AUGMENTED GROWTH OF *HAEMATOCOCCUS PLUVIALIS* USING NUTRIENTS FROM POST HYDROTHERMAL LIQUEFACTION WASTEWATER (PHWW)

BY

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THESIS

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ABSTRACT

This study was conducted to evaluate if a high-value algae species, *Haematococcus Pluvialis*, could be successfully cultivated utilizing nutrients derived from a concentrated wastewater source and produce the potent antioxidant astaxanthin at similar quantities to when it is grown on conventional media and nutrient sources. The primary wastewater used in this study was the aqueous product of hydrothermal liquefaction (HTL), which is an attractive process for the conversion of wet biomass to bio-crude oil. Using recycled nutrients from the post HTL wastewater (PHWW) or other wastewater sources for growing algae can reduce costs and synthetic nutrient inputs, which improves the sustainability of algae cultivation operations.

In the first part of this work, we determined an appropriate dilution of PHWW to support algal growth and astaxanthin production, while avoiding significant inhibitory effects of PHWW that have been previously reported in the literature. PHWW, was characterized for nitrogen and phosphorus content and compared with other wastewater sources and conventional algal media. Then, *H. Pluvialis* cells acclimated on Bold's Basal Media (BBM) were inoculated across a 0-2% PHWW gradient in a well plate to identify the algae's tolerance for the PHWW. This result determined that *H. Pluvialis* cells were able to grow well at concentrations of PHWW up to 0.25% when mixed with BBM.

In a second experiment designed to investigate algal preferences for different chemical forms of nutrients, *H Pluvialis* was inoculated in BBM having only nitrate or only ammoniacal nitrogen as the nitrogen source. The cell counts were measured daily with a hemocytometer, and nutrient removal was also measured. This experiment showed no significant differences in algal growth based on the chemical form of nitrogen provided.

Using the concentrations of 0.125% and 0.25% PHWW that were determined in the first experiment to support good algal growth, another larger scale cultivation experiment was performed in three 1L flasks to confirm algal growth results in comparison to the BBM only control. In each of the respective media compositions, the total amount of inorganic nitrogen was held constant by removing only nitrate from the baseline BBM media recipe, replacing it with the corresponding concentration of total ammoniacal nitrogen (TAN) from PHWW. For the 0.125% and 0.25% PHWW treatments, the corresponding amount of nitrogen replaced was 5 and 10%, respectively. During this experiment, nutrient concentrations were measured daily, and the algae were able to reduce nitrogen concentrations from the various media mixes by at least 80%. Based on cell counts, the PHWW at 0.125% and 0.25% augmented the algae growth by 44% and 17%, respectively.

The algae biomass from the BBM control media and the alternative media blends with PHWW nutrients was harvested and subjected to stress conditions of elevated light and salinity. Stressing the cells was intended to induce increased production of carotenogenic antioxidants, including astaxanthin, which was confirmed by a gradual reduction of the chlorophyll: carotenoid ratio. After encystment, astaxanthin production was quantified using high performance liquid chromatography (HPLC), and these data showed that it was reduced by 47.3% and 34.1% in the 0.125% and 0.25% PHWW augmented cultures, respectively. Even though the amount of astaxanthin was reduced for the PHWW treatments, the final harvest extracts of algae grown with PHWW presented a more diverse and complex carotenogenic profile.

In conclusion, this study demonstrates that PHWW nutrients can enhance *H. Pluvialis* cell growth, but further work is needed to better understand and control the amount and

types of carotenoids produced by *H. Pluvialis*. The long-term effects of acclimation and adaptation were not studied, and provide additional opportunities for improving overall system performance. In particular, acclimation could help mitigate inhibitory effects of the PHWW that were observed even when PHWW was added at very low levels. Finally, other sources of sustainable nutrients from wastewater with less inhibitory contaminants should be investigated to hopefully provide replacement of a larger fraction of the nutrients needed for large scale algae cultivation.

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CHAPTER 1: INTRODUCTION

As our population grows, there will be an ever-increasing demand to renewably create food, energy, and water resources to sustain life around the world. Water management is of special importance because access to fresh water is limited, and thus, innovative wastewater remediation technologies are needed to help recycle these resources sustainably. Wastewater treatment plants (WWTP) receive solid materials and more dilute concentrations of nutrients, including dissolved carbon, nitrogen, and phosphorous nutrients, among others, from natural sources, rainwater, households, and some commercial operations. Industrial and agricultural systems producing more concentrated wastewaters, which often include other inhibitory compounds and metals, often choose to perform on site treatment for economic reasons and the opportunity to reclaim and reuse certain resources.

High capital and operational costs for advanced remediation can limit the availability and effectiveness of wastewater treatment. The Environmental Protection Agency (EPA) estimates that in the U.S. more than $90B per year is needed to maintain and upgrade wastewater treatment infrastructure for drinking water to reduce the rising 900B gallons of untreated sewage that is discharged each year; however, only 40% of this is nationally funded for the mostly municipally-run WWTP operations across the country (ASCE, 2011). Municipal WWTP are often funded by a combination of local, state, and federal taxes, in addition to the local usage fees. In some cases, water treatment may be the only city service that generates income for small communities (Drinan et al., 2012).

Different treatment systems must use appropriate remediation strategies to clean wastewaters based on phase separation, pollutant concentrations, and economy of scale, while also being financially managed by the local municipality. Many modern wastewater treatment systems employ biological processes to remove pathogens and organic material to meet EPA and other global regulatory discharge standards. These systems have been developed to be robust and primarily for the purpose of reducing pollution discharge, in addition to the recovery of nutrients in different municipal, industrial, and agricultural streams. Pollutant concentrations and system design largely influence treatment costs, but technologies for resource and energy recovery, like methane production from anaerobic

digestion that is used in municipal and agricultural systems, can generate renewable value in waste management systems (Lettinga, 1995; Weiland, 2006).

Production of renewable resources from specialized wastewater remediation strategies can help to offset treatment costs and, if possible, generate energy or provide an additional revenue stream. In one case, the Innovation Center for US Dairy (2013) has reported billions of dollars in potential renewable revenue through the recovery of energy and nutrients, as presented in Figure 1. Given the additional potential revenue that could be recovered from wasted aquatic nutrients, new treatment technologies and systems should be developed to adequately remove chemicals of concerns at minimum costs while making operations more economically viable.

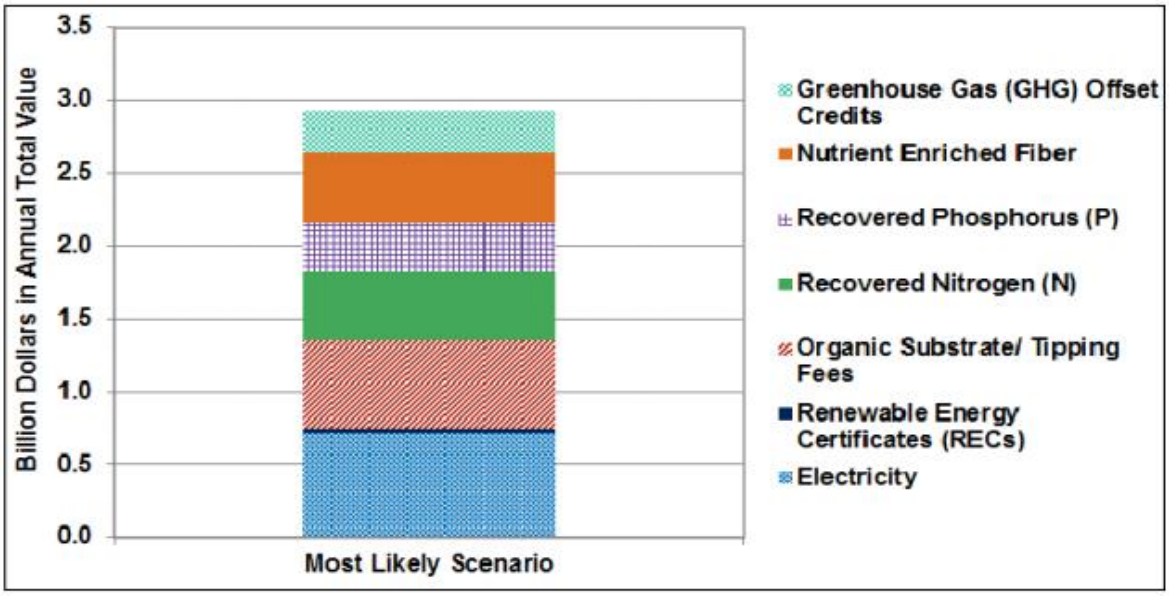


Figure 1: U.S. Dairy Industry's Report *National Market Value of Anaerobic Digester Products.* (Innovation Center for US Dairy, 2013)

Although biological systems can be some of the most difficult and costly to maintain, they have the advantages of being adaptable and cleaning water by assimilating waste nutrients to generate biomass. Carbon, nitrogen, and phosphorous, amongst other micronutrients in wastewaters, can be assimilated by microorganisms, most often bacteria, to remove pollutants. Such systems often use sequential biochemical processes performed by combined or separate biologically engineered processes, listed as equations 1-3 for

examples, in sequential tanks or constructed wetlands oxidize incoming nitrogen species for cellular uptake and clean water discharge (Water Environment Federation, 2007).

Eq. 1 $2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$

Eq. 2 $2NO_2^- + O_2 \rightarrow 2\ NO_3^-$

Eq. 3 Overall Reaction, $NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$

Regular biomass harvesting is necessary for a continuous system operation. However, these biomasses presenting less redeemable value are often discarded to landfills or, in other cases, applied to fields as a fertilizer, providing lesser return value for wastewater treatment plants than possible through newer renewable systems. High operational costs can be restrictive of industry development, and thus, advancement in the industry should aim to lower these costs or derive more renewable resources from treatment processes. Investing more energy and resources into wastewater treatment plants will improve water quality through nutrient removal (Burdick et al., 1982; Foley Haas, 2010), but water resource management must be improved to recover these nutrients making wastewater treatment more efficient and sustainable.

Technologies capable of remediating different types of wastewater through the recovery of nutrients and maximizing renewable resource production have the potential to benefit a wide range of waste producers and consumer markets. New biological systems need to be explored to succeed in different environments and to aid in diverse water management contexts around the world. Leveraging wastewater treatment operations to create resources through emerging biotechnology can contribute to future prosperity and alleviate growing concern for water scarcity.

