MINKERY WASTEWATER INTEGRATED INTO MICROALGAE AND CYANOBACTERIA PRODUCTION

by

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ABSTRACT

In Nova Scotia, the mink industry has become an essential component to the economic vitality of the agricultural sector. With emerging regulations from the Fur Industry Act of 2010, mink producers are required to identify means to reduce pollutants in effluent waters. Employing microalgae or cyanobacteria could serve a double purpose to generate high-value biomass while assisting in the remediation of wastewater. This study aims to investigate the potential of integrating minkery wastewater into microalgae and cyanobacteria production. Two strains, *Chlorella vulgaris* and *Anabaena sp.* were selected due to their prevalence. Based on the findings, this study proved the potential of utilizing minkery wastewater as an alternative medium for microalgae cultivation. The growth characteristics of *Chlorella vulgaris* in minkery wastewater were significantly better than those in modified Bold's basal medium. Although cyanobacteria cultivation in minkery wastewater remains a challenge, continued research may make minkery wastewater more competitive with other mediums.

LIST OF ABBREVIATIONS USED

A	Area under pigment peak
AU	Absorbance units
BBM	Modified Bold's basal medium
BOD	Biological oxygen demand
C	Pigment concentration (mg L^{-1}); coefficient
CC	Cell count
Chl-*a*	Chlorophyll *a*
COD	Chemical oxygen demand
CPCC	Canadian phycological culture centre
D	Dark
DW	Distilled water; dry weight
d	Index of agreement
E	Coefficient of efficiency; volume of acetone used (mL)
F	Calibration factor for sensitivity setting; standard response factor; dilution factor
FAO	Food and agricultural organization
HPLC	High-performance liquid chromatography
L	Light
L	The cell path length (cm)
MW	Minkery wastewater
NIR	Near-infrared
NTP	Nitrogen to protein conversion factor
OD	Optical density
R	Fluorometer reading for specific sensitivity
r	Pearson's correlation coefficient
TM	Traditional growth medium
TOC	Total organic carbon

TSS	Total suspended solids
UV-Vis	Ultraviolet-visible
V	Volume; the volume of water filtered (L)
VSS	Volatile suspended solids
WHO	World health organization
x	Growth measurement made on a day
$\bar{x}$	Mean of the values of the 1st method
y	Nutrient content measurement
$\bar{y}$	Mean of the values of the 2nd method
665	Absorptivity at 665 nm
663	Absorptivity at 663 nm

Greek letters

σ	Standard deviation
μ	Mean

Subscripts

a	Chlorophyll *a*; after
b	Before
E	Extraction
I	Injection
i	i^{th} value
n	n^{th} value
S	Sample
v	Variation
0	Day 0
6	Day 6

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CHAPTER I

INTRODUCTION

1.1 Background Information

The mink industry in Nova Scotia began in the 1930s and had grown at a high rate over the past 20 years. Today, there are around 100 mink farms, employing more than one thousand workers in Nova Scotia. According to the 2012 Statistics Canada census, Nova Scotian mink farming accounted for 54.3% of Canada's total mink production. Even further, the estimated value of the mink industry in Nova Scotia in 2012 was $140 million (Statistics Canada census, 2012). This value has dropped since then, due to weak demand and a problematic virus called Aleutian Mink Disease (AD) virus. Mink products are still consistently the largest agricultural export in Nova Scotia.

Amid the success of Nova Scotian mink industry, there are some concerns about the environmental impact of mink production. The main concerns are feces and urine produced by the mink. Rough estimates suggest that there are at least 18,000 tons of mink manure and 16 million litres of urine generated by mink farms in Nova Scotia each year (Boon, 2014). Many concerned citizens, academics, and journalists suspect that the impacts of mink production on adjacent surface waters are detrimental. One report concluded that multiple instances of eutrophication and algae blooms (Figure 1.1) were related to discharges from mink farms (Taylor, 2009). As with most agricultural wastewaters, mink discharges have high concentrations of pollutants, such as ammonium and phosphorus

(Ferguson, 2002). The composition of minkery wastewater will invariably have impacts on the local aquatic ecosystems, thereby damaging vital Nova Scotian resource. Fortunately, with emerging regulations from the Fur Industry Act of 2010, mink producers were required to identify means to reduce pollutants in effluent waters. This new government regulation of fur industry will have implications for operational practices in the future.

Figure 1.1. American Mink *Neovison vison* (left) and eutrophication of Mattatall Lake (right).

Minkery wastewater integrated into microalgae and cyanobacteria production as a means to reduce effluent water pollutants may provide a service to the mink industry as it tries to adapt to new regulations. Many studies reported microalgae and cyanobacteria assimilate various nutrients present in waste streams, which include ammonium, nitrate, nitrite, and phosphorus (Rai et al., 1981; Redalje et al., 1989). It is this characteristic that makes them obvious candidates for wastewater treatment. Studies on using the microalgae and cyanobacteria cultivation as a tertiary wastewater treatment process started in the early

1970s (McGriff and McKenney, 1971). The purpose was to treat secondary wastewater even further to mitigate the potential eutrophication of surface water bodies following discharge (Mcgriff and Mckenney, 1972). One study even concluded that freshwater microalga *Chlorella pyrenoidosa* removed nutrients from settled domestic sewage more efficiently than activated sludge process did, suggesting that it would be more economical and desirable to employ microalgae cultivation as the secondary rather than tertiary treatment process (Tam and Wong, 1989).

1.2 Introduction to Microalgae and Cyanobacteria

Microalgae have relatively simple structure lacking the complex tissues found in higher plants (Figure 1.2); however, play a vital role in biogeochemical cycles (Ramaraj et al., 2013). Microalgae sequester carbon dioxide by photosynthesis, supply food to the consumers of aquatic environments, and produce approximately half of the atmospheric oxygen on earth (Anesio et al., 2009).

Figure 1.2. Freshwater microalga *Chlorella vulgaris*.

In the past, cyanobacteria were included in the classification of 'algae'. However, the term 'algae' is now only reserved for eukaryotic organisms. Cyanobacteria are prokaryotic organisms and lack a defined nucleus. Therefore, cyanobacteria are now classified within the *Bacteria* domain. In contrast, microalgae are in the *Eukaryota* domain due to a membrane-enclosed nucleus, making them eukaryotic organisms.

For the last two decades, microalgae and cyanobacteria started to play an emerging role with increasing applications motivated by the increase in oil prices, the depletion of fossil-fuel reserves, and global warming (Safi et al., 2014). These dramatic thresholds are forcing researchers to find strategies by intensifying researches on the 'third-generation' biofuel feedstocks. Microalgae and cyanobacteria are now regarded as two promising 'third-generation' biofuel feedstocks (González-Fernández et al., 2012; Tran et al., 2010).

Similar to the crop-based 'first generation' biofuel feedstocks, microalgae and cyanobacteria capture sunlight and perform photosynthesis by producing oxygen and absorbing carbon dioxide as a primary feed. Therefore, growing them next to combustion power plants has an applicable potential (Banerjee et al., 2002; Lorenz and Cysewski, 2000; Spolaore et al., 2006). Different from the crop-based 'first generation' biofuel feedstocks, microalgae and cyanobacteria have ability to produce a volume of biomass hundreds of times greater (Brooker and Yildiz, 2014). Microalgae and cyanobacteria would not compete with agricultural lands; there would be no conflict with food production and especially would not cause deforestation.

1.3 Cultivation Challenges

At present, the large-scale microalgae and cyanobacteria production faces a number of cost related bottlenecks (Campbell et al., 2011; and Lee, 2001). The energy and fertilizer costs of the large-scale microalgae and cyanobacteria production have been too high to be economically feasible. Despite the tremendous opportunity and value of the large-scale microalgae and cyanobacteria production, it is still hampered by the nagging impediment of reaching economic viability (Lardon et al., 2009) which present a very challenging research topic to all investigators. This research topic is either escalation of efficiency by achieving higher microalgae and cyanobacteria biomass production, increasing in cellular lipid, protein and carbohydrate content, or to reduce infrastructural costs during the large-scale microalgae and cyanobacteria production (Bahadar and Khan, 2013; Delrue et al., 2013 and Goncalves et al., 2013).

1.4 Minkery Wastewater Opportunity

In most cases, the nutrients concentrations of agricultural waste streams were in excess of traditional culture mediums (Khademi et al., 2015). Extensive research has been conducted exploring using a variety of agricultural wastewaters to offset the financial burden of nutrient and carbon supplies of the large-scale microalgae and cyanobacteria production (Alkhamis and Qin, 2013; Lowrey and Yildiz, 2011b). Many studies have demonstrated that agricultural wastewaters have potential to be excellent culture mediums for microalgae and cyanobacteria cultivation with even improved yields when compared

to traditional culture mediums (Hammouda et al., 1995; Shi et al., 2007a). As with most agricultural wastewaters, minkery wastewater has high concentrations of nutrients, such as ammonium and phosphorus (Ferguson, 2002). While posing some serious environmental problems, minkery wastewater could potentially be an ideal culture medium for the large-scale microalgae and cyanobacteria production.

At present, the most common organic carbon source for the large-scale microalgae and cyanobacteria production is glucose, and it comes at an enormous expense. In the past few years, several economic feedstocks were proposed and tested for scale up viability and to reduce the costs of organic carbon sources, such as corn starch (Gao et al., 2012; Lu et al., 2010). However, they are still too expensive to be used for the large-scale microalgae and cyanobacteria production (Kaneko, 2013). Minkery wastewater is extremely rich in organic compounds that could potentially be an excellent organic carbon alternative for heterotrophic and mixotrophic cultivation.

1.5 Research Goal

This study aims to assess the viability of using minkery wastewaters for microalgae and cyanobacteria cultivation, identify the effect of different cultivation techniques on microalgae and cyanobacteria cultivation, quantity remediation efficiencies of wastewater treatment using microalgae and cyanobacteria, and evaluate the effectiveness of four different conventional methods (dry weight, optical density, cell count, and chlorophyll *a*) for microalgae and cyanobacteria biomass estimation. Our findings will provide the

framework for future investigation of using minkery wastewater as a potential resource in Nova Scotia. Specifically, four main objectives are addressed in this study:

1) To assess the viability of using minkery wastewaters for microalgae and cyanobacteria production.
2) To identify the effect of different cultivation techniques (light cycles) on microalgae and cyanobacteria production.
3) To quantify remediation efficiencies of wastewater treatment using microalgae and cyanobacteria.
4) To investigate the effectiveness of four different conventional biomass estimation techniques

1.6 Research Scope and Approach

In this study, the experiment started with the cultivation of microalgae and cyanobacteria. For each experimental unit, the growth period was six days, and a split-split-plot design was used to arrange each experimental unit. The whole plots were strains, the subplots were light cycles, and the sub-subplots were mediums. In total, the cultivation process had 24 different treatments with triplicate observations for each treatment. During the cultivation process, four conventional biomass estimation methods were performed to determine cell biomass generated in the experimental treatments. The efficiency of wastewater treatment was evaluated by comparing the selected nutrients' contents of wastewater samples before and after six-day microalgae and cyanobacteria cultivation. The

main chemical component of microalgae and cyanobacteria were also measured before and after six-day microalgae and cyanobacteria cultivation. Subsequently, to comprehensively compare the practical utility and analytical reliability of four different biomass estimation methods, the results of four conventional biomass estimation methods were compared in the category of cost and time, correlation, accuracy, and precision. This experiment formed four different statistical analysis techniques namely the Pearson's correlation coefficient, the coefficient of efficiency, the index of agreement, and the coefficient of variation, and it also recorded the cost and time consumed when performing four different biomass estimation methods.

1.7 Research Benefits and Impact

Due to the abundant agricultural sector in Nova Scotia, as well as the temperate climate, the province is an ideal location for an emerging mink, microalgae and cyanobacteria industry in Canada. This study confirmed the opportunity of associating minkery wastewater and microalgae and cyanobacteria production without detrimental impacts on final products. By testing the suitability of minkery wastewater as a growth medium for microalgae and cyanobacteria production, a promising green technology could potentially be employed in an industry with a spotty public relations image. The technical information generated in this study will be of tremendous value to developing economically, environmentally, and socially sustainable mink farming and microalgae and cyanobacteria industries.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Both developed and developing economies, and the growing world population highly rely on the natural resources of our planet. According to 2017 Revision of World Population Prospects, which are released every two years, the world population is expected to reach 8.6 billion in 2030, 9.8 billion in 2050 and 11.2 billion in 2100 (United Nations Population Division, 2017). For the human race, the demand for energy sources is greater than ever before, and sustainable techniques are necessary for energy production. The cultivation of microalgae and cyanobacteria have potential to make a significant contribution to the transition to a more sustainable production of energy (Figure 2.1).

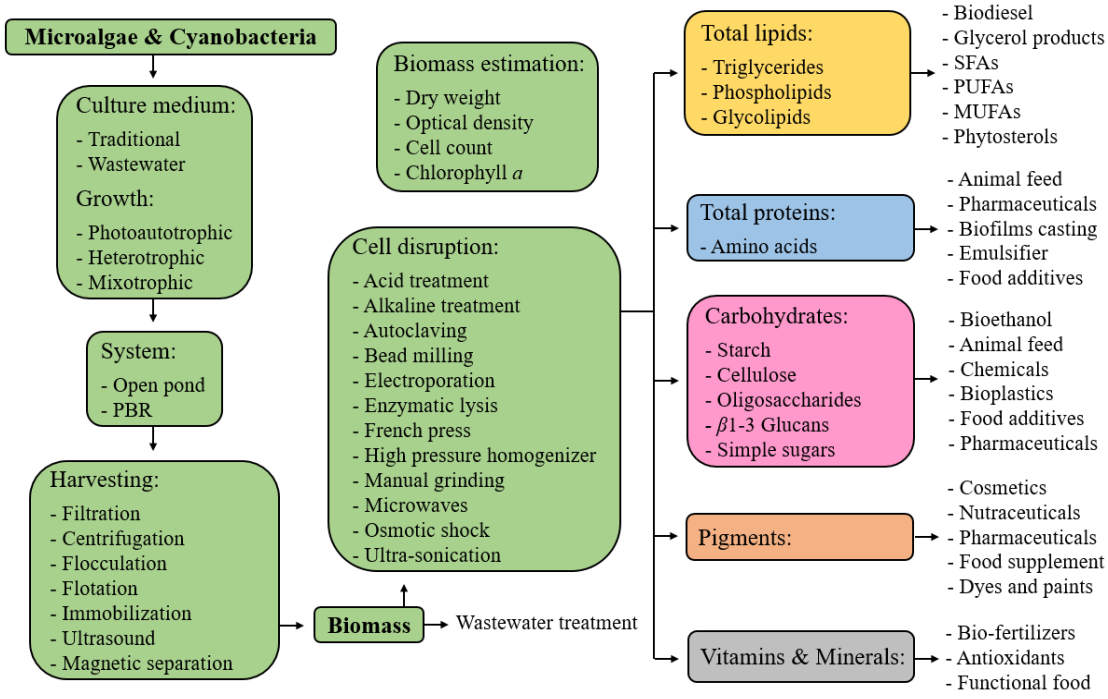

Figure 2.1. Algo-refinery concept from production to valorization.

2.2 Strain Selection

Chlorella is a genus of single-celled green microalgae measuring about 2 to10 μm in diameter and can be found in both fresh and marine water (Safi et al., 2014). When combined with biofuel production, microalgae species from the genus of *Chlorella* were most commonly used due to its high productivity of fatty acids relevant to transesterification reaction (Hempel et al., 2012). In our previous study, we found that microalga *Chlorella vulgaris* cultivated in municipal wastewater achieved approximately 60% total nitrogen removal and 90% total phosphorus removal in only six days.

Anabaena is a genus of filamentous cyanobacteria known for nitrogen-fixing abilities. They are also one of four genera of cyanobacteria that produce neurotoxins, which are harmful to local wildlife. In our previous study, cyanobacterium *Anabaena sp.* achieved over 80% total nitrogen removal through 6-day of cultivation in municipal wastewater.

Overall, both *Chlorella vulgaris* and *Anabaena sp.* (Figure 2.2) showed potential to simultaneously accumulate biomass and treat minkery wastewater. Thereby, *Chlorella vulgaris* and *Anabaena sp.* were selected representing highly valuable and viable microalgae and cyanobacteria, respectively.

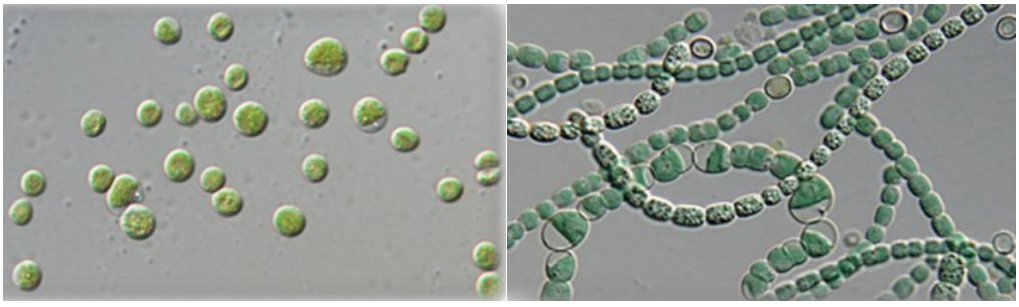

Figure 2.2. *Chlorella vulgaris* (left) and *Anabaena sp.* (right).

2.3 Cell Structure

Both microalgae and cyanobacteria can exist as individual cells, in cell colonies, or as long filamentous chains (Sheeler and Bianchi, 1987). In this study, microalga *Chlorella vulgaris* exist as individual cells, and the cyanobacterium *Anabaena sp.* exist as long filamentous chains.

2.3.1 Chlorella vulgaris

Chlorella vulgaris cells (Figure 2.3) are surrounded by a cell wall composed of polysaccharides. The rigidity of the cell wall preserves the integrity of the *Chlorella vulgaris* cell and is a protection against harsh environment or invaders (Safi et al., 2014). Cytoplasm is the gel-like substance confined within the barrier of the cell membrane, and it is composed of soluble proteins, minerals, and water. It hosts the internal organelles of *Chlorella vulgaris* such as a nucleus, mitochondria, vacuoles, a single chloroplast and the Golgi body (Kuchitsu et al., 1987; Solomon et al., 1999).

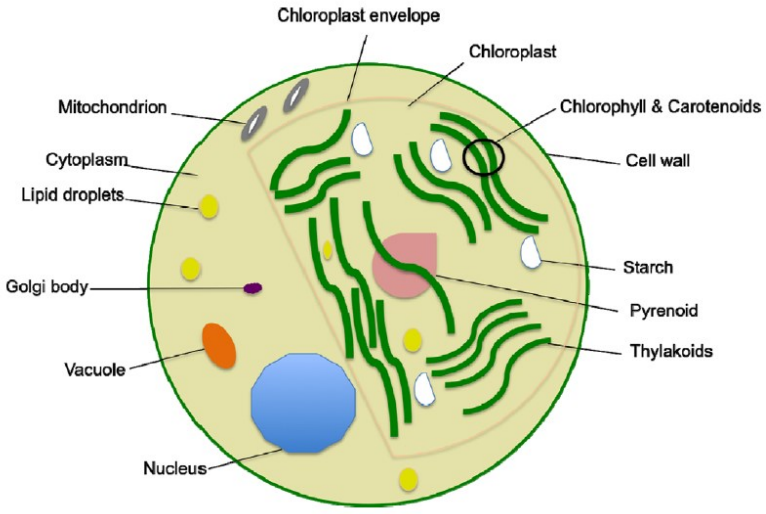

Figure 2.3. Schematic ultrastructure of *Chlorella vulgaris* representing different organelles (Adapted from Safi et al., 2014).

Chlorella vulgaris have a single chloroplast with a double enveloping membrane composed of phospholipids. The chloroplast stores a cluster of fused thylakoids where the dominant pigment chlorophyll is synthesized masking the color of other pigments such as lutein. The pyrenoid in the chloroplast is the center of carbon dioxide fixation. Starch granules can be formed inside the chloroplast, especially during unfavorable growth conditions. During nitrogen stress, lipid globules mainly accumulate in the cytoplasm and the chloroplast (Lee, 2008; Van den Hoek et al., 1995).

