ASSESSMENT OF THE POTENTIAL OF MINKERY WASTEWATERS FOR THE PRODUCTION OF *DUNALIELLA SALINA* AND β-CAROTENE.

by

Craig MacEachern

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ABSTRACT

Dunaliella salina is a species of green microalgae most prominently known for its ability to accumulate large quantities of β-carotene. As part of this study, mink wastewater was incorporated as a nutrient medium as a means of stimulating growth and reducing costs. Mink wastewater, when compared with standard Bold's Basal Medium saw no significant differences between the nutrient sources in terms of growth (P-value 0.880). This represents a savings of 0.21 CAD per litre of growth medium used at laboratory scale. Following the growth period, a light stress condition of 13 500 lux was introduced using LED bulbs and achieved a maximum β-carotene accumulation of 14.33% of total biomass in lower density cultures. For higher density cultures, maximum β-carotene accumulation would require an increase in illuminance. While *Dunaliella salina* was able to grow photoautotrophically and mixotrophically it demonstrated an inability to grow heterotrophically in mink wastewater.

LIST OF ABBREVIATIONS USED

AD	All Dark
AFDW	Ash free dry weight
AL	All Light
AU	Absorbance Units
BBM	Bold's Basal Medium
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
HPLC	High Performance Liquid Chromatography
Lsmeans	Least squares means
MMC	Multiple Means Comparison
NPP	Normal Probability Plot
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
WF	Water Footprint
WW	Wastewater
24L / 48D	24 hours light followed by 48 hours dark
48L / 24D	48 hours light followed by 24 hours dark

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CHAPTER 1 INTRODUCTION

1.1 Microalgae Basics

Microalgae are a diverse group of typically autotrophic microorganisms comprising in excess of 100,000 unique species (Amaro, Macedo, Malcata 2012). This species diversity is primarily made up of water-born microalgae but also sees a number of land-born species, such as those occurring in snow, sand and rock coverings. Water-born microalgae occur in fresh, brackish and marine water conditions with the species present in each, being highly influenced by water salinity. In the natural environment, microalgae function as primary producers thanks to their photosynthetic nature (Lee 2008). Microalgae are able to utilise the suns energy in combination with carbon dioxide and water to produce organic matter and replicate. As a primary producer, they operate at the lowest level of the food chain and are an essential component for ecosystems throughout the world.

1.2 Challenge of Water Usage in Microalgae Production

Microalgae represent a promising feedstock for a number of bioproduct sectors including biofuels, pharmaceuticals, fertilizers, feeds, cosmetics and dyes. While the products may be of high value, production of microalgae is not without significant challenge and economic barriers when it comes to the issue of microalgae production's water footprint (WF). Considering the water requirements for microalgae based biodiesel production, it has been observed that water usage could be as high as 3,726 kg_{water} $kg_{biodiesel}^{-1}$ (Yang et al. 2011). This is where water recycling measures can be implemented. Water recycling can come in multiple forms, whether it is recycling water following microalgae harvest or implementing water from other waste streams including

agricultural, municipal or industrial sources. Use of wastewaters as opposed to freshwater offers some unique advantages including a reduction in freshwater consumption as well as access to high value nutrient streams at a drastically reduced cost. This is important as nutrient costs make up the largest proportion of total production costs (Bilanovic, Holland, Armon 2012; Christenson and Sims 2011). Many wastewaters can be attained at little to no cost apart from the cost of transportation to the production facility making them an excellent candidate for microalgae production. Agricultural wastewaters seem the most appropriate as they are typically high in the same nutrients microalgae require for optimal growth. The nutrients of greatest importance include nitrogen, phosphorous and carbon. While microalgae can synthesize inorganic carbon dioxide from the air, this is a process, which occurs only under light conditions as part of photosynthesis and is known as photoautotrophic growth. A number of microalgae species have been observed to grow both mixotrophically (Bhatnagar et al. 2011; Ceron Garcia et al. 2006; Ceron Garcıa et al. 2005; Moraisa et al. 2009; Park et al. 2012) and heterotrophically (Agwa, Ibi, Abu 2013; Hsieh and Wu 2009; Jiménez Ruiz et al. 2009; Liu et al. 2011; Liu, Wang, Zhou 2008; Miao and Wu 2006; Xu, Miao, Wu 2006) and in doing so require the presence organic carbon. Heterotrophic growth occurs in compete darkness and substitutes the normal inorganic carbon dioxide used in growth for organic sources found in the nearby environment. Mixotrophic growth utilises a combination of both photoautotrophic and heterotrophic growth in order to grow. Organic carbon sources are readily available in many agricultural wastewaters and there exists the potential for improving total production through the optimization of metabolic pathways. Phosphorous is also a nutrient of particular interest as it is a finite resource. Phosphorous can be found

in mineral deposits however, it cannot be produced synthetically. By using wastewaters in microalgae production, a significant source of phosphorus can be attained without placing further stress on the resource (Lowrey, Brooks, McGinn 2015).

1.3 Mink Farming in Nova Scotia

1.3.1 Value of the Mink Industry

As recently as 2013, fur farming represented the most valuable sector of Nova Scotian agriculture with a capital value in excess of 90 million dollars. Since then, the industry has seen a significant decline in value with the most recent figures indicating a more than 50 percent decline in the value of the industry (Nova Scotia Department of Agriculture 2015). Despite the decline, the mink industry continues to remain at or near the top of the province's priority list. This is largely due to the over 1,200 people directly employed by the industry and the fact that mink production in Nova Scotia accounted for 60 percent of the national production in 2015 (Statistics Canada 2016). Table 1.1 shows the quantity and value of mink pelts produced both nationally and provincially within Nova Scotia.

Table 1.1: Quantity and value of mink pelts produced in Canada and Nova Scotia

Location	Quantity and Value	2011	2012	2013	2014	2015
Canada	Number of pelts	2,714,400	2,804,800	2,771,500	3,384,000	3,321,750
	Value per pelt (CAD)	81.87	90.56	42.88	49.57	29.57
Nova Scotia	Number of pelts	1,451,200	1,493,300	1,399,300	1,980,000	1,995,000
	Value per pelt (CAD)	80.33	85.91	38.57	43.94	27.28

(Statistics Canada 2016)

The data presented in Table 1.1 demonstrates the massive downturn, which the industry has experienced in recent years. In response to the economic downturn, the provincial government remains hopeful that the industry can recover and is actively looking for solutions to the industry's problems while addressing public concern.

1.3.2 Waste Generation and Public Concern

Mink waste handling remains one of the greatest challenges facing the mink industry in Nova Scotia. Due to the high concentration of mink farms in the southwestern portion of Nova Scotia, there simply is not enough land to accommodate spreading all of the mink waste (Nova Scotia Department of Agriculture and Marketing 1993). When spreading is utilised, there remains the evident side effect of nutrient runoff, which can result in eutrophication of nearby waterways. For each mink pelt produced there are 20

kg of manure and 18 L of urine produced. This same volume of manure and urine comprises 1.0 kg of total N and 0.3 kg total P (Impio 1993). Equating these figures to the 1,995,000 pelts produced in Nova Scotia in 2015 returns the figures of 39,900 tonnes of manure, 35,910,000 L of urine, 1,995 tonnes of N and 595.5 tonnes of phosphorous. If spreading alone cannot account for this significant amount of waste then alternate avenues such as microalgae production need to be explored as a means of handling this waste product.

Public concern surrounding the environmental impacts of mink farming remains one of the industry's greatest challenges. In years past, there were little to no regulations pertaining to waste handling for Nova Scotia's mink industry and multiple studies suggested that improper waste handling on mink farms was the most likely cause for eutrophication in surrounding water bodies within the province (Brylinksy 2011; Brylinsky 2012; Brylinsky 2014; Taylor 2009). In response to public outcry and evident environmental degradation, the province created and implemented the Fur Industry Regulations under the Fur Industry Act in 2010 and 2013 respectively (Province of Nova Scotia 2013). This act augmented the emphasis on improved waste handling techniques while increasing accountability for farmers to ensure waste is handled in an environmentally friendly manner. Farmers have been given 3 years to comply with the new regulations beginning in January of 2013 and reports are not yet available on their success. Regardless of the success, there remains the question of what to do with all of the mink wastewater. Using mink wastewater as a nutrient source for the cultivation of microalgae remains one possible avenue to which this waste resource could be directed. As mentioned, optimization of microalgae production requires supplementation of key

nutrients such as nitrogen, phosphorous and carbon and as mink wastewater is high in these same nutrients it is conceivable that microalgae could flourish if cultivated in mink wastewater. Utilising mink wastewater for microalgae production does have some key challenges though. Firstly, the dark coloration of the wastewater restricts the ability for light to penetrate the culture. Light is a critical component of photoautotrophic and mixotrophic growth through photosynthesis. For this reason, dilutions must be performed using clean waters. While some species may be able to utilise seawater other will require freshwater for this dilution. A second challenge is the elevated ammonia concentrations present in mink wastewater. Once again, this can be accounted for through dilution, but it does come at the expense of clean water resources. Thirdly, there is the concern of attempting to produce a food grade product through the use of a waste resource. In producing carotenoids, there is the end assumption that humans will consume this product. Ensuring the product is safe and free of any potential contamination which may come as a by-product of mink wastewater based microalgae production is critical. Finally, due to increasing concern over Aleutian Disease in mink, procurement of mink wastewater has become a slight challenge. Samples attained in this study required the signature of a waiver ensuring that the wastewater not be transported to other fur farms nor should those handling it visit other fur farms without proper sterilization. While these regulations were not a challenge to follow it might make farmers wary when deciding where their waste goes.

1.4 Research Gaps

There are a few critical research gaps, which this project will aim to fill in. The first is that there are no previously published works on the effect of microalgae and in

particular, green microalgae *Dunaliella salina* grown in mink wastewater. This is important due to the unique nutrient profile and dark coloration of the mink wastewater. It is unknown how *Dunaliella salina* will fare in a nutrient medium so drastically different from its natural growth conditions. Due to *Dunaliella salina's* extremophile nature (Oren 2005) however, it is expected that it will be able to adapt to this adverse growth condition.

The second gap is the lack of research pertaining to carotenogenesis in *Dunaliella salina* grown in agricultural wastewaters and in particular mink wastewater. Carotenoids in microalgae are photoinduced pigments, it is unknown what effect the dark coloration or composition of the mink wastewater might have on carotenogenesis. It was hypothesized that dilution of the mink wastewater should be able to account for any issues pertaining to the dark coloration of the raw mink wastewater. Initial tests indicated that a 1% mink wastewater solution absorbed at approximately 0.060 AU at 684 nm when blanked with Bold Basal Medium (BBM). Furthermore, it is unsure whether or not the unique nutrient composition of the mink wastewater will hinder carotenogenesis.

The final gap is the lack of research focusing on varied metabolic pathways in the production of *Dunaliella salina*. *Dunaliella salina* has been shown capable of growing both photoautotrophically as well mixotrophically however, there has not yet been any research on the heterotrophic production of the species in agricultural wastewaters. This is particularly important as agricultural wastewaters contain organic carbon which can stimulate dark cycle growth and it has been shown that some species of microalgae can grow better under these conditions than under conventional photoautotrophic conditions. The effects of high light exposure following a heterotrophic cultivation period have

likewise not been explored and may yield important results in understanding *Dunaliella salina* completely.

1.5 Specific Objectives and Project Importance

As evidenced by their recent implementation of new waste management guidelines in the mink sector, the province is aware of the issue the mink industry poses and the need to solve it. While waste management and wastewater treatment are expensive processes, microalgae production represents a means of cost offsetting through the production of valuable biomass and bioproducts. In looking at it from a phycological perspective, production of microalgae using wastewaters of any sort represents a potentially free nutrient resource that can be exploited. As finding acceptable and cheap nutrient resources can be one of the greatest challenges in large-scale microalgae production, wastewaters could be the solution. As mentioned, due to the abundance of mink agriculture in Nova Scotia, this is a readily available resource.(Miao and Wu 2006)

Through learning from and building upon existing research, this project will aim to provide a novel approach to the production of *Dunaliella salina* using mink wastewater. It also aims to provide a means of cost off setting through the production of valuable carotenoids. More specifically this project will address three key areas.

1. Assess the viability of *Dunaliella salina* to be grown photoautotrophically, heterotrophically and mixotrophically and compare between the techniques.
2. Assess the suitability of mink wastewater as a nutrient source for the cultivation of *Dunaliella salina*. To be deemed suitable, *Dunaliella salina* produced in mink wastewater should grow equally as well as conventional growth mediums.

3. Assess the potential for carotenogenesis in *Dunaliella salina* for cultures grown in mink wastewater. Evaluation of this potential will be based against existing research on a percentage of total biomass basis.

These three areas in combination, will aim to provide some indication of the feasibility of mink wastewater grown microalgae and set the foundation for future research into mink wastewater and microalgae based bioproducts.

CHAPTER 2 LITERATURE REVIEW

2.1 Microalgae Structure

When discussing microalgae there is an important distinction, which must be made between microalgae and cyanobacteria (blue-green algae). Cyanobacteria display many of the same characteristics as microalgae with the major difference that they are prokaryotic as opposed to eukaryotic. This means that cyanobacteria lack defined membrane bound organelles (Helliwell et al. 2016; Lee 2008; Wijffels, Kruse, Hellingwerf 2013). As a result, the term blue-green algae has been largely phased out, in favour of the more appropriate cyanobacteria. For the purposes of this paper, when microalgae are discussed it will not include cyanobacteria in the discussion.

As mentioned, microalgae are eukaryotic microorganisms meaning that they do have membrane bound organelles. The organelles of the microalga cell take a similar form to that of many other plant cells. A diagram of the microalga cell can be observed in Figure 2.1.

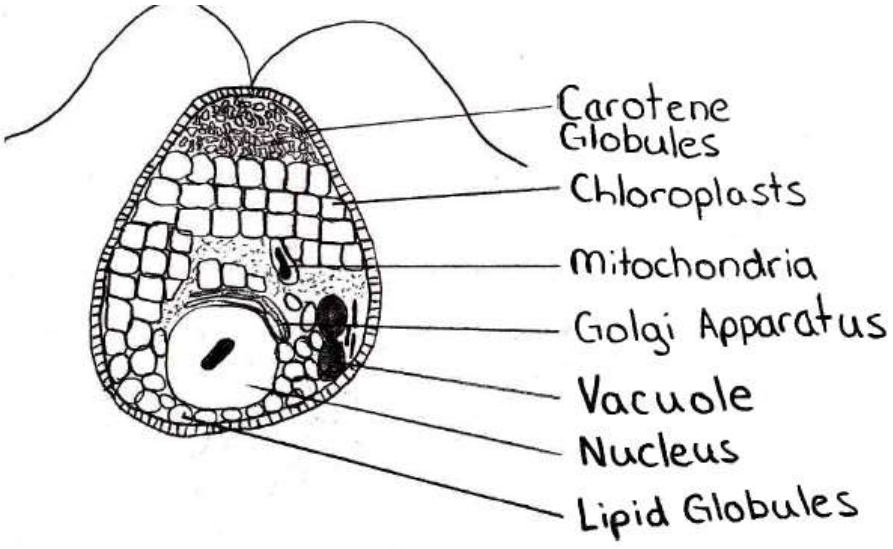

Figure 2.1: Sketch of Dunaliella salina cell

While microalgae are not plants, they do share a number of the same characteristics. Clearly defining microalgae from plants are their distinct lack of roots, stems, leaves, flowers, fruit, nuts, seeds or cones. While plants may only have some of these characteristics, microalgae are devoid of all of them.

Looking into eukaryotic microalgae further, there are three distinct groups into which microalgae can be divided. The first group encompasses those microalgae whose chloroplasts are only surrounded by the two membranes of the chloroplast envelope. This group can further be subdivided to into three categories, which are Glaucophyta, Rhodophyta (red algae) and Chlorophyta (green algae). The second group looks at those microalgae whose chloroplasts are surrounded by one membrane of the chloroplast endoplasmic reticulum. This group includes Euglenophyta (euglenoids), Dinophyta (dinoflagellates) and Apicomplexa. The final group comprises of microalgae whose chloroplasts are surrounded by two membranes of the chloroplast endoplasmic reticulum. This group includes Cryptophyta (cryptophytes), Heterokontophyta (heterokonts), Chrysophyceae (golden brown algae), Synurophyceae, Eustigmatophyceae, Pinguiophyceae, Dictyochophyceae (silicoflagellates), Pelagophyceae, Bolidophyceae, Bacillariophyceae (diatoms), Raphidophyceae (chloromonads), Xanthophyceae (yellow green algae), Phaeothamniophyceae, Phaeophyceae (brown algae) and Prymnesiophyta (haptophytes) (Lee 2008). While it is important to note the differences and variety in microalgae, this paper will solely focus on the Chlorophyta of group one.

2.1.1 Chlorophyta

Chlorophyta or green microalgae are a predominately freshwater microalgae, with around 90 percent of species occurring in such conditions (Smith and Allen 1955).

Freshwater, chlorophyta have a near global, cosmopolitan distribution, in that their range is extended across all appropriate habitats worldwide with significant species distribution (Garbary 2001). Marine Chlorophyta on the other hand are not distributed nearly as completely. While marine species nearer the poles have evolved and diversified, those found near the equator are largely the same across the globe. It is suggested that the warmer waters near the equator have acted as a geographical barrier in the evolution and diversification of species and genera in marine Chlorophyta (Lee 2008).