CHAPTER 2: LITERATURE REVIEW

Microalgae, one of the first life forms on Earth, were instrumental in fixing carbon dioxide through photosynthesis, and this process generates as much as 50% of the oxygen in our atmosphere. Some have estimated that algae account for approximately 50% of the total fixed organic carbon, although they represent <1% of the photosynthetically generated biomass annually (Darzins et al., 2010). Algal research for scalable fuel and food production dates as far back as the 1940's in the United States (Borowitzka et al., 2013). As its more diverse capabilities were studied, including wastewater treatment, scientists became increasingly interested in the engineering of algae in renewable and value recovery systems. Studies have expanded worldwide to optimize growth conditions in different environments and further understand the processes of nutrient assimilation and photosynthesis for cultivating algal biomass continuously at scale.

Different bioreactor systems have been proposed for maximizing algae production varying in temperature, pH, light intensity, mixing, as well as nutrient composition and sourcing, amongst other parameters (Razzak et al., 2013). Light, water, and carbon dioxide are the key components for photosynthesis and production of algal biomass (Equation 4). Sugars assembled through photosynthesis, along with nitrogen, phosphorous, and other micronutrients, are used to build fundamental cellular components including lipids, carbohydrates, and proteins, while also generating auxiliary biocompounds in lower concentrations. Grobbelaar et al. (2004) presents a general equation for algal biomass that can be used to perform mass balances of media nutrients for optimization of algal biomass production ($CO_{0.48}H_{1.83}N_{0.1}P_{.01}$). Concentration of growth nutrients, along with other requisites, directly influence cell doubling rate and total biomass production; thus, wastewaters could be combined with or replace conventional algal media to lower costs of algae production and renewable valuable biomass that could improve economics of treatment systems (Mallick, 2002; Grönlund et al. 2004; Behzadi et al. 2007).

Eq. 4 $$6CO_2 + 6H_2O + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2$$

Combining algae production with wastewater treatment was first proposed in California (Oswald, 1957). Since then, coupled systems have been proven to effectively remove nitrogen and phosphorous from wastewater streams meeting EPA standards while also replacing the need for synthetic nutrients in algae cultivation (Aresta et al., 2005; Woertz et al., 2009; Johnson et al., 2010). Woertz (2009) demonstrated ammonia and phosphate removal efficiencies up to >99% from municipal waters under a variety of operating conditions (Table 1). With respect to wastewater treatment costs, high production algal ponds have been estimated to have a cost of up to 70% less than activated sludge wastewater plants (Lundquist et al. 2010, EPA 2006), while also achieving enhanced effluent quality by removing nutrients more effectively (DOE, 2010; Mata et al., 2010; Yang et al., 2011). Continually improving results suggest that algae biomass production systems could be adapted to function as wastewater treatment systems and, thereby, beneficially reuse wastewater nutrients.

Table 1. Nutrient removal from municipal wastewaters (Woertz et al., 2009), HRT = Hydraulic Retention Time

Operating Conditions	Total Ammoniacal Nitrogen ($mg \cdot L^{-1}$)			Phosphate as P ($mg \cdot L^{-1}$)		
	Influent	Effluent	% Removal	Influent	Effluent	% Removal
CO_2, 4 Day HRT	39.0	<0.02	>99	2.1	<0.02	>99
CO_2, 3 Day HRT	39.0	<0.02	>99	2.1	<0.02	>99
Air, 3 Day HRT	39.0	6.1 (+/-0.89)	84	2.1	<0.02	>99
CO_2, 2 Day HRT	39.0	0.6 (+/- 0.57	98	2.1	0.15 (+/- 0.15)	93

There exists a wide variety of wastewaters with different potencies and compositions, each having particular advantages and disadvantages with respect to being used for growth of algae. Consideration should be especially given to the concentration of nitrogen and phosphorus species within different types of municipal, agricultural, and industrial wastewaters, as these nutrients are the most needed for biomass generation apart from carbon.

Expansion and implementation of algal biotechnology is hindered in many respects by the cultivation system. Open systems, such as lagoons and ponds, have similarities with existent wastewater treatment plant design, making them seemingly interchangeable;

however, open algal systems face additional complexities, such as limited light penetration, losses from evaporation, and limited diffusion of CO_2 from the atmosphere (Xu et al., 1999), as well as contamination by bacteria, protozoa, or fungi which can affect treatment efficiency and biomass yields (Chaumont, 1993). Closed photobioreactors have thus been increasingly explored as a solution to controlling contamination and other limiting effects of open systems for maximization of biomass productivity and, in the case of simultaneous wastewater treatment or carbon dioxide uptake, effective nutrient assimilation. While also presenting some financial difficulty, large capital costs of different renewable algal systems are hoped to be offset by the production of the biomass and its derivative biochemicals like bio-oils for fuels (Delrue et al., 2012; Fortier et al., 2014).

Over 35,000 natural species of algae, both unicellular and seaweed like polymers, have been identified and few studied for their different capacities to produce large amounts of biomass, provide food nutrition, treat wastewater, assimilate carbon dioxide, and generate a wide array of biochemicals (Razzak et al. 2013). Since 1980, the global algal biomass market, which does include some photosynthetic bacteria like Spirulina and Nostoc, has amounted to as much as 5000 tons of dry matter annually and approximately over a $1B USD industry, not including processed products (Spolaore et al., 2006). The most recent and innovative studies have aimed to improve the expanding algae sector by using alternative media to lower costs for biomass production. According to Lundquist (2010), algal derived biofuels from newly purchased inputs could cost more than $400/barrel; however, using wastewater nutrients and equipment as a substitute could bring production costs to lower than $30/barrel, as an example. The synergy of wastewater treatment with algal biomass production could bring further growth to the algae industry by replacing the existent supply chain for petroleum derived chemicals.

Algal biomass grown on municipal and agricultural wastewaters at scale for lipid production for biofuels have been extensively researched with species like *Chlorella* and *Scenedesmus,* amongst others (Jiang et al., 2011; Park et al., 2011; Bhatt et al., 2014). These species are most often utilized for their fast proliferation rate and ability to grow in a wide range of environmental conditions. Bhatt et al. (2014) reports the numerous species with their biomass and lipid production rates as well as providing nutrient removal efficiencies in Table 2 and 3, respectively.

Table 2. Biomass and lipid productivity of microalgae grown in different wastewaters (Bhatt et al., 2014)

Microalgae species	Wastewater type	Biomass Productivity (mg L^{-1} d^{-1})	Lipid Content (%DW)	Lipid Productivity (mg L^{-1} d^{-1})
Chlorella pyrenoidosa	Activated Sludge Extract	11.55	NA	NA
Chlorella pyrenoidosa	Digested Sludge Extract	51.82	NA	NA
Chlorella pyrenoidosa	Settled Sewage	275	NA	NA
Chlorella pyrenoidosa **and** ***Scendesmus sp.***	Activated Sewage	92.31	NA	NA
Botryococcus braunii	Secondarily Sewage	35.00	NA	NA
Scendesmus sp.	Artificial Wastewater	126.54	12.80	16.2
Poly culture of ***Chlorella sp.*** ***Micractinium*** **sp** ***Actinastrum*** **sp.**	Dairy Wastewater	NA	29.00	17
Poly culture of ***Chlorella sp.*** ***Micractinium*** **sp** ***Actinastrum*** **sp.**	Primary Clarifier Effluent	NA	9.00	24.4
Chlorella asccharophil	Carpet mill	23	18.10	4.2
Scendesmus sp.	Carpet mill	126.54	12.80	16.2
Chlorella **sp.**	Centrate	231.4	33.53	77.5
Hindakie **sp.**	Centrate	275.0	28.30	77.8
Chlorella **sp.**	Centrate	241.7	30.91	74.7
Scenedesmus **sp.**	Centrate	247.5	30.09	74.5
Auxenochlorella protothecoides	Concentrated Municipal Wastewater	268.8	28.9	77.7
Chlamdomonas Mexicana	Piggery Wastewater	NA	33 ± 3.4	0.31 ± 0.03
Scenedesmus obliquus	Piggery Wastewater	NA	31 ± 0.8	0.24 ± 0.03

Table 3. Nutrient removal efficiency of microalgal species (Bhatt et al., 2014)

Microalgal species	Wastewater type	Nitrogen removal efficiency	Phosphate removal efficiency	COD removal efficiency
Chlorella vulgaris	Textile wastewater	44.4-45.1%	33.1-33.3%	38.3-62.3%
Scenedesmus sp. LX1	Modified Effluent of a wastewater treatment plant of an electric factory by photo-membrane bioreactor	46%	100%	NA
Chlorella sorokiniana and aerobic bacteria	Potato processing industry	>95	80.7	84.8
Chlorella sorokiniana and aerobic bacteria	Pig manure	82.7	58.0	62.3
Chlamydomonas sp. TAI-2	Industrial wastewater	100%	33%	NA
Auxenchlorella protothecoides UMN280	Concentrated municipal wastewater	59%	81%	88%
Chlorella Mexicana	Piggery wastewater	62%	28%	NA
Scenedesmus obliquus	Piggery Effluent	23-58%	48-69%	NA
Chlamydomonas Polypyrenoideum	Dairy industry wastewater	74%-90%	70%	NA
Euglena	Sewage treatment plant	93%	66%	NA

Following biomass harvesting, numerous techniques for efficient drying and extraction have been investigated, as biomass collection and treatment prove to be the limiting process for these systems. Weschler et al. (2014) ran a study of 122 different production scenarios to compare the limitations in producing biofuels from algal biomass. They observed that scenarios that avoid thermal drying of wet biomass by using settling or membrane filtration presented the greatest energy efficiency. As an alternative, some wet extraction strategies have been investigated to remove biochemicals from biomass without the need for drying. Notably, more efficient extraction has been successfully performed using ultrasound waves to break cells (Adam et al., 2012), distillation procedures (Tanzi et al., 2013), or novel solvents (Olkiewicz et al., 2015), amongst others. Fortier et al. (2014) also concluded that greater energy efficiency could be achieved by using wet biomass in thermochemical conversion technologies like hydrothermal liquefaction (HTL). Moreover, production of oil from algal biomass via HTL has been shown as an effective way to recycle concentrated nutrients for production of more biomass (Zhou et al., 2013). Algal biomass producers must, therefore, be conscious of the species and, when necessary, appropriate extraction technology to maximize lipid production and energy efficiency for a resource recovery scenario.

Some biomass producers aim to grow algae for much higher value chemicals that include aquaculture nutrition, pigments, and pharmaceuticals, among others. More specific investigation on the biochemical composition of various species has derived more knowledge of low concentration metabolites and their production pathways for efficient harvest (Pignolet et al., 2013). Notably, different families of algae and the environmental conditions greatly affect their growth rates and the production of the desired chemicals. Cuellar-Bermudez et al. (2015) provide a list of some of the valuable microalgae species and their derivative high-value metabolites (Table 4).