2.3.2 Anabaena sp.

Anabaena sp. cells have an envelope organization with the cell wall, nucleoid, ribosomes, and absence of membrane-bound structures (Figure 2.4). The cell wall is four-layered with peptidoglycan present in the second layer. In most forms, the photosynthetic machinery is embedded into folds of the external cell membrane, called thylakoids. Attached to the thylakoid membranes are small granules known as phycobilisomes. They act as light-harvesting antennae for the photosystems (Grossman et al., 1993).

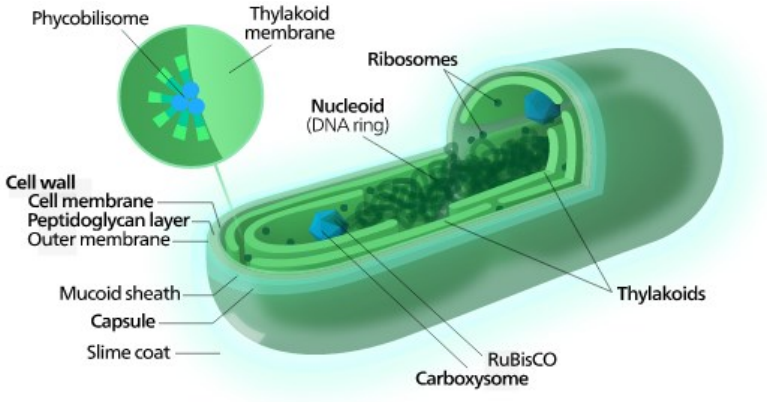

Figure 2.4. Schematic ultrastructure of *Anabaena sp.* cell representing different organelles.

Anabaena sp. can develop a specialized cell type named heterocyst from 5 to 10% of vegetative cells in times of nitrogen starvation. These cells are distributed in a semi-regular pattern along the filament (Figure 2.5) and are specialized in nitrogen fixation catalyzed by the oxygen-sensitive enzyme complex nitrogenase. Heterocysts supply vegetative cells with fixed nitrogen, whereas their function relies on sources of carbon and reductants provided by the vegetative cells. Hence, heterocyst development enables *Anabaena sp.* to adapt to nitrogen-limiting conditions and reflects the requirement for a micro-oxic environment for nitrogenase function (Nicolaisen et al., 2009).

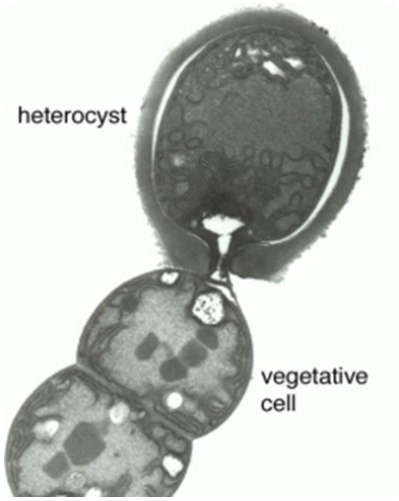

Figure 2.5. Ultrastructure of *Anabaena sp.* representing one terminal heterocyst and two vegetative cells (Adapted from Nicolaisen et al., 2009).

2.4 Growth Kinetics

The growth kinetics of microalgae and cyanobacteria can be determined in a homogenous batch culture, where the nutrient supply is limited, and nothing is added or removed from the culture medium. Figure 2.6 illustrates the growth curve of microalgae and cyanobacteria cultures associated with batch kinetics. In most cases, the phases were

not always as clear as shown, and the slope may vary in magnitude, length, and height. In short, the actual phase is based on the inoculation material, the nutrient concentration, and the environmental conditions.

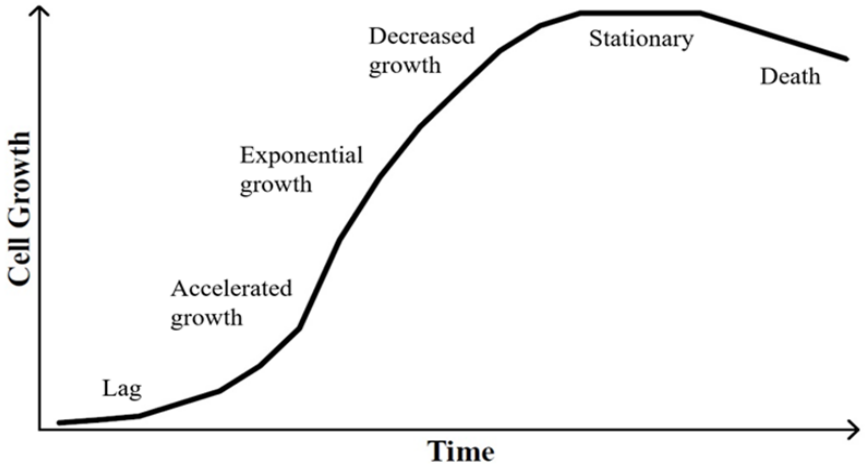

Figure 2.6. Growth curve of microalgae and cyanobacteria cultures.

1. *Lag phase*: The period during which the microalgae and cyanobacteria adapt to the new environment after being inoculated. The lag is caused by the cells adapting to new growth medium and reorganizing molecular constituents to uptake nutrients.

2. *Accelerating growth phase*: After the microalgae and cyanobacteria have adapted to the new environment, the growth rate begins to increase toward exponential growth.

3. *Exponential growth phase*: The microalgae and cyanobacteria have fully adapted to the environment and are reproducing exponentially. The growth curve of microalgae and cyanobacteria cultures approach the maximum growth rate in a closed environment. This phase is optimum to maintain microalgae and cyanobacteria production and commercial operations.

4. *Decreased log growth phase*: Microalgae and cyanobacteria begin to slow reproduction rates as nutrients and light become less abundant, and the growth curve of microalgae and cyanobacteria cultures becomes almost linear. In well-maintained environments, this phase continues, however, when one of the nutrients gets depleted, the microalgae and cyanobacteria population reaches carrying capacity.

5. *Stationary phase*: During this phase, growth rates of microalgae and cyanobacteria cultures are equal to the death rate of them, and the population is held constant. The growth curve of microalgae and cyanobacteria cultures approach the maximum biomass concentration in a closed environment.

6. *Death phase*: The death phase occurs once the maximum population has been supported for a period. Microalgae and cyanobacteria begin to release organic, growth-inhibiting, materials into the growth medium. The phase is caused by the nutrient depletion, limited supply of light, over-age of the cultures, and unfavorable conditions. As a result, the population leads to a complete breakdown, and infection from other microorganisms becomes more likely.

2.5 Growth Requirements

Microalgae and cyanobacteria have similar requirements for growth as any other terrestrial plants, including light, water, carbon, nutrients, and appropriate temperature. During the past decades, an enormous amount of research has been conducted on each input to microalgae and cyanobacteria growth to identify any possible opportunities to

maximize biomass yield and reduce costs. In this study, light intensity and temperature were controlled and maintained at constant levels during the experimental phase.

2.5.1 Light

Microalgae and cyanobacteria require both light and dark phases to conduct photosynthesis and respiration, respectively. Photosynthesis occurs when chlorophyll captures light and converts water, carbon dioxide, and minerals into energy-rich sugars (Smith and Smith, 2006).

$$6CO_2 + 12H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O$$

Photosynthesis in microalgae and cyanobacteria relies upon the conversion of light energy into chemical energy, most notably adenosine triphosphate (ATP), and this energy conversion is only possible within the photosynthetically active wavelength spectrum, 400 nm to 700 nm (Smith and Smith, 2006).

2.5.2 Temperature

Each microalgae and cyanobacteria strain has a specific requirement for optimum temperature for maximum growth rates. The biological activities of the cells increase with the growth temperature until an optimum temperature is reached. Above the optimum temperature, biological activities decline, sometimes abruptly, to zero (Darley, 1982).

Many researches have provided optimum temperature ranges for many well-known microalgae and cyanobacteria strains (Chisti, 2007). For instance, the growth temperatures

between 25 and 33 ℃ are most acceptable for the cultivation of freshwater microalga *Chlorella vulgaris*, as is described in Figure. 2.7.

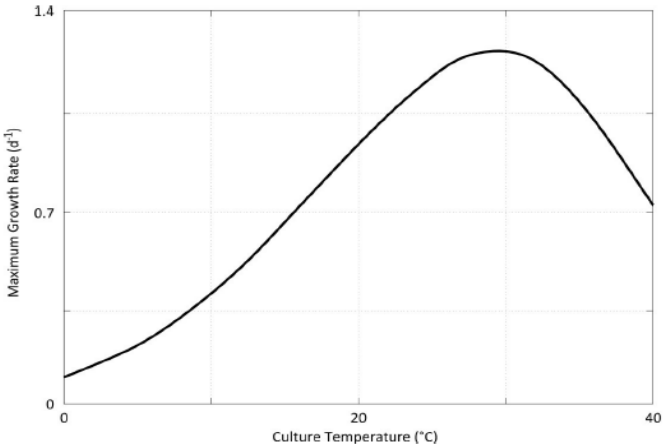

Figure 2.7. Variation of maximum growth rate with culture temperature for freshwater microalga *Chlorella vulgaris* (Adapted from Dauta et al., 1990).

2.5.3 Nutrients

Carbon is an essential nutrient for the growth of microalgae and cyanobacteria because carbon dioxide is a key ingredient driving photosynthesis. Besides carbon, the most significant nutrients are nitrogen, phosphorous, and potassium. They are all essential to the growth of microalgae and cyanobacteria as outlined in Table 2.1.

Table 2.1. Functions of each nutrient in microalgae and cyanobacteria metabolism.

Nutrients	Functions
Nitrogen	*Major metabolic importance as compounds*
Calcium	*Structural, enzyme activation, ion transport*
Magnesium	*Structural, enzyme activation, ion transport, ribosomal stability*
Phosphorus	*Structural, energy transfer*
Potassium	*Osmotic regulation, pH control, protein conformation and stability*
Sulfate	*Structural, active groups in enzymes and coenzymes*

Microalgae and cyanobacteria can assimilate both organic and inorganic nitrogen in the forms of urea, nitrate, nitrite, and ammonium. Nitrogen limitation during microalgae and cyanobacteria growth has been shown to increase lipid content within the cells, while reducing growth rates (Illman et al., 2000). Ammonium is preferred over nitrate; and in some cases, high concentrations of ammonium will inhibit nitrate uptake (Darley, 1982). Phosphorus, trace elements, and vitamins are also essential for microalgae and cyanobacteria growth. Orthophosphate is the only significant phosphorus source for microalgae and cyanobacteria production (Darley, 1982).

2.6 Cultivation Techniques

In order to maximize the cultivation potential of microalgae and cyanobacteria using minkery wastewater, three major cultivation techniques have to be studied first, including photoautotrophic growth, heterotrophic growth, and mixotrophic growth. In this study, all three cultivation techniques were performed to identify the effect of cultivation techniques on microalgae and cyanobacteria production.

2.6.1 Photoautotrophic growth

Most microalgae and cyanobacteria are commonly grown by fixing dissolved, inorganic carbon (carbon dioxide) and absorbing solar energy. As with most terrestrial plants, they perform photosynthesis and are photoautotrophs. Open ponds are the most common cultivation system for approaching photoautotrophic growth and the cheapest method for large-scale microalgae and cyanobacteria production. They are usually built

next to power plants or heavy industry with massive carbon dioxide discharge (Brennan and Owende, 2010). Open pond systems have some limitations because they require a strict environmental control to avoid the risk of pollution, water evaporation, contaminants, and invading bacteria. Temperature differences due to seasonal change cannot be controlled, and carbon dioxide concentration and excess exposure to sunlight are difficult to manage. Near the end of the exponential growth phase, most of microalgae and cyanobacteria cells are not sufficiently exposed to sunlight because other cells are floating near the surface over them, leading to low maximum biomass concentrations (Richmond et al., 1993). Using the cultivation technique of photoautotrophic growth, the maximum biomass concentrations of microalgae and cyanobacteria cultures were commonly reported ranging from 0.1 to 4g dried biomass per litre (Bhatnagar et al., 2011; De Swaaf et al., 2003).

2.6.2 Heterotrophic growth

One tremendously advantageous characteristic of certain microalgae and cyanobacteria strains is their ability to photosynthesize as well as utilize organic carbon in the absence of light (Bhatnagar et al., 2011; Cabanelas et al., 2013); therefore, light is not considered a limiting factor as either light or organic carbon substrates can support the growth (Martínez et al., 1991). In the presence of organic carbon sources, heterotrophic microalgae and cyanobacteria cultures can obtain a much higher maximum biomass concentration compared to photoautotrophic cultures (Zhang et al., 2013). Generally speaking, the maximum biomass concentration of heterotrophic microalgae and

cyanobacteria cultures could be higher than 100g dried biomass per litre (Doucha and Lívanský, 2012; Graverholt and Eriksen, 2007). As a consequence, microalgae and cyanobacteria can be grown in a stirred bioreactor or fermenter where a higher degree of growth is expected as well as low harvesting cost due to the higher maximum biomass concentration achieved and reduced energy costs of the cultivation (Liang et al., 2009). At present, carbon sources used for microalgae and cyanobacteria production are glucose, acetate, glycerol and glutamate with maximum specific growth rate obtained with glucose. Nevertheless, the major disadvantage of this heterotrophic growth system is the price and availability of those organic carbon sources.

2.6.3 Mixotrophic growth

Mixotrophic growth is the cultivation technique that microalgae and cyanobacteria make use of a combination of photoautotrophy and heterotrophy to optimize their growth when both light and organic carbons are present. This technique competes favorably with both photoautotrophic and heterotrophic growth, and many studies demonstrated higher biomass productivity under mixotrophic growth (Miao and Wu, 2004). Implementing mixotrophic cultivation is advantageous because it makes microalgae and cyanobacteria cultivation more flexible (Perez-Garcia and Bashan, 2015). Mixotrophic cultivation gather both carbon and energy demand by organic or inorganic sources and light simultaneously. Hence, microalgae and cyanobacteria cells are not strictly dependent on only light or organic substrates to grow (Safi et al., 2014).

2.7 Biomass Estimation Techniques

The methods of dry weight, optical density, cell count and chlorophyll *a* are commonly used to determine the biomass generated in the microalgae and cyanobacteria cultures. Each biomass estimation method has its pros and cons with regards to strain, culture size, growth medium, biomass concentration, cost, efficiency, desired output accuracy and precision, and time consumed. In this study, all four most recognized biomass estimation methods were used to monitor microalgae and cyanobacteria growth.

2.7.1 Gravimetric (dry weight) method

The gravimetric method of dry weight is considered one of the most accurate measurements of microalgae and cyanobacteria biomass. This technique measures the biomass directly if we assume that microalgae or cyanobacteria biomass is in the proportion of volatile suspended solids (VSS). Dry weight analysis involves a multistage process, which yields an end measurement of microalgae or cyanobacteria biomass in the unit of g/L. The major advantages of this method are the useful mass-based units, the simplicity of execution, and the inclusion of constituents based upon minimum particle size.

In the laboratory, the microalgae or cyanobacteria sample can be concentrated using multiple methods with the most popular being membrane filtration. One can pass a known volume of samples through a glass fiber filter with the aid of a vacuum pump. It should be noted that a small sample volume could introduce a significant amount of error into this measurement. In general, at least 25 mL of microalgae or cyanobacteria samples should be

used (APHA, 2005). Once completed, the concentrated wet sample will be placed in an oven at 103-105˚C to evaporate the remaining water from the concentrated sample so that only suspended particles remain, including microalgae or cyanobacteria biomass, suspended inorganic material, and suspended organic material. Once removed from the oven, the microalgae or cyanobacteria samples should be cooled in a desiccator to room temperature (20˚C), then be weighed to attain the dry weight. After this, samples will then be placed into a furnace at 550˚C. Eventually, depending on the growth medium, samples may simply be weighed to attain the ash weight (Figure 2.8), and the difference between the ash weight and the dry weight will account for the total amount of organic material (primarily microalgae or cyanobacteria biomass) present in the sample.

Figure 2.8. The ash left on the glass fiber filters.

Within microalgae and cyanobacteria research, it is an unfortunate reality that the terms dry weight and ash weight are sometimes used interchangeably to mean both the same and different things (Ji et al., 2017). Many published papers used the term dry weight

when in reality it was ash weight (Ben-Amotz et al., 1985; Chu et al., 1994; Reitan et al., 1994; Roleda et al., 2013), or performed an analysis only utilizing dry weight when ash weight would be more appropriate (Atta et al., 2013; Chevalier and De la Noüe, 1985). In this study, the microalgae and cyanobacteria were measured by the method of ash weight, even though the term dry weight was used.

2.7.2 Spectrophotometry (optical density)

The method of optical density, also known as absorbance or turbidity, is frequently used as a method for determining microalgae or cyanobacteria biomass. Using optical density as an indicator offers possibly the quickest and simplest means of quantifying the biomass accumulations (Ji et al., 2017). The optical measurements have the added benefit of being nondestructive and can easily be implemented into bioreactor systems as a means of providing constant monitoring for microalgae or cyanobacteria growth (Griffiths et al., 2011; Meireles et al., 2002; Sandnes et al., 2006).

It should be noted that there are some issues with the method of optical density. For instance, even if the incident wavelength is held constant, that is not to say that the nature of the microalgae or cyanobacteria cells is staying the same, especially when it comes to its absorbing characteristics (Ji et al., 2017). This can create a certain amount of bias when it comes to the absorbance readings. The most pertinent of this absorbing characteristic is the pigment concentration. As the growth conditions and light cycles change, so too do the pigment concentrations within the cells. For instance, the pigment content of freshwater

microalga *Chlorella vulgaris* cells varies between 0.5 and 5.5% dry weight with growth conditions and age (Griffiths et al., 2011). This is an aspect, which must be properly accounted for should spectrophotometry become a fully accurate predictor of microalgae and cyanobacteria biomass.

In the laboratory, optical density is measured through the use of the UV-Vis spectrophotometers. Wavelengths utilized range from 400 to 460 nm and 650 to 684 nm for microalgae and cyanobacteria measurements (Griffiths et al., 2011). Taking perhaps the most studied microalga *Chlorella vulgaris* as an example, maxima in the absorbance were found at wavelengths of 443, 487, and 684 nm (Griffiths et al., 2011). Other than absorbance, most UV-Vis spectrophotometers provide a reading of percent transmittance as well. It should be noted that percent transmittance is not often used in research because it does not vary linearly with concentration. This makes the development and implementation of standard curves impossible for comparing known values (Kenkel, 1988; Pitts, 2016). The most important of these circumstances is that there is some developed standard curve linking measured absorbance values to a more exact biomass estimation method (Griffiths, 2011). In this way, one can convert the measured absorbance units into more appropriate dry weight units, which are g/L and mg/L.

2.7.3 Microscopy (cell count)

For microalgae and cyanobacteria, the most basic method for biomass estimation comes in the form of cell count (Ji et al., 2017). Cell count makes use of microscopy as a

means of directly counting the number of cells within a known volume of cultures. This is done through the utilization of a hemocytometer. A hemocytometer is a specialized counting device used to help in counting microscopic cells (Salm et al., 2010). It was originally developed to aid in the counting of red blood cells and has since evolved to be applicable in a variety of fields including phycology (Aruoja et al., 2009; Berkson et al., 1939; Dragone et al., 2011; Heilmann et al., 2010; Tao et al., 2009). There are some advantages of using a hemocytometer as opposed to other counting mechanisms. The first is the ease in which a known volume can be counted (Ji et al., 2017). Provided the proper methods be followed then this volume will be specified by the particular hemocytometer which is employed. Following a thorough homogenization of the suspended cells within the microalgae or cyanobacteria culture, a Pasteur pipette can be used to transfer the culture to the hemocytometer. Once the culture is taken into the pipette, the pipette must be touched to the coverslip, and the culture must be allowed to fill the chambers by capillary action (Simu, 2016). In this way, any potential errors associated with under or overfilling of the chambers can be minimized.

The most commonly employed hemocytometer is the improved Neubauer. The improved Neubauer's central square is divided into 25 smaller squares, which are further divided into 25 smaller squares. The accepted counting pattern is to count the four corner squares within the large square along with the middle square and multiply the result by five (Simu, 2016). Figure 2.9 shows a schematic view of the improved Neubauer's counting

chamber. Squares outlined in red, are the squares, which are counted in samples where the cell counts exceed 100. The resulting count will then be multiplied by five to indicate the larger square. Should the cell count be fewer than 100, then all squares can be counted for the most accurate measurement (Simu, 2016).

