DHA. This process has shown to be prohibited by 40-50% by the presence of excess omega-6 PUFA (Gerster 1998). Direct consumption of longer chain omega fatty acids has become a huge market share of the nutraceutical markets because of this lack of omega-3 fatty acids in the western diet.

Nutraceuticals

Nutraceuticals are naturally sourced alternatives to pharmaceuticals that claim physiological benefits. The global nutraceutical market size by 2025 is projected to be 578 Billion. With the rise in healthcare costs, there is a health movement to find alternatives to pharmaceutical medications. In 2018, the market size of omega-3 supplements was 2.29 Billion, this is expected to increase by 7.4% by 2025 (Grand View Research, 2019) Most of these supplements are derived from Flax oils, fish oils, and algae oils because of their high omega-3 content.

The nutraceutical industry is far less regulated by the FDA than the pharmaceuticals, they are grouped with food products and dietary supplements therefore there has been some concern over the consistency and quality of these minimally regulated nutraceutical supplements. Poor quality and/or dosage inconsistencies were identified in St John's wart (Scotti et al. 2019, Booker et al. 2018), *Ginko biloba* (Booker et al. 2016a), ginger (Trucksess et al 2009), berberine (Funk et al. 2018), and *Rhodiola rosea* (Booker 2016b) nutraceutical supplements. Kleiner et al. found that over 70% of DHA and EPA supplements currently on the market did not contain as much active compound as the label stated (Kleiner et al. 2014). While the quality and contents of DHA and EPA supplements were compared across manufacturers, there have been no investigation into the specific fatty acid profile variability of these supplements across lots and across processes. Data strongly suggests that culture growth phase significantly effects diatom oil composition (chapter 2), an investigation into whether significant variability is currently occurring across manufacturer batches would be prudent.

Commercial Cultivation

In this evaluation two different commercially available algal omega-3 supplements will be evaluated for consistency and compared to *Cyclotella meneghiniana* oil. Three factors that could potentially affect the fatty acid composition and omega-3 content of commercially produced algal oil, include: growth style, bioreactor type, and harvest timing consistency. Two different growth styles will be represented, heterotrophic and autotrophic. Heterotrophic growth have a very high production cost due to large carbohydrate requirements in the growth matrix, but they can also reach much higher density because they are not reliant on light availability, autotrophic cultivation is illumination dependent so when the cell density

gets too high the cells closer to the light shade the rest of the cultivation, limiting growth. Three bioreactor types will be compared in this study: Fermenter, bioreactor, and photobioreactor. Fermenters facilitate heterotrophic growth and are impermeable to light. Bioreactors utilize natural light, and can be season or weather dependent, potentially contributing to product variability. Photobioreactors use artificial light for autotrophic growth which provides weather independent illumination, but wavelength and strength of light are dependent on equipment quality. Both heterotrophic and autotrophic algal growth will go through similar s-curve growth trends (chapter 1), and the fatty acid profiles and omega-3 concentrations will vary dependent on harvest timing. While not specifically evaluated in this study, harvesting timing has shown to have a significant impact on fatty acid profiles, specifically in omega-3 production which is highest at the transition point between exponential and stationary growth stages (Chapter 2). Depending on harvest timing in each respective process, inconsistencies would result in a variable omega-3 content of the algal biomass. The three taxa evaluated were *Schizochytrium* sp, *Nannochloropsis* sp. and *Cyclotella meneghiniana.*

Methods

Algal oil from three different taxa were evaluated, two commercially available omega-3 nutraceutical supplements, *Schizochytrium* sp. and *Nannochloropsis* sp. *Cyclotella meneghiniana* biomass produced by the SCSU Phytolab (Chapter 2) was also included for comparison.

Schizochytrium sp.

Schizochytrium sp. sourced oil was obtained from a commercially available omega-3 nutraceutical dietary supplement. This biomass was produced in fermenters and was grown with a heterotrophic growth strategy. Three

different bottles of this nutraceutical supplement were obtained from local pharmacies, each bottle contained a different lot number. This supplement has an added ingredient "High Oleic Sunflower oil" as a filler. Data was collected in duplicate from each lot, in the form of two separate pills from each bottle.

Nannochloropsis sp.