Table 4. Microalgae species of high-value compounds extraction and applications (Cuellar-Bermudez et al., 2015)

Species	Product	Application areas
Chlorella vulgaris	Biomass, pigments	Health food, food supplement
Coelastrella striolata var multistriata	Lutein, β-carotene	Pharmaceuticals, nutrition
Crypthecodinium conhi	Canthaxanthin, astaxanthin, β-carotene	Pharmaceuticals, nutrition, cosmetics
Diacronema vlkiamum	Docosahexaenoic acid	Pharmaceuticals, nutrition
Dunaliella salina	Fatty acids	Pharmaceuticals, nutrition
Caldiera suphuraria	Carotenoids, β-carotene	Health food, food supplement, feed
Haematococcus pluvialis	Phycocyanin	Pharmaceuticals, nutrition
Isochrysis galbana	Carotenoids, astaxanthin, canthaxanthin, lutein	Health food, pharmaceuticals, nutrition, feed additives
Lyngbya majuscule	Fatty acids, carotenoids, fucoxanthin	Pharmaceuticals, nutrition, cosmetics, animal nutrition
Muriellopsis sp.	Immune modulators	Pharmaceuticals, nutrition
Nannochloropsis gaditana	Lutein	Pharmaceuticals, nutrition
Nannochlorpsis sp.	Icosapentanoic acid	Pharmaceuticals, nutrition
Odontella aurita	Fatty acids	Pharmaceuticals, cosmetics, baby food
Parietochloris incise	Arachidonic acid	Nutritional supplement
Phaedactylum tricormutum	Lipids, eicosapentaenoic acid, fatty acids	Nutrition, fuel production
Porphyridium cruentum	Arachidonic acid, polysaccharides	Pharmaceuticals, cosmetics, nutrition
Scenedesmus almeriensis	Lutein, β-carotene	Pharmaceuticals, nutrition, cosmetics
Schizochytrum sp.	Docosahexaenoic acid	Pharmaceuticals, nutrition
Spirulina platensis	Phycocyanin, γ-Linolenic acid, biomass protein	Health food, cosmetics
Ulkenta spp.	Docosahexaenoic acid	Pharmaceuticals, nutrition

Production and recovery of high-value bioproducts from waste feedstocks is becoming increasingly studied, as researchers recognize the burgeoning population and high demand for agricultural production (Kaur et al., 2013). Amongst the organisms on this list, only a few have been studied for their simultaneous treatment of various wastewaters and production of bioproducts with value higher than that of lipids for biofuels. *Spirulina*

Platensis is a cyanobacteria that has been studied with respect to wastewater treatment in various parts of the world (Hong et al., 1993; Olguin et al., 2003; Wang et al., 2013); however, its production of phycocyanin when grown on wastewater was reported in one case (Chaiklahan et al., 2010). Considering this, further studies are needed for the production of high value algal species and their products when grown on wastewaters. Table 5 goes further to list some of the species with their valuable biocompounds, market values, and reported production.

Table 5. Different species of photosynthetic organisms with their respective valuable biocompounds, market value, and productivity rates (Borowitzka, 1992; Lee, 2001; Sreekumar et al., 2016)

Species	Valuable Compound	% Dry Weight	Market Value	Biomass Harvest Rate
Haematococcus Pluvialis	Astaxanthin	1-6	\$2000-7000 · kg^{-1}	5-13 g· m^{-2} · d^{-1}
Anthrospira Maxima	Phycocyanin	4-6	\$500-10000 · kg^{-1}	20-30 g· m^{-2} · d^{-1}
Nostoc Commune	UV A/B Absorbing Pigments	10	\$125· kg^{-1}	40 g· m^{-2} · d^{-1}
Dunaliella	β-Carotene	8-14	\$300-3000· kg^{-1}	30 g· m^{-2} · d^{-1}
Chlorella	Lipids (Oil)	20-50	\$1.60· kg^{-1}	26-30 g· m^{-2} · d^{-1}
Scenedesmus	Lipids (Oil)	31-60	\$1.60 · kg^{-1}	13-49 g· m^{-2} · d^{-1}

Coupling high value biomass production with wastewater nutrient recovery can serve 2 primary functions: First, the productivity of media for production of biomass containing valuable biocompounds could be improved, making these algae technologies more economical, and secondly, the production of potentially lucrative biomass could help to lessen the burden of wastewater treatment costs if performed at scale. This study investigates a new scenario for renewable biomass production using wastewater nutrients and defines some of the key cultivation parameters necessary to use wastewater for nutrient recovery and production of a high value compound.

Astaxanthin ($C_{40}H_{52}O_4$) is a xanthophyll pigment that presents in 3 isomeric forms: 3S-3S', 3R-3S', and 3R-3R, which are all oxygenated carotenoids synthesized from isoprenoid monomers. It is widely known for its antioxidant properties, being 10 times more effective

than other important carotenoids, such as zeaxanthin, lutein, canthaxanthin, and beta-carotene, and at least 100 times more than alpha-tocopherol (Shimidzu et al., 1996; Higuera-Ciapara et al., 2006). These various carotenogenic species have similar structures and are presented in Figure 2. Carotenoids serve to prevent reactive oxygen species and deactivate singlet oxygen during the photosynthetic processes. These molecules are produced by certain plants, some microbes, and other organisms as accessory pigments that provide the ability to harvest alternative light wavelengths and, thus, increase photosynthetic potential. As animals, including humans, cannot synthesize these compounds, they must be consumed through food including fruits, vegetables, and seafood (Jackson et al., 2008).

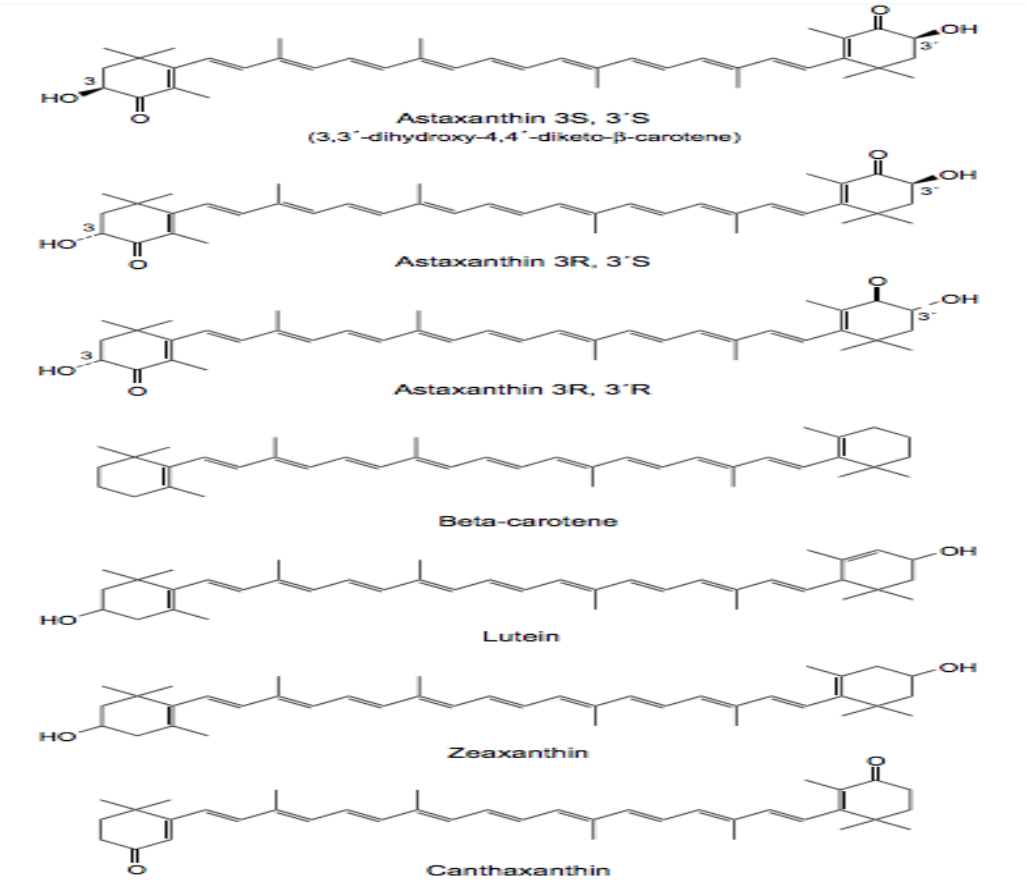


Figure 2. Carotenoids that are found in nature (Guerin et al., 2003)

Worldwide, astaxanthin is a carotenoid primarily used in feed mixtures for the aquaculture industry that helps to give salmon, trout, and crustaceans reddish colors. Although it is only 50-100ppm of salmon feed, it can constitute as much as 10-15% of the

total cost (Breithaupt, 2007). Its application as an animal and fish food ingredient has growing significantly since 1990, amounting to as much as 72% of the total astaxanthin market (Markets and Markets, 2017). More recently, it is also growing in use for pharmaceuticals and cosmetics, totaling a projected global market of 670 metric tons and a value of $1.1B USD by 2020 (Research and Markets, 2015). As of 2000, petroleum based synthetic astaxanthin occupied greater than 95% of the market (Lorenz et al., 2000), but this is lessening to closer to 55%, as consumers have shown preference for natural products (Markets and Markets, 2017). Additionally, studies have shown that the esterified form produced by algae, being more stable and less susceptible to oxidation, is preferable to the non-esterified synthetic version (Seabra et al., 2010). Astaxanthin, amongst other related carotenogenic compounds shown in Figure 2, has been shown to protect from UV damage, have benefits in immune response, and presented improved cellular reproduction, in addition to other health benefits (Guerin et al. 2003). More recent studies have shown increased application for astaxanthin as a nutritional supplement for its potential to serve as an anticancer agent, as well as prevent diabetes, cardiovascular diseases, and neurodegenerative disorders (Ambati et al., 2014). Furthermore, the authors reported it has new dosage forms that include tablets, capsules, syrups, oils, soft gels, creams, powders, and even raw biomass. As market demand and applications increase, industries and stakeholders have sought out more natural and sustainable suppliers to further replace the predominant petrochemically synthesized sources.