The Chlorophyta are differentiated from other microalgae based on a few key features. The first is the presence of both chlorophyll *a* and *b* within the chloroplast and the second is the formation of starch by the chloroplast. This is in contrast with other microalgae in that the storage product is formed in the chloroplast as opposed to the cytoplasm (Lee 2008). These two aspects in combination with the lack of endoplasmic reticulum surrounding the chloroplasts help to distinguish the Chlorophyta.

2.2 *Dunaliella salina*

The Chlorophyta genus *Dunaliella* was first identified in 1838 by French botanist Michel Felix Dunal. Dunal located the microalgae in a salt evaporation pond on the Mediterranean coast of France near Montpellier (Dunal 1838; Oren 2005). These ponds were generally not very friendly for life, which made the discovery of *Dunaliella* even more intriguing. Today, we know more specifically, what Dunal described in his discovery to be *Dunaliella salina*, a halophilic species of green microalgae responsible for most of the primary production in hypersaline environments worldwide. *Dunaliella* has since become a model species for the study of salt adaptation in microalgae (Oren 2005). A sketch of the *Dunaliella salina* cell can be observed in Figure 2.1.

2.2.1 Vitamin A, Carotenoids and their Importance

Vitamin A is a broad term that encompasses those compounds that have the biological activity of retinol. This includes not only preformed Vitamin A, such as retinyl esters and retinol, but also provitamin A carotenoids. The most prominent of these naturally occurring provitamin A carotenoids are β-carotene, α-carotene and β-cryptoxanthin which are most commonly found in yellow and orange fruits and vegetables as well as a number of green leafy vegetables (Ross and Harrison 2007). Comparatively, retinol itself is not derived from plants but rather, from animals. Foods such as liver, milk, butter, cheese and eggs are all known to be significant sources of vitamin A (Ball 2008; Ross and Harrison 2007). Liver is of particular note, as it is the part of the body that stores vitamin A derived largely from provitamin A sources. In fact, provitamin A sources are the main way through which most human populations satisfy their vitamin A requirements (Ball 2008).

Of the over 600 known carotenoids (Omaye and Zhang 1998), 50 are known as provitamin A carotenoids, with the most nutritionally important being β-carotene (Ball 1998; Ball 2008). β-carotene is a provitamin A carotenoid whose importance can be attributed to a unique trait; β-carotene contains two molecules of retinol joined at the tail to one another. In comparison, the structures of all other provitamin A carotenoids contain only one molecule of retinol and thus, in theory, only contribute 50% of the potential of β-carotene (Ball 1998; Ball 2008). In the majority of fruits and vegetables, β-carotene makes up more than 85% of the total provitamin A activity. Some exceptions to this include carrots, oranges and sweet corn whose provitamin A activity is largely influenced by α-carotene (carrots and oranges) and β-cryptoxanthin (orange juice and

sweet corn) in addition to β-carotene (Bureau and Bushway 1986; Lee, McCoon, LeBowitz 1981). In human and animal nutrition, β-carotene functions not only as a significant source of vitamin A but also serves as a powerful antioxidant (Omaye and Zhang 1998).

While it is generally not an issue for the more developed regions of the world, vitamin A deficiency remains one of the leading causes of malnutrition in the developing world (Ribeiro, Barreto, Coelho 2011). The first signs of vitamin A deficiency come in the form of night blindness and can escalate to more serious conditions such as keratomalacia, xerophthalmia and even death.

2.2.2 Carotenoid Production in *Dunaliella salina*

Dunaliella salina has garnered considerable attention in recent years, from researchers and consumers alike, as a result of its innate ability to accumulate carotenoids in elevated quantities. *Dunaliella salina* represents one of the richest sources of carotenoids on a per weight basis that can be found in the natural world (Lamers et al. 2008). *Dunaliella salina* is a halophilic species of microalgae, which has also been shown to thrive in elevated light intensities as well. In fact, it is this same ability to thrive in conditions of elevated light, which is primarily responsible for the massive accumulation of carotenoids that can occur in the species (Ben-Amotz and Avron 1983; Hejazi and Wijffels 2003; Lamers et al. 2008; Raja, Hemaiswarya, Rengasamy 2007; Ye, Jiang, Wu 2008). The primary carotenoid produced in *Dunaliella salina* is β-carotene. It is produced within the chloroplasts of the algal cell as a stress response to extended periods of elevated light intensity (Ben-Amotz and Avron 1983; Hejazi and Wijffels 2003; Lamers et al. 2008; Raja, Hemaiswarya, Rengasamy 2007; Ramos et al. 2008). The produced

carotenoids function as light harvesting pigments, which protect the photosynthetic mechanism from photoinduced oxidative damage (Telfer 2002). Carotenoids typically filter excess light in the range of 330 to 500 nm (Lamers et al. 2008). In the absence of these protective carotenoids, the chloroplasts will continue to absorb light even past the point where they are saturated and unable to process all of the absorbed light (Krieger-Liszkay 2004). Carotenoid production helps in quenching these excited chlorophyll states, allowing the cell to continue normal function in environments where other species would simply die off. In the absence of carotenoids, the excited chlorophyll are quenched by molecular oxygen, which results in the deadly by-product of singlet oxygen. This singlet oxygen is highly reactive and can cause pigment bleaching, protein oxidation and lipid peroxidation within the cell. In combination, this will result in the death of the cell (Formaggio, Cinque, Bassi 2001; Krieger-Liszkay, Fufezan, Trebst 2008; Triantaphylides et al. 2008; Triantaphylidès and Havaux 2009).

2.3 Metabolic Pathways in Microalgae Growth

As some of the oldest living organisms on earth, eukaryotic microalgae have evolved to thrive within a multitude of metabolic pathways. It is this ability coupled with their complex genetic makeup, which makes them such a suitable candidate for a variety of biotechnological products. Many microalgae species can easily transition between photoautotrophic (growth under light conditions using inorganic carbon) and heterotrophic growth (growth under dark conditions using organic carbon) and in doing so, cells can develop in a completely different manner (Brooijmans and Siezen 2010; Wijffels, Kruse, Hellingwerf 2013). For instance, by altering the metabolic pathway, one

can accentuate different aspects of development such as lipid, carbohydrate, protein or carotenoid production.

2.3.1 Photoautotrophic Microalgae Production

Photoautotrophic microalgae production is solely reliant on photosynthesis for the generation of microalgae biomass. Photosynthesis a process, which occurs within the algal cell whereby carbon dioxide, water and light are converted into sugars to fuel the cell, as well as the by-product oxygen. As all microalgae species photosynthesize, they can grow in this manner however, optimal growth is generally not achieved from photoautotrophic growth alone. For many species of microalgae, photoautotrophic growth results in lower biomass yields than optimized heterotrophic or mixotrophic (alternating between photoautotrophic and heterotrophic growth) conditions (Ogbonna and Tanaka 1998). Providing microalgae with a CO_2 rich environment can improve photoautotrophic productivity (Chiu et al. 2008; Gordillo et al. 1998) however, this too has its drawbacks. The drawback comes from the fact that light penetration decreases exponentially with culture turbidity. Turbidity in a microalgae culture comes largely from the microalgae cells themselves therefore, as the culture develops and cells reproduce, less light can penetrate the culture resulting in decreasing growth (Markou and Georgakakis 2011); this effect is known as photolimitation (Wang, Yang, Wang 2014). It is the above-described interaction, which is primarily responsible for photoautotrophic's decreased growth when compared with heterotrophic or mixotrophic growth.

Despite the lower potential yields, the ability to utilise readily available light and carbon dioxide as a means of producing valuable biomass is what largely maintains autotrophic growth as the most popular method for large-scale microalgae production

(Chen et al. 2011a). Both open (raceway ponds) and closed (photobioreactors) systems are employed as effective means of production.

While being more cost effective, open pond systems encounter two major hurdles. The first is that of contamination from both aerobic and anaerobic bacteria present in the pond as well as grazers such as protozoa and zooplankton. Bacteria are of concern as they directly compete for the same macronutrients the microalgae require to thrive. Many bacteria can be controlled by raising the pH above 8.3, which is generally still a comfortable range for the majority of microalgae species (Craggs 2005; Park, Craggs, Shilton 2011). Protozoa and zooplankton pose a far greater threat as they consume microalgae and can deplete a culture to near exhaustion in a matter of a few days. Zooplankton have been observed to deplete a culture by 90% in a 48-hour period while *Daphina* has been shown to reduce a culture by 99% in less than 3 days (Cauchie et al. 1995). The second issue of concern with open pond systems for photoautotrophic growth is that of surface exposure. Surface exposure is another major limiting factor in how much light can enter the system and will be a primary contributor to the maximum possible biomass obtainable by the system. When compared with photobioreactors this low surface area to volume ratio results in significantly lower yields (Carvalho and Malcata 2003; Posten 2009; Richmond 2004). It has been estimated the maximum biomass productivity achievable for photoautotrophic growth in an open pond system is 86.7 tons ha^{-1} yr^{-1}, which is far less than the laboratory predicted 130 tons ha^{-1} yr^{-1} outlined by Sheehan et al. 1998 (Chisti 2013; Sheehan et al. 1998; Wang, Yang, Wang 2014). Photobioreactors can aid in combatting these issues by providing optimal growth conditions in a contamination free environment and through improved surface exposure.

2.3.2 Heterotrophic Microalgae Production

Heterotrophic production of microalgae implies that microalgae grow and reproduce in the complete absence of light. To do this they utilise organic carbon found in their immediate environment as a substitute for inorganic carbon dioxide. Heterotrophic production is not reliant on light penetration into the culture and is therefore not affected by photolimitation. This results in theoretically and practically higher biomass productivities for algae cultures grown heterotrophically (Chojnacka and Noworyta 2004; Liang, Sarkany, Cui 2009; Liu et al. 2011; Miao and Wu 2004; Ogbonna and Tanaka 1998; Yu, Jia, Dai 2009). While sugar and organic, acid-based carbon sources are recognized as effective carbon sources (Liang, Sarkany, Cui 2009), municipal and agricultural wastewaters represent cheaper alternatives as carbon sources for heterotrophic growth (Devi, Subhash, Mohan 2012; Perez-Garcia et al. 2010; Perez-Garcia, Bashan, Esther Puente 2011; Zhou et al. 2011). In the case where a non-wastewater carbon source is used, then the cost of heterotrophic cultivation remains significantly higher than photoautotrophic production (Tabernero, del Valle, Eva M Martín, Galán 2012; Zhang et al. 2013). A further challenge to the utilisation of sugar and organic acid-based carbon sources is their inherent vulnerability to other microorganisms. These microorganisms create competition for the microalgae, hindering growth and potentially outcompeting the microalgae altogether (Chen et al. 2011a). It is for this reason that maintaining an axenic culture is of utmost importance for heterotrophic cultivation.

2.3.3 Mixotrophic Microalgae Cultivation

Mixotrophic cultivation is an anabolic pathway whereby microalgae can take best advantage of the aspects of both photoautotrophic and heterotrophic cultivation. In doing so, microalgae utilise both inorganic and organic carbon sources to stimulate their growth and reproduction (Kang et al. 2004; Martínez et al. 1997). There are two schools of thought when it comes to the combined effects of photoautotrophy and heterotrophy in microalgae production. The first is that the combined effect results in the sum of the photoautotrophic and heterotrophic cultivation methods (Marquez et al. 1993). This thought is less popular however as many researchers agree that there is a synergistic effect which occurs during mixotrophic growth that results in increased biomass production (Acién Fernández, Sevilla, Grima 2013; Vonshak, Cheung, Chen 2000; Wang et al. 2002; Yu, Jia, Dai 2009). The major benefit of mixotrophic growth is that microalgae are able to utilise their preferred growth method in photosynthesis but are not affected in the same way by photolimitation in that they can utilise organic carbon as the culture density increases and less light penetrates the culture. Further to this point, mixotrophy has been observed to enhance growth rates, augment maximum culture densities, reduce growth periods and lessen cellular losses during the dark cycles (Andrade and Costa 2007; Park et al. 2012). Biomass productivity is further enhanced during mixotrophic cultivation as CO_2 is released during respiration, which can be captured and used to enhance photosynthesis (Mata, Martins, Caetano 2010).

Mixotrophic cultivation is often considered as the most cost effective cultivation technique. This is because mixotrophic cultivation results in denser cultures in less time for the same amount of input (Zhang et al. 2013). While there may be some costs

associated with supplementing organic carbon, this can potentially be mitigated through the use of municipal and agricultural wastewaters.

2.4 Use of Photobioreactors

To combat the contamination and low productivity concerns associated with open ponds, modern research has transitioned towards photobioreactors both for research and market production. The most common of these designs include column, tubular and flat plate bioreactors (Amaro, Guedes, Malcata 2011; Brennan and Owende 2010). Photobioreactors offer a great deal of flexibility through their ability to utilise natural or artificial light, enhancing light qualities and altering photoperiods. Raes et al. 2014 found that for *Tetraselmis sp.* in a batch system with an initial cell density of 25 x 10^4 cells mL^{-1} photobioreactors performed far better than raceway pond systems for biomass production. In doing a partial harvest 3 times per week, they achieved maximum cell densities 97 x 10^4 cells mL^{-1} for race raceway ponds and 216 x 10^4 cells mL^{-1} in their tubular photobioreactors. This resulted in maximum productivities of 36 ± 2 mg AFDW L^{-1} day^{-1} for the raceway pond system and 67 ± 5 mg AFDW L^{-1} day^{-1} for the photobioreactors (Raes et al. 2014). A similar study performed by Eustance et al. on *Scenedesmus acutus* found that flat plate bioreactors could achieve biomass productivities up to 22.5 ± 4.5 g m^{-2} day^{-1} while maximum productivities from raceway systems were only 4.1 ± 4.8 g m^{-2} day^{-1}. This initial test was performed between mid-February and mid-March. In a second test performed between early-April and early-May biomass productivities of 21.9 ± 3.4 g m^{-2} day^{-1} and 8.7 ± 2.3 g m^{-2} day^{-1} were achievable for the photobioreactors and raceway ponds respectively.

2.5 Biomass Quantification Techniques

2.5.1 Microscopy

The simplest method for attaining reliable biomass estimations of a microalgae culture is done through microscopy and cell counting. This method employs a specialized counting device known as a hemocytometer. Hemocytometers were originally developed to aid in counting red blood cells however, their function translates well to phycological applications (Aruoja et al. 2009; Berkson, Magath, Hurn 1939; Dragone et al. 2011; Heilmann et al. 2010; Tao, Salihon, Meng 2009). Hemocytometers are often preferred over similar counting devices due to their ease of use and ability to contain a consistent known volume.

In order to estimate the total biomass following the cell count a quick calculation is required. Firstly, an estimate of the species-specific cell biomass is required which, can be estimated using a variety of biomass quantification techniques. Secondly, it is important to note that each large square of the improved Neubauer hemocytometer contains 10^{-4} mL of sample. Once the cell count is performed, one need simply multiply the cell count by the cell mass and divide by the chamber volume. While this method provides a consistent quantification of cell biomass, it relies heavily on the accuracy of other procedures for the determination of species specific cell biomasses. Furthermore, it is slower than spectrophotometry and requires a significant amount of manual input, thereby increasing the chance of human error.

2.5.2 Spectrophotometry

Spectrophotometry represents the quickest and most efficient means of estimating biomass for a microalgae culture. Along with the benefit of being quick, it is also non-

destructive to the cells and can be implemented into a bioreactor system as a means of constant monitoring (Griffiths et al. 2011; Meireles et al. 2002; Sandnes et al. 2006).

A spectrophotometer operates by directing a light of known intensity and wavelength towards a sample contained within a cuvette. As the light makes its way through the sample a percentage of that light is either absorbed or scattered as it comes into contact with microalgae cells suspended in the sample. The light that makes its way through the sample and contacts a detector, which results in the readouts of absorbance and percent transmittance (Kenkel 1988). Absorbance is measured in absorbance units (AU) and is in the majority of cases preferred over percent transmittance. This is because percent transmittance does not vary linearly with concentration, making the development of standard curves impossible, resulting in an inability to compare known values. In comparison, absorbance does vary linearly with concentration and standard curves can easily be developed to determine biomass in more appropriate units such as mg L^{-1}. Mirón et al. 2002 (Mirón et al. 2002), demonstrated that by using dry biomass as well as absorbance, a standard curve of the nature described above could be developed for *Phaeodactylum tricornutum*, a common marine microalgae. Their work determined that for *Phaeodactylum tricornutum*, $C_b = 0.38 * A_{625}$ where, C_b is an estimate of cellular biomass in and A_{625} (mg L^{-1}) is the absorbance (AU) reading at an incident wavelength of 625 nm. It is conceivable that this technique could be translated to any species of microalgae including *Dunaliella salina* so long as an appropriate incident wavelength is selected. If enough growth readings were collected throughout the cultivation period then a similar equation could be developed quite simply.

Despite the efficacy with which a standard curve can be generated, spectrophotometric determination of microalgae biomass is not without its issues. The most apparent of these challenges comes in the form of changing absorbing characteristics. Even if the incident wavelength does not fluctuate over time, the nature of the absorbing species may (American Public Health Association 2005; Nicholls and Dillon 1978). This is because, as microalgae grow, their pigment concentrations change, which can result in drastically different absorbance readings. Microalgae grow in four distinct phases, in order they are: lag, exponential, diminishing growth and stationary. These four phases can be observed in Figure 1.2.