Nannochloropsis sp. oil was extracted from a commercial omega-3 supplement obtained from local pharmacies. This biomass was grown autotrophically in outdoor raceway ponds and has no added ingredients or fillers. Two bottles with different lot numbers were purchased and two pills from each bottle were evaluated for fatty acid profiles.

Cyclotella meneghiniana

Cyclotella meneghiniana biomass was produced using a raceway photobioreactor and small-scale photobioreactors. Both small-scale and large-scale early stationary fatty acid data was used for analysis, no statistically significant differences were found between these two growth types (chapter 2) and they were used as 2 different lots when compared to commercial equivalents. Oil was extracted from biomass using a HLC direct derivatization method (Chapter 2).

Omega-3 Data

Bottle values for Omega-3 content of each supplement was given in milligrams per serving. contents were removed from pills and weighed resulting mass was used to determine serving size. With this baseline, listed omega-3 percentages were calculated by dividing omega-3 content per serving by serving size, the resulting ratios were used as the literature values for consistency comparisons. Oil was extracted directly from gel capsules of commercial pills using a syringe. Fatty acid profiles were obtained by HPLC,

methods outlined in Chapter 2. Data collected was compared to published omega-3 content located on bottle label and tested for significant differences using a two-way t-test. Consistency between and within bottles were compared using standard deviation.

Results

Schizochytrium *sp.*

The *Schizochytrium* sp. calculated literature value was used as a population mean for a two-way single sample t-test to determine if the mean of measured oil was statistically different than the claimed literature value across 6 samples representing 3 different production lots (Figure 3.1). The average value of total omega-3 content in sampled soft gels were 51.4%, which was not significantly different than the bottle value of 55% (p=0.45). However, the EPA (20:5ω3) content claimed on the bottle was 15% of total

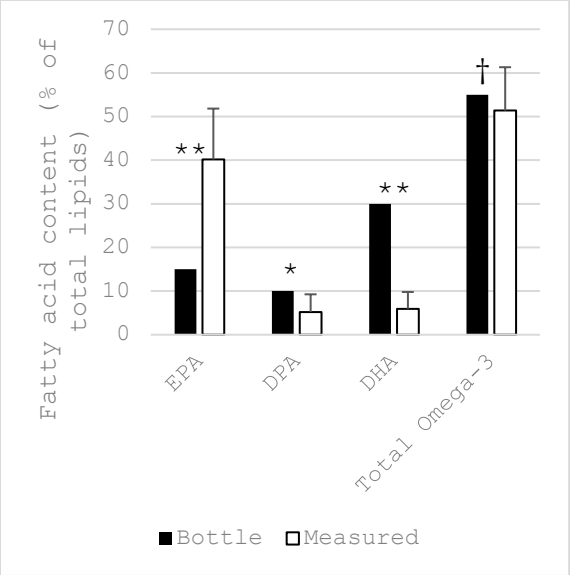

*Figure 3.1 Comparison of Schizochytrium sp. Omega-3 PUFA content between claimed values (bottle) and actual values (measured). *significant difference (p<0.05) **very significant difference (p<0.01) † indicates no statistically significant difference (p>0.05)*

lipids, significantly less than actual measured ratio of 40.2% this difference was statistically significant at a 99% confidence level (p=0.005). In contrast, the DPA (20:4ω3) content which was listed on the bottle as "other omega-3's was measured at 5.2% of lipids, which was less than the bottle's literature value of 10%, this difference was statistically significant at a 95% confidence level (p=0.045). Finally, DHA (22:6ω3) content was claimed by the vendor to be the most prevalent omega-3 PUFA present at 30% of total lipids, compared to the measured value which averaged at 6%, which was statistically significant at a 99% confidence level (p=0.00003). There was a very high variability of the oil samples across lots and within lots ranging from 20% to 80% of mean (Figure 3.1).

Nannochloropsis *sp.*

Calculated literature values were compared to sample values of 6 samples across 2 different production lots. Resulting values and standard deviation of the data is summarized on Figure 3.2. These data follow the same trends as the *Schizochytrium* sp. data in that the average EPA (20:5ω3) content in the samples was 18.3% of total lipids, significantly higher than the claimed value of 16% at a 99% confidence level (p=0.00001). in contrast, the DHA (22:6ω3) concentration measured were 10.7% which was significantly lower than the packaging claim at 12.5% of total lipids, this value was also significant at a 99% confidence level (p=0.0005). Like the previously analyzed strain the total omega-3 fatty acid content of *Nannochloropsis* sp. soft gels was not significantly different than the claimed content at the 95%

confidence level (p=0.05). The standard deviation of the *Nannochloropsis* sp. samples were very small, ranging from 1% to 4.5%.