Astaxanthin has been successfully derived from plants, yeast species, algae, and, at a research level, engineered species of *E. Coli*. However, the most potent natural producer of astaxanthin is the freshwater algae species *Haematococcus Pluvialis,* having up to 5% percent of its dry cell weight as astaxanthin when stress is induced to achieve its mature encysted form. Wayama et al. (2013) describes the unicellular Chlorophyta species as having two cell morphologies, a vegetative green flagellate stage known as a macrozoid and a larger encysted red state known as an aplanospore or akinete, as shown in Figure 3. Industrial scale production of natural astaxanthin began in the 1990s using *H. Pluvialis* (Lorenz et al., 2000; Oleizola, 2000). Lorenz et al. (2000) describe the mechanisms for carotenoid production and the profiles between cell types. Astaxanthin is synthesized through the isoprenoid pathway, which accounts for other lipid-soluble molecules like sterols, steroids, prostaglandins,

hormones, and some vitamins. Green vegetative cells contain between 75-80% lutein and 10-20% β-carotene, among which are low concentrations of astaxanthin, while red encysted **a**kinetes have as much as 80% astaxanthin within the carotenoid fraction of the cell. The astaxanthin produced within stressed *H Pluvialis* cells has multiple forms, being 70% monoesters, 25% diesters, and 5% free astaxanthin. As part of the process to produce algal biomass and derivatives, companies have sought innovative strategies to manipulate these pathways and compound profiles within the cells during their growth. Industrial systems have used multiple strategies, such as salt concentration, pH change, increased light intensity, and nutrient deprivation, amongst others, to induce encystment for enhanced accumulation of astaxanthin within akinete cells (Oleizola et al., 2005).

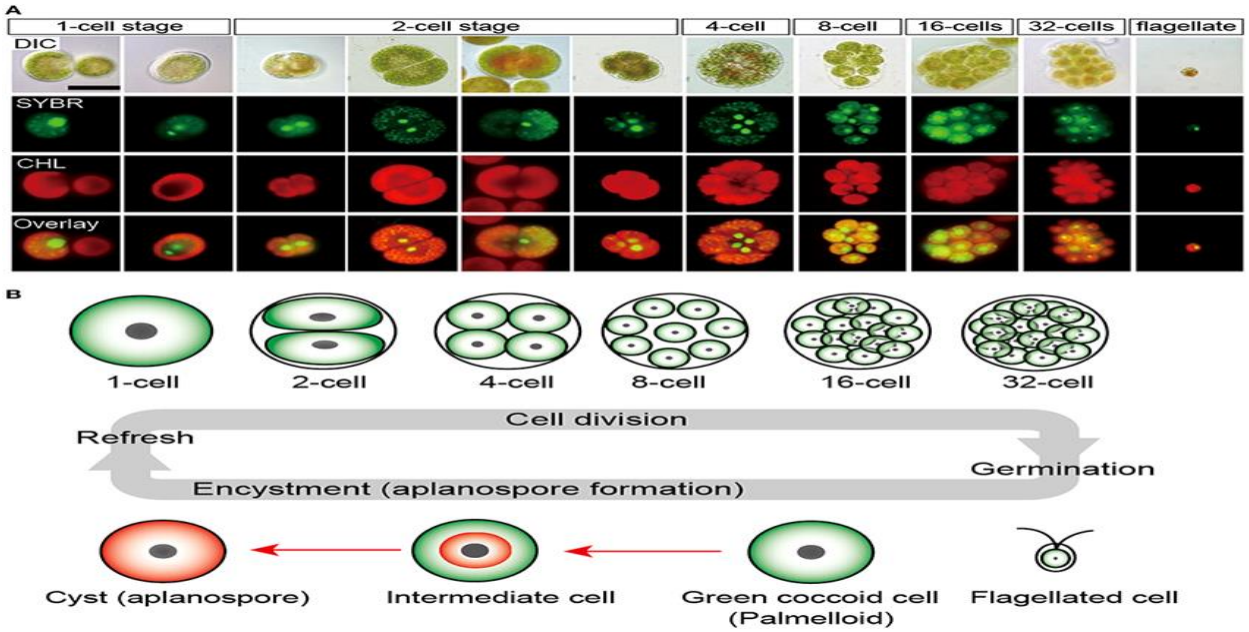


Figure 3. Life cycle and cell morphologies of *H. Pluvialis* (Wayama et al. 2013)

One of the biggest challenges when growing *H. Pluvialis,* as is with many other types of slow growing microbes, is susceptibility to contamination by other organisms. This can be the case with bacteria, fungi, protozoa, or other types of algae because contaminant species outcompete the organism of interest for available nutrients. A study comparing algal antibiosis used 5 different common freshwater algae at different inoculation ratios to determine which species could outcompete others for light and nutrients, as well as by secreting inhibitory compounds that prevented the growth of lesser dominant species. *H.*

Pluvialis was shown to be the least likely to thrive, especially against the two more robust growing species, *Chlorella* and *Scenedesmus* (Proctor, 1957). Thus, producers have had to implement considerable measures to limit the potential of such contaminants. Many strategies involve use of sub-micron filters or continual sterilization of bioreactor materials while working in scale up batches. For *H. Pluvialis,* vegetative cells are grown in smaller vessels with semi batch conditions to preserve an axenic culture before exposing them to stress conditions for encystment, usually in a more open environment. By this time in the industrial growth cycle, organisms do not need to compete for nutrients nor cultures need to be axenic because a contaminant cannot take over the culture before astaxanthin synthesis is complete (Shah et al., 2016). Upon contamination, however, it can be difficult to isolate *H. Pluvialis* due to its relative low cell density. As an alternative, Reinecke et al. (2009) sought mechanisms to eliminate contaminant species from large scale cultures. Despite giving *Haematococcus* an advantage in inoculation percentage, they also found that the species could not outcompete contaminant *Scenedesmus*. Several treatments including environmental stresses, such as high light intensity, nutrient deprivation, pH, or temperature, as well as inhibitory compounds like Rose Bengal and hydrogen peroxide, could eliminate most of the contaminant without also killing all *H Pluvialis* cells, however, this is not always successful nor the best strategy. Heliae (2015), an applied life sciences technology company that develops commercial scale platforms for algae systems, patented a hydrogen peroxide treatment for cultures to eliminate some contaminants. If a contaminant cannot be eliminated, algae batches and scale up process might need to be started a new, and additionally, potential revenue could be lost. These challenges present a significant bottleneck in the cultivation of some species of algae, including *H. Pluvialis*.

Nguyen (2013) performed a sustainability analysis to compare the different production methods, shown in Table 6. Production via algae has several benefits as compared to other microorganisms and the predominant chemical synthesis. Natural sources are seemingly healthier as they are safe for human consumption as well as having a higher oxygen radical absorbance capacity (ORAC) value (Chew et al, 2004; Palozza et al., 2009). Moreover, synthetic astaxanthin has not been approved for direct consumption by humans in food or supplements (Li et al., 2011). However, the table data suggests that natural production of astaxanthin via microorganisms might still be too costly but could be

more widely accepted if the production costs can be brought down to compete with the price of petrochemical synthesis. In Table 6, the difference in cost for raw materials and air emissions appear to be the major costs limiting factor for the algae derived astaxanthin; thus, eliminating or replacing these inputs could make these natural biological systems more competitive in the market. Moreover, biological production via algae could have additional environmental benefits if greenhouse gases like carbon dioxide can be sequestered or alternatively nutrient sources were used for biomass production.

Table 6. Comparison of Production Methods for Astaxanthin (Nguyen, 2013), ORAC = Oxygen Radical Absorbance Capacity

Per kg of Astaxanthin	Economical				Environmental		Societal		
Production Method	Raw Materials Cost ($)	Land Usage (sq. km)	Energy Usage (kWh)	Energy Cost ($)	Waste Water *	Emissions (Air)*	Cost($)	Human Consumption?	ORAC
Chemical Synthesis	40	10	170	26	0	2	2,000	No	33%
Yeast Fermentation	140	20	1,062	160	9	5	2,500	Most	66%
Algal Synthesis	164	25	796	120	2	9	>7,000	Yes	100%

Various studies have sought to optimize biomass production using different media and environmental conditions, as nutrient source, vitamin concentration, light, and aeration, among other factors, can all effect biomass production (Borowitzka et al, 1991; Gong et al., 1997; Goskan et al., 2011). Typically, conditions vary in nitrate compound concentrations between 0.25 and 1.0 $g \cdot L^{-1}$, vitamin concentrations for thiamine (B_1), biotin (B_7), and cyanocobalamin (B_{12}) of 0.3, 0.4, and 0.75 $mg \cdot L^{-1}$, respectively, and light irradiance between 10-150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, resulting in the highest growth rates and cell densities between 1-10 x 10^5 $cells \cdot mL^{-1}$. Treatments outside the range of these conditions resulted in lower cell densities and premature encystment of cells, due to environmental stress. Amongst the highest cell densities reported, Hong et al. (2010) compared the use of BG-11, OHM, and RM media and concluded that RM was the best for a strain isolated in Vietnam, growing to a maximum density of 5.1 x 10^4 $cells \cdot mL^{-1}$ when replenishing nutrients every other day. Imamoglu et al. (2007) also achieved a relatively high cell density of 9.5 x 10^5 $cells \cdot mL^{-1}$ when using RM media under a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$. These factors range widely across studies and are conducted for small, batch cultures. While some nutrients and vitamins are requisite and others irreplaceable, nitrogen and carbon sources appear to be

interchangeable for batch cultures. This suggests that media components could be substituted with less costly alternatives, without losing cell density or astaxanthin production.

Amongst studies manipulating different nutrient concentrations, light intensities, and other environmental factors to maximize cell production, researchers have measured chlorophyll and carotenoid densities, especially astaxanthin, to observe the growth of *H. Pluvialis*. One study that manipulated light intensity, nutrient concentrations of nitrate, phosphate, and iron, and salt concentration, concluded that the reduction in nutrients, especially in combination with increased light, were the most effective. Cell densities only reached 2.5 x 10^5 while astaxanthin concentration was greater than 300pg per cell when nitrate was limited in the original algal media (Harker et al., 1995). Boussiba et al. (1999) cultivated cells in a conventional media BG-11 while measuring cell concentration, dry weight, and astaxanthin and chlorophyll concentrations. Upon reaching the maximum cell concentration, cells were transferred to a nitrogen and phosphorous deprived medium to observe astaxanthin accumulation, reaching up to 4% of dry biomass. Orosa et al. (2005) studied different concentration of nitrate to observe the difference in cell numbers while also measuring the ratio of chlorophyll/carotenoids. Nitrogen deprivation, a commonly used cell stress mechanism, resulted in rapid production of carotenoids and a significant change in the ratio after an extended maturation period of over 15 days.