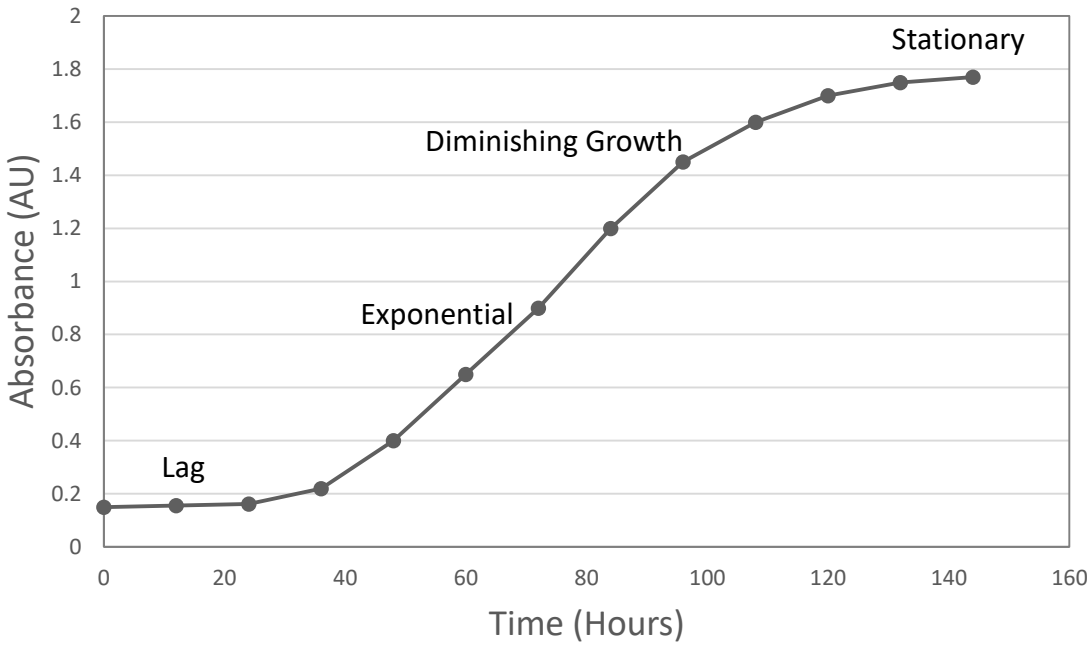

Figure 1.2: Plot of sample data for growth in absorbance in a microalgae culture displaying the four growth phases experienced by the culture. Data was attained by growing Dunaliella salina photoautotrophically in distilled water in 250 mL Erlenmeyer flasks

The lag and exponential phases are the most critical to the success and reproduction of any microalgae culture. Light is a critical element in this success and the concentration of

light harvesting pigments is much higher in these initial phases. As the culture grows, reproduction slows and the cells dedicate more of their available biomass to the accumulation of proteins, carbohydrates and lipids as opposed to photosynthetic pigments. In fact, it has been observed that there can be as much as a 30-fold fluctuation in the concentration of photosynthetic pigments within the alga cell throughout this sigmoidal growth phase (Healy 1975). As a result, the standard practice is to perform measurements within the range of maximum absorbance for chlorophyll in green microalgae. This is typically considered to be the ranges 400-460 nm and 650-690 nm (An et al. 2003; Bopp and Lettieri 2007; Chiu et al. 2008; De Morais and Costa 2007; Hsieh and Wu 2009; Linschitz and Sarkanen 1958; Piorreck, Baasch, Pohl 1984; Ras et al. 2011; Sung et al. 1999; Takagi et al. 2000). From these ranges, one can determine this maximum absorbance for any individual species by performing a series of readings and various incident wavelengths and recording the peak absorbance.

2.5.3 Chlorophyll-*a* analysis

A third useful method for biomass quantification comes in the form of chlorophyll-*a* analysis. Chlorophyll-*a,* is the primary pigment in microalgae and is responsible for the majority of the photosynthetic activity within the cell (Goericke and Montoya 1998). This technique sees its optimal application while the culture is still in its exponential phase. As mentioned earlier, once the culture enters the stationary phase the cells will begin to dedicate more of their biomass towards lipids, carbohydrates and proteins.

Chlorophyll-*a* analysis begins with the extraction of the chlorophyll. To do this, microalgae cells are filtered, ground and macerated. A solution of acetone and

magnesium carbonate is also added to complete the extraction. Once complete the resulting slurry can be centrifuged and left in a cold dark area for 24 hours. As the chlorophyll is highly susceptible to photodegradation it is imperative that the entire process is performed in low light conditions. Once the extraction is complete, the most common method for analysis is through the use of spectrophotometry. Measurements at wavelengths of 750 nm, 664 nm, 647 nm and 630 nm can be taken and the following equations can be used to determine chlorophyll a (C_a), b (C_b) and c (C_c) concentrations (American Public Health Association 2005):

$$\mathrm{Ca} = 11.85(\mathrm{OD664}) - 1.54(\mathrm{OD647} - 0.08(\mathrm{OD630}) \quad \text{(Eq. 1)}$$

$$Cb = 21.03(OD647) - 5.43(OD664) - 2.66(OD630) \quad \text{(Eq. 2)}$$

$$Cc = 24.52(OD630) - 7.60(OD647) - 1.67(OD664) \quad \text{(Eq. 3)}$$

It should be noted that the reading taken at 750 nm is a turbidity correction factor and should be subtracted from the other readings prior to calculation. While this method accounts for chlorophyll *b* and *c* as well, these are minor pigments in the pigment profile for microalgae and as a result do not have a major impact on the biomass calculation. As with the absorbance method, a standard curve can be developed with an established method such as dry biomass for comparing amongst methods. While this is a generally employed and accepted method, there exist variations of this method, which may be used in specific scenarios (Holm-Hansen et al. 1965; Sartory and Grobbelaar 1984; Seely, Duncan, Vidaver 1972; Shoaf and Lium 1976; Simon and Helliwell 1998; Wisconsin State Lab of Hygiene 1991).

2.5.4 Gravimetric Methods (Dry Biomass)

The most reliable method for algae biomass quantification is through gravimetric methods, specifically dry biomass. This reliability comes from the fact that it is the only one of the four proposed methods which directly measures cell biomass and does not rely on a calculation or conversion in its quantification. The gravimetric method employs a number of concentrating, weighing and heat exposure steps in order to determine both the total suspended solids (TSS) as well as volatile suspended solids (VSS) and uses the difference to determine total cellular biomass (American Public Health Association 2005). In calculating the VSS a process known as ashing is employed which, returns the measurement of ash weight. Ashing involves the application of 550^{o}C temperatures in order to volatilize any organics in the sample. The difference between the weights before and after this process can be used to calculate VSS. It should be noted that ash weight is often used interchangeably with dry weight and is not always reflective of its true methodology. Dry weight should be used to describe the weight after the initial heat application (TSS) while ash weight or dry biomass can be used to describe the weight after the second heat application. For marine microalgae species such as *Dunaliella salina* there is the added complication of salt in the growth medium. This salt has a significant effect on dry weight measurements and serves to demonstrate the benefits of ashed weight. The ashed weight measurement is unaffected by salt in the growth medium while dry weight could see a significant error should the salt concentration be high enough. Examples of this terminology being used interchangeably can be observed in a number of published works (Atta et al. 2013; Chevalier and De la Noüe 1985; Yoo et al. 2010).

While gravimetric methods remain the most accurate means of biomass determination, their major drawback is the time that is required to perform the measurement. In comparison to simpler methods such as cell counting or spectrophotometry, the time commitment is significantly longer. For this reason, gravimetric is often performed for exact measurements while those mentioned previously are generally used as monitoring techniques.

2.6 Microalgae growth in Wastewater

While cultivating microalgae in mink wastewater is a new approach to limiting production costs, microalgae growth has seen success through the use of a variety of other wastewater sources. This is important to note as many wastewaters, particularly agricultural wastewaters, share many similar characteristics in regards to coloration and nutrient composition. These sources have also seen success through the integration of all three of the previously discussed metabolic pathways. Table 2.1 shows a variety of microalgae species along with the wastewater sources and metabolic pathways in which they have been successfully cultivated under.

Table 2.1: Microalgae strain, wastewater source and growth condition of cultivation (modified after Khademi et al. 2014)

Strain	Wastewater Source	Metabolic Pathway
Aphanothece microscopica Nageli	Fish processing	Heterotrophic[1]
Chlamydomonas globosa	Poultry	Mixotrophic[2]
Chlamydomonas reinhardtii	Domestic (concentrated)	Photoautotrophic[3]
Chlorella kessleri	Domestic (concentrated)	Mixotrophic[4]
Chlorella minutissima	Poultry	Mixotrophic[2]
Chlorella protothecoides	Domestic (concentrated)	Mixotrophic[4]
Chlorella pyrenoidosa	Swine (diluted)	Mixotrophic[5]
Chlorella pyrenoidosa	Olive Mill	Mixotrophic[6]
Chlorella pyrenoidosa	Cassava fermentation	Photoautotrophic[7]
Chlorella sorokiniana	Synthetic	Heterotrophic[8]
Chlorella sp.	Municipal	Photoautotrophic[9]
Chlorella vulgaris	Synthetic	Photoauto, hetero and mixotrophic[10,11]
Chlorella vulgaris	Brewery	Photoauto, hetero and mixotrophic[12]
Chlorella vulgaris	Dairy digestate	Photoautotrophic[13]
Chlorella vulgaris	Dairy slurry	Heterotrophic[14]
Chlorella vulgaris	Poultry	Heterotrophic[14]
Chlorella vulgaris	Fish (concentrated)	Heterotrophic[14]
Desmodesmus sp.	Raw Municipal	Photoautotrophic[15]
Monoraphidium sp.	Municipal	Photoautotrophic[16]
Scenedesmus bijuga	Poultry	Mixotrophic[2]
Scenedesmus sp.	Fermented Swine Urine	Photoautotrophic[17]
Tetraselmis chuii	Dairy	Heterotrophic[14]
Tetraselmis chuii	Poultry	Heterotrophic[14]
Tetraselmis chuii	Fish	Heterotrophic[14]
Tetraselmis sp.	Dairy	Photoautotrophic[13]

1 (Queiroz et al. 2013), *2* (Bhatnagar et al. 2011), *3* (Kong et al. 2010), *4* (Li et al. 2012), *5* (Wang et al. 2012), *6* (Sánchez et al. 2001), *7* (Yang, Ding, Zhang 2008), *8* (Ogbonna and Tanaka 1998), *9* (Wang et al. 2010), *10* (Feng, Li, Zhang 2011), *11* (Perez-Garcia et al. 2010) *12* (Farooq et al. 2013), *13* (Lowrey 2011), *14* (Lowrey and Yildiz 2013), *15* (Komolafe et al. 2014), *16* (Holbrook et al. 2014), *17* (Kim et al. 2007)

What makes wastewaters such an excellent candidate for microalgae growth is that they are generally high in a number of essential nutrients for optimal microalgae growth. In many cases, these nutrient levels are higher than traditional microalgae culture

mediums, though dilutions can be easily employed when this is the case. In addition to this, trace metals such as manganese, copper, zinc and iron can also be up taken by microalgae during their growth (Cabanelas et al. 2013; Pires et al. 2013). For many industrial and municipal wastewaters, this is a particularly attractive feature, especially if implemented as part of a treatment system. In theory, wastewaters also serve as a significant source of cost offsetting for heterotrophic and mixotrophic cultivation. Liquid waste streams are often high in organic carbon, which would otherwise have to be supplemented at an increased cost to the producer (Khademi et al. 2014). With that being said, some researchers argue that in many wastewaters, organic carbon in high concentrations may become inaccessible and even toxic to microalgae (Perez-Garcia et al. 2010).

2.7 Potential of Mink Wastewater for Microalgae Cultivation

Regardless of whether or not the waste is being handled in an environmentally friendly manner, there remains the question of what to do with the waste. Mink waste is high in a number of important nutrients including ammonia and phosphorous. Table 2.2 shows the crucial nutrient profile for a mink wastewater sample diluted to 1%, attained from The Canadian Centre for Fur Animal Research in Bible Hill, Nova Scotia.

Table 2.2: Nutrient profile of mink wastewater attained from the Canadian Centre for Fur Animal Research in Bible Hill, Nova Scotia

Nutrient	**Concentration (mg L^{-1})**
Ammonia (NH_3)	60
Nitrate (NO_3^-)	12
Phosphorous (Total P)	10
COD	125

As can be seen from Table2.2, values for ammonium, phosphorus and COD are quite high given the size of the sample. This is important, as N, P and COD have been shown to directly influence growth rates in microalgae (Chen et al. 2011b; Huang et al. 2015; Xin et al. 2010b). It should also be noted that *Dunaliella tertiolecta* was shown to be capable of using either ammonium or nitrate as its primary nitrogen source (Chen et al. 2011b). The importance of this is that nitrate levels in mink wastewater are quite low in comparison to ammonia and the indication that *Dunaliella* can grow in ammonium rich and nitrate poor ratios is promising. A further aspect to consider is the dark color of the mink wastewater, which can be seen in Figure 2.2. This dark color will inhibit light penetration giving further benefit to diluting the sample beyond simply lowering the nutrient concentrations.

Figure 2.2: Undiluted mink wastewater attained from the Nova Scotia Fur Animal Research Centre, Bible Hill Nova Scotia

CHAPTER 3 METHODS

3.1 Procedure

3.1.1 Culturing

Microalgae culture *Dunaliella salina* was attained from the Canadian Phycological Culture Centre at the University of Waterloo as a 10 mL frozen sample. Cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of modified Bold's Basal Medium and 5 mL of the initial frozen sample. Cultures were maintained under a consistent photoperiod of 12 hours light followed by 12 hours dark. Cultures were also provided with mechanical agitation in the form of magnetic stirrers operating at 250 rpm and were maintained at room temperature with a pH of 7.0. The flasks were sealed with plugs and manually mixed twice daily. Samples on day 0 and day 7 following inoculation can be observed in Figure 3.1.

Figure 3.1: Inoculation of Dunaliella salina in Bold's Basal Medium of day 0 (left) and day 7 (right)

Once the culture reached an absorbance of 0.500 AU at 684 nm on day 7, it was transferred to a 1 L Erlenmeyer flask containing 700 mL of BBM. An absorbance of .500 AU was selected based on previous work to ensure the best chance of culture survival upon increasing the culture volume. More important than the time it takes to do so is actually reaching this density and for that reason, the time period of seven days used in this study is not critical. Once the single 1 L Erlenmeyer flask reached a culture density of 0.500 AU at 684 nm it was then split into 4, 1 L Erlenmeyer flasks (Figure 3.2) to attain enough volume for the treatments. Table 3.1 shows the nutrient concentrations for stock solutions in modified BBM. One mL of each stock solution was added to 1L of distilled water to produce the modified BBM. All BBM solutions were then autoclaved to ensure sterility.

Table 3.1: Nutrient concentrations for stock solutions in modified Bold's Basal Medium

Nutrient	**Stock Solution Concentration ($g\ L^{-1}$)**
$NaNO_3$	250
KH_2PO_4	175
$MgSO_4 \cdot 7H_2O$	75
K_2HPO_4	75
$CaCl_2 \cdot 2H_2O$	25
NaCl	25
$FeSO_4 \cdot 7H_2O$	9.96
H_3BO_3	8.05
KOH	12.4
H_2SO_4	2
H_3BO_3	2.86
$MnCl_2*4H_2O$	1.81
$ZnSO_4*7H_2O$	0.222
$Na_2MoO_4*2H_2O$	0.39
$CuSO_4*5H_2O$	0.079
$Co(NO_3)2*6H_2O$	0.0494

3.1.2 Experimental Design

Experimental culture inoculation began by starving the dense microalgae cultures used as inoculant, (Figure 3.2) for a week in order to reduce the nutrient levels within the samples and better observe the effect of the treatments. Starvation implies that no additional nutrients were added during this period. In this way, nutrient levels would be as low possible prior to inoculation into one of the treatment conditions.

Figure 3.2: Dense microalgae cultures from which the experimental inoculation material was drawn

100 mL of each dense microalgae culture was combined in a 2 L Erlenmeyer flask and diluted with distilled water to approximately 0.400 AU at a wavelength of 684 nm to ensure consistency across replications. The eight bioreactors were then randomly assigned one of the 8 treatments, one for each of the four photoperiods under each nutrient condition. For the samples grown in BBM, 100 mL of diluted microalgae was combined with 900 mL of BBM within the assigned bioreactor. BBM was used as a control and was not supplemented with organic carbon, in this way no heterotrophic growth was expected. For the samples grown in mink wastewater, 100 mL of diluted microalgae was combined with 890 mL of distilled water and 10 mL of mink wastewater. Cultures were then left to grow for a six-day period under each of the photoperiods. Each treatment was run in three replications.

3.1.3 Statistical Analyses Software

All statistical analyses performed in this study were carried out using SAS 9.4. All ANOVA were carried out at a 95% confidence level ($\alpha = 0.05$) and all MMC were done using Duncan's multiple range test. All statistical plots were generated using Minitab 17 and Excel 2013.