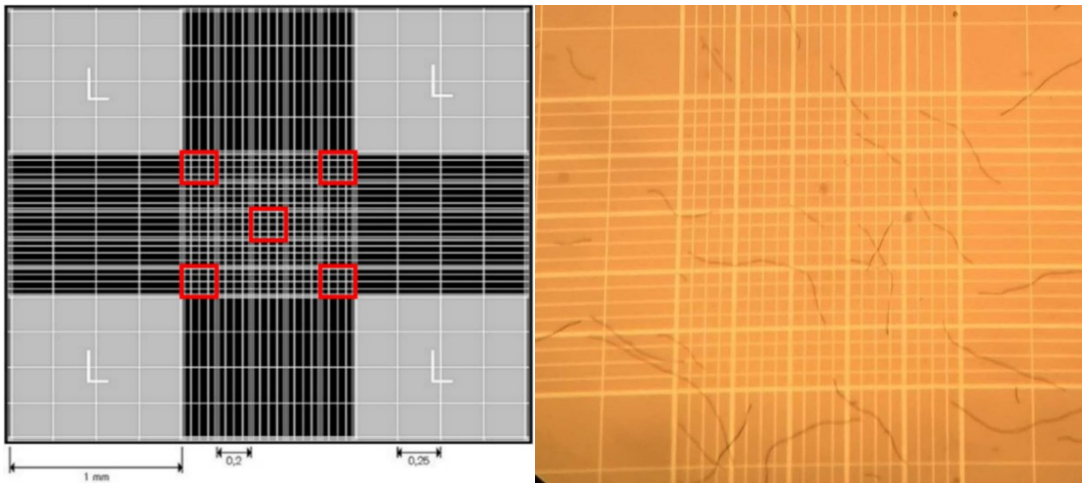

Figure 2.9. Schematic view of the improved Neubauer's counting chamber (edited to show general counting pattern in samples of more than 100 cells) (left) and counting *Anabaena sp.* (right).

It is important to note that hemocytometers hold a three-dimensional volume despite the fact that they appear two-dimensional under the microscope (Salm et al., 2010). In this way, the number of cells per mL can easily be determined based on a quick calculation. For example, each large square holds 10^{-4} mL of culture solution with the improved Neubauer. Therefore, based on the cell count, the number of cells per litre can be quickly determined (Simu, 2016; Sigma-Aldrich, 2016). Last but not least, the method of cell count requires a microscope with a minimum of 100 times magnification for microalgae or cyanobacteria applications (Ji et al., 2017).

2.7.4 Chlorophyll content (chlorophyll a)

Chlorophyll *a* is the predominant pigment in microalgae and cyanobacteria, and as a result of the ever-changing chlorophyll concentration, another employable technique, which aims to combat this, is that of chlorophyll *a* content analysis. The greatest advantage of this approach is that it directly targets the biomass. However, a substantial margin of error exists in the underlying assumption about the chlorophyll *a* concentration, because it is highly variable in microalgae and cyanobacteria cells.

The first step in analyzing chlorophyll *a* concentration is the pigment extraction. It is imperative that throughout the extraction process the cultures are kept in the dark. Light levels during this process should be maintained to a minimum as exposure to electromagnetic radiation will cause a deterioration and loss of chlorophyll (APHA, 2005). Once the pigment has been extracted, there are three analytical instrumentations that can be used to determine the chlorophyll *a* content (Ji et al., 2017).

The first of these instrumentations is spectrophotometry. A detailed explanation of spectrophotometry is described in the previous section. Also, the fluorometric approach can also be used, and in fact often yield more sensitive results than spectrophotometric determinations (APHA, 2005). Last but not least, the high-performance liquid chromatography (HPLC) is perhaps the most effective instrumentation in the accurate determination of chlorophyll *a* content. With that being said, with greater accuracy comes a more complex analytical process along with a greater time and economic cost. As such,

HPLC is only used when one wishes to determine the complete pigment profile and not only for biomass determination (Ji et al., 2017).

Regardless of the employed method, the resulting measurement is a chlorophyll *a* content. A conversion must be performed to determine microalgae and cyanobacteria biomass. The standard method uses the assumption that chlorophyll *a* makes up 1.5% dry weight within the microalgae and cyanobacteria cells (APHA, 2005). The obvious problem is that not all microalgae and cyanobacteria strains are comprised of 1.5% chlorophyll *a* by dry weight, and the estimate may be very rough or entirely false in many cases. Therefore, this method must be adapted to the particular strain being studied along with the specific set of growth conditions. From this point, the method of chlorophyll *a* content could be used for microalgae and cyanobacteria biomass estimation.

2.8 Harvesting Techniques

With the increased demand of microalgae and cyanobacteria biomass comes to an equivalent increase in production and production practices. Harvesting remains one of the most important and challenging practices of production. A variety of conventional harvesting technologies currently available for microalgae and cyanobacteria production, such as membrane filtration, centrifugation, flocculation, air flotation, immobilization, magnetic separation, ultrasound wave, and even a combined harvesting system. In this study, only techniques of membrane filtration and centrifugation were performed to harvest the biomass of microalgae and cyanobacteria.

2.8.1 Membrane filtration

Membrane filtration is one of the most common harvesting techniques used for microalgae and cyanobacteria production. Membranes provide a thin physical barrier that selectively restricts the passage of solvents and solutes, depending on membrane characteristics and properties of solvents and solutes (Drexler and Yeh, 2014). The pore size of membranes governs which constituents can pass through, and the rejection of solutes by macrofiltration, microfiltration, ultrafiltration, nanofiltration and reverse osmosis (also known as hyperfiltration) is described below (Figure 2.10).

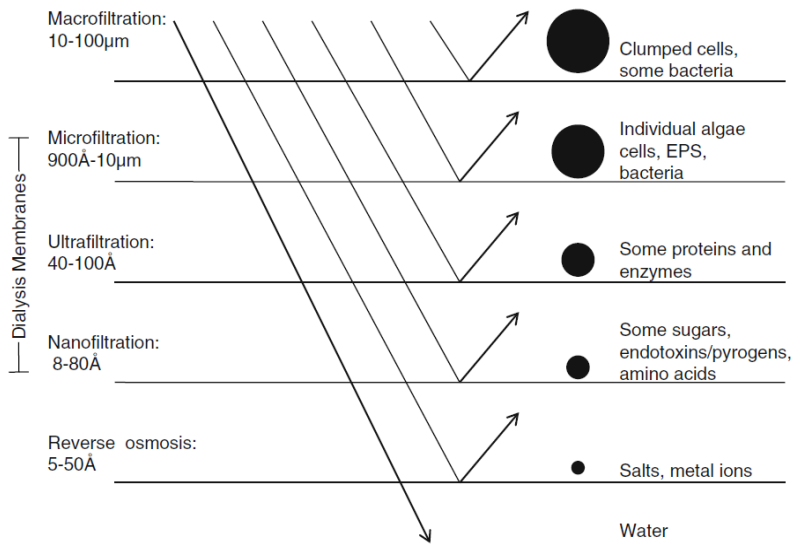

Figure 2.10. Filtration spectrum based on membrane pore size (Adapted from Drexler and Yeh, 2014).

The main advantages of membrane filtration are high harvesting efficiency, continuous operation, and no chemical requirement in the process (Khademi et al., 2015). When it comes to the production of the bioproducts, it provides another advantage by enabling the use of the recycled growth medium for continuous cultivation (Chen et al.,

2011). In the laboratory, membrane filtration is a cost-efficient technique, but membrane fouling may be the critical restriction and principal challenge in large-scale filtration system (Grima et al., 2003).

Fouling propensity can be mitigated with proper membrane selection and operation system. Membrane selection has two components, which including pore size and membrane materials. Two studies concluded that comparing all pore sizes, those membranes with a pore size range from 40 kDa to 100 kDa (about the range of ultrafiltration membranes) were recommended for long-term operation (Rossi et al., 2004; Rossignol et al., 1999). Another study found a similar result, ultrafiltration membranes showing a better fouling resistance than microfiltration membranes (DeBaerdemaeker et al., 2013). Common membrane materials include cloth and nonwoven fabric for macrofiltration; polyvinylidene fluoride, polyacrylonitrile, polyethersulfone, and polysulfone for microfiltration and ultrafiltration; cellulose acetate and polyamide for nanofiltration and reverse osmosis (Drexler and Yeh, 2014). One study concluded that polyvinylidene fluoride membranes maintained the highest permeability compared to other membrane materials after critical fouling resistance tests (Rossi et al., 2004).

Proper operation system can help minimize membrane fouling as well. Operation system also has two principal components, which including membrane modules and flow configurations. There are seven common membrane modules (Figure 2.11) including the string wound cartridge, pleated sheet cartridge, spiral-wound element, dialysis tubing, flat

sheet, hollow fiber, and tubular (Bilad et al., 2014). Selecting proper membrane modules are mainly depending on the pore size selection of membrane, which already discussed in the previous paragraph. There are two typical flow configurations for membrane filtration, which are passive filtration and active filtration (Figure 2.11).

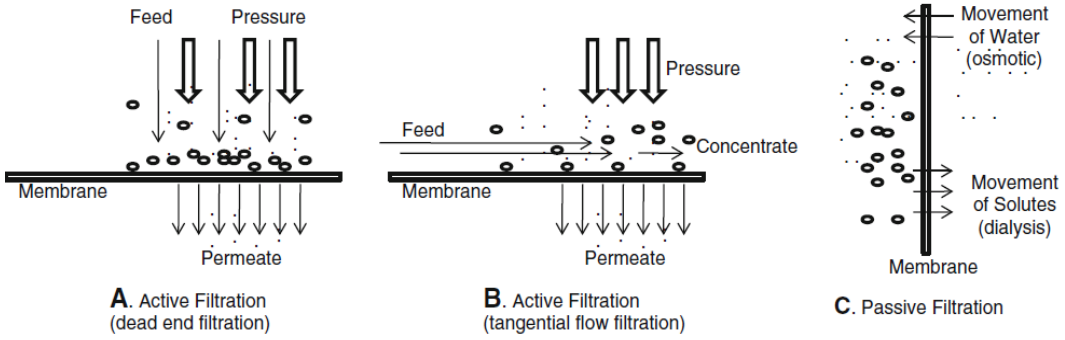

Figure 2.11. Various flow configurations for membrane filtration (Adapted from Drexler and Yeh, 2014).

Passive filtration, such as dialysis or forward osmosis, relies on the movement of a solute or solvent across the membrane as a result of concentration gradients (Drexler and Yeh, 2014). Passive filtration systems are particularly attractive due to their lower energy costs; however, solutions that contain divalent magnesium ions can cause severe and irreversible fouling due to reverse diffusion (Zhou et al., 2013).

Active filtration is characterized by a pressure gradient, where the solute is selectively rejected, and the filtrate is either drawn across the membrane with positive feed-side pressure, negative permeate-side pressure, or even both (Bilad et al., 2013). Active filtration includes dead-end filtration and tangential flow filtration. Dead-end filtration is typically more successful when filtering low concentration microalgae and cyanobacteria

solutions, as fouling can occur quickly. Tangential flow filtration can create shear due to the parallel movement of the feed flow. Maintaining a high fluid velocity bubbling across the membrane surface can reduce fouling potential (Rossi et al. 2008). Compared with other operating systems, it reduces fouling potential the most by shearing the membrane (Bilad et al., 2013).

Overall, membrane filtration is an advantageous harvesting technique, even though membrane fouling remains a challenge to large-scale harvesting and continued research in system configuration and design may make it more competitive with other technologies.

2.8.2 Centrifugation

Centrifugation is another common technique used either on its own or as a second step for harvesting microalgae and cyanobacteria biomass from dilute suspension. Continuous-flow centrifuge systems allow microalgae and cyanobacteria cultures to be pumped continuously through the bowl assembly, forcing cells to the wall while clarified growth medium passes through the overflow (Rees et al., 1991). The centrifuge was switched on and allowed to reach its maximum speed before inflow was initiated. The cultures from the secondary sump were drawn by a rotary vane pump connected to the stem on top of the centrifuge bowl. Flow rates varied using the flow regulator, and water is leaving the outlet was allowed to drain to the ground (Figure 2.12). Compared with other techniques, centrifugation is the most efficient technique to recover microalgae and cyanobacteria without chemical and bacterial contamination (Sim et al., 1988).

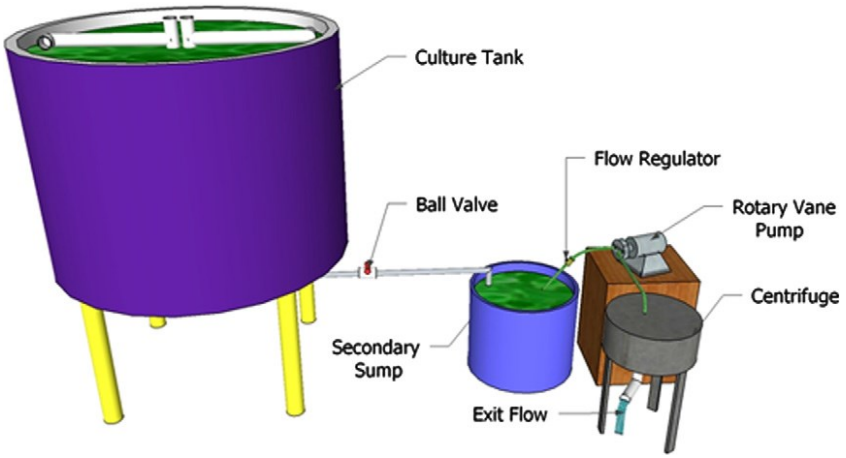

Figure 2.12. A typical setup for continuous-flow centrifuge system (Adapted from Dassey and Theegala, 2013).

High harvesting efficiency and no chemical requirement in the process are the two most significant advantages of this technique (Khademi et al., 2015). One study observed that the harvesting efficiency of microalga *Chlorella vulgaris* varied from 66.66 to 99.6% by using centrifugation and the maximum harvesting efficiency was observed with 7000 rpm (Xavier et al., 2014). Another study also reported that the harvesting efficiency is evident with 84% of 0.2 g/L culture at a flow rate of 379 L/min under a rotational velocity of 3000 rpm (Kothandaraman and Evans, 1972).

The energy requirement of centrifugation still has an adverse impact on the process of biomass harvesting (Chen et al., 2011). The use of centrifugation for harvesting microalga *Chlorella vulgaris* (0.04 to 4g of dried biomass per liter on average) costs 1.3 kW h/m3 of culture water (Sim et al., 1988). In comparison, techniques such as membrane filtration, which are capable of consuming 0.25 kW h/m3 of culture water, would appear to be more suitable for biomass harvesting (Bilad et al., 2012). At present, the harvesting

technique of centrifugation may be feasible for high-value bioproducts but is far too costly in an integrated system producing lower-value products, such as biofuels (Pienkos and Darzins, 2009).

2.9 Cell Disruption Techniques

Both microalgae and cyanobacteria cells have a resistant cell wall, which is a major barrier for extraction process of the main chemical components. Breaking the cell wall is one of the most significant challenges that require a thorough understanding of the ultrastructure and composition of the cell wall to select the appropriate cell disruption technique and to improve the cell disruption efficiency. Depending on the microalgae and cyanobacteria strains and on the product nature to be obtained, 12 different techniques are commonly used for cell disruption, namely acid treatment, alkaline treatment, autoclaving, bead milling, electroporation, enzymatic lysis, French press, high-pressure homogenizer, manual grinding, microwaves, osmotic shock, and ultrasonication (Lee et al., 2010; Zheng et al., 2011). In short, the cell disruption is done by either mechanical action based or non-mechanical action based techniques. During the mechanical action based cell disruption, a cooling system is always required, because the high-energy input of the process can overheat the broken microalgae or cyanobacteria cells and compromises the integrity of target components by damaging or oxidizing them. During the non-mechanical action based cell disruption, the quality of the target molecules is most likely to be different from the cell disruption technique applied. For example, the amino acid profile of proteins

obtained after conducting alkaline treatment on microalgae or cyanobacteria is distinct from the amino acid profile obtained after high-pressure homogenizer (Safi et al., 2014). In this study, acid treatment, autoclaving, and manual grinding were performed before the extraction of lipid, protein, and carbohydrate.

2.10 Primary Composition

The main chemical components of microalgae and cyanobacteria cells were proteins, lipids, and carbohydrates. Some microalgae and cyanobacteria strains can have significant differences in these main chemical component through simple manipulations of the cultivation techniques (Behrens and Kyle, 1996).

2.10.1 Proteins

Proteins are of central importance in the chemistry and composition of microalgae and cyanobacteria cells. Total proteins content in most microalgae and cyanobacteria ranges from 42 to 58% of dry biomass (Safi et al., 2014). They are involved in principal roles such as growth, repair, and maintenance of the cell as well as serving as cellular motors, chemical messengers, regulators of cell activities and defense against foreign invaders (Solomon et al., 1999).

Protein nutritional quality is determined by its amino acid profile (Becker, 1994; Safi et al., 2013), and the amino acid profile of the majority of microalgae and cyanobacteria compares favorably and even better with the standard profile for human nutrition proposed by World Health Organization (WHO) and Food and Agricultural Organization (FAO),

because the cells of microalgae and cyanobacteria synthesize essential and non-essential amino acids. Regardless of the extraction procedure, microalgae and cyanobacteria proteins showed excellent emulsifying capacity (Ursu et al., 2014) that is comparable and even better than the commercial ingredients. Therefore, microalgae and cyanobacteria proteins open the gate for additional valorization options in the market, especially in the food sector (Safi et al., 2014).

Protein extraction is technically the same for all microalgae and cyanobacteria and is mainly conducted by solubilization of proteins in alkaline solution (Bajguz, 2000; Rausch, 1981). Further purification can be followed by precipitating the solubilized proteins with trichloroacetic acid (Barbarino and Lourenço, 2005) or hydrochloric acid (Oliveira et al., 1999). Quantification is carried out by elemental analysis, Kjeldahl, Lowry assay, Bradford assay or the dye binding method.

The first two analyses take into consideration total nitrogen present in the microalgae, and multiplying it by the standard nitrogen to protein conversion factor (NTP) 6.25 may lead to overestimation or underestimation of the correct protein quantity. The Lowry assay is more acceptable than the Bradford assay because the latter does not react with all the amino acids present in the extract, thus giving lower protein concentrations. The colorimetric method of Lowry was also considered as one of the most accurate methods to quantify proteins, but with time this method showed only to quantify hydrosoluble proteins, which represents the major part of proteins.

2.10.2 Lipids

Lipids are a heterogeneous group of compounds that are defined not by their structure but rather by the fact that they are soluble in nonpolar solvents and relatively insoluble in water (Bajguz, 2000). During favorable growth conditions, most microalgae and cyanobacteria can reach 5 to 40% lipids per dry weight of biomass (Becker, 1994) and are mainly composed of glycolipids, waxes, hydrocarbons, phospholipids, and small amounts of free fatty acids (Hu et al., 2008; Lee, 2008). These components are synthesized by the chloroplast and also located on the cell wall and membranes of organelles. Nevertheless, during unfavorable growth conditions, lipids content (mainly composed of triacylglycerols) can reach up to 58% for many microalgae and cyanobacteria (Becker, 1994; Mata et al., 2010; Stephenson et al., 2009). Unlike other lipids, triacylglycerols do not perform a structural role but instead accumulate as dense storage lipid droplets in the cytoplasm and the inter-thylakoid space of the chloroplast (Hu et al., 2008).

The fatty acid profile changes on growth conditions and is suitable for different applications. For instance, according to one study, the fatty acid profile of freshwater microalga *Chlorella vulgaris* grown under mixotrophic and heterotrophic conditions contain about 60 to 68% saturated and monounsaturated fatty acids (Yeh and Chang, 2012). Such a fatty acid profile is more suitable for biodiesel production (Yeh and Chang, 2011). On the contrary, if it is grown under photoautotrophic conditions, its fatty acid profile is

unsuitable for biodiesel but more appropriate for nutritional uses because it is more concentrated in polyunsaturated fatty acids (Stephenson et al., 2009).