Some researchers have investigated the possibility of using less costly nutrients as an alternative to the conventional media. Tocquin et al. (2011) performed a medium screening with hydroponic fertilizer resulting in a higher than normal cell density of 2x10^6 cells·mL^{-1}. It is also noteworthy that they determined lower N:P levels caused vegetative cells to have a prolonged state and, thus, achieve higher cell density. Sipauba-Tavares et al. (2015) used NPK fertilizer as an alternative to commercial media WC, while evaluating the uptake of nutrients and growth rate of *H Pluvialis* cells. The NPK media proved to give a slightly greater cell productivity of 5.4 x 10^5 cells·mL^{-1}, while reducing the cost of media by 65%. Moreover, it should be noted that the cells were able to use ammonia in lieu of nitrate, more commonly used in media, as a nitrogen source; however, utilization of phosphorus seemed to be poor in this study. This media proved to be efficient in a batch scenario without the high cost of

commercial media nutrients. Further experimentation with alternative media could yield more results to lower the cost of natural algae based astaxanthin.

Further studies are needed to determine how production of valuable chemicals like astaxanthin could make these waste-to-energy systems more environmentally sustainable and cost effective. Studies on *H. Pluvialis* growth in waste streams are significantly limited as compared to other species studies with less valuable algal biomass production with wastewater nutrients. There is only one published study in which a wastewater was used as alternative media to cultivate *H Pluvialis*. Kang et al. (2006) used different concentrations of primary treated sewage and piggery wastewaters to test the assimilation of nitrogen and phosphorus into biomass for the production of biomass and subsequent extraction of astaxanthin. For wastewaters diluted at least 4 times, nitrate was consumed completely within 7 days, being the primary nitrogen source, and up to 12 $mg \cdot L^{-1}$ of phosphate was consumed during the same interval. Cell biomass more than tripled during encystment upon depletion of nitrogen resources within the previously mentioned diluted media cultures, and the resulting astaxanthin concentrations reached between 5-6% (Kang et al., 2006). It is thus promising to further evaluate the potential for *H. Pluvialis* to assimilate nutrients from other potentially valuable waste sources, using similar methods to maintain high productivity of astaxanthin.

Studies overview a variety of stress mechanisms and intensities to induce enhanced growth or specific metabolite production in microorganism. Minhas et al. (2016) published a recent review suggesting that temperature, light, salinity, and carbon, nitrogen, or phosphate deprivation, can affect autotrophic growth, while carbon sources like glucose and glycerol, amongst others, can change metabolic rates for mixotrophic growth in algal species. Several of these factors during the growth phase or encystment stage of *H Pluvialis* biomass production can ultimately influence the production of astaxanthin in matured cells. Sarada et al. (2002) proposed pH as an environmental stressor and concluded that biomass and astaxanthin production could be limited outside of the range of pH 6-8. Amongst the different stress mechanisms reported in the literature, nutrient deprivation and high light intensity were the most productive (Brinda et al., 2004). Recht et al. (2014) used metabolic models to explain that the deprivation of nitrogen and high light environment change stimulates

accumulation of carbohydrates and fatty acids, along with astaxanthin, because of increased carbon repartitioning in the TCA cycle.

Given the complexities of algal cultivation in general, studies focused on nutrient recovery from wastewater that also potentiate diverse value recovery from wastewater systems are needed. Moreover, since some higher value species and production require further consideration for growth research development, this study evaluated at a new nutrient source for algae *H. Pluvialis* and astaxanthin production.

CHAPTER 3: MATERIALS AND METHODS

The set of experiments to evaluate the growth of *H. Pluvialis* using alterative nutrients from wastewater were conducted between the laboratories of the Agricultural and Biological Engineering Department of University of Illinois Urbana-Champaign in Urbana (UIUC), Illinois, USA and the Food Engineering Department of the University of Sao Paulo, Faculty of Animal Sciences and Food Engineering (USP-FZEA) in Pirassununga, São Paulo, Brazil.

Experiments were conducted first to characterize wastewaters for comparison of different concentrations and nutrient recovery potentials. The high value algae, *H Pluvialis*, was chosen for its limited application in wastewater growth and grown across a dilution gradient to assess its tolerance for inhibitory compounds in Post Hydrothermal Liquefaction Wastewater (PHWW). Later, repeating this experiment at a larger scale allowed for larger samples to monitor growth, nutrient reductions, carotenogenesis, and lastly collect biomass to study the comparative production of the desired extract, astaxanthin.

Wastewater Nutrient Comparison

Several wastewaters were evaluated using Hach kit methods (Total Ammoniacal Nitrogen (TAN): 8038, Nitrate: 8039, Total Nitrogen: 10072, Total Phosphorus: 10127; Water Analysis Handbook, 2003) to determine the concentration of nutrients for comparison to a standard algal growth medium BBM, as well as the other wastewaters. Measurements were performed with 3-5 replicates amongst different experiments to determine average values and standard deviations.

PHWW Inhibition Assay

Once PHWW was determined to be the most potent source of nitrogen nutrients, an inhibition assay was performed in a Thermo Scientifc microplate at varying concentrations of the wastewater (0, 0.125, 0.25, 0.5, 1, and 2%) mixed with BBM, without nutrient substitution., to ensure that ideal conditions would support growth under normal conditions. The growth was measured over 10 days with a 2800 Tecan Spectrophotometer at 680nm in triplicate.

H Pluvialis Reactor Design

A Biological Oxygen Demand (BOD) refrigerator was cleaned and sterilized with bleach prior to experimentation. All materials, including the media, glassware, and tubing were sterilized in an autoclave at 115˚C at 15 psi prior to placing into the sterile space for algal growth. Mixing was provided by an aerator connected to a carbon dioxide tank, which was passed through a filter before being evenly distributed to the cultures inside the refrigerator. A light fixture was constructed to provide light to cultures and, then, be intensified for the maturation phase. This reactor configuration is presented in Figure 4 below.

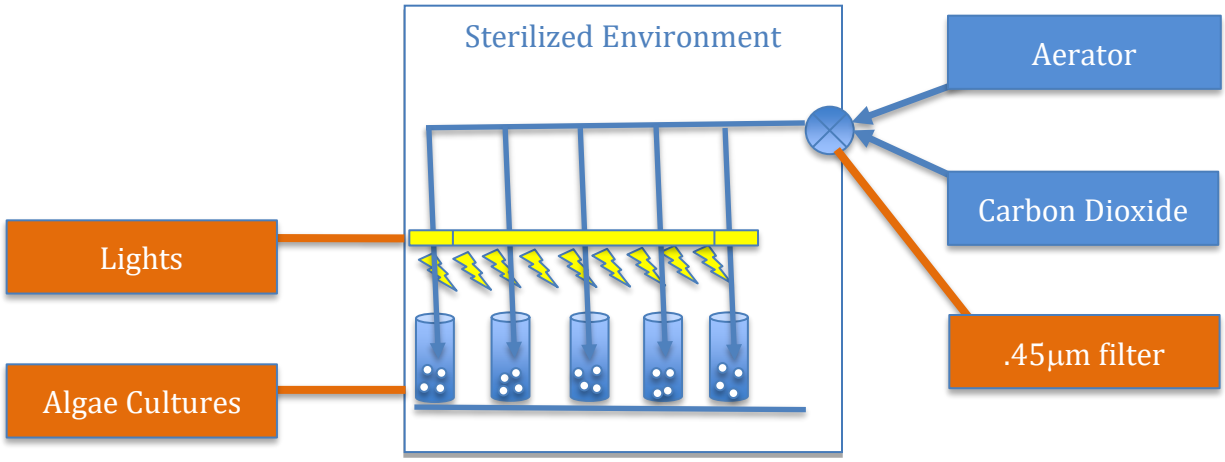


Figure 4. Schematic and pictures of BOD Refrigerator used as growing algae cultures under controlled environmental conditions

H Pluvialis Growth Conditions

Haematococcus pluvialis Flowtow culture was obtained from the Sipauba-Tavares laboratory at the São Paulo State University (UNESP) campus in Jaboticabal, São Paulo, Brazil. The algae inoculum was seeded in Bold's Basal Media (BBM) containing the following nutrients at the given concentrations: 250 mg·L^{-1} Sodium Nitrate ($NaNO_3$), 25 mg·L^{-1} Calcium Chloride ($CaCl_2{\cdot}2H_20$), 75 mg·L^{-1} Magnesium Sulfate ($MgSO_4{\cdot}7H_20$), 75 mg·L^{-1} Potassium Phosphate Dibasic (K_2HPO_4), 175 mg·L^{-1} Potassium Phosphate Monobasic (KH_2PO_4), 25 mg·L^{-1} Sodium Chloride (NaCl), 25 mg·L^{-1} Ethylenediaminetetracetic acid (EDTA), 25 mg·L^{-1} Potassium Hydroxide (KOH), 5 mg·L^{-1} Ferric Chloride ($FeCl_3{\cdot}6H_20$), 11.5 mg·L^{-1} Boric Acid (H_3BO_3), with trace elements: 8.75 mg·L^{-1} Zinc Sulfate ($ZnSO_4{\cdot}7H_20$), 1.4 mg·L^{-1} Manganese Chloride ($MnCl_2{\cdot}4H_20$), 0.7 mg·L^{-1} Molybdenum Trioxide (MoO_3), 1.6 mg·L^{-1} Cupric Sulfate ($CuSO_4{\cdot}5H_20$), 0.5 mg·L^{-1} Cobalt Nitrate ($Co(NO_3)_6{\cdot}6H_20$), and a vitamin mix containing 7 µg·L^{-1} Vitamin B_1, 7 µg·L^{-1} Vitamin B_2, 5 µg·L^{-1} Vitamin B_6, 5 µg·L^{-1} Vitamin B_{12}, and 7 µg·L^{-1} Vitamin H. Cultures were maintained under light conditions of 10 µmol·m^{-2}·s^{-1} in 12:12 light dark cycle at 24˚C in the temperature-controlled BOD refrigerator, which also served to provide the sterile environment. For the following growth experiments, the cell cultures were agitated by 0.45µm filtered air, augmented to have 5% CO_2 (w/v), at a rate of 50 mL·min^{-1} (0.005g $CO_2 \cdot min^{-1}{\cdot}L^{-1}$) per flask, which helped to maintain the pH at approximately 6.5-7.

H Pluvialis Growth on Nitrate vs Ammoniacal Nitrogen

A growth comparison was conducted to evaluate the growth of *H Pluvialis* using either nitrate (as normally provided by BBM and other algal media) or ammoniacal nitrogen (typical nitrogen species measured in wastewater). BBM was prepared without nitrate and an equivalent concentration of total ammoniacal nitrogen (TAN) was provided to the culture. Growth was measure with cell counts using a Brightline Neubauer Hemocytometer and nutrient reduction of nitrate, ammoniacal nitrogen, and phosphorous using calorimetric methodologies (Cataldo et al., 1975; Hach Method 8038; Chen et al., 1956) were measured daily over 7 days, described in further detail below.