3.1.4 Growth Quantification

Measurements of absorbance were taken spectrophotometrically twice daily at 0630 and 1830 at a wavelength of 684 nm to monitor growth. Dry weight measurements from 50 mL of culture were taken on day 0, 3 and 6 of the growth period. Dry weight measurements were performed using the following method. A 0.6 µm glass fibre filter was placed in a furnace at 550ºC for 15 minutes in order to remove any organic contamination present on the filter. Filters were then weighed (w_1) and placed in a filter apparatus equipped with a vacuum pump. A volume of 50 mL of sample was then pumped through the filter in order to separate the solids from the liquid medium. Filters were then placed in an oven at 105ºC for one hour in order to evaporate any of the remaining liquid medium. Filters were once again weighed (w_2) and then placed back in the furnace at 550ºC for 15 minutes in order to volatilize any organics on the filter. Filters were weighed (w_3) a final time and the TSS and VSS were calculated using the following method:

$$TSS\ \left(\frac{mg}{L}\right) = (((w_2 - w_1)\ x\ 1000)/50)\ x\ 1000 \quad \text{(Eq. 4)}$$

$$VSS\ \left(\frac{mg}{L}\right) = (((w_2 - w_3)\ x\ 1000)/50)\ x\ 1000 \quad \text{(Eq. 5)}$$

While each of the above discussed quantification techniques has its advantages and disadvantages, selection of the best technique remains of vital importance. This is particularly the case when it comes to the time and monetary costs associated with microalgae production. Based on previous work (Ji, Yildiz, MacEachern 2017), it was determined that gravimetric quantification resulted in the most precise measurements while spectrophotometry was the most accurate to that same gravimetric value. While gravimetric methods are expensive and more time consuming, they represent the best method for properly quantifying growth. Spectrophotometry is much quicker and cheaper but does not sacrifice accuracy in doing so. Additionally, spectrophotometry does not return the most useful unit of mg L^{-1} however, when used in combination with gravimetric quantification an equation can easily be developed to convert to mg L^{-1}. For these reasons, the two methods in combination allow for the best pairing of accuracy, precision, cost and time efficiency. In this project, a gravimetric method was used to determine initial and final biomasses only due to its elevated cost and time commitment while spectrophotometry was used to monitor growth in 12-hour periods thanks to its non-invasiveness, simplicity and low cost.

3.1.5 Nutrient Analysis

Filtrate attained from the dry weight analyses filtering process was collected and used to perform the nutrient analysis. Filtrate for each of the samples was collected on day 0 and day 6 in order to determine the change in nitrate, ammonia and phosphorous levels within the culture. The nutrient analyses were performed using a Hanna spectrophotometer (HI 83200 Hanna Instruments) along with the corresponding reagents.

3.1.6 Carotenoid Production and Analysis

After the 6-day growth period was completed and all appropriate measurements and samples had been taken and obtained, 25 mL of sample was transferred to a 100 mm by 15 mm sterile petri dish and placed in the high light intensity chamber (Figure 3.3).

Figure 3.3: High light intensity chamber producing an illuminance of approximately 13,500 lux

Samples were left in the chamber for a further 6-day period in order to observe their carotenoid accumulation. After the six-day period under high light intensity, a carotenoid analysis was performed on the samples.

A standard curve was developed using β-carotene (>95%) standard solution with pure acetone as a solvent. The solution was measured spectrophotometrically at 453nm, the peak range for β-carotene absorbance (Zhu and Jiang 2008). Following the development of the standard curve, a 2 mL sample of day 12 microalgae was collected and centrifuged at 4,000 rpm for 5 minutes. Following centrifugation, the supernate was removed and 2 mL of pure acetone was added to the precipitate. Samples were then

shaken for one minute and left to sit until separated. The samples were then centrifuged at 3,500 rpm for 5 minutes. Separated pigments in the supernate were moved to another tube (Lorenz 2001). The process was repeated a second time to ensure full extraction of pigments. In tests, it was determined that a third extraction was not necessary as it yielded insignificant pigment extraction. The extracted pigments were then analyzed spectrophotometrically at 453nm and referenced against the standard curve.

3.1.7 Proximate Analysis

Proximate analysis was performed as a point of discussion to determine potential areas where *Dunaliella salina* could be directed in addition to carotenoid production. As a maximum carotenoid accumulation of only 10-14% is achievable (Ramos et al. 2011) there remains a large portion of the biomass, which could be directed to other bioproducts. Due to storage limitations, only one replication could be analyzed and for that reason, any presented data is only used as a point of discussion and makes no conclusions or comparisons between treatments.

3.1.7.1 Lipid Analysis

Total lipid analysis was performed using a modified version of the Bligh-Dyer lipid extraction method (Bligh and Dyer 1959). One gram of centrifuged microalgae sample was collected and to that 10 mL of distilled water was added to aid in grinding. The cell suspension was ground using a mortar and pestle for 10 minutes to ensure maximum cell rupturing. From the ground sample, 2 mL were added to a separatory funnel. In addition to the algae, 4.5 mL of a 1:2 solution of chloroform:methanol was also added to the separatory funnel (Figure 3.4).

Figure 3.4: Lipid extraction process showing the separation of the lipids from the algae biomass

The contents were then mixed and excess gasses were released. Following the mixing, 1.25 mL of chloroform was added and the contents were mixed and vented again. Finally, 1.25 mL of distilled water was added and the contents were mixed and vented a final time. The solution was then left to sit in the separatory funnel until separated. Following separation the bottom layer was added to a tared test tube. The samples were then placed in a nitrogen evaporator and left until dry. The nitrogen evaporator blows nitrogen gas over the sample via a needle placed into the test tube while also heating the sample in order to encourage vaporization of liquids in the sample. The dried samples were then weighed and the total lipid percentage was calculated using the following equation:

$$Lipids\ (\%) = \frac{weight\ of\ extracted\ lipids}{weight\ of\ dried\ biomass}\ x\ 100 \qquad \text{(Eq. 6)}$$

3.1.7.2 Protein Analysis

Total protein analysis was performed using a modified version of the Lowry protein assay (Lowry et al. 1951). One gram of centrifuged microalgae sample was

weighed and to that 10 mL of distilled water was added to aid in grinding. The cell suspension was then ground using a mortar and pestle for 10 minutes to ensure maximum cell rupturing. The 2 mL of the ground algae was then combined in a test tube with 2 mL of 2M NaOH. The solution was then vortexed and boiled for 10 minutes. As the solution was allowed to cool, a separation occurred and the top protein containing layer was transferred to another test tube.

Figure 3.5: Protein extraction process showing how boiling the sample induces the separation (left) as well as the extracted protein-containing top layer (right)

From the transferred top layer, 0.5 mL was then combined with 5 mL of complex forming reagent (solution composition available in Table 3.2).

Table 3.2: Composition of complex forming reagent

Complex Forming Reagent
Solution A: 2% (w/v) Na_2CO_3 in distilled water
Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water
Solution C: 2% (w/v) $KNaC_4H_4O_6 \cdot 4H_2O$ (Rochelle salt) in distilled water

**Solutions should be combined in the following proportions (w/v) 100:1:1, A:B:C*

The solution was then vortexed and left for 10 minutes. To the solutions, 0.5 mL of 1M Folin reagent was then added and left for 45 minutes. It should be noted that this reaction period can range from 30 to 60 minutes but should not exceed 60 minutes. Samples were then read spectrophotometrically at 750 nm and referenced against a standard curve developed with bovine serum albumin (BSA standard). Total protein percentage was then calculated using the following equation:

$$Protein\ (\%) = \frac{weight\ of\ extracted\ protein}{weight\ of\ dried\ biomass}\ x\ 100 \qquad \text{(Eq. 7)}$$

3.1.7.3 Ash Analysis

Total ash percentage was calculated by centrifuging 100 mL of microalgae cell suspension and placing the separated microalgae cells into a tared porcelain crucible (w_1). The contents were then left to dry in an oven at 105ºC for 12 hours to ensure the evaporation of all moisture in the sample. The samples were weighed once again (w_2) and placed into a furnace at 550ºC for one hour. The samples were weighed a third time (w3) and the total ash content was calculated using the following equation:

$$Ash\ (\%) = \frac{w_3 - w_1}{w_2 - w_1}\ x\ 10 \qquad \text{(Eq. 8)}$$

3.1.7.4 Carbohydrate Analysis

All biomass, which was not determined to be lipid, protein or ash, was then assumed to be carbohydrate. As a result, total carbohydrate percentage was calculated using the following formula:

$$Carbohydrate\ (\%) = 100 - \%\ Lipid - \%\ Protein - \%\ Ash \quad \text{(Eq. 9)}$$

3.2 Growth Chambers, Conditions and Materials

To accommodate the 6-day growth stage of the study, eight vertical column photobioreactors were constructed. Each photobioreactor consisted of a clear PVC tube with an inner diameter of 7 cm and a total volume of 2.25 L. The columns were capped at each end with cone head piling caps and fitted for an air sparging hose at the bottom. The top cap was also fitted with a hole to allow the escape of excess gasses. The columns were mounted vertically to a backing and were each fitted with a light fixture. The light fixtures were constructed by mounting bi-pin T8 lampholders onto a reflector, which directed the incident light towards the bioreactor tube. Each light fixture was equipped with a T8 ballast and powered using a 120V power source. The fixtures were fitted with a single 18 W, 5000 K, 2 ft fluorescent bulb that was the only source of light for the photobioreactors throughout the growth period. Air sparging was provided at the bottom of the reactor to stimulate photosynthesis and ensure that the culture remained suspended and homogeneous. The deconstructed bioreactors and light sources along with the completed bioreactors can be observed in Figure 3.6 and Figure 3.7 respectively.

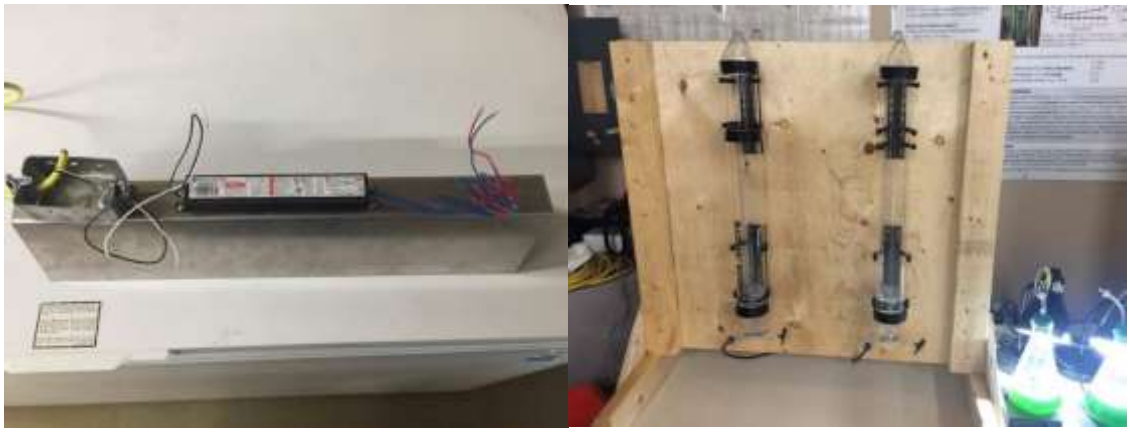

Figure 3.6: Deconstructed light fixture and reflector for photobioreactors (left) and mounted growth columns of the photobioreactors (right)

Figure 3.7: Front and side angles of the completed photobioreactor systems

A high light intensity chamber was also constructed by orienting two, two bulb, four-foot t8 fixtures towards one another with 4 cm of clearance between the bulbs. The fixtures were fitted with four 18 W, 5000 K T8 LED bulbs. Reflectors were placed around the chamber to ensure the highest light intensity possible from the system. Overall the

chamber had an approximate illuminance of 13,500 lux. The chamber with the reflectors removed can be observed in Figure 3.3.

3.3 Statistical Design

3.3.1 Experimental Design and Model

The objective of this study was to determine the effect of two factors across multiple response variables. The first factor (Factor A), type of nutrient, had two levels, which were BBM and mink wastewater. The second factor (Factor B), photoperiod, had four levels, all light, 48 hours light/24 hours dark, 24 hours light/48 hours dark and all dark. The design utilised eight experimental units in the form of the individual photobioreactors. This allowed for the design to be easily split into three batches testing each of the treatment combinations in each batch. This design is known as a 2 by 4 factorial in three blocks with the three batches representing the blocks. This was done to account for any differences, which might be present during the collection of inoculant microalgae. This factorial design can be represented by the following equation:

$$y_{ijk} = \mu + \gamma_i + \alpha_j + \beta_k + \alpha\beta_{jk} + \varepsilon_{ijk} \text{ where } i = 1, 2; j = 1, 2, 3, 4 \text{ and } k = 1, 2, 3 \qquad \text{(Eq. 10)}$$

In this model y_{ijk} represents the response variable of interest, μ is the overall mean, γ_i is the block effect, α_j is the main effect of nutrient type, β_k is the main effect of photoperiod, $\alpha\beta_{jk}$ is the interaction effect between nutrient type and photoperiod and ε_{ijk} is the error term (Montgomery 2013).

3.3.2 Interaction Effect

The major advantage in using the factorial design is that the potential interaction effect ($\alpha\beta_{jk}$) can be observed and handled should it be present. An interaction effect

occurs when the impact of one factor is dependent on the level of another factor (Montgomery 2013). Interaction effects are ignored by one-factor-at-a-time designs and doing so can lead to inaccurate conclusions about the data. Interaction effects are particularly important to consider because, should they be present, inference cannot be made about the main effects but rather only about the interaction.

3.3.3 Hypotheses and Assumptions

The hypotheses for this factorial design are as follows:

H_o: $\alpha_1 = \alpha_2 = 0$ (There is no significant difference between the nutrient sources)
H_a: At least one $\alpha_j \neq 0$ (There is a significant difference between the nutrient sources)

H_o: $\beta_1 = \beta_2 = \beta_3 = \beta_4 = 0$ (There is no significant difference between the any of the photoperiods)
H_a: At least one $\beta_k \neq 0$ (At least one of the photoperiods is significantly different)

H_o: $\alpha\beta_{jk} = 0$ for all j and k (There is no significant higher order interaction effect)
H_a: at least one $\alpha\beta_{jk} \neq 0$ (There is a significant higher order interaction effect)

Prior to performing the ANOVA there were three assumptions which had to be met. The first of these assumptions was normality of the error terms. Normality of error terms was be checked by creating a Normal Probability Plot (NPP) of residuals and performing the pen-test. Since all points along the plot could be covered by a normal sized pen when printed on a half sheet of paper, then the data was said to have a normal distribution. In addition to the pen-test, the Anderson-Darling test was also used to gain an indication of normality. The pen-test however, is always the superior indicator of normality despite its subjectivity (Montgomery 2013). If the data is found to be non-normal then a transformation must be utilised, however this was not required in this experiment as all data was normally distributed. This is crucial, as non-normal data would result in an invalid F-value when performing the ANOVA. The second assumption,

which, had to be met is that of constant variance of error terms. Constant variance was checked by plotting the residuals versus the fitted values and checking for the impression of an even band amongst the plotted points. If the impression of an even band were not present, then a transformation would be required. Once again, this was not required as constant variance was achieved in all data. It should be noted that the consequence of violating constant variance is not as detrimental as violating normality. The final assumption is that of independence. Being that this experiment used proper statistical randomization and blocking is assured that the independence assumption was met.

3.3.4 Statistical Analyses

Once all of the assumptions were met, the first step in the analyses was to perform an ANOVA to observe whether or not there were any significant differences amongst the treatments. As there was found to be significance amongst the main effects but not the interaction effect an appropriate multiple means comparison (MMC) was selected to determine where the significance lies. In this case, the Duncan's Multiple Range Test was used to determine statistical significance. For this study, it should be noted that no significant higher order interactions were present, rendering any comparisons of that nature redundant.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Microalgae Growth

4.1.1 Absorbance

As part of the initial six-day growth period where cultures were grown under low light intensity, varying nutrient sources and each of the four photoperiods, absorbance readings were taken spectrophotometrically at 684 nm every twelve hours. The mean results of these measurements are displayed in Figure4.1.