The lipid extraction process from microalgae and cyanobacteria is conducted by the method of Bligh and Dyer, or by hexane, or petroleum ether (Safi et al., 2014). Quantification of total lipids is conducted gravimetrically after evaporating the extracting solvent. Column chromatography is carried out to separate different lipid constituents followed by evaporating the solvent and then weighing the remaining lipid extract (Olmstead et al., 2013). Total lipids are composed of three major fractions, which is phospholipids, glycolipids, and neutral lipids. These fractions are fractionated by sequential elution of chloroform and acetic acid for neutral lipids, acetone, and methanol for glycolipids, and methanol for phospholipids recovery (Olmstead et al., 2013).

2.10.3 Carbohydrates

Carbohydrates represent a group of reducing sugars and polysaccharides such as starch and cellulose. During nitrogen limitation, most microalgae and cyanobacteria can reach 12 to 55% total carbohydrates per dry weight of the biomass (Branyikova et al., 2011; Choix et al., 2012). Starch is the most abundant polysaccharide in most microalgae and cyanobacteria. It is located in the chloroplast and is composed of amylose and amylopectin, and together with sugars, they serve as energy storage for the microalgae and cyanobacteria cells. Cellulose is a structural polysaccharide with high resistance, which is located on the cell wall of microalgae and cyanobacteria as a protective fibrous barrier. One of the most

valuable polysaccharides present in many microalgae and cyanobacteria is the $\beta 1 \rightarrow 3$ glucan, which has many health and nutritional benefits for humans (Lordan et al., 2011).

Total carbohydrates of microalgae and cyanobacteria can be quantified by the phenol-sulfuric acid method (DuBois et al., 1956; Shi et al., 2007b), yielding simple sugars after hydrolysis at 110℃, then quantification by HPLC (Shi et al., 2007b). Starch quantification is better using the enzymatic method compared to the acidic method (Dragone et al., 2011; Fernandes et al., 2012).

2.11 Applications and Potential Interest

Applications of microalgae and cyanobacteria are numerous, ranging from bioproduct to human nutrition to animal feed and agrochemical applications (Breuer et al., 2013; Raposo et al., 2013).

2.11.1 Biofuel

Crop-based 'first generation' biofuel systems are already extensively used for the biofuels production in Brazil, USA, Southeast Asia, and Europe (Keeler et al., 2013, Li et al., 2013). Increasing pressure on food supplies has led to a heated 'food and land versus fuel' debate (Frank et al., 2013). In contrast, microalgae and cyanobacteria based 'third generation' biofuel systems are considered as promising alternatives to current biofuel crops such as soybean, corn, rapeseed and lignocellulosic feedstocks because it does not compete with food and does not require arable lands to grow (Singh et al., 2011). Microalgae and cyanobacteria also compete favorably with crops by their potential of

producing a volume of biomass hundreds of times greater. For instance, microalga with a lipid content of 30% can produce over 300 times more biofuel than corn on a per weight basis (Chisti, 2007).

2.11.2 Wastewater treatment

Many studies demonstrated the extraordinary wastewater treatment potential of microalgae and cyanobacteria cultivation in absorbing nitrogen by 45 to 97%, and phosphorus by 28 to 96% and in reducing 61 to 86% of the chemical oxygen demand (*COD*) from municipal, textile, sewage, recalcitrant, and agricultural wastewaters (Safi et al., 2014). In our previous study, freshwater microalga *Chlorella vulgaris* cultivated in municipal wastewater achieved approximately 60% nitrogen removal rate and 90% phosphorus removal rate in 6 days, and freshwater cyanobacterium *Anabaena sp.* even achieved over 80% nitrogen removal rate through 6-day of cultivation in municipal wastewater. Saving and requirements for nutrients remediation and minimization of freshwater use are the main drivers for growing microalgae and cyanobacteria as part of a wastewater treatment process (Brennan and Owende, 2010).

CHAPTER III

MATERIALS AND METHODS

3.1 Strain Selection and Culture Maintenance

Two strains used in this study, namely *Chlorella vulgaris* and *Anabaena sp*., were purified and inoculated in 1 L Erlenmeyer flasks at approximately 20˚C and illuminated with a cool-white fluorescent light (32 W, 6500K) with a light cycle of 16-hour light and 8-hour dark. Both flasks were sealed with a plug, allowing for aseptic gas exchange and placed on a mechanical stirrer (HI 190, Hanna Instruments, U.S.A.) with 150 rpm rotation (Figure 3.1).

Figure 3.1. Cultures of *Chlorella vulgaris* (left) and *Anabaena sp.* (right).

Table 3.1 summarizes the nutrient content of these two traditional culture mediums. Both strains and mediums were purchased from the Canadian Phycological Culture Centre (CPCC), Department of Biology, University of Waterloo, Waterloo, Ontario, Canada.

Chlorella vulgaris was cultured in a modified Bold's basal medium, and *Anabaena sp.* was grown in BG-11_o medium modified by J. Acreman.

Table 3.1. Nutrient concentrations of two traditional culture mediums.

Nutrients	Concentration (mg/L)	
	Bold's basal	BG-11_o
$NaNO_3$	*250*	*0*
$MgSO_4 \cdot 7H_2O$	*75*	*75*
$CaCl_2 \cdot 2H_2O$	*25*	*36*
K_2HPO_4	*75*	*30*
$Na_2EDTA \cdot 2H_2O$	*10*	*1*
H_3BO_3	*10.91*	*2.86*
$MnCl_2 \cdot 4H_2O$	*1.81*	*1.81*
$ZnSO_4 \cdot 7H_2O$	*0.222*	*0.222*
$Na_2MoO_4 \cdot 2H_2O$	*0.390*	*0.390*
$CuSO_4 \cdot 5H_2O$	*0.079*	*0.079*
$Co(NO_3)_2 \cdot 6H_2O$	*0.0494*	*0.0494*
KH_2PO_4	*175*	*0*
KOH	*6.2*	*0*
$FeSO_4 \cdot 7H_2O$	*4.98*	*0*
NaCl	*25*	*0*
Na_2CO_3	*0*	*20*
Ferric Ammonium Citrate	*0*	*6*
Citric Acid	*0*	*6*

3.2 Pretreatment of Minkery Wastewater

In this study, the raw minkery wastewater (Cage-washing wastewater) was obtained from the Dalhousie Fur Animal Research Centre in Bible Hill, Nova Scotia. All minkery wastewater samples were taken on the same day to ensure homogeneity amongst samples. Eventually, the raw minkery wastewater was frozen and then thawed as needed to discourage biological activity.

The raw minkery wastewater collected had an extremely high concentration of some major nutrient, and these nutrient concentrations were higher than what would normally be needed for microalgae or cyanobacteria cultivation. Therefore, the dilution of raw minkery wastewater should be employed to provide an optimum nutrient content for *Chlorella vulgaris* and *Anabaena sp.* cultivations. A number of pre-tests were employed to investigate the optimal minkery wastewater concentration for *Chlorella vulgaris* and *Anabaena sp.* cultivations (Figure 3.2). Both strains were cultivated in various concentrations of diluted minkery wastewaters under the light cycle of 16-hour light and 8-hour dark, and it demonstrated that the most advantageous minkery wastewater concentration for *Chlorella vulgaris* cultivation ranges from 1% to 3%, and the most favourable concentration for *Anabaena sp.* cultivation was between 0.5% and 1%. As a consequence, in this study, the raw minkery wastewater was diluted with 99% autoclaved distilled water before each experimental unit.

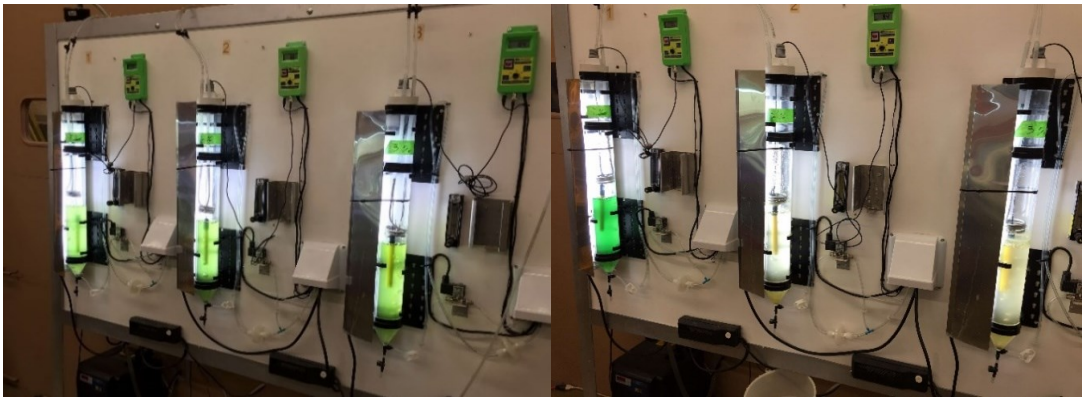

Figure 3.2. *Chlorella vulgaris* (*left*) and *Anabaena sp.* (*right*) cultivated in the 1, 2, and 3% pre-treated minkery wastewaters, respectively.

After dilution, the minkery wastewater was thoroughly homogenized and filtered through a filter cloth to remove large solid particles. Then, the minkery wastewater was filtered one more time using 1.5 um glass microfiber filters (691, VWR, UK). In this way, all side effects of solid particles and indigenous bacteria in minkery wastewater on the cultivation of microalga *Chlorella vulgaris* and cyanobacterium *Anabaena sp.* were eliminated. After filtration, the minkery wastewater was autoclaved at a sustained temperature of 121°C and 15 psi for 20 minutes. Eventually, this pre-treated minkery wastewater was stored at 4°C in a refrigerator and prepared for the experiment.

3.3 Cultivation Equipment

For each experimental unit, cultures were transferred from Erlenmeyer flasks to three transparent plastic cylinders (Aqua Medic GmbH, Plankton Reactor, Bissendorf, Germany) which were 2.25-litre vertical column controlled environment photobioreactors (PBR). These three photo-bioreactors essentially served as a holding tank, allowing microalgae or cyanobacteria cultivation and wastewater treatment (Figure 3.3). Three photo-bioreactors were employed under three independent cool-white fluorescent lights (8 W, 6700K) at 20˚C. Each fluorescent light was able to provide enough light for photosynthesis to occur. Three independent ambient air pumps (Fusion Quiet Power, 400, Taiwan) were used to continuously agitate the solution of each photo-bioreactor to keep the microalgae or cyanobacteria culture homogeneous. Injecting air into the aqueous solution forms carbonic acid, thus lowering the pH. Consequently, three independent pH meters (Milwaukee, SMS

122, Romania) continuously monitored the pH of the microalgae and cyanobacteria solutions. They were coupled with carbon dioxide control valves (Red Sea, CO_2 Magnetventil, Israel), which acted as the gas dosing solenoid, in essence maintaining a constant pH level by supplying the proper amount of carbon. Therefore, the pH was in constant balance between carbon fixation and air injection. Last but not least, daily monitoring of temperature was performed to ensure no adverse effects on the cultivation. Overall, combining this set of cultivation equipment allowed the major growth conditions to be controlled and maintained at constant levels during the experimental phase.

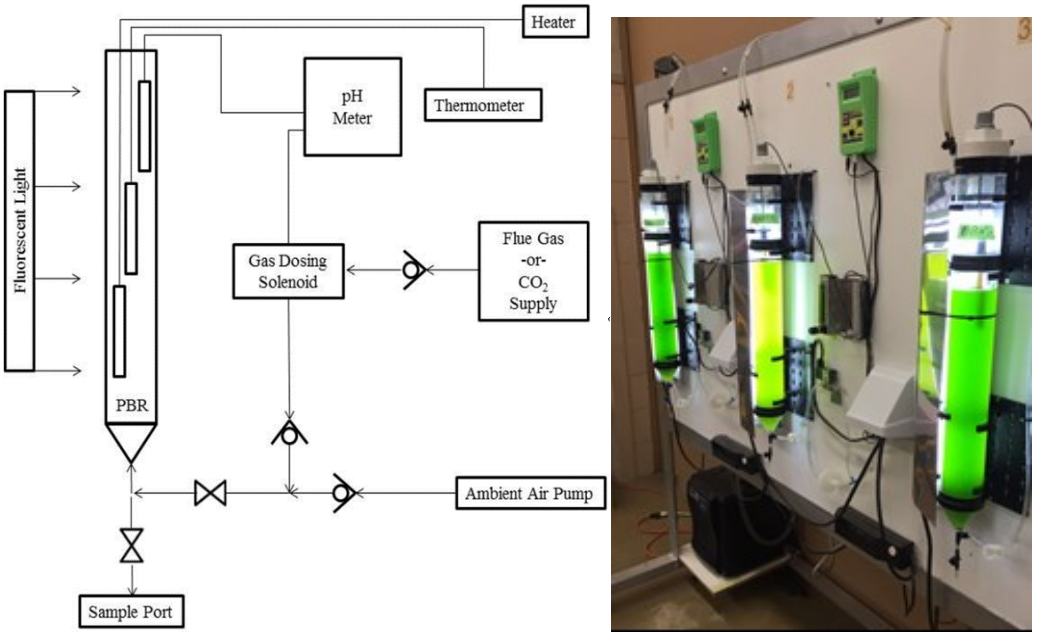

Figure 3.3. Laboratory PBR schematic (left) and Laboratory PBRs in use (right).

3.4 Experimental Conditions

Each experimental unit was limited to 6 days of cultivation. Once all three photo-bioreactors were inoculated, the growth period began. For each photo-bioreactor, exactly

400 mL purified *Chlorella vulgaris* or *Anabaena sp.* inoculum (0.3-0.4 g/L) (20%), and 1600 mL of certain medium (80%) were inoculated. As mentioned above, these mediums were minkery wastewater, traditional culture mediums (modified Bold's basal medium / $BG\text{-}11_o$ medium), and distilled water. Upon analyzing cultures at the end of the growth period, all three photo-bioreactors were taken apart, sanitized carefully, and put back together for the proceeding experimental units.

For each experimental unit, one of four light cycles was employed, including 6-day continuous light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuous dark. For 6-day continuous light, photo-bioreactors were placed under fluorescent light for six days. For 6-day continuous dark, three photo-bioreactors were wrapped with aluminum foil for six days. For 48-hour light and 24-hour dark, photo-bioreactors were wrapped with aluminum foil at days 3 and 6. For 24-hour light and 48-hour dark, photo-bioreactors were wrapped with aluminum foil at day 2, 3, 5, and 6.

3.5 Experimental Design

In some experiments, researchers may be unable to randomize the order of the runs completely. This results in a generalization of the factorial design called a 'split-plot design'. The concept of split-plot designs can be extended to situations in which randomization restrictions may occur at any number of levels within the experiment.

In this study, there were two levels of randomization restrictions which were strains and light cycles. Within a replicate, the experiment was better performed by employing

one particular strain first only to prevent the contamination between two strains. Within a strain, once a particular light cycle was employed, all three growth mediums had to be tested at that light cycle because of the limitation of cultivation equipment (Figure 3.4). Because of these two levels of randomization restrictions, the design used was called a 'split-split-plot design'.

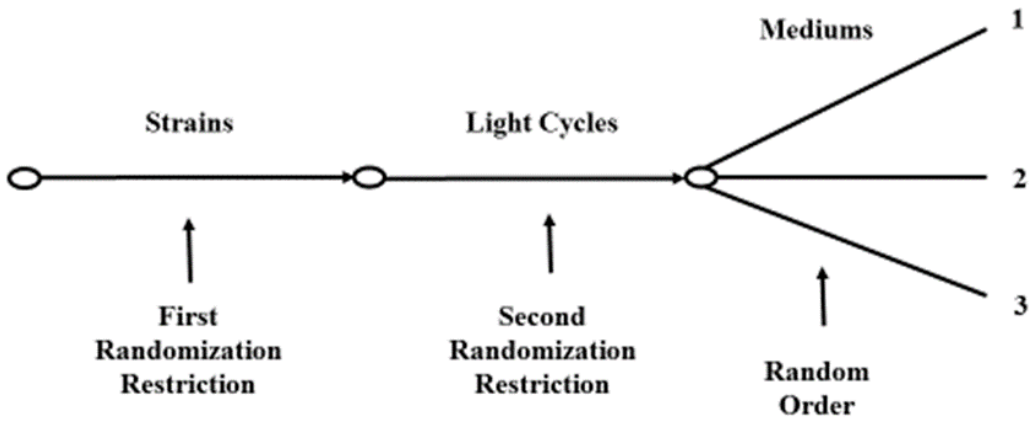

Figure 3.4. Randomization restrictions and experimental layout in this design.

A split-split-plot design was used to arrange each experimental unit and illustrated in Table 3.2. The whole plots corresponded to the strains, the order in which the strains were employed first was randomly determined. The light cycles form four subplots. Each light cycle was randomly assigned to a subplot. Within a particular light cycle, the three growth mediums were tested in random order, forming three sub-subplots. In total, this statistical design had twenty-four different treatments, and three replications for each treatment were used to ensure the adequate integrity of findings. Eventually, all statistical analyses were performed using a combination of the *Minitab 17* and *SAS* software.

Table 3.2. The split-split-plot design used in this study.

		Strains							
		1				*2*			
Replicates	Light cycles	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*
1	Mediums	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
		2	*2*	*2*	*2*	*2*	*2*	*2*	*2*
		3	*3*	*3*	*3*	*3*	*3*	*3*	*3*
2	Mediums	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
		2	*2*	*2*	*2*	*2*	*2*	*2*	*2*
		3	*3*	*3*	*3*	*3*	*3*	*3*	*3*
3	Mediums	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*
		2	*2*	*2*	*2*	*2*	*2*	*2*	*2*
		3	*3*	*3*	*3*	*3*	*3*	*3*	*3*

Whole plots (Strains): *Chlorella vulgaris* (1) and *Anabaena sp.* (2)

Subplots (Light cycles): All light (1), 48h light + 24h dark (2), 24h light + 48h dark (3), and all dark (4)

Sub-subplots (Mediums): Minkery wastewater (1), traditional culture mediums (2), and distilled water (3)

Note that the table does not show any randomization used in the study

3.6 Quantification of Growth Kinetics

In this study, four biomass estimation methods were used to determine biomass generated in microalgae and cyanobacteria cultivation, including dry weight, optical density, cell count and chlorophyll *a*. Each method has an ideal sampling condition with regards to strain selection, culture size, growth medium, and desired output accuracy and precision. Methods of optical density and cell count were performed every 12 hours, while dry weight and chlorophyll *a* content methods were carried out at the beginning and end of each growth period to determine the biomass generated in the microalgae and cyanobacteria cultures.

Once these measurements were taken, biomass increase in the percentage of each growth period was calculated using the following Equation 3.1:

$$Biomass\ \ increase = \left(\frac{x_6 - x_0}{x_0}\right) \times 100\% \qquad 3.1$$

Where:

x_0 = *biomass at the beginning of each growth period (day 0)*

x_6 = *biomass at the end of each growth period (day 6)*

3.6.1 Dry weight

The gravimetric method of dry weight is an intuitive method that directly measured the biomass of microalgae and cyanobacteria. In this study, dry weight of the samples was measured from the triplicate averages of volatile suspended solids (VSS) obtained using membrane filtration according to the Standard Methods for the Examination of Water and Wastewater, Method 2540 (APHA, 1998). The detailed procedure is as follows:

1) Prepare three 0.4 um glass microfiber filters (GB-140, ADVANTEC, Japan) by rinsing with distilled water under Millipore 47mm vacuum filter assembly (Figure 3.5) until all water is pulled through the filter.
2) Place three filters in three aluminum foil containers and bake in the furnace (550°C) for approximately 15 minutes.
3) Remove three filters with a container from furnace and place in bell jar desiccator until the sample reaches room temperature.

4) Weigh three filters with the container and record weight (A).

5) A filter uniform aqueous sample of known volume (V) through the filter under vacuum filter assembly.

6) Return three filters to the corresponding container.

7) Place three filters with the container in the oven at 103-105°C for 1 hour.

8) After baking removes from oven and allows cooling in a desiccator.

9) Re-weigh three filters with dry residue and container and record weight (B).

10) TSS is then calculated using Equation 3.2 below:

$$TSS = \frac{B-A}{V} \quad 3.2$$

11) Place three filters with dry residue and container in a furnace at 550°C for 5 minutes.