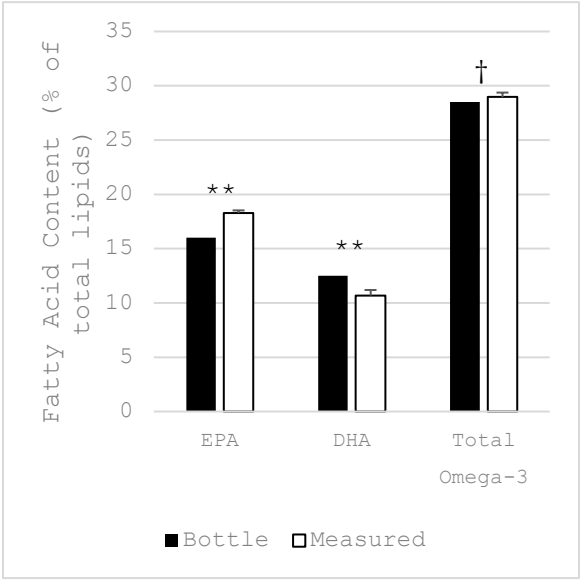

*Figure 3.2 Comparison between Nannochloropsis sp. commercial omega-3 supplement's claimed oil content (bottle) and actual (measured). *significant difference (p<0.05) **very significant difference (p<0.01) † indicates no statistically significant difference (p>0.05)*

Product Comparison

In a comparison across commercial omega-3 producing taxon, there were significant differences between the mean EPA concentrations (Figure 3.3). There were statistically significant differences at the 99% confidence level between Schiz*ochytrium* sp. and both the *Nannochloropsis* sp. and *Cyclotella meneghiniana* (p=0.0012 and p=0.0047 respectively). However, the difference between the mean EPA levels of *Nannochloropsis* sp. and *Cyclotella meneghiniana* were less pronounced but still significant at the 95% confidence level (p=0.0109). The DHA ratios between the three taxon were distributed differently (Figure 3.3), *Nannochloropsis* sp. had the highest mean DHA concentration at 10.7%, there were significant differences between the

Nannochloropsis sp. and the *Schizochytrium* sp. at the 95% confidence level ($p=0.0213$); as well as the *Cyclotella meneghiniana* at the 99% confidence interval ($p<0.0001$). There was no significant difference between the mean DHA content in *Schizochytrium* sp. and *Cyclotella meneghiniana* ($p=0.0770$).