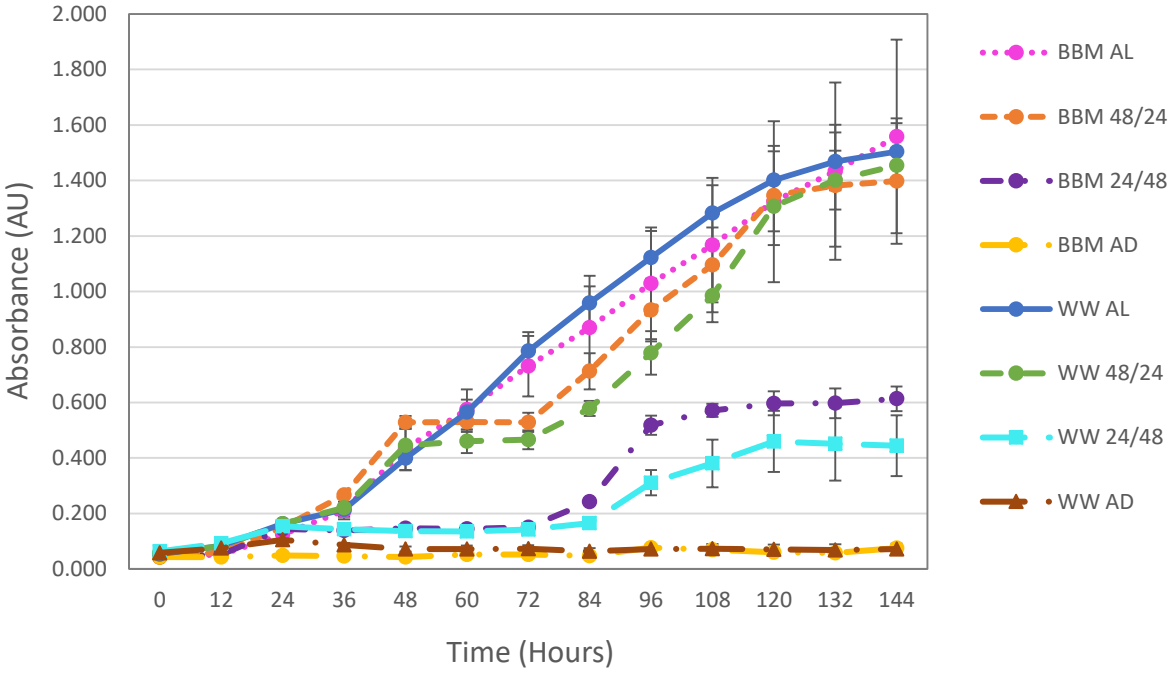

Figure 4.1: Mean absorbance at 684nm by hour for each treatment combination of photoperiod and nutrient source. In the legend, BBM and WW denote Bold's Basal Medium and Mink wastewater, while AL, 48L/24D, 24L/48D and AD denote the four different light/dark cycles along with exposure time for each over the 6-day growth period

In looking at Figure 4.1, there are a few aspects that quickly become apparent. The first of these aspects is the minimal growth, which is observed during the dark cycles of each of the photoperiods that incorporate a dark cycle. This effect can be observed throughout the entirety of the AD photoperiod, between hours 24 - 72 and 96 - 144 for 24L / 48D as

well as between hours 48 - 72 and 120 - 144 for 48L / 24D. During each of these periods there is little to no change in absorbance, which implies that the cultures are not actively reproducing during the dark cycle. This suggests that the cultures did not metabolize heterotrophically by utilising the organic carbon present in the mink wastewater. A potential reason for the lack of heterotrophic growth comes from the findings of Perez-Garcia et al. 2010 (Perez-Garcia et al. 2010) who suggested that organic carbon may become inaccessible to microalgae in large enough concentrations. COD of the 1% diluted mink wastewater was measured at 125 mg L^{-1} which can be considered as very high even by wastewater standards (Environment Canada 2006). A second suggestion comes from Gordillo et al. 1998 (Gordillo et al. 1998) who looked at the effect of light intensity on *Dunaliella viridis*. In this study, it was concluded that the cells grown in darkness remained in a state similar to the lag state of sigmoidal growth. In this way, very little growth is observed during the dark period, similar to what can be seen in Figure 4.1. The study goes on to describe that even though critical nutrients are available in abundance, the cells are unable to enter the exponential phase of growth in the absence of light. *Dunaliella viridis* and *Dunaliella salina* are of the same genus and are structurally quite similar, it is conceivable that they would behave similarly in dark conditions. This result is similar to what is observed in the work of Liang et al. 2009 (Liang, Sarkany, Cui 2009) who found that supplementing glucose in concentrations of 2 and 5 percent (w/v) resulted in optimal mixotrophic growth of *Chlorella vulgaris* however, and increase to 10 percent (w/v) drastically hindered growth. It must also be considered that *Dunaliella salina* may have a preference for certain organic carbon sources and an inability to utilise others, which are present in the mink wastewater. Therefore, despite the high COD, a

lack of dark cycle growth was observed. This sort of preference for certain organic carbon sources has been explored in other microalgae species through the supplementation of glycerol, acetate (Liang, Sarkany, Cui 2009), glucose (Bhatnagar et al. 2011; Garcıa et al. 2005; Liang, Sarkany, Cui 2009) sodium acetate, starch, glycerol, glycine, sodium lactate, glycerol + urea (Garcıa et al. 2005) and sucrose (Bhatnagar et al. 2011). It is most likely that one or more of these reasons led to the insignificant growth observed during the dark cycles of this study. It should also be noted that COD was not monitored throughout the growth period and some assimilation may have occurred that was not reflected in the growth. Any future work on heterotrophic production of *Dunaliella salina* should consider measuring COD throughout its cultivation to rule out any possible heterotrophic growth.

The second aspect that becomes apparent from Figure 4.1 is the vastly superior growth observed from the AL and 48L / 24D photoperiods for both nutrient sources. Table 4.2 and Table 4.3 show the effect of photoperiod on growth for each of the nutrients. Comparisons were made in this manner, as there was no significant higher order interaction present amongst the treatments. This conclusion was drawn from a P-value of 0.9076 for the interaction between the photoperiod and nutrient source. The complete ANOVA table for the interaction effect can be observed in Table 4.1.

Table 4.1: ANOVA to determine whether a significant higher order interaction was present amongst the treatments for absorbance

Source	Degrees of Freedom	F-value	P-value
Nutrient	1	0.14	0.7095
Photoperiod	3	39.56	<0.0001
Nutrient x Photoperiod	3	0.18	0.9076

Table 4.2: Duncan's multiple range test of Day 6 absorbance for the main effect of photoperiod for Bold's Basal Medium

Photoperiod	Mean (AU)
All Light	$1.464 \pm 0.349_a$
48 Light / 24 Dark	$1.361 \pm 0.227_a$
24 Light / 48 Dark	$0.610 \pm 0.044_b$
All Dark	$0.074 \pm 0.009_c$

Means, which do not share a letter in the subscript, are an indication of a statistical difference. Means, which do share a letter in the subscript, indicate a lack of statistical difference.

Table 4.3: Duncan's multiple range test of Day 6 absorbance for the main effect of photoperiod for mink wastewater

Photoperiod	Mean (AU)
All Light	$1.505 \pm 0.102_a$
48 Light / 24 Dark	$1.454 \pm 0.063_a$
24 Light / 48 Dark	$0.444 \pm 0.109_b$
All Dark	$0.072 \pm 0.020_c$

Means, which do not share a letter in the subscript, are an indication of a statistical difference. Means, which do share a letter in the subscript, indicate a lack of statistical difference.

From the MMC it can be concluded that the AL and 48L / 24D photoperiods yielded significantly greater growth than the other two photoperiods for both nutrients. 24L / 48D yielded greater growth than the AD photoperiod for both nutrients. There was no significant difference between the AL and 48L / 24D photoperiod and therefore the recommendation would be to utilise the 48L / 24D photoperiod as a cost and energy saving production method if culture growth is the only aspect of concern. It was also

concluded from the ANOVA that there was no significant difference between the nutrient sources in terms of growth with a P-value of 0.7095. This is a particularly interesting result, as it indicates that mink wastewater shows little difference from BBM in terms of total biomass yield. This represents massive cost saving potential when it comes to nutrient supplementing in the production of *Dunaliella salina.*

4.1.2 Dry Biomass

Dry biomass measurements were taken on day 0, 3 and 6 of the growth period as a means of attaining the optimal precision for the quantification of biomass. Results of these measurements can be observed in Figure 3.

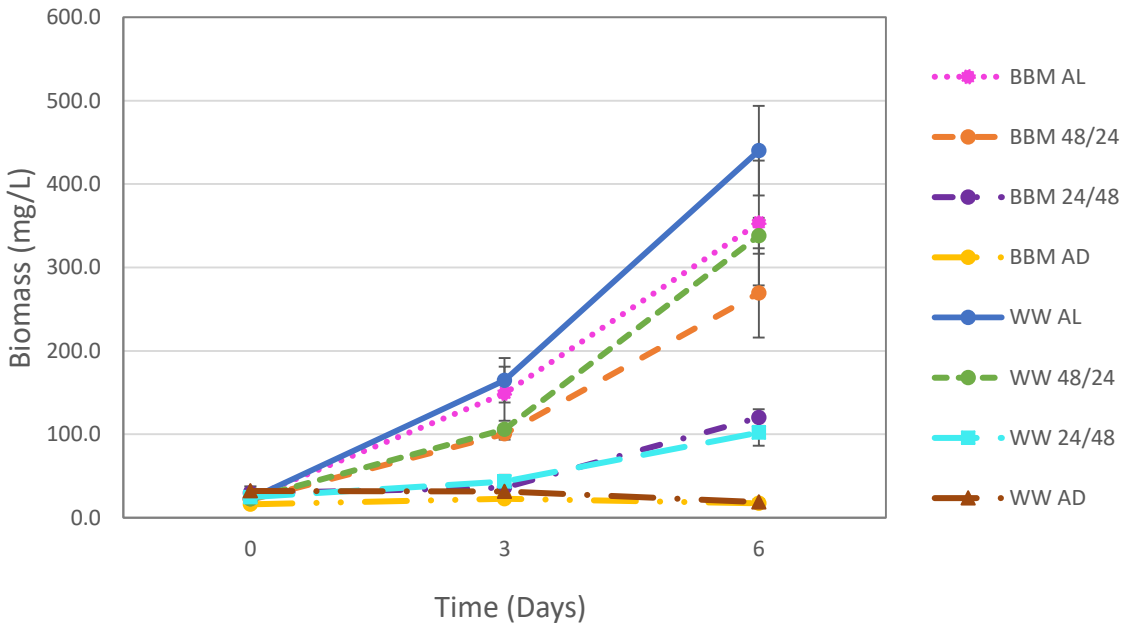

Figure 3: Dry biomass measurements for each combination of photoperiod and nutrient source. In the legend, BBM and WW denote Bold's Basal Medium and Mink wastewater, while AL, 48L/24D, 24L/48D and AD denote the four different light/dark cycles along with exposure time for each over the 6-day growth period

Dry biomass followed a similar trend to that of absorbance and being that the two methods are indications of the same parameter the result is understandable. The Pearson

correlations for each nutrient between the absorbance and dry weight was 0.984 for BBM and 0.971 for mink wastewater. This result confirms what was outlined by Ji et al. 2017 (Ji, Yildiz, MacEachern 2017), who cited the accuracy of the two methods to one another in the quantification of microalgae growth. Despite this strong correlation, there were some differences between the MMC for each of the methods although there was still no significant higher order interaction effect present, which can be observed in the ANOVA seen in Table 4.4.

Table 4.4: ANOVA to determine whether a significant higher order interaction was present amongst the treatments for dry biomass

Source	Degrees of Freedom	F-value	P-value
Nutrient	1	1.58	0.226
Photoperiod	3	39.59	<0.0001
Nutrient*Photoperiod	3	0.85	0.487

Duncan's MMC can be observed in Table 4.5 for cultures grown in BBM and Table 4.6 for cultures frown in mink wastewater under each of the photoperiods.

Table 4.5: Duncan's multiple range test of dry biomass for the main effect of photoperiod for Bold's Basal Medium

Photoperiod	Mean (mg L^{-1})
All Light	$353.33 \pm 74.8_{a}$
48 Light / 24 Dark	$269.33 \pm 53.5_{ab}$
24 Light / 48 Dark	$120 \pm 9.87_{bc}$
All Dark	$17.33 \pm 2.67_{c}$

Means, which do not share a letter in the subscript, are an indication of a statistical difference. Means, which do share a letter in the subscript, indicate a lack of statistical difference.

Table 4.6: Duncan's multiple range test of dry biomass for the main effect of photoperiod for mink wastewater

Photoperiod	Mean (mg L^{-1})
All Light	$440 \pm 53.7_a$
48 Light / 24 Dark	$338 \pm 21.4_b$
24 Light / 48 Dark	$102 \pm 15.5_c$
All Dark	$18.67 \pm 3.53_c$

Means, which do not share a letter in the subscript, are an indication of a statistical difference. Means, which do share a letter in the subscript, indicate a lack of statistical difference.

As with absorbance, there was no significant interaction effect between the photoperiod and the nutrient source, nor was the main effect of nutrient source significant. Therefore, MMC was only performed on the photoperiods within each nutrient source. For BBM it can be concluded that AL and 48L / 24D performed the better than the other two photoperiods although 24L / 48D was not significantly different from 48L / 24D for BBM. This is an interesting result as it does vary slightly from what was concluded in the absorbance analysis. In looking at the analysis of dry biomass for mink wastewater, it was concluded that AL performed the best followed by 48L/24D. There was no significant difference between the 24L/48D and the AD condition in this case.

4.1.3 Growth Conclusions

Taking everything into account it can be stated that *Dunaliella salina* performed far better under mixotrophic growth than under heterotrophic growth. This was evidenced by the minimal change in absorbance during the dark cycles of photoperiods containing dark cycles and the insignificant growth achieved during the AD cycle. Despite the success of a number of green microalgae which have been cultivated heterotrophically (Farooq et al. 2013; Feng, Li, Zhang 2011; Lowrey and Yildiz 2013; Ogbonna, Yoshizawa, Tanaka 2000; Queiroz et al. 2013), results indicate that *Dunaliella salina* is

not among them. This idea is supported by Heredia-Arroyo et al. 2010 (Heredia-Arroyo, Wei, Hu 2010) who stated that *Dunaliella salina* can only grow autotrophically. This statement would also apply for the autotrophic component of mixotrophic growth. A second possibility for the lack of heterotrophic growth is that there is an aspect of the mink wastewater, which inhibits microalgae growth. As previously discussed, this could be a result of the high organic content of the mink wastewater or as a result of another unknown and unaccounted for factor. With that being said, this conclusion would not explain the significant growth observed during periods of light and therefore, the suggestions that *Dunaliella salina* does not grow well heterotrophically seems far more likely especially when a similar trend was observed for BBM which does not have the same elevated organic content.

While research the effect of photoperiod on the growth of *Dunaliella salina* is scarce, comparisons can be drawn between the results of this study and work on other microalgae species. In a similar result to this study, Walidin et al. 2013, (Wahidin, Idris, Shaleh 2013) concluded that *Nannochloropsis sp.* achieved its best growth under continuous light for lower incident light intensities. A light intensity of 50 μmol m^{-2} s^{-1} was utilised to attain this result and is similar to the light provided in this study. As light intensity increased however, there was a reduction in growth under the AL condition in favor of the other photoperiods. This is likely the case of saturating the chlorophyll with too much light, resulting in a negative effect on growth and a reduction in cell density as a result of cell death. This is the same concept used to achieve carotenogenesis in *Dunaliella salina* however; it is generally not employed until cultures have reached an acceptable cell density and not for growth itself. While the Walidin et al. 2013 study did

not explore the effects of 48L/24D it did look at both 18L/6D and 12L/12D, both of which were inferior for growth at lower incident light intensities but increased in effectiveness as this intensity was increased. *Nannochloropsis sp.* represents a suitable comparison for *Dunaliella salina* as it is a marine species with carotenogenesis potential. While *Nannochloropsis sp.* is more known for its accumulation of astaxanthin, zeaxanthin and canthaxanthin (Lubián et al. 2000) its behaviour in producing these carotenoids as a result of stressful environmental conditions is similar to that of *Dunaliella salina*.

Another study by Tang et al. 2011 (Tang et al. 2011) looked at the effect of photoperiod on *Dunaliella tertiolecta.* Being that this is from the same genus as *Dunaliella salina* it makes for an excellent comparison. Similar to what was observed in this study as well as the Walidin et al. 2013 study, it was concluded that for lower light intensities, the AL photoperiod attained the greatest biomass increase among all tested photoperiods. Due to the inherent similarities between *Dunaliella tertiolecta* and *Dunaliella salina* it can be concluded that under these lower light intensity an AL photoperiod will best optimize growth however given the insignificant difference between AL and 48L/24D there is reasonable evidence to suggest that 48L/24D could be used in place as a means of cost and energy reduction. These results make sense in that *Dunaliella salina* is a near equator species and as a result, has adapted to handle greater quantities of incident light.

Each of these results is further supported by the work of Baroli and Melis 1996 (Baroli and Melis 1996) who looked at the effect of irradiance on the growth of *Dunaliella salina.* What they found is that *Dunaliella salina* will see increased growth for

irradiances up to 800 μmol m^{-2} s^{-1}. For irradiances ranging from 800 μmol m^{-2} s^{-1} to 1500 μmol m^{-2} s^{-1} the chloroplasts in *Dunaliella salina* will reach their upper limit in their ability to respond to increasing irradiances and beyond 1500 μmol m^{-2} s^{-1} the rate of cellular replication decreased due to an inability to properly process the incident light. Their work goes on to note that alterations in photoperiod will result in a correlated change in the noted irradiances (Baroli and Melis 1996). This indicated that there is a significant margin to which irradiance could be increased in order to attain even greater growth in *Dunaliella salina* than was attained in this study. Further research would be required to outline the relationship between altering photoperiods and irradiances.

4.2 Nutrient Utilization

4.2.1 Nitrate

Nitrogen is the primary nutrient in microalgae growth and understanding where each species of microalgae best attains their nitrogen from, is an essential component in optimizing growth. Nitrate is one of the primary nitrogen sources for many green microalgae and evaluating its uptake can give indication as how to best optimize species growth. The two nutrient sources used in this study offered drastically different nitrate concentrations with BBM having an average inoculation concentration of 84.36 mg L^{-1} nitrate and mink wastewater having an average inoculation concentration of 10.88 mg L^{-1} nitrate. Table 4.7 demonstrates the 6-day removal for nitrate.