H Pluvialis Growth on PHWW

A four-day old seed culture in its exponential growth phase was inoculated at a 10% (v/v) portion into 1L flasks containing BBM with different concentrations of sterilized PHWW of 0%, 0.125%, and 0.25% (BBM, $mBBM_{0.125}$, $mBBM_{0.25}$). For the conditions containing PHWW, the BBM media was maintained with respect to all components except for nitrogen. The nitrate in the original BBM was partially replaced with the corresponding ammoniacal nitrogen sourced from the wastewater, being 5 and 10% of the inorganic nitrogen for the 0.125 and 0.25% PHWW conditions, respectively. As presented in Table 7, this setup was chosen to balance the available inorganic nitrogen to the algae, noting that the PHWW also contains additional nutrients that could improve cell production via mixotrophic growth. Cultures were grown in triplicate with the same aeration parameters. It should also be noted that at this dilution, many other constituents of the PHWW, including organics and inhibitory compounds, are considerably reduced and should not affect the balance of nitrogen proposed for this study.

Table 7. Balance of available inorganic nitrogen sources in 3 media compositions for comparison of *H Pluvialis* growth on a BBM control and BBM augmented with PHWW

Treatment	% PHWW	Growth Type	Total Ammoniacal Nitrogen (TAN) ($mg \cdot L^{-1}$)	Nitrate NO_3-N ($mg \cdot L^{1}$)	Total Inorganic Nitrogen ($mg \cdot L^{-1}$)
BBM	0	Autotrophic	0	75	75
$mBBM_{0.125}$	0.125	Mixotrophic	3.75	71.25	75
$mBBM_{0.25}$	0.25	Mixotrophic	7.5	67.5	75

Daily samples of 10mL were taken to measure biomass growth and nutrient content. Cells were counted using a Brightline Neubauer Hemocytometer with a BEL Phototonics brightfield microscope, and pictures were taken using a Digilab microscope camera. In addition to total cell counts, different cell types (macrozoid, palmelloid, and akinete) were counted as an indicator of cell maturity and stress.

In regard to the cell doubling time, the daily cell counts applied to the following equation and doubling rate was calculated:

Eq. 5 $$\mu = \text{doubling} \cdot \text{day}^{-1} = \ln N(n) - \ln N(i) / \ln 2(t_n - t_i)$$

where t_i and t_n are the start and end times of parts of the growth phase, expressed in hours, and *N(i)* and *N(n)* are the starting and ending cell counts, respectively.

Afterwards, cells were centrifuged at 5˚C at 3000rpm for 5 minutes to spin down biomass. Supernatant was collected for measuring nutrient concentrations and diluted as necessary to achieve concentrations within the ranges for each of the tests.

H Pluvialis Nutrient Assimilation from PHWW Augmented Media

Colorimetric nitrate (NO_3^--N) determination was performed using salicyclic acid according to Cataldo et al. (1975). Reagent 1 was prepared with 5% (w/v) in concentrated sulfuric acid and stored in a dark bottle. Reagent 2 was 2M NaOH made with 40g of solid NaOH in 500 mL of deionized water. For each sample or standard, .2mL was mixed with .8mL of reagent 1 in a clean tube and mixed with a vortex. Tubes were let to react for 20 minutes at room temperature. Next, 19 mL of reagent 3 was added and mixed with a vortex. Tubes were left to cool to room temperature. Tubes were measured for absorption at 410nm. A calibration curve was performed resulting in R^2=0.99435 and the regression equation y=359.84x-9.1587.

Ammoniacal nitrogen was measured using a modified version of the Hach Method 8038 using Nessler's Reagent. 2.5mL of sample or standard was pipetted into a clean tube. 3 drops of mineral stabilizer were added and tube contexts were mixed. 3 drops of polyvinyl alcohol were added and tube contexts were mixed. Then, 1mL of Nesslers reagent was added to each tube and vortex again. Samples were left to react for 1 minute prior to measuring absorbance at 425nm. The calibration curve performed for testing ammoniacal nitrogen resulted in R^2=0.9995 and the regression equation y=6.5355x-0.0061.

An ascorbic acid method was used to measure phosphate (PO_4^-) according to Chen et al. (1956). Reagent A was prepared as 2% (w/v) of ascorbic acid to be mixed with Reagent B which was 0.5% ammonium molybdate and diluted with concentrated sulfuric acid upon testing of samples or standards. Curves were performed individually with each of the readings for this test as it is dependent on the reaction time. Samples were read between 3-5 minutes after mixing samples with reaction solution. The calibration curve derived for the

main experiment, which paralleled the measurements for all other phosphate measurements, resulted in R^2=0.9997 and the regression equation y = 2.6633x + 0.0202.

Each nutrient was calibrated with standard solutions of the nutrients and tested with the media, as well as deionized water as a blank, to ensure dependable measurements during algal growth phases. All calibration curves are presented in the discussion section with their respective R^2 values and regression equations. All nutrient measurements were conducted using a Hach DR 2800 spectrophotometer.

H Pluvialis Carotenogenic Profile when Grown on PHWW Augmented Media

During the secondary phase after assimilation of detectable nutrients and visible encystment phase of maximized cell density, cultures were harvested from original wastewater by centrifugation at 5˚C at 3000rpm for 5 minutes. Original nutrient-rich and wastewater media were replaced with 2.5% salt water at pH 7 for nutrient deprivation and cells were set in increased light conditions of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ while aeration and temperature was maintained the same as in vegetative growth conditions. Daily samples were taken and used for quantification of astaxanthin within cells.

Cell pellet biomass was treated with N,N-dimethtlformamide (DMF) and measured photometrically with the same Hach DR 2800 spectrophotometer to quantify chlorophyll a/b ($C_{a/b}$) and carotenoids using the following equations, per Lichtenthaler et al. (1987).

Eq. 6 $C_a\ (\mu g \cdot mL^{-1}) = 11.24\ A_{661.6} - 2.04\ A_{644.8}$

Eq. 7 $C_b\ (\mu g \cdot mL^{-1}) = 20.13\ A_{644.8} - 4.19\ A_{661.6}$

Eq. 8 $\text{Concentration X+C}\ (\mu g \cdot mL^{-1}) = (1000\ A_{470} - 1.90\ C_a - 63.14\ C_b)/\ 214$

A sample of the final biomass harvest was taken from each replicate and centrifuged at 5˚C at 3000 rpm for 5 minutes to concentrate biomass to cell pellet. Supernatant was discarded and cells were resuspended in 70˚C 2M Hydrochloric Acid (HCl) for ten minutes to disrupt the cell wall, in accordance with findings of Sarada et al. (2006). After heated acid treatment, cells were recentrifuged at 5˚C at 3000 rpm for 5 minutes, and HCl was discarded. Cells were resuspended in acetone before being centrifuged again to separate biomass from the extract to perform High Pressure Liquid Chromatography (HPLC). Injections of 20µL

were run in a mobile phase of 90% acetonitrile and 10% methanol at $1mL \cdot min^{-1}$ through a C_{18} Column Ultra C18 100A (250mm x 4.6mm). Resultant peaks were compared against a standard astaxanthin sample purchased from Sigma Aldrich (SML0982). The calibration curve for astaxanthin was run over the concentrations of $0\text{-}20mg \cdot L^{-1}$, resulting in $R^2=0.98678$ and a linear regression of $y=8.0 \cdot 10^{-5} x - 0.1691$, also presented in the discussion.

Statistical analysis was performed using Excel 2016. The final cell count and astaxanthin product analysis were subjected to a one-way ANOVA ($p \geq 0.05$) to test for differences between means of conditions.

CHAPTER 4: RESULTS AND DISCUSSION

As a means of comparing alternative sources of nutrients for algae, a range of wastewaters were measured for pH, as well as nitrogen and phosphorus content. Table 8 presents different types of wastewaters as compared to conventional algae media, BBM. It is evident that these wastewaters present a wide range of nutrient concentrations. In consideration of these various levels of nutrient concentrations, different wastewater nutrient sources can be identified as suitable sources for augmented algal growth. Post Hydrothermal Liquefaction wastewater (PHWW), while being more variable in its composition dependent on the feedstock used for the thermochemical process, has the greatest density of nutrients that could be utilized in cultivation of algae. This nutrient-rich PHWW media, however, has been reported to have certain toxic or inhibitory compounds that can significantly inhibit growth of a variety of microorganisms. (Pham et al., 2013; Garcia Alba et al., 2013; Gai et al., 2015; Zheng et al., 2017).

Table 8. Different wastewater sources nutrient concentrations in comparison to a standard algae growth medium, Bold's Basal Media (BBM).

Nutrient Source	pH	Total Ammoniacal Nitrogen (TAN) ($mg \cdot L^{-1}$)	Nitrate NO_3-N ($mg \cdot L^{-1}$)	Total Nitrogen ($mg \cdot L^{-1}$)	Total Phosphorus ($mg \cdot L^{-1}$)
Bold's Basal Media (BBM)	6.5	.5	75	80	150
Urbana-Champaign Sanitary District (UCSD) Primary Clarifier Effluent	8.65 ± 0.20	25.0 ± 2.12	1.18 ± 0.22	26.1 ± 0.78	0.52 ± 0.11
UCSD Tertiary Effluent	8.61 ± 0.34	0.25 ± 0.09	35.23 ± .89	35.67 ± 1.91	1.67 ± 0.22
Swine Lagoon Wastewater	8.74 ± 0.15	462.67 ± 13.51	20.45 ± .63	683.23 ± 14.52	111.58 ± 5.48
Anaerobic Digester (AnMBR)	9.13 ± 0.41	580.14 ± 15.62	40.76 ± 2.03	842.09 ± 23.65	213.11 ± 6.73
Post Hydrothermal Liquefaction Aqueous Phase (PHWW) from Swine Manure	8.25 ± 0.31	4133.2 ± 156.1	85.83 ± 3.14	5305.4 ± 116.2	2187.9 ± 58.61

As a preliminary test for tolerance to the mentioned inhibitory compounds, *H Pluvialis* cells were inoculated into various concentrations (0, 0.125, 0.25, 0.5, 1, 2%) of PHWW diluted into BBM. 680nm was chosen as the wavelength to measure the growth of algae in the various media compositions (Biller et al., 2012; Alba et al., 2013). As shown in Figure 5, comparing culture growth with PHWW to a control, the cells only tolerated up to 0.25% PHWW without inhibition. The absorbance data are accompanied with pictures of the growth plates on day 0, 5, and 10 (Figure 6), where it can also be observed that the conditions permitting cell growth resulted in a green coloration of the triplicated wells. It should also be noted that the 0.5% concentration seemed to present some growth after 150 hours based on the increased absorbance values, but total growth was still much smaller in this case. Thus, 0, 0.125, and 0.25% PHWW was chosen for further experiments. The resultant concentrations were similar to previous findings for growth of *Chlorella (*Jena et al., 2011; Biller et al., 2012) and *Scenedesmus* (Biller et al., 2012) on PHWW. Recognizing that there are many types of possible inhibitory compounds in the PHWW (phenols, cyclic nitrogen compounds, concentrated ammonia, etc.) (Pham et al. 2013), it was necessary to determine whether *H. Pluvialis* was intolerant of ammoniacal nitrogen concentrations or the other compounds generated by the liquefaction process.