12) After ignition removes from the furnace and allows cooling in a desiccator.

13) Re-weigh three filters with ash residue and container and record weight (C).

14) VSS can be calculated using Equation 3.3 below:

$$VSS = \frac{B-C}{V} \quad 3.3$$

Figure 3.5. Millipore 47mm vacuum filter assembly.

3.6.2 Optical density

Optical density is frequently used as a rapid measurement for biomass estimation of microalgae and cyanobacteria. In this study, the optical density of microalga *Chlorella vulgaris* was measured by a UV-Vis spectrophotometer (Cole-Parmer, USA) at 684 nm wavelength (Griffiths et al., 2011) with a light path of 10 mm, and the optical density of cyanobacterium *Anabaena sp.* was measured with a light path of 10 mm at 683 nm wavelength (Yoon et al., 2002) using the UV-Vis spectrophotometer (Figure 3.6).

Before each measurement, the readings of the UV-Vis spectrophotometer were blanked by a purpose-made sample, which had traditional mediums (modified Bold's basal medium for *Chlorella vulgaris* samples; BG-11_0 medium for *Anabaena sp.* samples) instead of microalgae or cyanobacteria cultures. As an example, blanks for measuring minkery wastewater samples contained exactly 80% minkery wastewater and 20% traditional mediums.

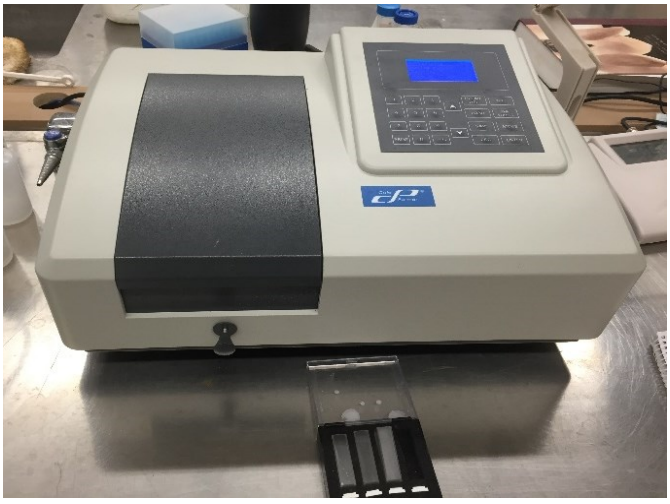

Figure 3.6. Cole-Parmer UV-Vis spectrophotometer.

3.6.3 Cell count

Cell count is another widely used method for estimating microalgae and cyanobacteria biomass. In this study, a Bright-Line™ hemocytometer was used to count the number of cells under the microscope (Motic, BA310) (Figure 3.7) and has a counting chamber defined by a known depth and a grid with the known surface area. The counting method was taken from Standard Methods for the Examination of Water and Wastewater, Method 10200E and 10200F (APHA, 1998). Before each sample was loaded for counting the hemocytometer, the coverslip was rinsed with distilled water and dried via lens paper. Ensuring the hemocytometer and the coverslip was clean, the sample was ready to be loaded. After mixing the sample well, a sterile Pasteur pipet was used to dispense the sample into the counting chamber. Caution was taken upon injecting the sample into the counting chamber because if the sample spills over the grid surface, the chamber volume becomes compromised and the process must be repeated.

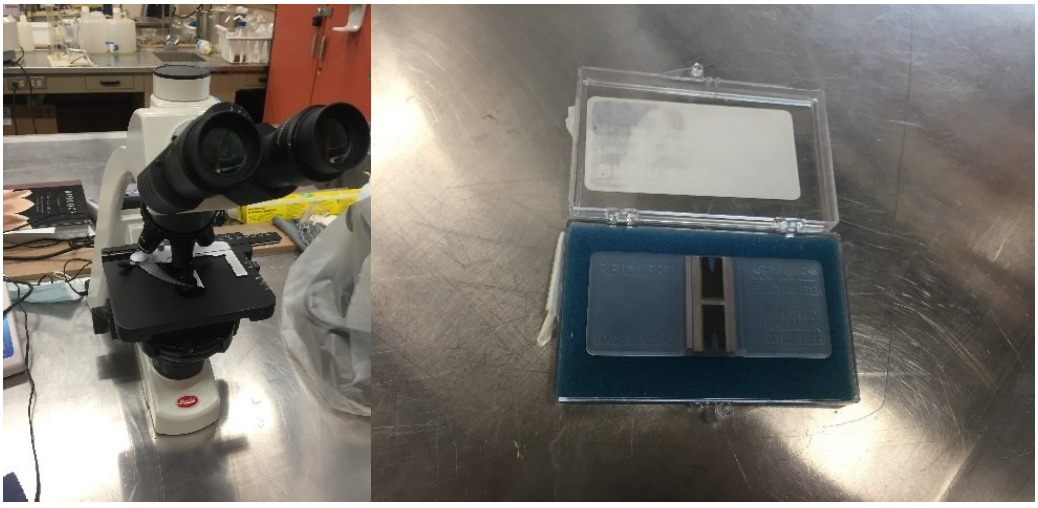

Figure 3.7. Motic BA310 microscope (left) and Bright-Line™ hemocytometer (right).

3.6.4 Chlorophyll a

Chlorophyll a is regularly used as an estimator of microalgae and cyanobacteria biomass. In this study, chlorophyll *a* content of the samples was measured according to the standard method (ESS, 1991). The detailed procedure is as follows:

1) Place the filter containing the concentrated microalgae or cyanobacteria residue in a centrifuge tube.
   a) Add about 10 mL of aqueous acetone solution and cap tightly and place in the dark box.
2) Repeat Step 1) until the desired number of samples have been processed.
3) Remove the cap from the centrifuge tube, insert the microtip, and sonify for 20 seconds at the five setting.
   a) Rinse the microtip into the centrifuge tube with approximately 1 mL of an aqueous acetone solution.
   b) Bring the extract to a volume of 13.0 mL with the acetone solution, cap, mix and return to the dark box.
   c) Repeat the steps outlined in Step 3) until all of the samples have been sonified.
4) Place the dark box in the 4°C cold room and allow the extract to steep overnight.
5) Clarify the extract by centrifuging the extract for 20 minutes at 4000 rpm (Figure 3.8).

6) Carefully transfer the clear extract to a 5.0 cm cell and using the multi-wavelength mode on the spectrophotometer, measure the absorbance at 750, 663, 645, and 630 nm.

   a) Use a shorter or longer cell as necessary to maintain absorbance between approximately 0.1-1.0 at 663 nm.

   b) Note: Operate the spectrophotometer at a slit width no wider than 2 nm for maximum resolution.

7) For corrected samples: Immediately after measuring the absorbance, add 0.1 mL of 0.1 N HCl to the spectrophotometer cell, mix, wait 90 seconds and measure the absorbance specified in Step 6).

8) Discard the sample, rinse the cell two times with 5 mL portions of an aqueous acetone solution.

9) Repeat Steps 6) through 8) until all of the samples have been measured.

10) Determine the absorbance at 750, 663, 645, and 630 nm directly from the printout.

11) Subtract the absorbance at 750 nm from the 630, 645, and 663 nm values (turbidity correction).

12) Calculate the uncorrected chlorophyll *a* concentration by inserting the corrected absorbance values in the following Equation 3.4.

$$Uncorrected\ \ Chlorophyll\ \ a\ \ (\mu/L) = \frac{[11.64\ \ (Abs663) - 2.16\ \ (Abs645) + 0.10\ \ (Abs630)]\ \ E(F)}{V(L)}$$

Where: 3.4

F = Dilution Factor

E = The volume of acetone used for the extraction (mL)

V = The volume of water filtered (L)

L = The cell path length (cm)

13) For corrected samples, determine the absorbance at 665 nm and 750 nm after acidification.

14) Subtract the absorbance at 750 nm from the absorbance at 665 nm (turbidity correction).

15) Calculate the corrected chlorophyll *a* concentration by inserting the turbidity corrected absorbance readings in the following Equation 3.5.

$$Corrected\ \ Chlorophyll\ \ a\ \ (\mu g/l) = \frac{26.73(663b - 665a)E(F)}{V(L)}$$

Where: 3.5

F = Dilution Factor (if the extract requires dilution)

E = The volume of acetone used for the extraction (mL)

V = The volume of water filtered (L)

L = The cell path length (cm)

665_a *= The turbidity corrected Abs at 665 nm after acidification*

663_b *= The turbidity corrected Abs at 663 nm before acidification*

Figure 3.8. Thermo Scientific CL2 centrifuge.

3.7 Determination of the Main Chemical Components

In this study, microalgae and cyanobacteria cultures at the beginning and end of the growth period were utilized for dry weight measurement, and biomass productivity was calculated using the Equation 3.6 below:

$$Biomass\ \ productivity = \frac{DW_6 - DW_0}{t} \qquad 3.6$$

Where:

DW_0 = *dry weight of biomass at the beginning of each growth period (day 0)*

DW_6 = *dry weight of biomass at the end of each growth period (day 6)*

t = *cultivation period (day)*

On the other hand, the cultures of microalgae and cyanobacteria at the beginning and end of the growth period were also used for the determination of the main chemical components.

3.7.1 Extraction of crude lipids

The lipids in microalgae and cyanobacteria cells can be obtained by wet extraction

and dry extraction. In this study, the crude lipids were directly extracted from wet biomass using a method adapted from Bligh and Dyer (1959), and the solvents were chloroform: methanol: water (2: 2: 1.8). The lipid content of microalgae and cyanobacteria cells was calculated following Equation 3.7 below:

$$C_{Lipid} = \frac{Weight\ of\ extracted\ lipid}{Weight\ of\ dried\ biomass} \times 100\% \quad (3.7)$$

Lipid productivity is another factor related to both lipid content and biomass productivity. It was calculated as follows Equation 3.8 below:

$$Lipid\ \ productivity = \frac{WL_6 - WL_0}{t} \quad (3.8)$$

Where:

WL_0 = *weight of extracted lipid at the beginning of each growth period (day 0)*

WL_6 = *weight of extracted lipid at the end of each growth period (day 6)*

t = *cultivation period (day)*

3.7.2 Crude protein analysis

In this study, the crude protein was determined by a dye-binding method described by Servaites et al. (2012), and the protein content of microalgae and cyanobacteria cells was calculated using Equation 3.9 below:

$$C_{Protein} = \frac{Weight\ of\ extracted\ protein}{Weight\ of\ dried\ biomass} \times 100\% \quad (3.9)$$

Protein productivity is another factor related to both protein content and biomass productivity. It was calculated as follows Equation 3.10 below:

$$Protein\ \ productivity = \frac{WP_6 - WP_0}{t} \quad 3.10$$

Where:

WP_0 = *weight of extracted protein at the beginning of each growth period (day 0)*

WP_6 = *weight of extracted protein at the end of each growth period (day 6)*

t = *cultivation period (day)*

3.7.3 Total carbohydrate measurement

In this study, the total carbohydrate was measured by the method described by Miao et al. (2003). An amount of 0.5 g dry microalgae or cyanobacteria biomass was acidified with HCl to a final concentration of 2.5 N and hydrolyzed at 100 ℃ for 30 min. After neutralization, the volume was adjusted to 100 ml and then filtered. The resulting solution was used for the assay of carbohydrate. The carbohydrate content of microalgae and cyanobacteria cells was calculated using Equation 3.11 below:

$$C_{carbohydrate} = \frac{Weight\ of\ total\ carbohydrate}{Weight\ of\ dried\ biomass} \times 100\% \quad 3.11$$

Carbohydrate productivity is another factor related to both carbohydrate content and biomass productivity. It was calculated as follows Equation 3.12 below:

$$Carbohydrate\ \ productivity = \frac{WC_6 - WC_0}{t} \quad 3.12$$

Where:

WC_0 = *weight of total carbohydrate at the beginning of each growth period (day 0)*

WC_6 = *weight of total carbohydrate at the end of each growth period (day 6)*

t = *cultivation period (day)*

3.8 Evaluation of Wastewater Treatment

The efficiency of wastewater treatment was evaluated by comparing the selected nutrients' contents of wastewater samples before and after microalgae and cyanobacteria cultivation. All samples were filtered using a Millipore 47mm vacuum filter assembly (Figure 3.4) with a 0.4 um glass microfiber filter (GB-140, ADVANTEC, Japan) to remove all microalgae or cyanobacteria cells and other large suspended solids before analysis. Nutrient removal analysis was performed using Hanna multiparameter photometer (HI 83200, Hanna Instruments) (Figure 3.9) and corresponding reagents, according to the instruction manual. The tested nutrients were ammonium, nitrate, nitrite, and phosphorus. The chemical oxygen demand (*COD*) removal of minkery wastewater was measured by the UV-Vis spectrophotometer (DR 6000™, HACH) and TNT 822 reagent, (Figure 3.10) according to the HACH procedure manual.

Figure 3.9. Hanna HI 83200 multiparameter photometer.

Figure 3.10. HACH DR 6000™ spectrophotometer (left) and TNT 822 reagent (right).

Once these measurements have been taken, efficiencies of wastewater treatment were calculated using the Equation 3.13 below:

$$Nutrient\ \ removal = \left(\frac{y_0 - y_6}{y_0}\right) \times 100\% \qquad 3.13$$

Where:

y_0 = *content at the beginning of each growth period (day 0)*

y_6 = *content at the end of each growth period (day 6)*

Also, measurements of alkalinity, ammonium, calcium, dissolved oxygen, magnesium, nitrate, nitrite, phosphorus, sulfate, and chemical oxygen demand of employed minkery wastewater were performed using aforementioned Hanna multiparameter photometer and UV-Vis spectrophotometer (DR 6000^{TM}, HACH).

3.9 Comparison of Biomass Estimation Methods

In this study, the methods of dry weight, optical density, cell count and chlorophyll *a* were performed to determine the biomass generated in the same microalgae and cyanobacteria culture during the cultivation process. This study aims to investigate the

effectiveness of four biomass estimation methods by their time and cost requirements, as well as their accuracy and precision in multiple growth conditions. The methodology adopted is illustrated in Figure 3.11.

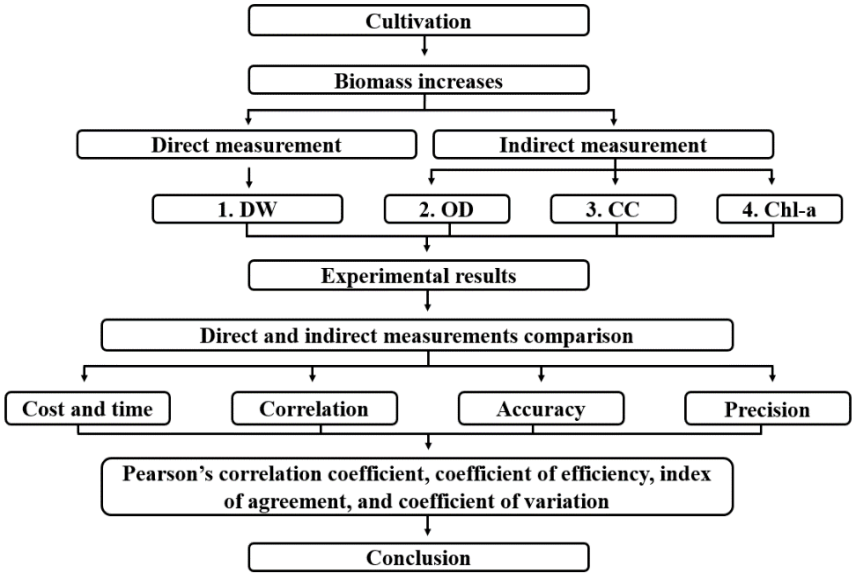

Figure 3.11. A flowchart of methodology (1. Dry weight; 2. Optical density; 3. Cell count; 4. Chlorophyll *a*).

This experiment recorded the cost and time consumed when performing four different methods, and it also formed four different statistical analysis techniques namely Pearson's correlation coefficient, coefficient of efficiency, index of agreement, and coefficient of variation. Compared with other three methods, the gravimetric method of dry weight was the only measurement that directly measured the microalgae and cyanobacteria biomass. Therefore it was considered as the only direct measurement and most accurate measurement, and other three methods were considered as the indirect measurements. As a consequence, the accuracy analyses in this study were only designed for those indirect measurements, which were the methods of optical density, cell count, and chlorophyll *a*.

3.9.1 Pearson's correlation coefficient

The Pearson's correlation coefficient (r), developed by Pearson (1895), is a measure of the linear dependence (correlation) between two quantitative variables, and it does not matter what units are used. It is given Equation 3.14 as follows:

$$r = \frac{\sum_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i-\bar{y})^2}} \quad 3.14$$

Where n values of one method for biomass measurement were denoted by x_i, and the corresponding values of another method were denoted by y_i. $\bar{x}$ is the mean of the values of the first method, and $\bar{y}$ is the mean of the values of the second method. The value of r ranges from -1 to 1, and 1 is a perfect positive linear correlation, 0 is no linear correlation, and -1 is a perfect negative linear correlation.

3.9.2 Coefficient of efficiency

Nash and Sutcliffe (1970) proposed an efficiency (E) criterion for objective assessment of measurement performance presented in Equation 3.15 as:

$$E = 1 - \frac{\sum_{i=1}^{n}(x_i-y_i)^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2} \quad 3.15$$

Where n values of direct measurement (dry weight) were denoted by x_i, and the corresponding values of one indirect measurement (optical density, cell count, or chlorophyll *a*) were denoted by y_i. The $\bar{x}$ is the mean of the values of direct measurement. The value of E ranges from minus infinity to 1, with higher values indicating better performance of the indirect method employed. If $E > 0$, that indirect measurement gives

better results than representing all values by the mean ($\bar{x}$); $E = 0$ means there is no difference between that indirect measurement and the mean; and $E < 0$ means that indirect measurement is worse than representing all values by the mean.

3.9.3 Index of agreement

The index of agreement (d), developed by Willmott (1981), is another relative performance measure. It is given in Equation 3.16 as:

$$d = 1 - \frac{\sum_{i=1}^{n}(x_i - y_i)^2}{\sum_{i=1}^{n}(|x_i - \bar{x}| + |y_i - \bar{x}|)^2} \quad 3.16$$

Where $\bar{x}$ is the mean of the values of direct measurement, and *n* values of direct measurement were denoted by x_i, and the corresponding values of one indirect measurement were denoted by y_i. The value of *d* ranges from 0 to 1, and values closer to one are desirable. However, one problem with d is usually that its values are very high.

3.9.4 Coefficient of variation

The coefficient of variation (C_v) is a standardized measure of dispersion of a probability distribution or frequency distribution. In this study, the coefficient of variation was calculated as an indicator of within method precision for biomass measurement. It is defined in Equation 3.17 as:

$$C_v = \frac{\sigma}{\mu} \quad 3.17$$

Where σ is the standard deviation of the values of each method, and μ is the mean of the values of each method.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Minkery Wastewater Characteristics

In this study, the nutrients content of the 1% pre-treated minkery wastewater was measured (Table 4.1). Compared to the traditional culture mediums mentioned in the previously chapter, the characteristic of the minkery wastewater is very different in term of nitrogen form. In minkery wastewater, about 99 % of total nitrogen was in the form of ammonium, which makes it an excellent growth medium for strains with a high ammonium demand, such as microalgae *Chlorella vulgaris* and *Chlorella sp.* (Šoštarič et al., 2009; Wang et al., 2009). Whereas it may not be an ideal growth medium for strains with a high demand in nitrate and nitrite, such as cyanobacteria *Anabaena sp.* and *Anabaena cylindrica* (Oliveira et al., 2015; Weare and Benemann, 1972).

Table 4.1. Nutrient content of the 1% pre-treated minkery wastewater.

Nutrients	Concentration (mg/L)
Alkalinity ($CaCO_3$)	*160*
Ammonium (NH_4^+)	*73.27*
Calcium (Ca^{2+})	*10*
Chemical oxygen demand (*COD*)	*126*
Dissolved oxygen (DO)	*8.9*
Magnesium (Mg^{2+})	*5*
Nitrate (NO_3^-)	*0.1*
Nitrite (NO_2^-)	*0.18*
Total phosphorus (P)	*13.6*
Sulfate (SO_4^{2-})	*10*

With emerging government regulation (Fur Industry Act of 2010), the surface and ground water monitoring were required following recommendations from the designated professionals, and the owner must sample for total phosphorus, nitrate, and ammonia concentrations of the surface and ground water at the mink farm. If the surface or ground water sample results come back higher than any of the substances listed, the owner of that mink farm may require measures to be taken to reduce concentration levels.