Table 4.7: 6-day mean nitrate removal in Bold's Basal Medium and mink wastewater expressed as percent removed

Photoperiod	Mean Removal in Bold's Basal Medium (%)	Mean Removal in Mink Wastewater (%)
All Light	$61.90 \pm 1.78_a$	$94.70 \pm 4.26_a$
48 Light / 24 Dark	$25.20 \pm 2.02_b$	$96.00 \pm 2.67_a$
24 Light / 48 Dark	$19.30 \pm 6.49_b$	$87.30 \pm 12.7_a$
All Dark	$0.70 \pm 0.53_c$	$84.70 \pm 15.3_a$

Values sharing a letter in the same column shows no statistical significance. Values in the same column, which do not share a letter, shows statistical significance. Values were not compared across columns

Nitrate removal in BBM was observed in three distinct levels with the AL condition showing the greatest removal with an average of 61.9%. There was little significant difference between the 48L / 24D and the 24L / 48D condition with means of 25.2% and 19.3% removed respectively. The AD photoperiod showed the smallest average removal and it is possible that what difference was detected comes down to error more so than actual removal. Mean nitrate removal in mink wastewater showed no significant difference among the photoperiods with means of 94.7%, 96%, 87.3% and 84.7% for each of the AL, 48L / 24D, 24L / 48D and AD photoperiods respectively. No comparison was made between BBM's and mink wastewater's nutrient removals as the initial concentrations and relative composition of the two nutrient sources are drastically different. Mean nitrate utilization rates for BBM and mink wastewater expressed as

concentrations can be observed in Figure 4 and Figure 5.

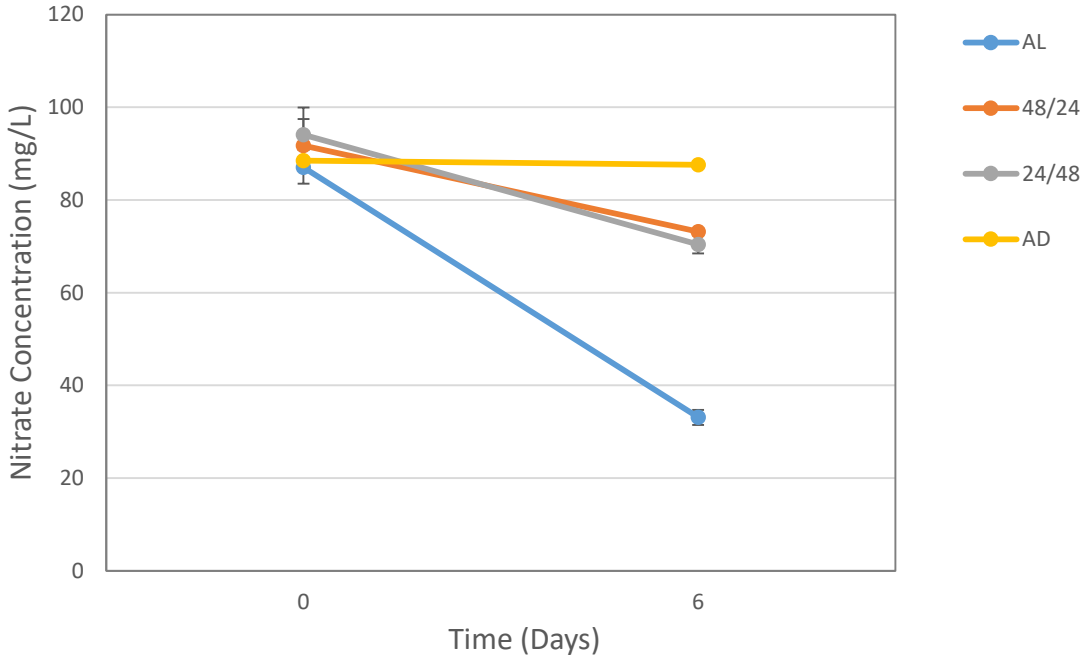

Figure 4: Mean nitrate concentration on day 0 and day 6 for cultures grown in Bold's Basal Medium

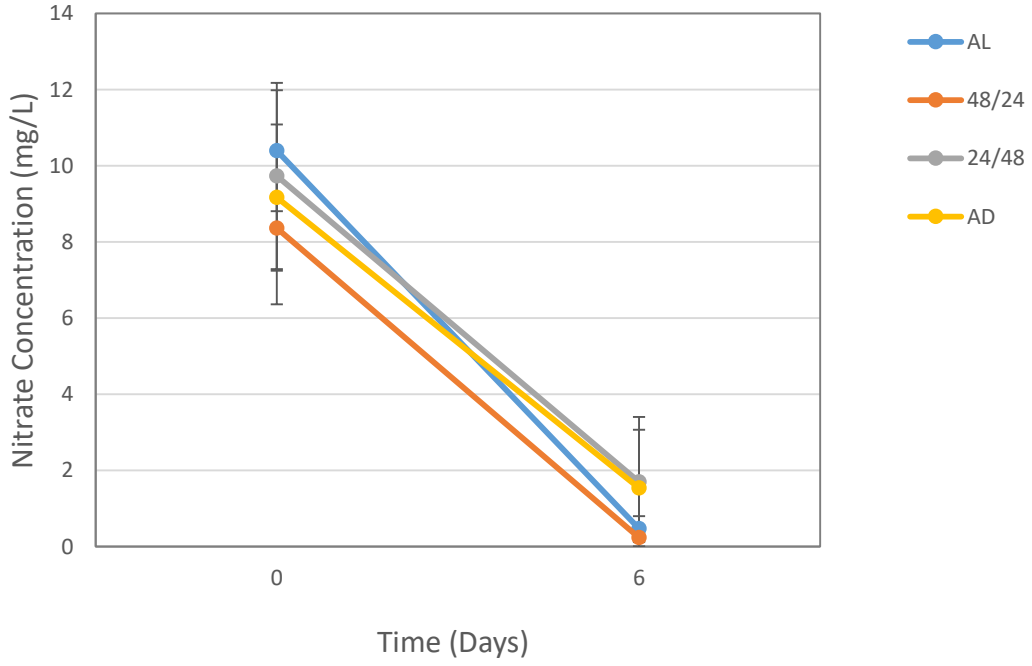

Figure 5: Mean nitrate concentration on day 0 and day 6 for mink wastewater

Inference can however, be made based on photoperiod. For BBM the effect of light has a significant effect on nitrate uptake. This result makes sense since, as microalgae grow and reach higher culture densities, they will inevitably uptake more nitrate. It has already been discussed that greater growth was observed for those photoperiods experiencing light over half of the time. Despite this seemingly important link, nitrate removal and dry biomass were only moderately correlated for BBM with a Pearson correlation of 0.711 making it an inadequate predictor of growth. The Pearson correlation was even lower for nitrate removal and dry biomass in mink wastewater with a value of 0.319 being calculated. This result, while still being moderately correlated may be somewhat misleading due to the 100% removal of nitrate observed in some replications. This is important, as it is unknown when the entirety of the nitrate was removed and if further uptake might have occurred if there were more available. The benefit of this result is that nitrate uptake does not seem to be inhibited by high concentrations of ammonia. This result is in accordance with what was concluded by Dortch 1990 (Dortch 1990). Additionally, it is important to note that a potential method for improving mink wastewater as a nutrient source in microalgae production could be to increase the nitrate concentration through supplementation. Further study coupled with a cost-benefit analysis would need to be performed to see whether the benefit of supplementation would make financial sense. Thakur and Kumar 1999 (Thakur and Kumar 1999) studied nitrate uptake in *Dunaliella salina* and found that nitrate reduction of 45% could be achieved over a 36 hour period for free cells. It should be noted that the Thakur and Kumar 1999 study looked at inoculation concentrations of 180 mg L^{-1}, which is far in excess of the

mean nitrate inoculation concentrations of 87.06 mg L^{-1} and 10.40 mg L^{-1} used in this study for BBM and mink wastewater respectively. While hourly nutrient data was not taken in this study, it would seem that *Dunaliella salina* in the Thakur and Kumar 1999 study took up nitrate at a rate greater than what was observed in this study. This serves to suggest that there could be a number of other factors that could influence nitrate uptake. This could include everything from the cultivation environment to the presence of other nitrogen sources. With that being said there also remains the possibility that nitrate accumulation decreases with time though this hypothesis seems unlikely so long as culture density is increasing as it was in this study.

4.2.2 Ammonia

Ammonia is another common nutrient through which many microalgae attain their nitrogen. As with nitrate, the two different nutrient sources used in this study offered drastically different ammonia concentrations. BBM had a mean inoculation concentration of 1.77 mg L^{-1} while mink wastewater had a mean inoculation concentration of 60.94 mg L^{-1}. 6-day removal for ammonia can be observed in Table 4.8.

Table 4.8: 6-day mean ammonia removal in Bold's Basal Medium and mink wastewater expressed as percent removed

Photoperiod	Mean Removal in Bold's Basal Medium (%)	Mean Removal in Mink Wastewater (%)
All Light	$79.60 \pm 7.88_a$	$95.90 \pm 1.89_a$
48 Light / 24 Dark	$81.50 \pm 6.21_a$	$86.50 \pm 2.05_a$
24 Light / 48 Dark	$71.70 \pm 11.1_a$	$46.60 \pm 7.93_b$
All Dark	$0.80 \pm 0.76_b$	$23.90 \pm 10.4_c$

Values sharing a letter in the same column shows no statistical significance. Values in the same column, which do not share a letter, shows statistical significance. Values were not compared across columns

BBM saw uptake rates greater than 70% for each of the light containing photoperiods. For these photoperiods, there was no significant difference in uptake rates. The AD photoperiod saw what was effectively no ammonia reduction with a mean less than one percent. Once again, what difference was detected could easily come down to experimental error. For mink wastewater, the AL and 48L / 24D photoperiods show the greatest removals with means of 95.9% and 86.5% respectively though these means were not deemed to be significantly different from one another. The 24L / 48D photoperiod was in the middle in terms of uptake with a mean of 46.6% while the AD photoperiod performed the worst having only a 23.9% removal. Ammonia utilization rates for BBM and mink wastewater expressed as concentrations can be observed in Figure 6 and Figure 7.

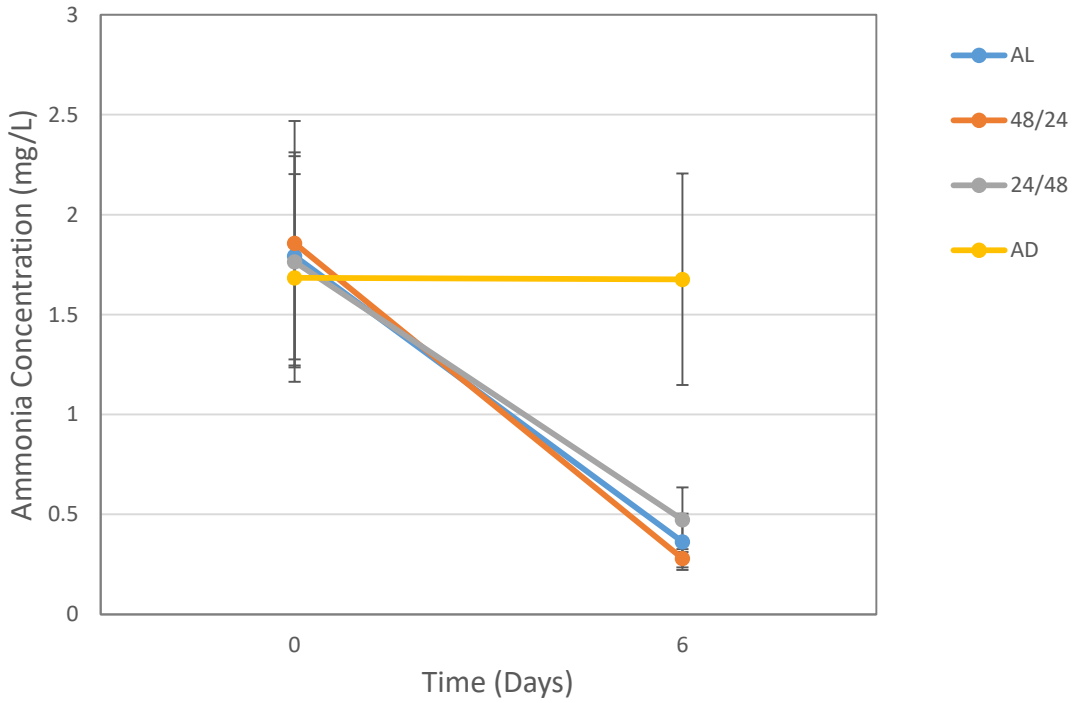

Figure 6: Mean ammonia concentration on day 0 and day 6 for cultures grown in Bold's Basal Medium

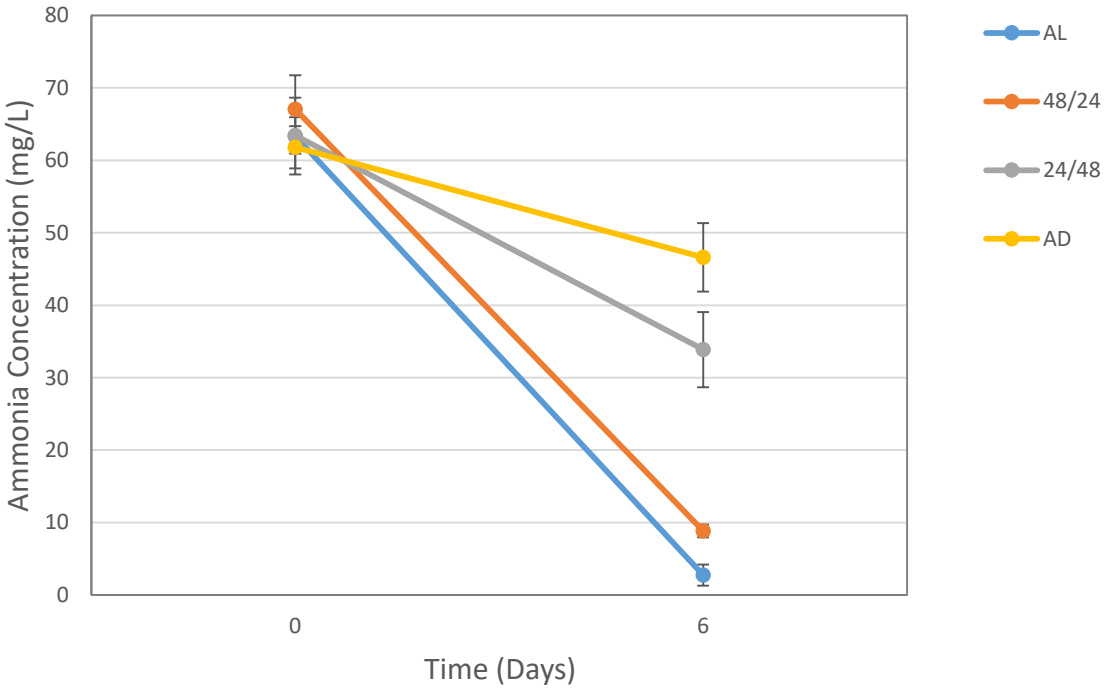

Figure 7: Mean ammonia concentration on day 0 and day 6 for mink wastewater

Removals in excess of 70% were not surprising for light containing photoperiods grown in BBM. If a culture is healthy and growing well and the strain being grown will actively uptake ammonia then it is conceivable that this will occur even when nitrate is readily available. In fact, it has been discussed that the presence of ammonia may even significantly reduce nitrate uptake in many species of algae though this concept has been largely disproven (Dortch 1990) and would not be in line with the results of this study. What is surprising are that the removals observed in BBM are not higher given the drastic difference in total ammonia reduction observed in mink wastewater. In looking simply at the AL photoperiod for both nutrient sources, 79.6% in BBM corresponds to 1.409 mg of ammonia removed while 95.9% in mink wastewater corresponds to 58.441 mg removed on average. This difference clearly shows the ability for *Dunaliella salina* to uptake ammonia and also serves to insist that there is some element of BBM, which is resulting

in reduced ammonia uptake. While it is well established that some species of cyanobacteria have been shown to fix nitrogen (Allen and Arnon 1955; Watanabe, Nishigaki, Konishi 1951), this is not the case for green microalgae. A possible cause for this difference is the link between ammonia uptake and the presence of phosphate established by Santos et al. 2001 (Santos et al. 2001). Their study found that the presence of phosphate in increasing quantities inhibited the uptake of ammonia in *Dunaliella salina*. Given that the average phosphate concentrations used in this study were 144.92 mg L^{-1} and 32.85 mg L^{-1} for BBM and mink wastewater respectively this could be a contributing factor to the lower total uptake observed in the BBM grown cultures.

Looking at the correlation between ammonia removal and dry biomass in BBM yields a Pearson correlation of 0.740, making ammonia removal a moderate but inadequate predictor of growth. In contrast, the correlation between ammonia removal and dry biomass in mink wastewater was 0.931. For this reason, ammonia removal in mink wastewater could be used as an adequate predictor of growth.

4.2.3 Nitrate and Ammonia Reduction Comparison

When discussing the uptake of nitrogen in this study, the term preference is used to describe the degree to which *Dunaliella salina* assimilates nitrate. As microalgae convert nitrate to ammonia for assimilation, ammonia will always be preferred in the true sense. Despite the collected data, making a definitive conclusion on which nitrogen source is preferred by *Dunaliella salina* would be impossible without proper study. In the case where two completely different nutrient sources were studied as in this one, there are simply too many uncontrollable factors and possible interactions to consider in making a justifiable conclusion. As this was not one of the specific objectives for this study, it was

not looked into any further. If one were to design such an experiment, it would need to look at varying the ratios of nitrate and ammonia from high nitrate and no ammonia to no nitrate and high ammonia. In doing so, all other factors would need to be held constant and within optimal ranges for *Dunaliella salina*. In this sort of experiment, it would be possible to determine which ratio of nitrate to ammonia *Dunaliella salina* prefers. What can be discussed is the relationship between nitrogen uptake and growth. As was determined through ANOVA, there was no significant difference between the two nutrient sources in terms of either absorbance or dry biomass. For this reason and by considering the drastically different nitrogen compositions of the two nutrient sources it would seem that *Dunaliella salina* can operate and grow well under high nitrate / low ammonia as well as high ammonia / low nitrate ratios. Further to this, a study by Chen et al. 2011 (Chen et al. 2011b) looked at *Dunaliella tertiolecta* and how growth was affected by altering nitrate and ammonia levels while maintaining all other factors the same. The study concluded that the best growth occurred under high nitrate (≥1,420 mg L^{-1}) and no ammonia. Given the similarities between *Dunaliella salina* and *Dunaliella tertiolecta*, it is conceivable that *Dunaliella salina* may operate similarly. Despite the fact that neither of the nutrient sources had nitrate levels near what optimised growth, the study also concluded that high levels of environmental ammonia (≥17 mg L^{-1}) inhibited cell growth in *Dunaliella tertiolecta*. Mink wastewater used in this study has ammonia concentrations far in excess of this concentration and despite this, showed no significant differences in growth when compared with BBM, which has ammonia concentrations well below 17 mg L^{-1}.