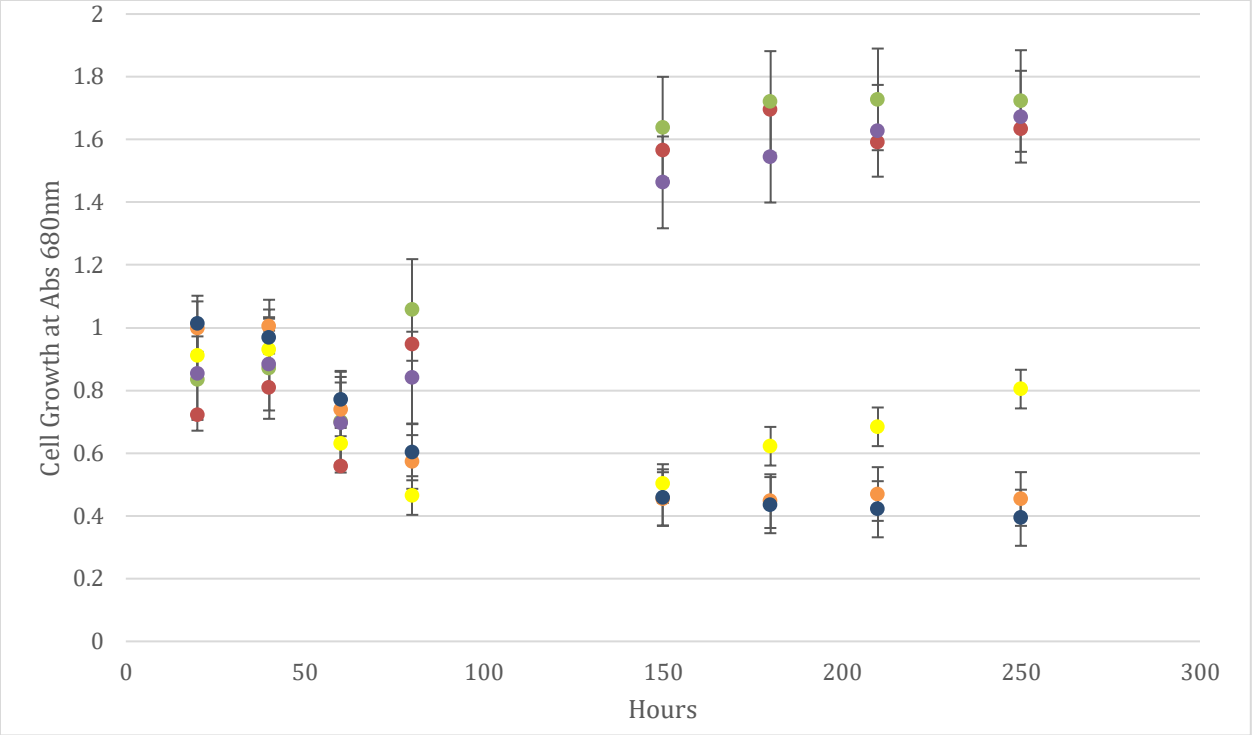


Figure 5. Absorbance readings at 680nm for *H. Pluvialis* grown at different concentrations of PHWW mixed in BBM (0% = Red, 0.125% PHWW = Green, 0.25% PHWW = Purple, 0.5% PHWW = Yellow, 1% PHWW = Orange, 2% PHWW = Dark Blue)

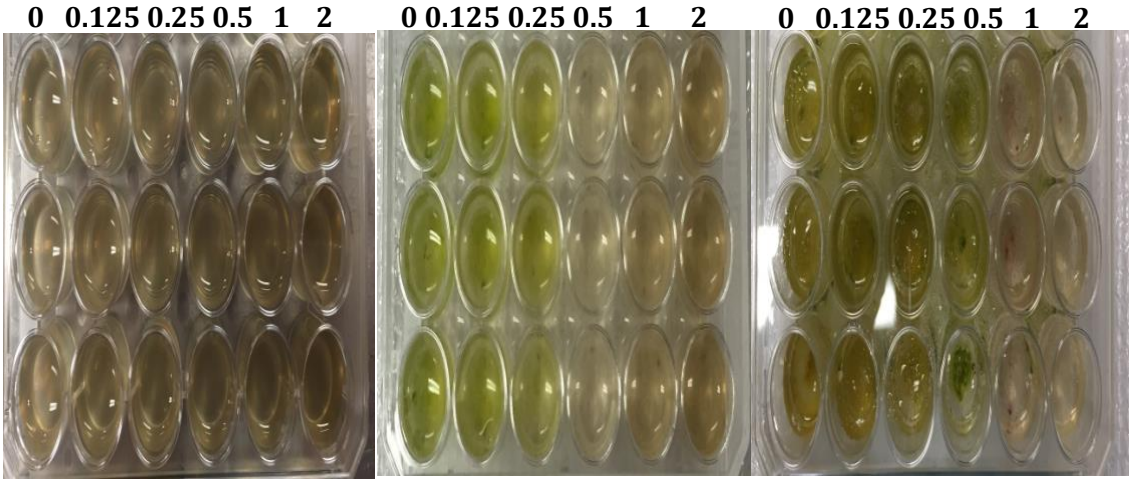


Figure 6. Pictures of PHWW inhibition assay taken on (a)day 0, (b) day 5, and (c) day 10

Referring back to Table 7, wastewaters predominantly contain ammoniacal nitrogen instead of nitrate as the readily available form of inorganic nitrogen. Thus, in a second growth experiment, it was necessary to compare the growth of *H. Pluvialis* utilizing the two different nitrogen sources, determining the suitability of using ammoniacal nitrogen from

wastewater for cultivation of this species of algae, *H Pluvialis*. The results for growth, measured in terms of cells·mL^{-1} with a hemocytometer, are presented in Figure 7. As can be seen, there was little difference obtained for growth of *H Pluvialis* when varying the nitrogen source. Moreover, this growth is within the range of reported concentration for cells grown in BBM and other common algal media (Tripathi et al., 1999; Goksan et al., 2011; Sipauba-Tavares et al., 2013;).

It should be noted that the pH drops as a result ammoniacal nitrogen assimilation by algae, which releases H^+ ions, and on the other hand, pH can increase when OH^- is expelled by cells during the uptake of NO_3 (Goldman et al., 1982). As such, it is necessary to control the pH. CO_2 is continuously added to prevent the pH from increasing above 7 when cultivating algae, as suggested in the review by Singh and Singh (2013). For the following growth experiments, the cell cultures were agitated by a 0.45µm filtered mixture of air augmented to have 5% CO_2 (m/v) at a rate of 50 mL· min^{-1} (0.02g CO_2 · min^{-1}·L^{-1}), which helped to maintain the pH at approximately 7.