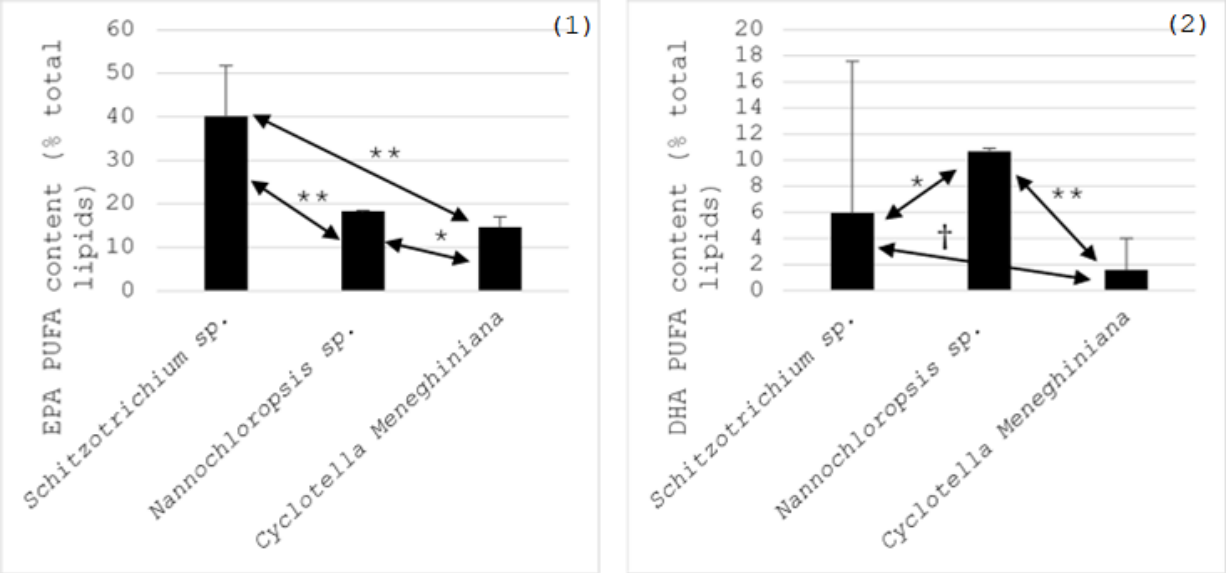

*Figure 3.3 Eicosapentaenoic acid (EPA) and polyunsaturated fatty acid (PUFA) content of different commercial algal oil feedstocks represented in figure (1). Docosahexaenoic acid (DHA) polyunsaturated fatty acid content in figure (2). Error bars represent standard deviation across samples. *significant difference ($p<0.05$) **very significant difference ($p<0.01$) † indicates no statistically significant difference ($p>0.05$)*

Discussion

Schizochytrium *sp.*

Even though the total omega-3 content of the *Schizochytrium* sp. based algal supplement were similar to the claims on the nutrition label, the specific fatty acid claims were very different. This manufacturer claims that the most prevalent type of fatty acids in their oils are DHA, the longest chain omega-3 with the highest degree of unsaturation (30% of oil) but that

was simply not true, the highest concentration of DHA found in these supplements was 11%, the lowest was 0.8%. In contrast these supplements had the highest amount of EPA, the shorter chain omega-3 fatty acid, the lowest concentration of EPA was 23% and the highest was 56% which is a much larger concentration than the bottle's claim of 15%. The combination of large variability and inaccurate labeling on the bottle suggests that the commercial growth and harvest strategy do not provide optimal or consistent results in their heterotrophic cultivation process.

Nannochloropsis *sp.*

Similar to the commercial omega-3 oil produced by *Schizochytrium* sp. biomass, the *Nannochloropsis* sp. oil's total omega-3 content was not significantly different than the concentrations claimed on the label. However, the specific ratios of omega-3 PUFA differ significantly from the claimed values, the EPA was significantly higher than claimed and the DHA was significantly lower. Although the fatty acid ratios are not what the bottle claims, the standard deviation of the tested samples was very low, suggesting a stable process strategy with regular harvest intervals and consistent biomass harvest.

Product Comparisons

The differences between *Cyclotella meneghiniana* and the current omega-3 producing commercial strains are significant. However, Cyclotella has not gone through a product optimization process like *Nannochloropsis* sp. and *Schizochytrium* sp. Table 3.1 outlines the difference in omega-3 production between the published literature values and the current measured values from a commercial production.

Table 3.1 Estimate of current omega-3 biomass yield of optimized commecial strains (Schizochytrium sp. and Nannochloropsis sp.) and potential commercial strain Cyclotella meneghiniana.

	Published Omega-3 content (% lipids)	Measured Omega-3 content (% lipids)	Optimization difference (% Change)	
Schizochytrium sp.	31.5%*	51.4%	**+63%**	*(Sahin et al. 2018)
Nannochloropsis salina	8.4%**	29%	**+345%**	**(Hoffmann et al. 2010)
Cyclotella meneghiniana	18.2%***	16.2%	**-12%**	***(Sicko-goad 1989)

The commercial *Schizochytrium* producers increased omega-3 concentration in the total lipids by 63% from published values and *Nannochloropsis* manufacturers successfully increased omega-3 production by approximately 345%. This type of optimization can be achieved a few different ways, strain selection or modification, process optimization, growth and harvesting strategies.