Looking at the total nitrogen removal in both nutrient sources leads to some interesting conclusions. Despite the similar growth between the growth mediums, cultures grown in mink wastewater removed on average 52.04 mg L^{-1}, 49.67 mg L^{-1}, 26.17 mg L^{-1} and 14.14 mg L^{-1} of nitrogen under All light, 48L/24D, 24L/48D and All dark respectively. Comparatively cultures grown in BBM removed on average 13.38 mg L^{-1}, 7.70 mg L^{-1}, 6.34 mg L^{-1} and 0.14 mg L^{-1} of nitrogen under All light, 48L/24D, 24L/48D and All dark respectively. While nitrite was not measured in this study; based on previous work with mink wastewater and BBM (Ji 2017; Liu 2018) it is unlikely that this concentration would be enough to make up this difference. A far more likely explanation for the nitrogen removal discrepancy is the fact that mink wastewater was not autoclaved in this study, in an attempt to further reduce costs. As a result, undetected microbial activity may account for the difference. These microbes may have assimilated nitrogen, resulting in the apparent imbalance of the system. While cultures were monitored daily for large-scale microbial blooms, it is not to say that their presence was entirely absent. Comparatively, BBM was autoclaved as part of its production procedure, effectively eliminating the possibility for microbial activity. It is also unclear whether this hypothesized microbial activity might have hindered the growth of *Dunaliella salina* despite its similar growth to BBM. Further study with autoclaved samples would be required to truly understand the effect; however, this would result in an increase of costs.

4.2.4 Phosphorous

While nitrogen may be the primary nutrient in microalgae growth, phosphorous is the limiting nutrient, in that for the majority of microalgae species, lack of phosphorous will greatly hinder growth (Juneja, Ceballos, Murthy 2013; Larned 1998; Xin et al.

2010a). For phosphorous (expressed as total P), mean inoculation concentrations were 47.33 mg L^{-1} and 10.72 mg L^{-1} for BBM and mink wastewater respectively. 6-day removals for phosphorous can be observed in Table 4.9.

Table 4.9: 6-day mean phosphorous (Total P) removal in Bold's Basal Medium and mink wastewater expressed as percent removed

Photoperiod	Mean Removal in Bold's Basal Medium (%)	Mean Removal in Mink Wastewater (%)
All Light	$7.90 \pm 1.06_{a}$	$8.30 \pm 1.96_{a}$
48 Light / 24 Dark	$6.70 \pm 2.43_{ab}$	$10.80 \pm 0.98_{a}$
24 Light / 48 Dark	$3.40 \pm 1.07_{ab}$	$2.60 \pm 0.37_{b}$
All Dark	$2.00 \pm 1.03_{b}$	$0.30 \pm 0.32_{b}$

Values sharing a letter in the same column shows no statistical significance. Values in the same column, which do not share a letter, shows statistical significance. Values were not compared across columns

Both nutrient sources saw uptake rates of less than 11 percent for all photoperiods. For BBM, the AL, 48L / 24D and the 24L / 48D photoperiods were not significantly different from one another while the 48L / 24D, 24L / 48D and the AD photoperiods were not significantly different from one another. For mink wastewater, the AL and 48L / 24D saw the highest uptakes while the 24L / 48D and the AD photoperiods saw the lowest uptakes. Mean utilization rates for BBM and mink wastewater expressed as concentrations can be observed in Figure 8 and Figure 9. It should be noted that the Day 0 concentrations for BBM does vary as a result of inaccuracies with the Hanna spectrophotometer (HI 83200 Hanna Instruments) while measuring diluted samples. All samples of BBM had to be diluted as the maximum range for phosphorous measurements is 15 mg/L. The device, which was used for all nutrient measurements performed in this study, showed a lack of precision when it came to phosphorous measurements. All samples were measured three times and an average was taken from the three.

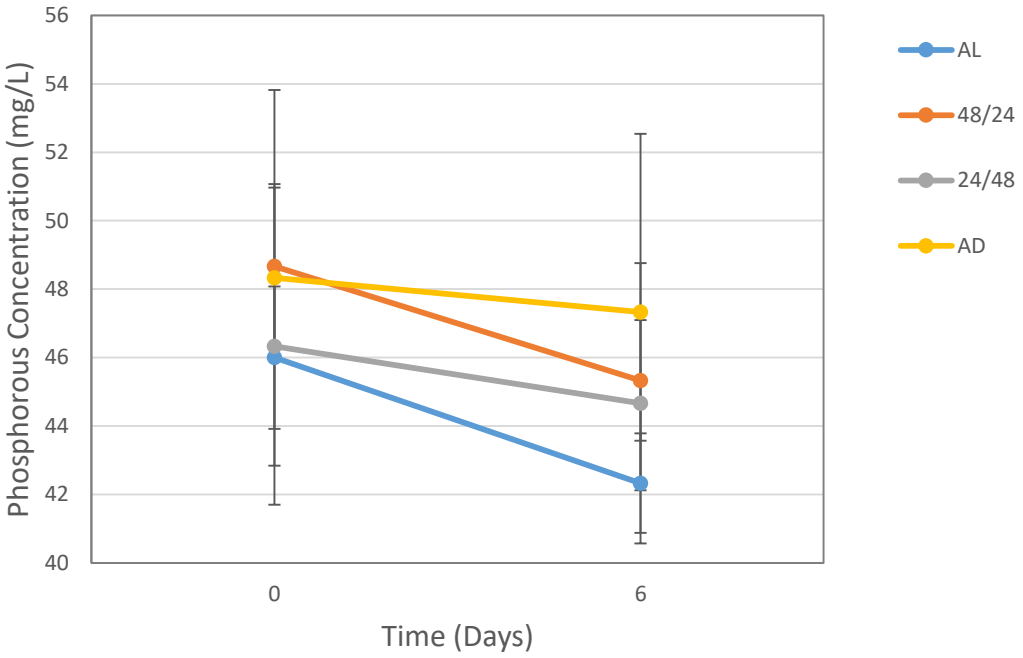

Figure 8: Mean phosphorous concentration on day 0 and day 6 for cultures grown in Bold's Basal Medium

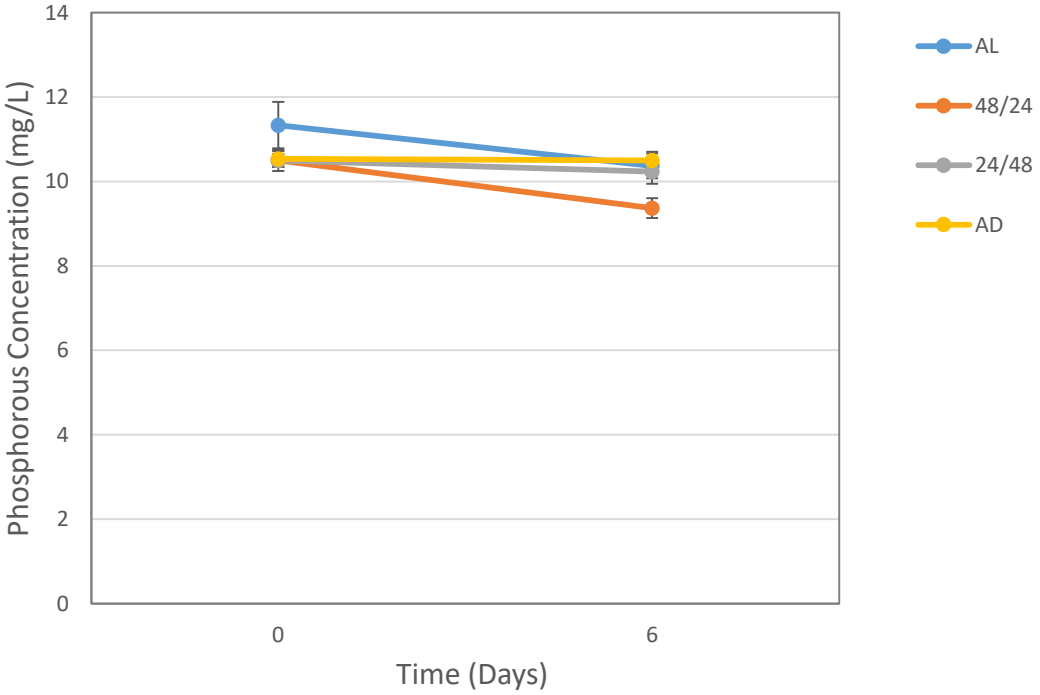

Figure 9: Mean phosphorous concentration on day 0 and day 6 for mink wastewater

In the absence of published data on total phosphorous uptake in *Dunaliella salina*, phosphate data is used for comparison purposes. First, taking a look at a study by Thakur and Kumar 1999 (Thakur and Kumar 1999) who found uptake of phosphate across their studies to be, 34.8% in 36 hours for suspended *Dunaliella salina*. Given that the 6-day uptakes are far lower than this figure suggests that there are other aspects to consider. Mean phosphate inoculation concentrations in BBM and mink wastewater were 144.91 mg L^{-1} and 32.85 mg L^{-1} respectively. The mean phosphate inoculation concentration used in the Thakur and Kumar study was 115 mg L^{-1} indicating that this is not likely to be an issue of concentration. In contrast, a study on green microalgae *Chlorella vulgaris* by Aslan and Kapdan 2006 (Aslan and Kapdan 2006) found that for phosphate concentrations greater than 23.61 mg L^{-1}, removals were less than 30% and decreased with increasing concentrations. The study cited light limitation due to increasing chlorophyll α concentrations as the primary reason for the reduction in phosphorous removal at higher densities. They went on to conclude that removal efficiencies could vary drastically dependant on a variety of factors including nitrogen to phosphorous ratio, photoperiod, light intensity and initial nutrient concentration. A further study on *Chlorella kessleri* by Lee and Lee 2001 (Lee and Lee 2001), found removal efficiencies ranging from 8-20% for initial phosphate concentrations of 30.66 mg L^{-1}. While these two studies were not performed on *Dunaliella salina*, they were still green microalgae and do serve to reinforce the idea that elevated phosphorous inoculation concentrations as well as a variety of other factors can result in low removals as was seen in this study. It should also be brought to attention that all phosphorous conclusions rely of the accuracy

and precision of the Hanna spectrophotometer, which as previously discussed had issues of precision.

4.3 Carotenoid Analysis

A standard curve was developed to analyze the accumulation of carotenoids following 8 days of high light intensity exposure for those cultures grown under the varying nutrient and photoperiod combinations over the previous 6-day period. The developed standard curve yielded a regression equation of y = .1606 + 45.21x where y is the absorbance at 453 nm and x is the biomass in g/L. Table 4.10 and Table 4.11 show the quantity of accumulated carotenoids as well as the percentage of total biomass for each of the nutrient and photoperiod combinations.

Table 4.10: Mean carotenoid accumulation and percentage of total biomass for Bold's Basal Medium by photoperiod

Photoperiod	Carotenoid Accumulation (mg L^{-1})	Percentage of total biomass (%)
AL	$14.50 \pm 6.25_a$	4.10
48L/24D	$20.25 \pm 1.40_a$	7.52
24L/48D	$11.79 \pm 3.50_{ab}$	9.82
AD	$1.99 \pm 0.64_b$	11.50

Values sharing a letter in the same column shows no statistical significance. Values in the same column, which do not share a letter, shows statistical significance

Table 3: Mean carotenoid accumulation and percentage of total biomass for mink wastewater by photoperiod

Photoperiod	Carotenoid Accumulation (mg L^{-1})	Percentage of total biomass (%)
AL	$16.81 \pm 2.02_a$	3.82
48L/24D	$12.74 \pm 3.04_a$	3.77
24L/48D	$13.821 \pm 4.65_a$	13.55
AD	$2.67 \pm 0.53_b$	14.31

Values sharing a letter in the same column shows no statistical significance. Values in the same column, which do not share a letter, shows statistical significance

There was no significant higher order interaction between nutrient source and photoperiod observed. The interaction was deemed insignificant after calculating a p-value of 0.478 for the interaction. The complete ANOVA table for this test can be observed in Table 4.12.

Table 4.12: ANOVA to determine whether a significant higher order interaction was present amongst the treatments for carotenoid absorbance

Source	Degrees of Freedom	F-value	P-value
Nutrient	1	0.06	0.807
Photoperiod	3	6.86	0.0045
Nutrient*Photoperiod	3	0.87	0.478

The same ANOVA also determined that the effect of nutrient source on carotenoid accumulation was insignificant and for that reason, the comparison is not shown here. Photoperiods were analyzed within each nutrient source using Duncan's multiple range test. For BBM, AL, 48L/24D and 24L/48D were not significantly different from one another while 24L/48D and AD were not significantly different from one another. In mink wastewater, AL, 48L/24D and 24L/48D were not significantly different from one another and performed better that AD which was significantly different from all other values. In looking at the percentage of total biomass, which the carotenoids comprised, the results fall into line with what was expected. Those samples with lower culture densities tend to accumulate a higher percentage of carotenoids despite the lower total values. There are two main reasons why this occurs. The first is that total carotenoid accumulation is limited by the amount of algal biomass that is present. It is for this reason that a greater percentage is observed despite the significantly lower total carotenoid accumulation. The findings of Ben-Amotz 1995 (Ben-Amotz 1995) offer an intriguing

insight here as well, as they conclude that for *Dunaliella bardawil*, total carotenoid production was highest for non-stressed cells, in greater number with lower cellular β-carotene content than for stressed cells in lower number with higher β-carotene contents. This study looked at nitrate starvation as a means of cellular stress but, the results of this study indicate that a similar pattern could be present for light induced stress. This is possible because, lower culture densities exposed to the same high light intensity as higher culture densities, are exposed to more light on a per cell basis and do not benefit as much from mutual shading as higher culture densities. In this way, there is more stress placed on individual cells in lower culture densities and therefore they accumulate more carotenoids on a per cell basis as is observed in Table 4.10 and Table 4.11. This concept is supported by the findings of Grobbelaar 1995 (Grobbelaar 1995), who concluded similarly that β-carotene accumulation was higher on a per cell basis for lower culture densities thanks to the increased stress exposure placed on the individual cells. Grobbelaar also goes on to conclude on the importance of a minimum residence time of at least one week to allow for larger cells to develop and accumulate greater quantities of β-carotene. This effect has the additional benefit of making harvest somewhat easier as well as improving the potential for further processing beyond β-carotene extraction as the cells are given more time to mature and therefore are larger and more robust.

Studies performed on maximum β-carotene accumulation in *Dunaliella salina* range in conclusion from as much as 10% (Lamers et al. 2012; Prieto, Pedro Cañavate, García-González 2011) to more than 10% (Macías-Sánchez et al. 2009), from 10 to 14% (Ramos et al. 2011) and up to 14% (Borowitzka and Borowitzka 1990). From these studies, a general value ranging from 10 to 14 % is accepted as the maximum achievable

accumulation. Data collected in this study falls within this range and demonstrates one of two possibilities as to why higher accumulations were not observed in those cultures containing greater biomass. The first potential reason is that the provided light intensity of approximately 13,500 lux was not high enough to properly stimulate β-carotene production in *Dunaliella salina*. This however would not seem to be the case as those cultures of lower biomass did accumulate carotenoids in quantities within the accepted maximum range. For this reason, it is most likely that mutual shading did have a significant effect on carotenoid accumulation by lessening the effective light intensity within the culture and reducing the total carotenoid production. For this reason, an increase in light intensity beyond 13,500 lux would be suggested as a means of maximizing carotenoid production in cultures of greater cell density. Abu-Rezq et al. 2010 (Abu-Rezq et al. 2010) looked at the effect of light intensity on carotenoid accumulation in *Dunaliela salina* and found that their highest intensity of 50,000 lux produced the greatest accumulation of β-carotene. As this was their highest illuminance, it is unknown what the results would be of a further increase however, *Dunliella salina* has been observed in healthy cultures in the salt marshes of Khiran under light intensities up to 150,000 lux (Al-Hasan and Sallal 1985). In addition, the work of Baroli and Melis 1996 indicates that the light intensity could be increased as high as 111,000 lux before a decrease in cellular replication would be observed in *Dunaliella salina* (Baroli and Melis 1996). While the study also suggested an optimal growth range of approximately 60,000 lux (Baroli and Melis 1996) this is still far in excess of what was provided in this study, giving further credence to the notion that increasing the light intensity could be an important factor to consider for β-carotene accumulation in denser *Dunaliella salina*

cultures. All of this indicates that there is certainly a large range to which illuminance could be increased however, the cost associated with running and operating such a system would need to be considered. The system used in this study incorporated four 18 W LED bulbs in order to maximize energy efficiency while still providing 13,500 lux, an illuminance which resulted in maximum β-carotene production in some cultures. A system capable of providing over four times this illuminance would come at a significant cost and further research would be needed to determine whether the additional β-carotene production justified the total energy costs.