Table 4.2 shows the concentration targets the surface water and groundwater at mink farm should meet, and the nutrient concentrations of the 1% pre-treated minkery wastewater. Compared to the concentration limits of the surface water and groundwater for mink farms, the 1% pre-treated minkery wastewater used in this study already had a much lower nitrate concentration. However the ammonium concentration and total phosphorus concentration of the 1% pre-treated minkery wastewater were much higher than those concentration limits. It means that a further wastewater treatment would be required to reduce ammonium and total phosphorus concentrations of the 1% pre-treated minkery wastewater.

Table 4.2. Concentration limits of the surface water and groundwater for mink farms, and the corresponding nutrient concentration of the 1% pre-treated minkery wastewater.

Substances	Concentration limits		Concentration
	Surface water	Groundwater	Minkery wastewater
Ammonium (NH_4^+)	1 mg/L	4 mg/L	73.27 mg/L
Nitrate (NO_3^-)	3 mg/L	13 mg/L	0.1 mg/L
Total phosphorus (P)	20 μg/L	0.1 mg/L	13.6 mg/L

4.2 Biomass Accumulation Comparison

In this study, the gravimetric method of dry weight was the only measurement that directly measured the microalgae and cyanobacteria biomass, and the methods of optical density, cell count, and chlorophyll *a* were indirect measurements.

4.2.1 Direct measurement

The means of biomass increase in dry weight of *Chlorella vulgaris* and *Anabaena sp.* in various medium under different light cycles was provided by Table 4.2. Based on the findings, *Chlorella vulgaris* achieved the highest biomass increase in minkery wastewater under the light cycle of 6-day continuous light. This particular treatment yielded a biomass increase of 834% over the 6-day growth period, which was significantly higher than those under any treatment associated with traditional medium and distilled water.

While *Chlorella vulgaris* did experience the largest biomass accumulation in minkery wastewater under light cycle of 6-day continuous light, the Tukey's test (with a confidence coefficient of 95%) determined that there was no significant difference between the mean biomass increase under the light cycles of 6-day continuous light and 48-hour light and 24-hour dark. This leads to the conclusion that both light cycles, 6-day continuously light and 48-hour light and 24-hour dark, could be employed for achieving highest biomass cultivation of *Chlorella vulgaris* in minkery wastewater; and using 48-hour light and 24-hour dark instead of 6-day continuous light has a potential to reduce energy costs of microalgae and cyanobacteria cultivation.

Table 4.3. Means of biomass increase (%) in dry weight of *Chlorella vulgaris* and *Anabaena sp.* in minkery wastewater, traditional mediums, and distilled water under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark. Means that do not share a letter are significantly different.

No.	Light cycles	Mediums	Strain I *Chlorella vulgaris* Mean (%)	Strain II *Anabaena sp.* Mean (%)
1	All light	Minkery wastewater	*834 a*	*108 hi*
2	All light	BBM / BG-11_0	*695 b*	*427 cd*
3	All light	Distilled water	*677 b*	*201 gh*
4	48h light + 24h dark	Minkery wastewater	*831 a*	*117 hi*
5	48h light + 24h dark	BBM / BG-11_0	*529 c*	*449 cd*
6	48h light + 24h dark	Distilled water	*475 c*	*201 gh*
7	24h light + 48h dark	Minkery wastewater	*418 c-e*	*86 hi*
8	24h light + 48h dark	BBM / BG-11_0	*341 d-f*	*283 fg*
9	24h light + 48h dark	Distilled water	*301 e-g*	*99 hi*
10	All dark	Minkery wastewater	*83 hi*	*53 i*
11	All dark	BBM / BG-11_0	*3 i*	*13 i*
12	All dark	Distilled water	*4 i*	*9 i*

In contrast, the biomass accumulation of *Chlorella vulgaris* cultivated in traditional culture medium and distilled water were observed to be highly dependent on the light cycles. Li et al. (2012) reported similar finding in the biomass accumulations of microalgae *Chlorella protothecoide* and *Chlorella kessleri* cultured in highly concentrated municipal wastewater correlated to the light cycles.

In comparing these experimental results to the minkery wastewater characteristics, these findings could be easily explained. Both traditional mediums and distilled water used

in this study were short of organic carbon (Less than 30mg/L); however, minkery wastewater contained a certain amount of organic carbon (126 mg/L), which makes it a better organic carbon substrate for the heterotrophic cultivation of *Chlorella vulgaris*. The only deficiency of minkery wastewater used in this study was that its content of organic carbon was too low to support a 6-day heterotrophic cultivation due to the dilution of minkery wastewater before the experiment. As a consequence, although mean biomass increase of *Chlorella vulgaris* under the light cycle of 6-day continuous dark in minkery wastewater was higher than those in traditional medium and distilled water under the same heterotrophic condition, the Tukey's test did not determine that there was a significant difference between these findings.

In comparing the mean biomass increase in minkery wastewater between *Chlorella vulgaris* and *Anabaena sp.* across each of the light cycles, it was found that there was no significant difference for the 6-day continuous dark. There was, however, a significant difference for the rest of light cycles. *Chlorella vulgaris* showed a much better adaptation than *Anabaena sp*. in minkery wastewater because the mean biomass increase of *Anabaena sp*. was significantly lower than those of *Chlorella vulgaris*. The growth characteristics of *Anabaena sp.* in BG-11_o medium was significantly better than those in minkery wastewater. Oliveira et al. (2015) reported that *Anabaena sp.* has a high demand in nitrate. Insufficient nitrate of minkery wastewater made the minkery wastewater an inappropriate substrate for *Anabaena sp.* cultivation.

4.2.2 Indirect measurements

In this study, the means of biomass increase determined by the method of the optical density of *Chlorella vulgaris* and *Anabaena sp.* in minkery wastewater, traditional medium, and distilled water under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark was provided by Table 4.4.

Table 4.4. Means of biomass increase (%) in optical density of *Chlorella vulgaris* and *Anabaena sp.* in minkery wastewater, traditional mediums, and distilled water under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark. Means that do not share a letter are significantly different.

No.	Light cycles	Mediums	Strain I *Chlorella vulgaris* Mean (%)	Strain II *Anabaena sp.* Mean (%)
1	All light	Minkery wastewater	*820 a*	*-61 h*
2	All light	BBM / BG-11$_0$	*699 b*	*417 b-d*
3	All light	Distilled water	*524 b*	*143 fg*
4	48h light + 24h dark	Minkery wastewater	*772 a*	*-61 h*
5	48h light + 24h dark	BBM / BG-11$_0$	*546 b*	*417 b-d*
6	48h light + 24h dark	Distilled water	*446 bc*	*143 fg*
7	24h light + 48h dark	Minkery wastewater	*332 c-e*	*-68 h*
8	24h light + 48h dark	BBM / BG-11$_0$	*252 ef*	*276 d-f*
9	24h light + 48h dark	Distilled water	*190 ef*	*22 gh*
10	All dark	Minkery wastewater	*33 gh*	*-70 h*
11	All dark	BBM / BG-11$_0$	*-6 gh*	*-11 h*
12	All dark	Distilled water	*-3 gh*	*-18 h*

Biomass accumulation varied much among different treatments. While biomass increases of *Anabaena sp.* in minkery wastewater under four light cycles were negative numbers, indicating that *Anabaena sp.* biomass at the end of each growth period was lower than that at the beginning of each growth period. In reality, the biomass accumulation of any microalgae and cyanobacteria strain has to be great than or at least equal to zero when cultivating in a completely closed environment. Therefore, the indirect measurements of optical density were incapable of determining the biomass increase if the majority of microalgae and cyanobacteria cells were already dead at the end of each growth period.

The growth of *Chlorella vulgaris* in minkery wastewater under different light cycles measured by the method of optical density was provided by Figure 4.1 as well. The effect of light cycles has been demonstrated in Figure 4.1 clearly.

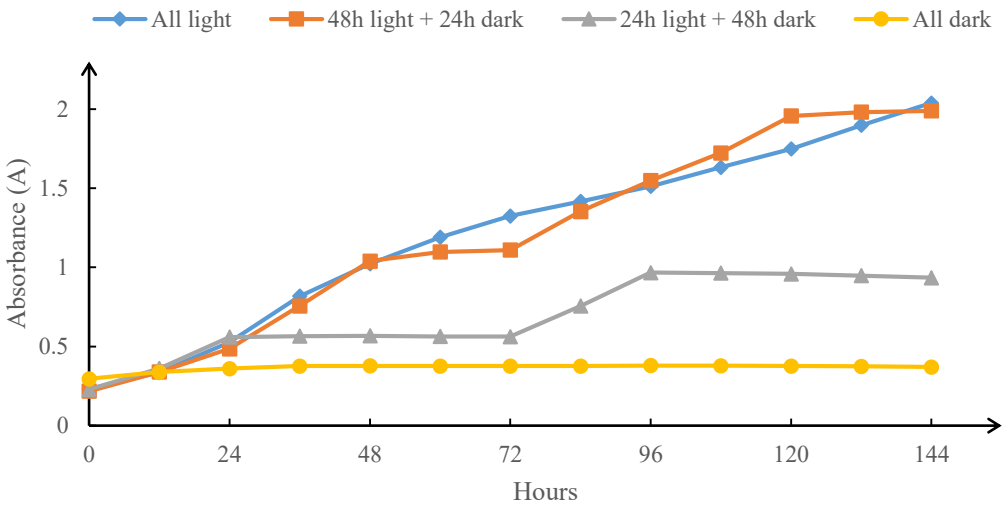

Figure 4.1. Growth of *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater measured by the method of optical density.

The finding is supported by the biomass accumulation data determined by the method of dry weight. Both light cycles, 6-day continuously light and 48-hour light and 24-hour dark, could be performed for achieving highest biomass cultivation of *Chlorella vulgaris* in minkery wastewater.

In this study, means of biomass increase in cell count of *Chlorella vulgaris* and *Anabaena sp.* under different light cycles in various mediums were provided in Table 4.5.

Table 4.5. Means of biomass increase (%) in cell count of *Chlorella vulgaris* and *Anabaena sp.* in minkery wastewater, traditional mediums, and distilled water under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark. Means that do not share a letter are significantly different.

No.	Light cycles	Mediums	Strain I *Chlorella vulgaris* Mean (%)	Strain II *Anabaena sp.* Mean (%)
1	All light	Minkery wastewater	*812 a*	*-100 i*
2	All light	BBM / BG-11_0	*732 b*	*365 d*
3	All light	Distilled water	*536 c*	*137 f*
4	48h light + 24h dark	Minkery wastewater	*763 ab*	*-100 i*
5	48h light + 24h dark	BBM / BG-11_0	*533 c*	*362 d*
6	48h light + 24h dark	Distilled water	*418 d*	*135 f*
7	24h light + 48h dark	Minkery wastewater	*390 d*	*-100 i*
8	24h light + 48h dark	BBM / BG-11_0	*258 e*	*224 e*
9	24h light + 48h dark	Distilled water	*232 e*	*-8 gh*
10	All dark	Minkery wastewater	*43 g*	*-100 i*
11	All dark	BBM / BG-11_0	*-5 gh*	*-49 hi*
12	All dark	Distilled water	*-3 gh*	*-56 hi*

Different from other indirect measurements, the mean biomass increases of *Anabaena sp.* in minkery wastewater under all four light cycles were exactly negative one hundred, showing that no living *Anabaena sp.* cell could be found under microscopy at the end of each growth period.

In this study, means of biomass increase in chlorophyll *a* of *Chlorella vulgaris* and *Anabaena sp.* under different light cycles in various mediums were provided in Table 4.6.

Table 4.6. Means of biomass increase (%) in chlorophyll *a* content of *Chlorella vulgaris* and *Anabaena sp.* in minkery wastewater, traditional mediums, and distilled water under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark. Means that do not share a letter are significantly different.

			Strain I *Chlorella vulgaris*	Strain II *Anabaena sp.*
No.	Light cycles	Mediums	Mean (%)	Mean (%)
1	All light	Minkery wastewater	*806 a*	*-44 h*
2	All light	BBM / BG-11_0	*613 b*	*393 d*
3	All light	Distilled water	*567 bc*	*105 g*
4	48h light + 24h dark	Minkery wastewater	*730 a*	*-47 h*
5	48h light + 24h dark	BBM / BG-11_0	*548 bc*	*385 d*
6	48h light + 24h dark	Distilled water	*339 de*	*127 g*
7	24h light + 48h dark	Minkery wastewater	*519 c*	*-24 h*
8	24h light + 48h dark	BBM / BG-11_0	*256 f*	*275 ef*
9	24h light + 48h dark	Distilled water	*322 d-f*	*13 h*
10	All dark	Minkery wastewater	*4 h*	*-45 h*
11	All dark	BBM / BG-11_0	*-34 h*	*-20 h*
12	All dark	Distilled water	*-30 h*	*-19 h*

4.3 Chemical Component Comparison

Based on the biomass accumulation data discussed above, the main chemical components of *Anabaena sp.* had not been measured because not even one living *Anabaena sp.* cell could be found in minkery wastewater under microscopy at the end of each growth period. In this study, the contents of main chemical components of *Chlorella vulgaris* cells in minkery wastewater under different light cycles were provided by Table 4.7.

Table 4.7. Contents of main chemical components of *Chlorella vulgaris* cells under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater.

Component (%)	All light	48h L + 24h D	24h L + 48h D	All dark
Protein	*51.35 ± 0.31*	*48.79 ± 0.43*	*41.67 ± 0.37*	*36.24 ± 0.28*
Lipid	*18.46 ± 0.19*	*20.16 ± 0.22*	*25.04 ± 0.16*	*28.73 ± 0.21*
Carbohydrate	*14.32 ± 0.16*	*14.96 ± 0.26*	*16.17 ± 0.15*	*17.15 ± 0.18*
Ash	*4.94 ± 0.06*	*6.63 ± 0.07*	*5.79 ± 0.06*	*4.59 ± 0.04*
Others	*10.93 ± 0.63*	*9.46 ± 0.57*	*11.33 ± 0.65*	*13.29 ± 0.61*

The main chemical components of *Chlorella vulgaris* cells were proteins, lipids, and carbohydrates. They accounted for 84.13% in photoautotrophic cells, 83.91 and 82.88% in mixotrophic cells, and 82.12% in heterotrophic cells. Miao and Wu (2004) reported similar finding in the main chemical components accounted for 83 to 86% of the dry biomass in *Chlorella protothecoides* cells.

This study also suggested that heterotrophic cells contained a higher lipid and carbohydrate content than photoautotrophic and mixotrophic cells, whereas protein content of photoautotrophic cells was much greater than that of mixotrophic and heterotrophic cells.

Miao and Wu (2004) reported similar trend that *Chlorella protothecoides* showed an increase in total lipid content from 14.57 to 55.20%, an increase in total carbohydrate content from 10.62 to 15.43%, and a decrease in total protein content from 52.64 to 10.28% under heterotrophic conditions as opposed to photoautotrophic conditions.

4.3.1 Extraction of lipids

In this study, the comparison of lipid contents and lipid productivities of *Chlorella vulgaris* in minkery wastewater under different light cycles was provided by Figure 4.2. It was observed that lipid productivity increased while lipid content decreased. Theoretically, it was because increases in biomass productivity outweighed decreases in lipid content. It was also demonstrated that the light caused an adverse effect on lipid content.

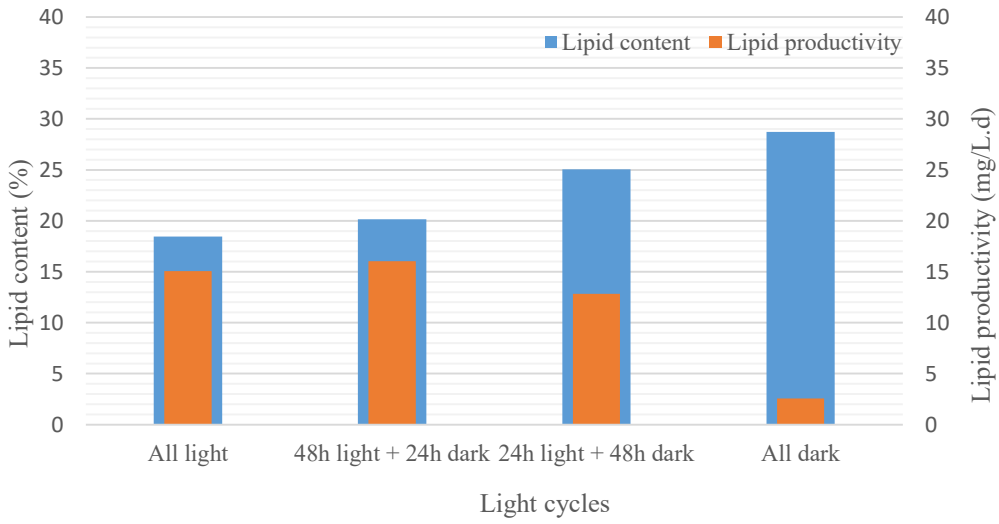

Figure 4.2. Comparison of lipid contents (% dry weight biomass) and lipid productivities of *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater.

More specifically, the biomass and lipid productivities of *Chlorella vulgaris* in minkery wastewater under four different light cycles were shown in Table 4.8. The highest biomass productivity of *Chlorella vulgaris* in minkery wastewater was achieved under light cycle of 6-day continuous light, however the Tukey's test (with a confidence coefficient of 95%) determined that there was no significant difference between this and the biomass productivity of *Chlorella vulgaris* under the light cycle of 48-hour light and 24-hour dark. This result is supported by the biomass accumulation data discussed above in which both of the light cycles also experienced two largest biomass accumulations.

Table 4.8. Biomass and lipid productivities of *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater. Means that are in the same column but do not share a letter are significantly different.

Light cycle	Lipid productivity (mg/L.d)	Biomass productivity (mg/L.d)
All light	*15.06 a*	*82.50 a*
48h light + 24h dark	*16.03 a*	*79.50 a*
24h light + 48h dark	*12.82 a*	*49.22 b*
All dark	*2.57 b*	*3.56 c*

In comparison, the highest lipid productivity of *Chlorella vulgaris* in minkery wastewater was achieved under light cycle of 48-hour light and 24-hour dark instead of 6-day continuous light, and the Tukey's test (with a confidence coefficient of 95%) determined that there be no significant difference between this and the lipid productivity of *Chlorella vulgaris* under the light cycle of 6-day continuous light and 24-hour light and 48-hour dark. This leads to the conclusion that three different light cycles, 6-day continuous

light, 48-hour light and 24-hour dark, and 24-hour light and 48-hour dark could be employed for achieving highest lipid productivity of *Chlorella vulgaris* in minkery wastewater, and using light cycle of 24-hour light and 48-hour instead of 6-day continuous light or 48-hour light and 24-hour dark has a potential to reduce energy costs of cultivation.

4.3.2 Crude protein analysis

In this study, the comparison of protein contents and protein productivities of *Chlorella vulgaris* in minkery wastewater under different light cycles was provided by Figure 4.3. It was observed that protein productivity increased while protein content increased as well. It was also demonstrated that the light increased the protein content of microalga *Chlorella vulgaris*.