Conclusion

This market analysis of current commercial omega-3 supplements has shown that while both supplement oils contain the same concentration of omega-3 PUFA that they claim on the label, neither of them were accurate in the fatty acid composition contained in their supplement. In both cases there were more EPA fatty acids than claimed and less DHA fatty acids than the label stated. This discrepancy could be from several factors including growth

and harvest timing and inconsistencies in their processes. There was also a very large amount of variation in the heterotrophically grown *Schizochytrium* sp. suggesting the need for additional process optimization. When these currently commercially produced strains were compared to the potential commercial omega-3 producing strain *Cyclotella meneghiniana*, the differences were pronounced, however, with a concerted strain and process optimization strategy *Cyclotella meneghiniana* could be competitive in the omega-3 nutraceutical market, it can also generate additional revenue streams in the joint supplement and nutraceutical additive markets, which would increase profit margins and decrease waste streams by utilizing more of the algal biomass. This commercial omega-3 supplement research further affirms the conclusion that *Cyclotella meneghiniana* has the potential to perform competitively in the omega-3 nutraceutical market space; solidifying the scale-up potential of this organism for commercial algal cultivation with strategic utilization of strain productivity and versatility.

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Appendix: Start-up Summary for a Pilot Facility

Objectives

- Apply for an agricultural grant for the establishment of a sustainable, female owned agricultural business.
- Rent greenhouse facility for pilot facility.
- Implement cultivation strategy that results in biomass containing at least 50% lipids (w/w), 5% EPA (w/w), and 10% chitin (w/w) with 90% harvest recovery rates.

Mission

To provide high quality sustainable algal biomass for human health and nutrition; grown using environmentally and economically sustainable growth and harvest techniques.

Keys to Success

- Establishing effective cultivation and harvest strategy that demonstrates achievability of harvest yields.
- Leverage local and national agricultural grant programs to establish small scale algal growth facility.

Company Summary

Phycologics inc. is a small, family owned and operated business. We grow local strains of algae to prevent negative environmental impact due to invasive species. We also only use sustainable growth and harvesting techniques to achieve our goal to be carbon neutral and environmentally responsible. Phycologics inc. provides omega-3 enriched oil and glucosamine for use in the nutraceutical market. these products are sourced from pure algal cultures, they are sustainable, vegan, and shellfish free.

Company Ownership

Phycologics inc. is a privately family owned for-profit corporation with the following executive structure.

- CEO: Mary Calva
- CFO: Kristine Holmes
- COO: DeAnna Dvorak

All three officers are owners of this corporation and maintain equal voting rights.

Startup Summary

Three major factors will contribute to the initial start-up financing of $215,000 (table 4.1) for this pilot facility: a strategic location, local and state grant awards, and small business loans. It is our intention to identify and rent a commercial greenhouse facility for the pilot phase of this start-up, this will provide a relatively protected environment for the growth and harvest of the algae. These rented greenhouses will be used for the pilot facility, including: Inoculate production tanks, raceways, and harvesting equipment. All this equipment will be housed in commercial greenhouse structures. There are numerous local and state grants available for sustainable agriculture as well as bioremediation and biochemical innovation. One opportunity of interest is the MN department of agriculture's "AGRI Bioenergy/Biochemical Pilot Project Grant" that provides up to $150,000 to small agricultural startup facilities. In addition to private small business loans, we intend to pursue a MN department of agriculture reduced interest loan program (eg. "beginning farmer loan program"). Utilizing these resources for the startup of the initial pilot plant will enable the completion of a proof of concept effort that can be leveraged to secure

startup financing for Phycologics inc to launch an agricultural commercial scale algal growth facility.

Start-up Financial Requirements

Approximate financial start up requirements for pilot scale algal growth facility are outlined below. Legal expenses include contract reviews and incorporation fees. Insurance includes liability coverage for high risk, health supplement manufacturer up to $150,000 revenue. Rent and utilities was estimated for a 4,500 ft^2 commercial greenhouse facility for 1 year. Long term Assets include, Small scale inoculate tanks, Raceway commercial bioreactors, light banks, harvesting centrifuge and pump. All costs are estimates and will depend upon available financing.

Startup Requirements	
Startup Expenses	
Legal	$5,000
Stationery etc.	$500
Insurance	$7,500
Rent and Utilities	$30,000
Computer	$2,500
Other	$1,000
Total Expenses	*$46,500*
Startup Assets	
Cash Required	$5,000
Chemicals and Nutrients	$1,500
Long-term Assets	$162,000
Total Assets	*$168,500*
Total Requirements	***$215,000***