A secondary hypothesis is that there are other significant factors at play beyond simply light intensity when it comes to carotenoid accumulation in *Dunaliella salina*. Nitrogen deprivation represents one of these factors. Lamers et al. 2012 (Lamers et al. 2012) found that carotenoid accumulation increased drastically at the point where nitrogen was completely depleted from the system. As nitrogen never reached full depletion in this study, the effect was unobservable. Leaving the culture for a longer period of time or selecting a growth medium with reduced nitrogen content, represents a potential option for increasing carotenoid accumulation. Further reinforcing the suitability of nitrogen deprivation is the work of Pisal and Lele 2005 (Pisal and Lele 2005) who found that the greatest carotenoid accumulation occurred under nitrogen deprivation at an illuminance of only 6,000 lux. It should be noted that this study utilised metal halide bulbs as opposed to LED's which may factor into their observed carotenoid accumulations. This factor is important to note as metal halide bulbs can range in color temperature from 3,000 to 20,000 K. As the color temperature is not mentioned in the study of Pisal and Lele 2005 (Pisal and Lele 2005) it is unknown what potential influence

this may have. Several other studies have also cited the positive effect of nitrogen starvation on carotenoid accumulation (Ben-Amotz 1987; El-Baky, El-Baz, El-Baroty 2004; Marín et al. 1998; Ribeiro, Barreto, Coelho 2011). The link between nitrogen starvation and irradiance remains prevalent however, and some studies have found that it is more complex than simply limiting nitrogen, stating that nitrogen limitation in the absence of high irradiance would not be wise for the production of β-carotene (Grobbelaar 1995). As one of the outcomes for this study was to assess the viability of mink wastewater as a nutrient source in the production of *Dunaliella salina*, nitrogen starvation was not a feasible approach due to the inherent nitrogen in both the control BBM as well as the mink wastewater itself.

4.4 Proximate Analysis

Proximate analysis was used as a means of observing protein, lipid and carbohydrate concentrations in *Dunaliella salina*. Due to the limited storage capabilities, replications were not run for these tests and results are expressed as mean values across all treatments (one sample for each treatment). The complete results of the proximate analysis can be observed in Figure 10 and Figure 11.

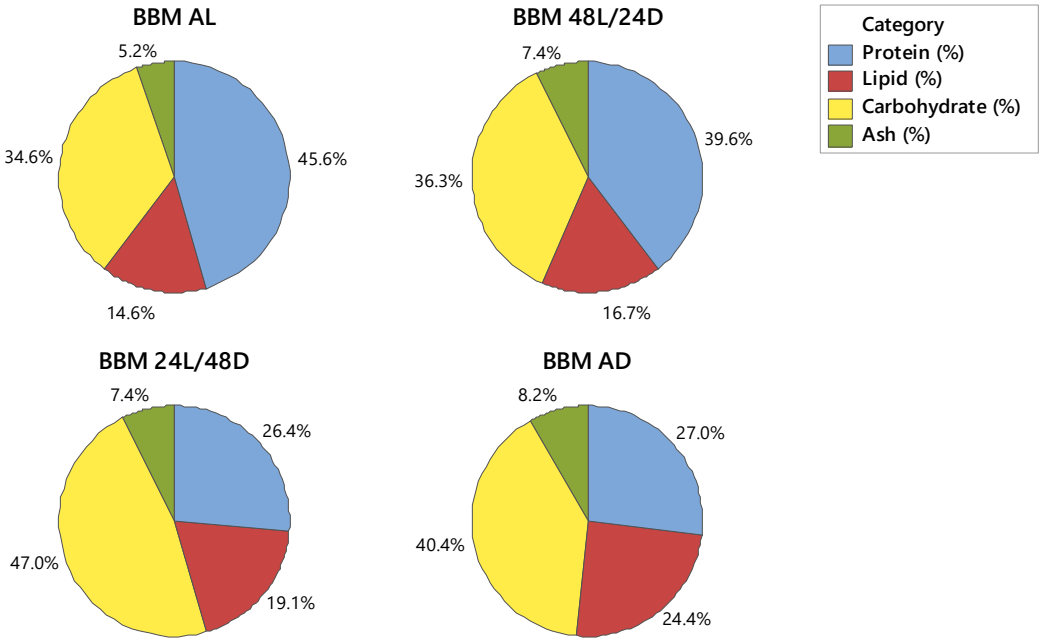

Figure 10: Results of proximate analysis for Dunaliella salina in Bold's Basal Medium for each of the treatment combinations displaying protein, lipid, carbohydrate and ash contents as a percentage of total cell biomass

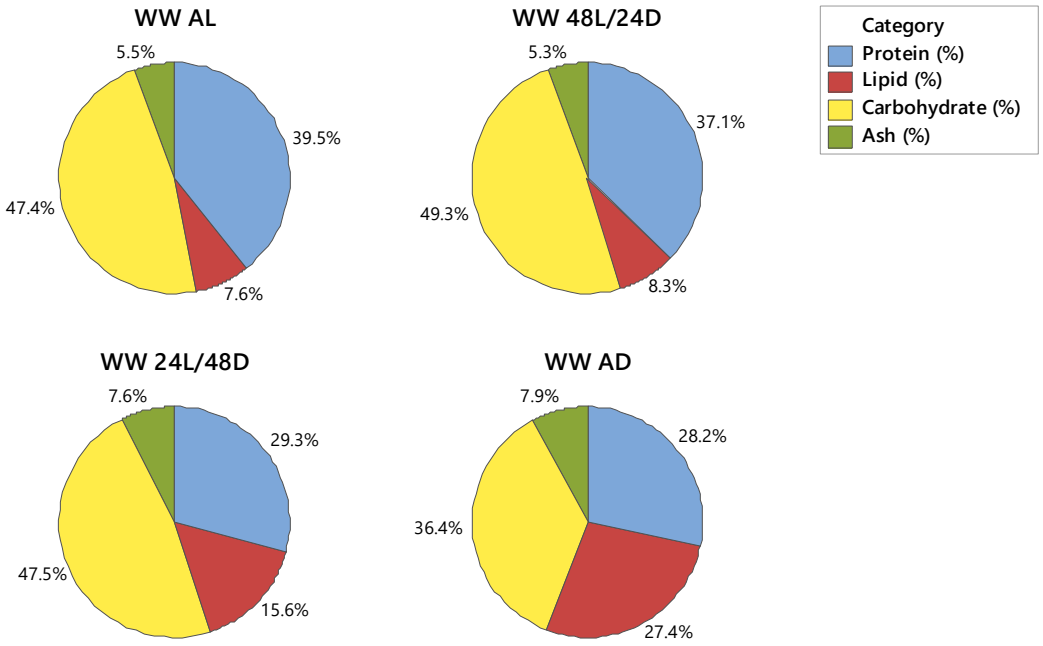

Figure 11: Results of proximate analysis for Dunaliella salina in mink wastewater for each of the treatment combinations displaying protein, lipid, carbohydrate and ash contents as a percentage of total cell biomass

From the analysis, *Dunaliella salina* was observed to have mean concentrations of 34.07%, 16.75% and 42.37% for protein, lipid and carbohydrate percentages respectively. These values are expressed as percent of total cellular biomass. The remaining percentage is ash weight, which had a mean value of 6.82%. While the values obtained in this analysis, do seem to vary somewhat from published values obtained by Thomas et al. 1984 (Thomas et al. 1984), the lack of statistical replication coupled with the differing growth conditions and photoperiods likely account for these differences. Thomas et al. stated that for *Dunaliella sp*., protein content could be as high as 64%, lipid content could range from 20 to 29% and carbohydrates could range from 11 to 23%. Despite the differences, it should be noted that photoperiod and nutrient availability have been widely

observed to have a major impact on microalgae characteristics (Adams et al. 2013; Cheirsilp and Torpee 2012; George et al. 2014; González-Fernández and Ballesteros 2012; Khoeyi, Seyfabadi, Ramezanpour 2012; Seyfabadi, Ramezanpour, Khoeyi 2011; Uslu et al. 2011; Wahidin, Idris, Shaleh 2013). This is true for single species as well as across species and as a result, one could expect significantly different characteristics for different treatment combinations. Additionally, as algae grow they will dedicate more of their biomass to certain characteristics. For instance, as an algae culture reaches the diminishing growth stage, it will begin to dedicate less biomass towards photosynthetic pigments and more towards other protein, lipids and carbohydrates (Healy 1975). This would indicate that stage of harvest also has a major effect on cellular composition in microalgae.

Protein concentration in BBM was higher than in mink wastewater for the All light and 48L/24D photoperiods despite the significantly lower nitrogen removals observed as part of the nutrient analysis. This observation gives further credence to the hypothesis that microbial activity was present in the mink wastewater samples. While protein was not higher in the 24L/48D and All dark photoperiods, these treatments did not remove nitrogen to nearly the same degree. Taking all conclusions and hypotheses into account, the presence of microbial activity in the mink wastewater cultures seems highly likely.

4.5 Analysis of Mink Wastewater Potential

There are a number of factors to consider when looking at the potential for mink wastewater to serve as an acceptable culture medium for microalgae growth. The first of these factors is how well it compares to the existing standard, in this case BBM. In

running the initial ANOVA for both the absorbance data as well as the dry biomass data it was concluded that there was no significant difference between the two nutrient sources in terms of growth. This is important to note due to the drastically different costs of the two nutrient sources. The cost breakdown for BBM can be observed in Table 4.13.

Table 4.13: Cost breakdown for Bold's Basal Medium

Nutrient	**Cost ($/500g)**	**Cost ($/$L_{media}$)**
$NaNO_3$	49.77	0.02489
KH_2PO_4	171.62	0.06007
$MgSO_4*7H_2O$	157.54	0.02363
K_2HPO_4	173.92	0.02609
$CaCl_2*2H_2O$	106.38	0.00532
NaCl	125.31	0.00627
$FeSO_4*7H_2O$	185.69	0.00370
H_3BO_3	154.31	0.00248
KOH	255.85	0.00635
H_2SO_4	95.57	0.00038
	Trace metal solution	
H_3BO_3	154.31	0.00062
$MnCl_2*4H_2O$	274.38	0.00110
$ZnSO_4*7H_2O$	227.46	0.00091
$Na_2Mo_4*2H_2O$	233.25	0.00093
$CuSO_4*5H_2O$	61.41	0.00025
$Co(NO_3)_2*6H_2O$	315.98	0.00126
Total	**2742.75**	**0.16423**

**All prices were attained from Fisher Scientific (https://www.fishersci.ca/ca/en/home.html)*

A quick analysis of the cost to produce BBM yielded a price of 0.16 USD (0.21 CAD) per litre. Additionally, one must account for the time required to produce the media. Typically, a batch of BBM will require half an hour to generate with the volume being determined by the capacity of the autoclave system in use. While the cost may be minimal on a small scale, this is not the case on scale up and as one moves towards industrial level production. Consider even a 200L batch system, which would require 32 USD (41.52 CAD) of BBM for each batch. If a batch takes a week to produce the desired culture density then this would result in a yearly expense of 1,664 USD (2,159.41 CAD).

This example is still a fairly small system, which would be difficult to draw profit from simply due to the significant nutrient cost. Scale up to an industrial size would require even further expense with profitability needing to be assessed on a case-by-case basis. Such a system would require a drastically increased containment volume, larger lighting system, greater air supplementation and more thorough sterilization regimes in addition to the nutrient costs. This all serves to make the requirement for affordable nutrients all the more important. It is equally important to note that buying bulk chemicals could represent a significant cost savings over the values presented here. Despite this, assembling BBM still remains far cheaper than purchasing it where costs can be as high as 9 USD (11.68 CAD) per litre (Canadian Phycological Culture Centre). It has been estimated that cost reductions over 50% are possible for microalgae production systems with access to low cost nutrients, water and CO_2 (Slade and Bauen 2013). The mink wastewater sample used in this study was attained from The Canadian Centre for Fur Animal Research at no expense beyond the time it took to collect and pick up. It is also conceivable that Nova Scotia farmers could be willing to pay and industry to collect mink wastewater and remove it from their farms given the new guidelines outlined in the 2013 Fur Industry Act (Province of Nova Scotia 2013). If this arrangement were to be organised then it could represent a significant source of cost offsetting in microalgae production. While the mink wastewater used in this study required dilution with fresh water, unpublished work suggests that this can be done with seawater in the case of *Dunaliella salina.* The benefit of utilising wastewaters as a nutrient source for carotenogenesis in *Dunaliella salina* has also been noted in previous researches (Oren

2010; Santos et al. 2001) and in particular comparing it with known fresh mediums (Santos et al. 2001) as was done in this study.

If the utilization of mink wastewater is to be profitable on a large scale, there remain some practical issues of how this would be achieved. Firstly is the concern over procurement of mink wastewater in that farmers might be wary to release their waste to industry due to potential repercussions associated with the spread of Aleutian disease. More so that this is, the practical challenge associated with the dilution requirement. The need for a 1:100 dilution might rule out freshwater species, as it simply would place too much strain on freshwater resources at a larger scale. Marine species such as *Dunaliella salina* remain a promising option though. The final issue is the location of the mink wastewater. Nova Scotia's climate is not friendly for year round outdoor production and therefore any production system would need to be inside. This comes at the increase cost of heating and lighting, which would need to be factored into any profitability analysis. If these issues could be overcome however, there is certainly significant potential for mink wastewater to replace traditional nutrient mediums as a source of significant cost reduction.

CHAPTER 5 CONCLUSIONS

5.1 Experimental Conclusion

The first specific objective of this study was to assess the potential for *Dunaliella salina* to be grown photoautotrophically, heterotrophically and mixotrophically. Results for *Dunaliella salina* grown in both BBM and mink wastewater indicate an ability to grow photoautotrophically and mixotrophically but an inability to attain comparable growth under heterotrophic conditions. With the best growth being achieved under the AL and 48L/24D photoperiods, the observations of this study reinforce the importance of light in the growth of *Dunaliella salina.*

The second specific objective was to assess the viability of mink wastewater as a nutrient source in the production of *Dunaliella salina*. Based on the insignificant difference between mink wastewater and the standard growth medium, BBM, it can be concluded that diluted mink wastewater could be utilised as an alternative nutrient source to already in place industry standards such as BBM. This is particularly important as it represents a significant potential cost savings in the production of *Dunaliella salina.* If mink wastewater can be attained, free of charge as it was in this study, then its usage represents a cost saving potential of 0.16 USD (0.21 CAD) per litre of medium in comparison to BBM. It is also an important result as it offers a potential pathway through which mink wastewater can be diverted. As mink wastewater handling is near the top of Nova Scotian agricultural concerns, microalgae production could be the answer to at least a portion of this issue.

The final specific objective was to assess the potential for carotenogenesis in *Dunaliella salina* for species grown in mink wastewater. While typically accepted maximum carotenoid contents were achieved for the 24L/48D and AD photoperiods they were not achieved for the AL and 48L/24D photoperiods. This result however is most likely an issue of light intensity and culture density. If light intensity were to be increased, it is conceivable that those cultures of greater density might have achieved maximum carotenogenesis as mentioned earlier (Abu-Rezq et al. 2010; Al-Hasan and Sallal 1985; Baroli and Melis 1996). Longer residence time in the growth stage as a means of further reducing the nitrogen levels in the culture could also play a significant effect on carotenoid accumulation thanks to the noted benefits of nitrogen starvation.

In addition to the specific objectives, there are a number of other significant results, which can be discussed. The first is the notion that *Dunaliella salina* can grow in both ammonia rich and nitrate poor as well as ammonia poor and nitrate rich conditions. As mink wastewater is rich in ammonia and poor in nitrate and BBM is rich in nitrate and poor in ammonia and there was no significant difference between the growth observed in both mediums, it can be concluded that *Dunaliella salina* can have significant growth under both sets of conditions.

This study also serves to reinforce the correlation between absorbance and gravimetric growth quantification. As was determined previously, these two methods represent the optimal combination of accuracy, precision, time and cost savings. The high correlation achieved in this study justifies their usage and the omission of other methods of growth quantification.

5.2 Future Research

Based on the findings of this research there remain a few areas which could be explored further in future studies. The first of these considerations would be to observe the effect of further increasing the light intensity during the carotenogenesis portion of the method. In doing this, it is hypothesized that it would increase the rate of carotenogenesis in the denser samples. The denser samples of this study did not have carotenoid levels in accordance with maximum literature values and this can most likely be attributed to the illuminance.

A second area, of potential exploration, would be to examine the effect of increasing the concentration of mink wastewater in the sample. The 1% dilution was selected due to its minimal effect on the color of the medium and the hypothesis that darkening the medium might effect carotenogenesis. This however did not seem to be a factor and experimenting with greater wastewater concentrations might prove prosperous. With that being said, increasing the concentration too much might have other toxic effects on the microalgae, especially when it comes to the high levels of ammonia found in mink wastewater

Branching this study out into other species and applications might also be of interest. Being that *Dunaliella salina* saw such great success it is conceivable that other green microalgae species might as well. In this way, future studies might focus on other bioproducts such as protein, biofuels or bioplastics.

Regardless of where future research may lead, mink wastewater as a nutrient source for the production of *Dunaliella salina* provides a promising alternative to conventional nutrient mediums.

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