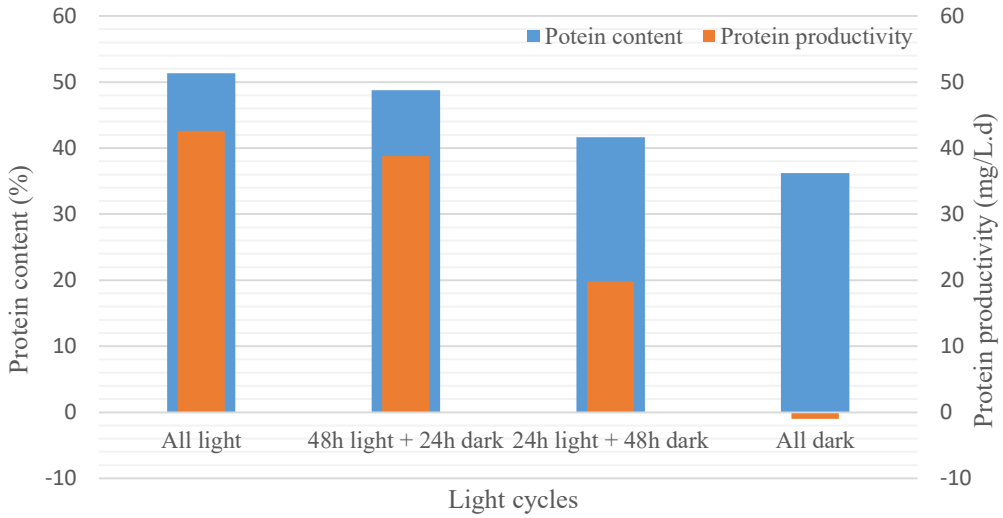

Figure 4.3. Comparison of protein contents (% dry weight biomass) and protein productivities of *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater.

Table 4.9 provides the biomass and protein productivities of *Chlorella vulgaris* in minkery wastewater under different light cycles. The highest protein productivity of *Chlorella vulgaris* in minkery wastewater was achieved under light cycle of 6-day continuous light, and the Tukey's test (with a confidence coefficient of 95%) determined that there be no significant difference between this and the protein productivity of *Chlorella vulgaris* under the light cycle of 48-hour light and 24-hour dark. This leads to the conclusion that both of light cycles, 6-day continuous light, and 48-hour light and 24-hour dark could be employed for achieving highest protein productivity of *Chlorella vulgaris* in minkery wastewater, and using light cycle of 48-hour light and 24-hour dark instead of 6-day continuous light reduced energy costs of microalgae and cyanobacteria production.

Table 4.9. Biomass and protein productivities of *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater. Means that are in the same column but do not share a letter are significantly different.

Light cycle	Protein productivity (mg/L.d)	Biomass productivity (mg/L.d)
All light	*42.62 a*	*82.50 a*
48h light + 24h dark	*38.79 a*	*79.50 a*
24h light + 48h dark	*19.80 b*	*49.22 b*
All dark	*-0.98 c*	*3.56 c*

4.3.3 Total carbohydrate measurement

In this study, the comparison of carbohydrate contents and carbohydrate productivities of *Chlorella vulgaris* in minkery wastewater under different light cycles was provided by Figure 4.4. Similar to lipid productivity, it was observed that carbohydrate productivity

increased while carbohydrate content decreased. Theoretically, it was because increases in biomass productivity outweighed decreases in carbohydrate content. It was demonstrated that the light caused an adverse effect on carbohydrate content as well.

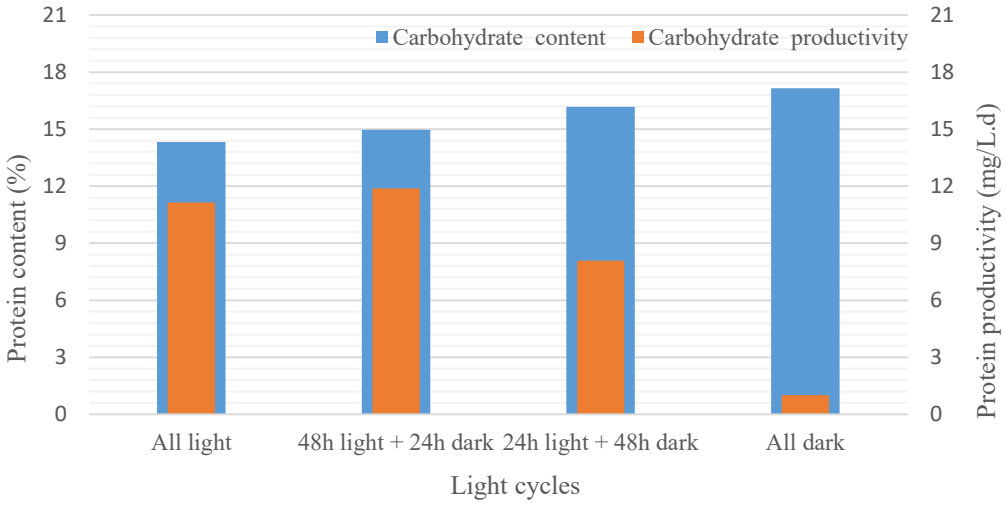

Figure 4.4. Comparison of carbohydrate contents (% dry weight biomass) and carbohydrate productivities of *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater.

More specifically, the biomass and carbohydrate productivities of *Chlorella vulgaris* in minkery wastewater under four different light cycles were shown in Table 4.10. The highest carbohydrate productivity of *Chlorella vulgaris* in minkery wastewater was achieved under light cycle of 48-hour light and 24-hour dark, and the Tukey's test (with a confidence coefficient of 95%) determined that there was no significant difference between this and the carbohydrate productivity of *Chlorella vulgaris* under the light cycle of 6-day continuous light and 24-hour light and 48-hour dark. This result is similar to the lipid productivity data discussed above in which the light cycles of 6-day continuous light, 48-

hour light and 24-hour dark, and 24-hour light and 48-hour dark also experienced three highest lipid productivity.

This leads to the conclusion that three different light cycles, 6-day continuous light, 48-hour light and 24-hour dark, and 24-hour light and 48-hour dark could be employed for achieving highest carbohydrate productivity of *Chlorella vulgaris* in minkery wastewater, and using light cycle of 24-hour light and 48-hour instead of 6-day continuous light or 48-hour light and 24-hour dark reduced energy costs of microalgae and cyanobacteria cultivation.

Table 4.10. Biomass and carbohydrate productivities of *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark in minkery wastewater. Means that are in the same column but do not share a letter are significantly different.

Light cycle	Carbohydrate productivity (mg/L.d)	Biomass productivity (mg/L.d)
All light	*11.15 a*	*82.50 a*
48h light + 24h dark	*11.89 a*	*79.50 a*
24h light + 48h dark	*8.08 a*	*49.22 b*
All dark	*1.01 b*	*3.56 c*

In conclusion, for the cultivation of *Chlorella vulgaris* in minkery wastewater, the most appropriate cultivation techniques (light cycles) for the production of microalgae biomass and crude protein was the light cycles of 48-hour light and 24-hour dark, and the light cycle of 24-hour light and 48-hour dark was the most appropriate cultivation techniques for the production of lipid and carbohydrate.

4.4 Nutrient Removal Comparison

The remediation efficiencies of wastewater treatment were evaluated by comparing the selected nutrients' contents of wastewater samples before and after the cultivation.

4.4.1 Total Nitrogen Removal

The means of total nitrogen removal from various mediums via cultivation of *Chlorella vulgaris* and *Anabaena sp.* under different light cycles were shown in Table 4.11.

Table 4.11. Means of total nitrogen removal (%) from minkery wastewater, traditional mediums, and distilled water by culturing *Chlorella vulgaris* and *Anabaena sp.* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark, and means that do not share a letter are significantly different.

No.	Light cycles	Mediums	Strain I *Chlorella vulgaris* Mean (%)	Strain II *Anabaena sp.* Mean (%)
1	All light	Minkery wastewater	*96.2 a*	*29.2 f*
2	All light	BBM / BG-11_0	*82.8 b*	*94.8 a*
3	All light	Distilled water	*85.2 b*	*92.6 a*
4	48h light + 24h dark	Minkery wastewater	*94.8 a*	*30.9 f*
5	48h light + 24h dark	BBM / BG-11_0	*52.4 e*	*95.0 a*
6	48h light + 24h dark	Distilled water	*51.6 e*	*91.9 a*
7	24h light + 48h dark	Minkery wastewater	*57.4 d*	*28.5 f*
8	24h light + 48h dark	BBM / BG-11_0	*22.9 g*	*65.2 c*
9	24h light + 48h dark	Distilled water	*20.3 gh*	*61.2 cd*
10	All dark	Minkery wastewater	*21.4 gh*	*18.4 h*
11	All dark	BBM / BG-11_0	*1.6 i*	*4.5 i*
12	All dark	Distilled water	*1.6 i*	*3.6 i*

In comparing the total nitrogen removal from minkery wastewater between *Chlorella vulgaris* and *Anabaena sp.* across each of the light cycles, it was found that *Chlorella vulgaris* has higher potential than *Anabaena sp.* to treat minkery wastewater and accumulate highly valuable biomass simultaneously. The total nitrogen removal from minkery wastewater via cultivation of *Chlorella vulgaris* was significantly higher than that of *Anabaena sp.* under three of the four light cycles, including 6-day continuous light, 48-hour light and 24-hour dark, and 24-hour light and 48-hour dark.

The highest total nitrogen removal from minkery wastewater in this study was achieved through the cultivation of *Chlorella vulgaris* under light cycle of 6-day continuous light. This particular treatment achieved a nitrogen removal of 96.2% over the 6-day growth period, and the Tukey's test (with a confidence coefficient of 95%) determined that there be no significant difference between this and the total nitrogen removal from minkery wastewater by cultivating *Chlorella vulgaris* under the light cycle of 48-hour light and 24-hour dark. This leads to the conclusion that *Chlorella vulgaris* showed the highest total nitrogen removal from minkery wastewater under the light cycle of 6-day continuous light and 48-hour light and 24-hour dark, and using light cycle of 48-hour light and 24-hour dark instead of 6-day continuous light reduced energy costs.

In comparison, Li et al. (2012) reported that mixotrophic microalgae *Chlorella kessleri* and *Chlorella protothecoide* cultivated in highly concentrated municipal wastewater achieved 62.2 and 64.5% total nitrogen removal in only 4 days, respectively.

Wang et al. (2009) reported that microalga *Chlorella sp.* cultivated in municipal wastewater from sludge centrifuge achieved 82.8% total nitrogen removal in 9 days. It should be noted that minkery wastewater used in our study had a higher initial nitrogen concentration than municipal wastewaters used in those studies, which makes these number even more impressive.

Figure 4.5 provided the ammonium concentrations of minkery wastewater before and after 6-day treatment by *Chlorella vulgaris* under four light cycles. It demonstrated that the minkery wastewaters after 6-day treatment by *Chlorella vulgaris* under light cycles of 6-day continuously light and 48-hour light and 24-hour dark had a lower ammonium concentration than both surface water and groundwater concentration limits. As mentioned previously, ammonium concentration limits of the surface water and groundwater for mink farms were 1 mg/L and 4 mg/L, respectively.

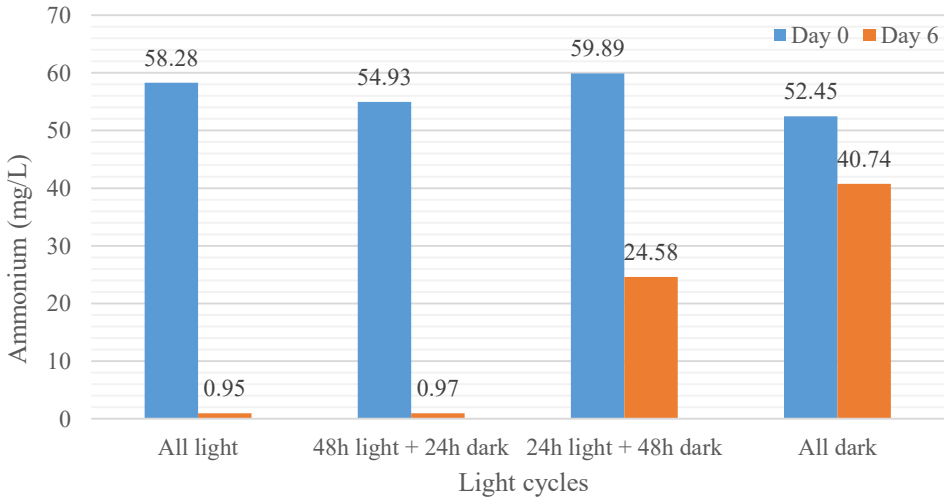

Figure 4.5. Ammonium concentrations of minkery wastewater before and after 6-day treatment by *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark.

4.4.2 Total Phosphorus Removal

The means of total phosphorus removal from various mediums by culturing *Chlorella vulgaris* and *Anabaena sp.* under different light cycles were shown in Table 4.12. In comparing the total phosphorus removal from minkery wastewater between *Chlorella vulgaris* and *Anabaena sp.* across each of the light cycles. It was found that the total phosphorus removal from minkery wastewater through cultivation of *Chlorella vulgaris* was significantly higher than that of *Anabaena sp.* under all four light cycles.

Table 4.12. Means of total phosphorus removal (%) from minkery wastewater, traditional mediums, and distilled water through the cultivation of *Chlorella vulgaris* and *Anabaena sp.* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark, and means that do not share a letter are significantly different.

No.	Light cycles	Mediums	Strain I *Chlorella vulgaris* Mean (%)	Strain II *Anabaena sp.* Mean (%)
1	All light	Minkery wastewater	*29.7 a*	*5.9 j*
2	All light	BBM / BG-11_0	*23.4 b*	*12.1 ef*
3	All light	Distilled water	*23.5 b*	*12.5 ef*
4	48h light + 24h dark	Minkery wastewater	*28.8 a*	*6.1 ij*
5	48h light + 24h dark	BBM / BG-11_0	*17.4 cd*	*12.5 ef*
6	48h light + 24h dark	Distilled water	*17.7 c*	*11.8 fg*
7	24h light + 48h dark	Minkery wastewater	*14.8 de*	*5.8 j*
8	24h light + 48h dark	BBM / BG-11_0	*9.2 gh*	*8.4 h-j*
9	24h light + 48h dark	Distilled water	*8.8 hi*	*8.6 h-j*
10	All dark	Minkery wastewater	*7.7 h-j*	*3.1 k*
11	All dark	BBM / BG-11_0	*1.6 k*	*1.4 k*
12	All dark	Distilled water	*1.5 k*	*1.5 k*

The highest total phosphorus removal from minkery wastewater was performed by cultivating *Chlorella vulgaris* under 6-day continuously light, and the Tukey's test did not determine that there was a significant difference between this and the total phosphorus removal from minkery wastewater via culturing *Chlorella vulgaris* under 48-hour light and 24-hour dark. This leads to the conclusion that *Chlorella vulgaris* showed the highest total phosphorus removal from minkery wastewater under the light cycles of 6-day continuous light and 48-hour light and 24-hour dark, and using light cycle of 48-hour light and 24-hour dark instead of 6-day continuous light reduced energy costs of the cultivation.

These two particular treatments achieved phosphorus removals of 29.7% and 28.8% over the 6-day growth period, respectively. In comparison to other studies, these phosphorus removal rates were relatively low. Li et al. (2012) observed that mixotrophic microalgae *Chlorella kessleri* and *Chorella protothecoide* cultivated in highly concentrated municipal wastewater achieved 87.4 and 86.1% total phosphorus removal in only 4 days. Wang et al. (2009) reported that microalga *Chlorella sp.* cultivated in municipal wastewater from sludge centrifuge achieved 85.6% total phosphorus removal in 9 days. It should be noted that minkery wastewater used in our study had a much higher initial phosphorus concentration than municipal wastewaters used in those studies. When it comes to amount of phosphorus removal (mg/L), the results were much closer.

The total phosphorus concentrations of minkery wastewater before and after 6-day treatment by *Chlorella vulgaris* under different light cycles were provided by Figure 4.6.

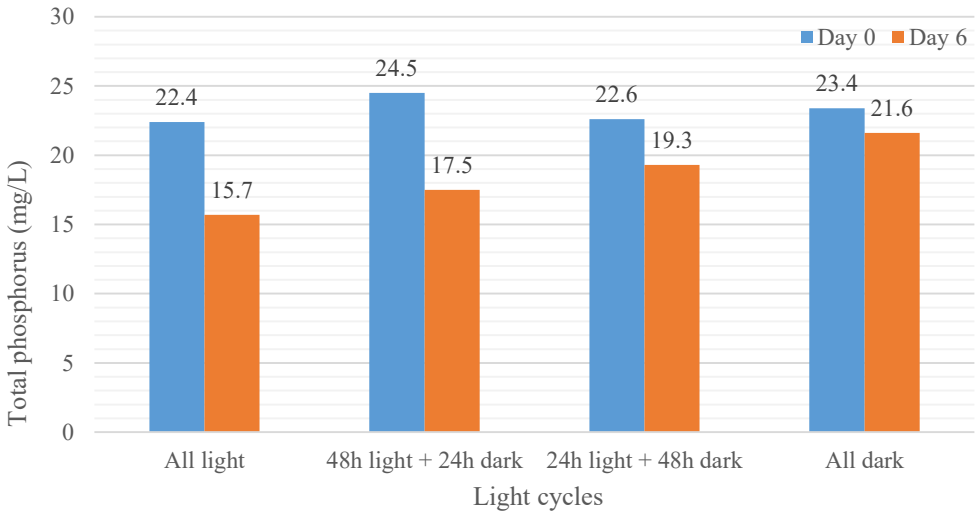

Figure 4.6. Total phosphorus concentrations of minkery wastewater before and after 6-day treatment by *Chlorella vulgaris* under light cycles of 6-day continuously light, 48-hour light and 24-hour dark, 24-hour light and 48-hour dark, and 6-day continuously dark.

As mentioned in the previous chapter, total phosphorus concentration limits of the surface water and groundwater for mink farms were 20 μg/L and 0.1 mg/L, respectively. Unfortunately, even after 6-day treatment by *Chlorella vulgaris*, the total phosphorus concentration of minkery wastewater was still much higher than the surface water and groundwater concentration limits, which means that further treatment will be required to reduce the concentration levels of total phosphorus in minkery wastewater.

4.4.3 Correlation between nitrogen removal and phosphorus removal

In this study, the value of r investigating the relationship between total nitrogen removal and total phosphorus removal of *Chlorella vulgaris* was 0.983, which was extremely close to one, suggesting that total nitrogen removal had a very strong positive linear relationship with total phosphorus removal for *Chlorella vulgaris* cultivation. Furthermore, the value of r measuring the correlation between total nitrogen removal and

total phosphorus removal of *Anabaena sp.* was 0.985, which was also extremely close to one, suggesting that total nitrogen removal and total phosphorus removal of *Anabaena sp.* were highly correlated. However, the value of r determining the linear dependence between total nitrogen removal and total phosphorus removal of both *Chlorella vulgaris* and *Anabaena sp.* was only 0.781, which was much lower, suggesting that total nitrogen removal of *Chlorella vulgaris* or *Anabaena sp.* was only highly correlated to its own total phosphorus removal.

In conclusion, for the cultivation of *Chlorella vulgaris* or *Anabaena sp.* in this study, the total nitrogen removal and total phosphorus removal were highly correlated. However, each strain has a specific nitrogen and phosphorus removal ratio, which is unique and different from one another. This finding was supported by many studies (Darley, 1982; Illman et al., 2000; Safi et al., 2014).

4.4.4 COD Removal

As mentioned in the previous section, in comparing the total nitrogen removal and total phosphorus removal from minkery wastewater between *Chlorella vulgaris* and *Anabaena sp.*, it was found that *Anabaena sp.* has much lower potential than *Chlorella vulgaris* to treat minkery wastewater simultaneously. This conclusion is supported by the chemical oxygen demand removal data in this section. Compared to *Chlorella vulgaris* cultivation, a much lower chemical oxygen demand removal from minkery wastewater was achieved through the cultivation of *Anabaena sp.* under both photoautotrophic and

heterotrophic condition. The mean of chemical oxygen demand removal from minkery wastewater by culturing *Anabaena sp.* under photoautotrophic condition was 7.3%, and a mean chemical oxygen demand removal of 53.7% was achieved from minkery wastewater via culturing *Anabaena sp.* under heterotrophic condition.

Table 4.13 provides the average of chemical oxygen demand removal from minkery wastewater through the cultivation of *Chlorella vulgaris* under photoautotrophic condition. A relatively low chemical oxygen demand removal of 40.5% was achieved from minkery wastewater through the cultivation of *Chlorella vulgaris* under photoautotrophic condition. The concentration of chemical oxygen demand decreased very slightly in the first three days however followed by rapid decreases in the last three days.

Table 4.13. Mean of chemical oxygen demand removal (%) from minkery wastewater by culturing *Chlorella vulgaris* under photoautotrophic condition.

	COD (mg/L)			Removal (%)
Replicates	Day 0	Day 3	Day 6	
1	99	91	62	
2	103	86	57	40.5
3	97	88	59	

As mentioned in the previous chapter, organic carbon is not essential for the growth of microalgae and cyanobacteria under photoautotrophic condition (Perez-Garcia and Bashan, 2015). Nevertheless, in the last three days of photoautotrophic cultivation, the cell density of the *Chlorella vulgaris* culture increased tremendously, and light became less abundant, then the metabolic pathway of *Chlorella vulgaris* altered with a supply of organic substrates, which means that they performed heterotrophic growth besides the

photoautotrophic growth. This conclusion is supported by the biomass accumulation data discussed above in which mean biomass increases of *Chlorella vulgaris* in minkery wastewater were significantly higher than those in traditional medium and distilled water under three of the four light cycles because minkery wastewater was the only organic carbon substrate in this study.

The mean of chemical oxygen demand removal from minkery wastewater through the cultivation of *Chlorella vulgaris* under heterotrophic condition was provided in Table 4.14. In six days, a mean chemical oxygen demand removal of 80.6% was achieved from minkery wastewater via the cultivation of *Chlorella vulgaris* under heterotrophic condition. Totally different from photoautotrophic cultivation, the concentration of chemical oxygen demand decreased rapidly in the first three days followed by slight decreases in the last three days due to fast assimilation by *Chlorella vulgaris* under heterotrophic condition, suggesting that a treatment period of three days is enough to achieve a very high chemical oxygen demand removal from minkery wastewater when culturing *Chlorella vulgaris* under heterotrophic condition.

Table 4.14. Mean of chemical oxygen demand removal (%) from minkery wastewater by culturing *Chlorella vulgaris* under heterotrophic condition.

	COD (mg/L)			Removal (%)
Replicates	Day 0	Day 3	Day 6	
1	102	34	18	
2	105	20	24	80.6
3	97	28	17	

Many studies argued that the organic carbon forms may not be accessible or toxic in agricultural wastewaters (Bhatnagar et al., 2011; Perez-Garcia and Bashan, 2015). In comparison to other studies, the COD removal from minkery wastewater by culturing *Chlorella vulgaris* in this study was as good as many studies using municipal wastewater. Li et al. (2012) reported that mixotrophic microalgae *Chlorella kessleri* and *Chlorella protothecoide* cultivated in highly concentrated municipal wastewater achieved 77.0 and 70.3% COD removal in only 4 days. Wang et al. (2009) observed that microalga *Chlorella sp.* cultivated in municipal wastewater from sludge centrifuge achieved 83.0% COD removal in 9 days. It should be noted that minkery wastewater used in our study had a much higher initial COD concentration than those municipal wastewaters.

4.5 Correlation between Biomass Accumulation and Nutrient Removal

Table 4.15 provides values of r investigating the relationship between the biomass accumulation of *Chlorella vulgaris* determined by four biomass estimation methods and the nutrient removal of *Chlorella vulgaris*.

Table 4.15. Pearson's correlation (r) matrix investigating the correlation between the biomass accumulation of *Chlorella vulgaris* determined by the biomass estimation methods of *dry weight*, *optical density*, *cell count*, and *chlorophyll a*, and the total nitrogen removal and total phosphorus removal of *Chlorella vulgaris*.

Method	Nitrogen removal	Phosphorus removal
Dry weight	*0.959*	*0.965*
Optical density	*0.937*	*0.950*
Cell count	*0.956*	*0.964*
Chlorophyll *a*	*0.937*	*0.938*

It demonstrates that values of r were all above 0.9, which was very close to one, suggesting that regardless of biomass estimation methods used, the biomass accumulation of *Chlorella vulgaris* had a very strong positive linear relationship with the total nitrogen and total phosphorus removal of *Chlorella vulgaris*. This result was supported by many studies (Pate et al., 2011; Safi et al., 2014). Aside from carbon sources, microalgae and cyanobacteria need nutrients to grow and reproduce, and it is expected that the higher the nutrient uptake, the better the biomass growth. As a consequence, the biomass accumulation and nutrient removal of microalgae and cyanobacteria should be correlated.

Table 4.16 provides values of r measuring the correlation between the biomass growth of *Anabaena sp.* quantified by four biomass estimation methods and the nutrient removal of *Anabaena sp.*. It shows that values of r were all above 0.7, which was not as high as that of *Chlorella vulgaris*, however still suggesting that regardless of biomass estimation methods used, the biomass accumulation of *Anabaena sp.* had a strong positive linear relationship with the total nitrogen and total phosphorus removal of *Anabaena sp.*.

Table 4.16. Pearson's correlation (r) matrix investigating the correlation between the biomass accumulation of *Anabaena sp.* determined by the biomass estimation methods of *dry weight*, *optical density*, *cell count*, and *chlorophyll a*, and the total nitrogen removal and total phosphorus removal of *Anabaena sp.*.

Method	Nitrogen removal	Phosphorus removal
Dry weight	*0.846*	*0.823*
Optical density	*0.809*	*0.750*
Cell count	*0.847*	*0.787*
Chlorophyll *a*	*0.791*	*0.733*

In conclusion, the biomass accumulation and nutrient removal are highly correlated for both *Chlorella vulgaris* and *Anabaena sp.* cultivated in this study. The reason correlation between biomass accumulation and nutrient removal of *Anabaena sp.* was lower than that of *Chlorella vulgaris* is that insufficient nitrate and redundant toxic compounds in minkery wastewater made it an inappropriate substrate for *Anabaena sp.* cultivation. The indirect measurements of optical density, cell count, and chlorophyll *a* methods were incapable of determining the biomass accumulation correctly when the majority of *Anabaena sp.* cells were already dead, and chlorophyll concentration of *Anabaena sp.* cells was changed. In evidence, the biomass accumulation of *Anabaena sp.* determined by the methods of dry weight, which is a direct measurement, showed a much stronger positive linear dependence with the nutrient removal of *Anabaena sp.* than that of other three indirect measurements.

4.6 Comparison between Biomass Estimation Methods

This study measured the time and cost requirements, as well as accuracy and precision of each indirect biomass estimation methods, and cost and time consumed and precision of the direct biomass estimation method.

4.6.1 Cost and time comparison

Cost and time requirements reported below were recorded during execution of each method in the laboratory. The only direct method, dry weight, was limited by the spatial capacity of the furnace to only three samples per measurement. Therefore, the time required

to measure three samples was also recorded for other methods as a comparison. In this way, the most reliable and reasonable time required per sample could be obtained for all four methods. In addition, the 'warming-up' time of each equipment required before measurements was included as well. In detail, the time required per sample for each of the biomass estimation methods were shown in Table 4.17.

Table 4.17. Time required per sample for each of the biomass estimation methods.

Method	Time required (per sample)	
Dry weight	*3.0 hr – 3 samples*	*1.00 hr*
Optical density	*0.4 hr – 3 samples*	*0.13 hr*
Cell count	*0.5 hr – 3 samples*	*0.17 hr*
Chlorophyll *a*	*1.5 hr – 3 samples*	*0.50 hr*

Selection of the methods was not only based upon minimizing the time requirement, but also the total cost for each measurement. In this case, it was assumed that the labor rate was $15.00 per hour, and the total cost for each sample was the combination of labor cost and material cost. The materials consumed during execution of measurement included glass microfiber filters, aluminum foil containers, disposable Pasteur pipets and pipette tips. Overall, estimated cost per sample for each of the biomass estimation methods was provided in Table 4.18.

Table 4.18. Estimated cost per sample for each of the biomass estimation methods.

Method	Labour	Material	Total
Dry weight	*$15.00*	*$3.00*	*$18.00*
Optical density	*$2.00*	*$1.00*	*$3.00*
Cell count	*$2.50*	*$0.30*	*$2.80*
Chlorophyll *a*	*$7.50*	*$3.00*	*$10.50*

4.6.2 Correlation between methods

In this study, the values of r comparing biomass estimation methods for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.* were provide by Table 4.19. It demonstrated that values of r were all very close to one, suggesting that regardless of strains, culture mediums, and light cycles, experimental results of four different biomass estimation methods were all highly correlated.

Table 4.19. Pearson's correlation (r) matrix comparing the *dry weight*, *optical density*, *cell count*, and *chlorophyll a* methods for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.* in various mediums under different light cycles.

Method	Chlorophyll *a*	Dry weight	Optical density
Dry weight	*0.969*	-	-
Optical density	*0.965*	*0.971*	-
Cell count	*0.978*	*0.974*	*0.990*

Table 4.20 provides values of r comparing biomass estimation methods for only measuring the biomass growth of *Chlorella vulgaris*. Methods of dry weight, optical density, and cell count demonstrated excellent correlations between one another. This finding supports the common practice of relying solely on these three methods to monitor *Chlorella vulgaris* cultivation (Safi et al., 2014).

Table. 4.20. Pearson's correlation (r) matrix comparing the *dry weight*, *optical density*, *cell count*, and *chlorophyll a* methods for only measuring the biomass growth of *Chlorella vulgaris* in various mediums under different light cycles.

Method	Chlorophyll *a*	Dry weight	Optical density
Dry weight	*0.961*	-	-
Optical density	*0.938*	*0.972*	-
Cell count	*0.965*	*0.982*	*0.990*

In conclusion, the resulting matrices proved the excellent correlations between the methods for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.* in various growth mediums under different light cycles. This finding was supported by another study (Lowrey and Yildiz, 2011a).

4.6.3 *Assessment of method accuracy*

The methods of optical density, cell count, and chlorophyll *a* were measurements that indirectly measured the *Chlorella vulgaris* and *Anabaena sp.* biomass. One should consider relative measures to determine the accuracy of those three indirect measurements using the only direct measurement, dry weight, as a reference. One such relative performance measure was an index of agreement (d), however a potential problem with d is usually that its values are high, and might give a false impression of good performance. Therefore, another relative performance measure, coefficient of efficiency (E), was also performed. Both E and d were calculated as an indicator of the accuracy of indirect methods.

Table 4.21 demonstrates that including all strains, mediums, and light cycles, values of E in the second column are all above zero, suggesting that all three methods give better results than representing all values by the mean of dry weight method. The values of E identified the method of optical density as the most accurate indirect measurement for determining the biomass growth of *Chlorella vulgaris* and *Anabaena sp.* in various growth mediums under different light cycles. On the other hand, according to d shown in the third column of Table 4.21, the method of optical density also gives better performance than

other methods. In short, the results prove that all three indirect measurements were quite accurate for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.* in various growth mediums under different light cycles, however the method of optical density was more accurate than the methods of cell count and chlorophyll *a*.

Table 4.21. Coefficient of efficiency (E) and index of agreement (d) for *optical density*, *cell count*, and *chlorophyll a* methods for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.* in various mediums under different light cycles.

Method	E	d
Optical density	*0.879*	*0.972*
Cell count	*0.850*	*0.966*
Chlorophyll *a*	*0.878*	*0.970*

In addition, values of E and d of three methods for only measuring the biomass growth of *Anabaena sp.* were provided in Table 4.22. Obviously, values of E in the second column were quite different in magnitude. The value of E of optical density and chlorophyll *a* methods were around 0.5 and 0.6, respectively, whereas the value of E of cell count method was only around 0.2, which means that the method of cell count was not as accurate as the other two methods for measuring the biomass growth of *Anabaena sp.*. Based on the experience, the reason was related to the filamentous structure of *Anabaena sp.* The filaments could be straight, circinate or even irregular, which makes the execution of cell count extremely difficult. As a result, the method of cell count should be avoided for measuring the biomass of microalgae and cyanobacteria with a filamentous structure.

Table 4.22. Coefficient of efficiency (E) and index of agreement (d) for *optical density*, *cell count*, and *chlorophyll a* methods for only measuring the biomass growth of *Anabaena sp.* in various mediums under different light cycles.

Method	E	d
Optical density	*0.522*	*0.911*
Cell count	*0.206*	*0.853*
Chlorophyll *a*	*0.581*	*0.913*

4.6.4 Assessment of within method precision

In this study, the coefficient of variation (C_v) was performed as a technique for determining the precision of the biomass estimation methods. Each C_v was compared to those of other strains and mediums to assess the potential impacts of different strains and mediums upon the precision of the biomass estimation methods. Figure 4.7 shows that values of C_v of the method of dry weight were smaller than any other methods in both strains, which means dry weight was the most precise method for measuring the biomass of *Chlorella vulgaris* and *Anabaena sp.* in various mediums under different light cycles.

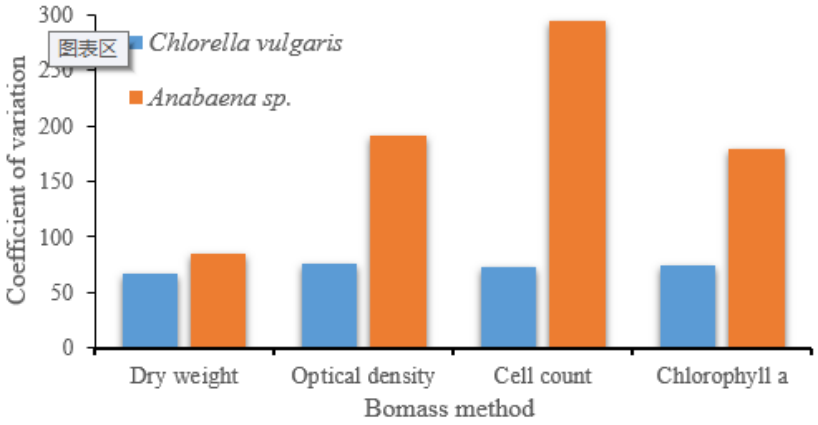

Figure 4.7. Coefficient of variation (C_v) for *dry weight*, *optical density*, *cell count*, and *chlorophyll a* methods for measuring the biomass of *Chlorella vulgaris* and *Anabaena sp.* in various mediums under different light cycles.

A much higher value of C_v in *Anabaena sp.* for cell count method can be observed in Figure 4.7. As mentioned above, it can be explained by potential influence of the filamentous structure of *Anabaena sp.*. The execution of cell count method for microalgae and cyanobacteria with a filamentous structure is much more difficult than of that for unicellular microalgae and cyanobacteria. Because of that, the method of cell count was more precise for measuring the biomass of *Chlorella vulgaris* rather than for measuring the biomass of *Anabaena sp.* in this study.

Although contradicting the expected interferences associated with chromophoric dissolved organic matter in minkery wastewater, Figure 4.8 reinforces the utility of the method of dry weight for measuring biomass in minkery wastewater. There is no evidence that organic matter in minkery wastewater increased the variability of this method.

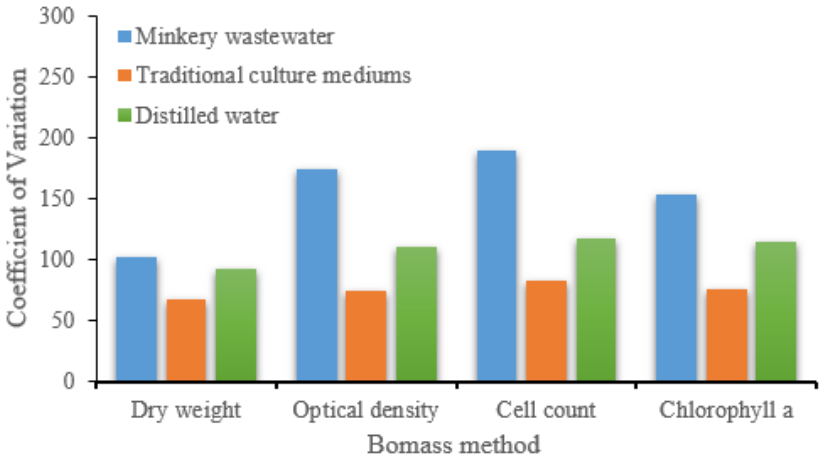

Figure 4.8. Coefficient of variation (C_v) for *dry weight*, *optical density*, *cell count*, and *chlorophyll a* methods for measuring the biomass of *Chlorella vulgaris* and *Anabaena sp.* in *minkery wastewater, traditional culture mediums, and distilled water* under different light cycles.

In conclusion, this study suggests the practice of performing dry weight measurements at the beginning and end of the growth period partnered with more frequently optical density measures and cell counts (daily), as a means of monitoring microalgae and cyanobacteria biomass growth in minkery wastewater. The chlorophyll *a* method for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.* appears to be not as practical as the other three biomass estimation methods employed, because of the low precision and high cost. Ramaraj et al. (2013) reported similar finding that Chlorophyll a is not an appropriate measurement for microalgae and cyanobacteria biomass.

CHAPTER V

CONCLUSION

5.1 Experimental Conclusion

Chlorella vulgaris demonstrated better cultivation potential than *Anabaena sp.* in controlled environment minkery wastewater. For *Chlorella vulgaris* cultivation, the minkery wastewater was a superior medium than modified Bold's basal medium under most of the light cycles. In contrast, the growth characteristics of *Anabaena sp.* in BG-11_o medium were significantly better than those in minkery wastewater under most of the light cycles. Based on the findings, this study showed the potential of using minkery wastewater as an alternative medium for microalgae and cyanobacteria production, even though the cultivation of *Anabaena sp.* in minkery wastewater remains a huge challenge due to the insufficient nitrate levels of minkery wastewater.

Both light cycles, 6-day continuous light and 48-hour light and 24-hour dark, achieved the largest biomass accumulation and crude protein productivity of *Chlorella vulgaris* in minkery wastewater and using 48-hour light and 24-hour dark instead of 6-day continuous light reduced energy costs of the cultivation. The light cycle of 6-day continuous light, 48-hour light and 24-hour dark, and 24-hour light and 48-hour dark achieved the largest lipid and carbohydrate productivity and of *Chlorella vulgaris* in minkery wastewater, and using light cycle of 24-hour light and 48-hour instead of 6-day continuous light or 48-hour light and 24-hour dark reduced energy costs of the cultivation.

The highest nitrogen and phosphorus removal from minkery wastewater were achieved using *Chlorella vulgaris* under light cycles of 6-day continuous light, and 48-hour light and 24-hour dark, and using 48-hour light and 24-hour dark instead of 6-day continuous light reduced energy costs of the cultivation. It was supported by the biomass increase data discussed above in which both of the light cycles also experienced two largest biomass accumulations of *Chlorella vulgaris* in minkery wastewater.

The cost and time analyses suggested that cell count was the most cost-effective method with a total cost of $2.80 per sample, and the most time-effective method was optical density with an average time required for 0.13 hour per sample. The resulting correlation matrix demonstrated excellent correlations between each of biomass estimation methods. The coefficient of efficiency and index of agreement suggested that optical density was the most accurate indirect method for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.*. In contrast, the method of cell count demonstrated a lack of accuracy compared to the other two indirect methods for the measurement of *Anabaena sp.* with a filamentous structure. The method of dry weight was identified as the most precise method for measuring the biomass growth of *Chlorella vulgaris* and *Anabaena sp.*. Overall, this study suggests the practice of performing dry weight measurements at the beginning and end of the growth period partnered with more frequently optical density measures and cell counts (daily), as a means of monitoring microalgae and cyanobacteria biomass growth in minkery wastewater.

5.2 Future Research

As mentioned in the previous chapter, before the experiment, all raw minkery wastewater were diluted with 99% autoclaved distilled water. In reality, the utilization of freshwater should be minimized to reach a commercial scale in the future truly. Therefore, some studies can be conducted to investigate the cultivation and wastewater treatment potential of seawater microalgae and cyanobacteria strains in controlled environment minkery wastewater.

Furthermore, many research can be carried out on the cultivation and wastewater treatment potential of microalgae and cyanobacteria in minkery wastewater with a higher concentration. Based on the biomass accumulation data discussed above, mean biomass increase of *Chlorella vulgaris* under heterotrophic condition (6-day continuous dark) in minkery wastewater was not significantly higher than those in modified Bold's basal medium and distilled water. This result may change with the increased minkery wastewater concentration, because of the higher organic carbon content of 3% minkery wastewater.

Last but not least, in this study, the most appropriate cultivation techniques (light cycles) were identified for the production of each main chemical component of *Chlorella vulgaris* in minkery wastewater. Based on this design, a similar study can be conducted to determine the best growth medium for the production of each main chemical component of *Chlorella vulgaris* under different light cycles.

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