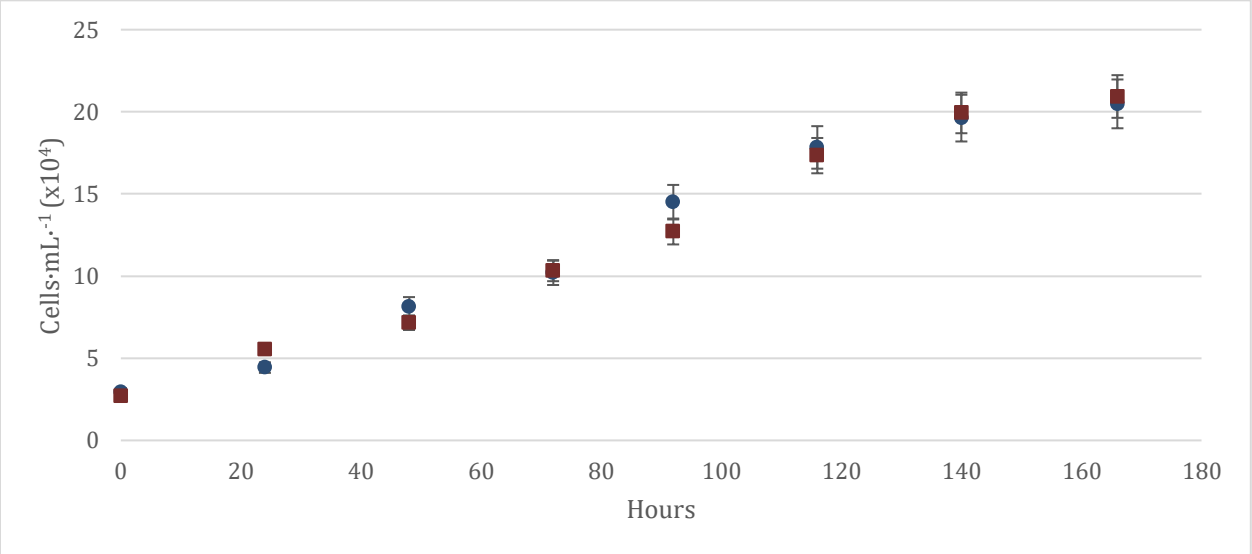


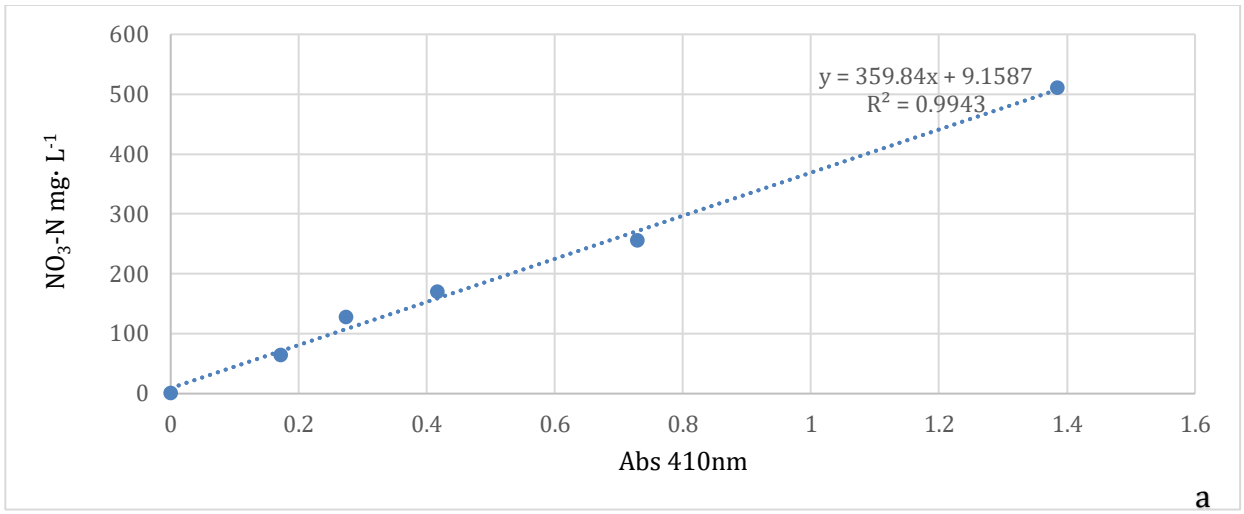
Figure 7. Hemocytometer cell counts for *H. Pluvialis* grown in Bold's Basal Media with either Nitrate or Ammoniacal Nitrogen as Nitrogen Source (Squares =TAN Treatment; Circles = NO_3 Treatment)

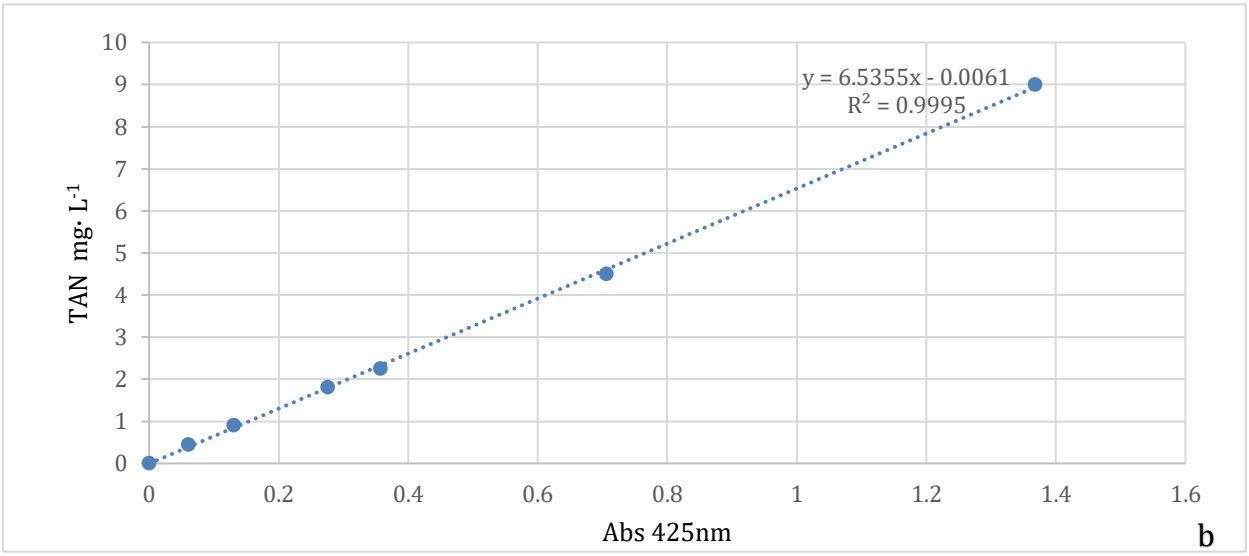
The nutrient concentrations observed during the cultivation phase were measured with the previously described Hach methods (Water Analysis Handbook, 2003). A standard solution was prepared for each nutrient and diluted to varying concentrations for reaction

and subsequent reading of absorbance at the corresponding wavelength. The following calibration results, presented below in Figure 8, gave reasonable certainty that the measured absorbance values were reliable for calculating concentration of the nutrients in the media samples: Nitrate calibration: R^2=0.99435; Ammoniacal nitrogen calibration: R^2=0.9995; and Phosphate calibration: R^2=0.9997.

Regarding the reduction of nutrients from the medium, this was also very promising as both ammoniacal nitrogen and nitrate were reduced between 85-90% across all the replicates, as shown in Figures 9 and 10, respectively. However, it was noticed that on day 7 in the ammoniacal nitrogen treatments, there was an increase at the end of the growth. This might suggest that any nitrogen assimilated by the algae from the media would be converted into organic compounds, such as proteins, which would be expelled in the event of cell death or lysing. Dong et al. (2007) suggested that ammonium species can be excreted by cells in different nitrogen and light intensities. If the cells were reaching the end of the growth phase and entering the maturation phase, degradation of proteins to channel carbon into astaxanthin with subsequent expulsion of ammoniacal nitrogen species could explain this increase.

In comparison with other algae and wastewaters, more complete reduction of nitrogen species can be achieved (Woertz et al., 2009). With regard to recovery of nutrients in this study, it could be suggested that the environmental conditions were limiting for this species or that other microorganisms might be more effective for the reduction of these constituents in a wastewater remediation scenario.





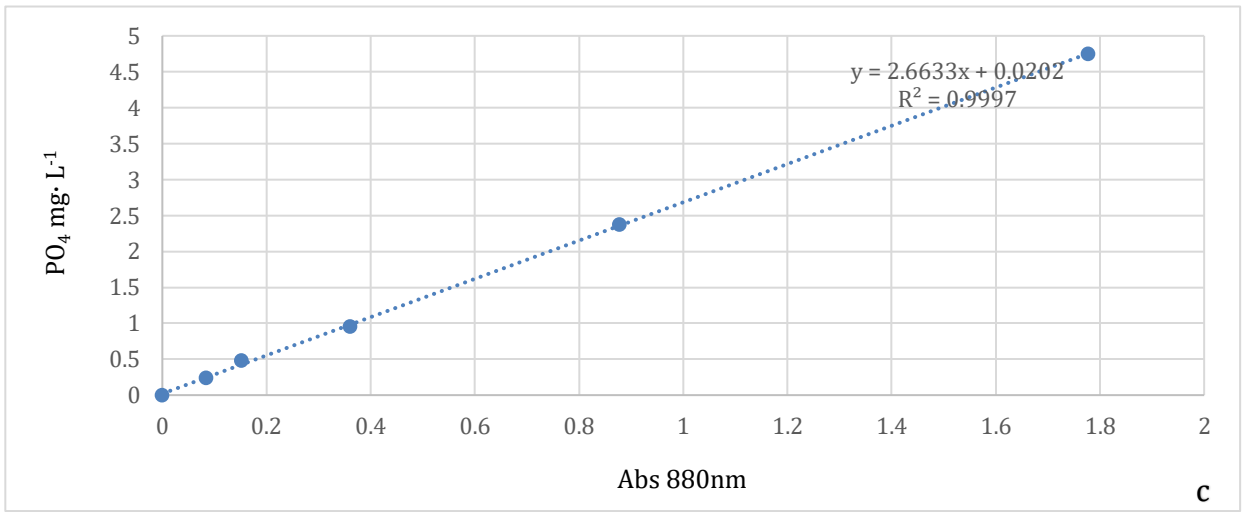


Figure 8. Calibration curves for (a)NO_3, (b) TAN, and (c) PO_4, accompanied by corresponding R^2 values and regression equations.

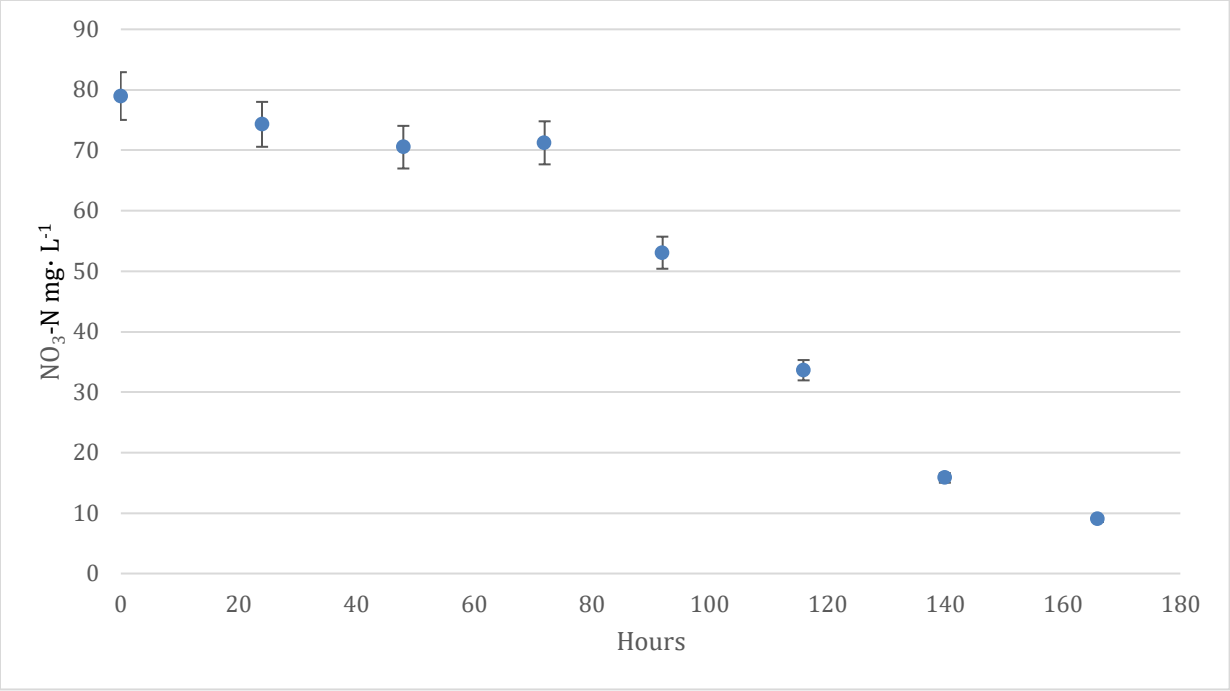


Figure 9. Concentration of Nitrate in media for *H. Pluvialis* grown in BBM with Nitrate as Nitrogen Source (Circles = NO_3 Treatment)

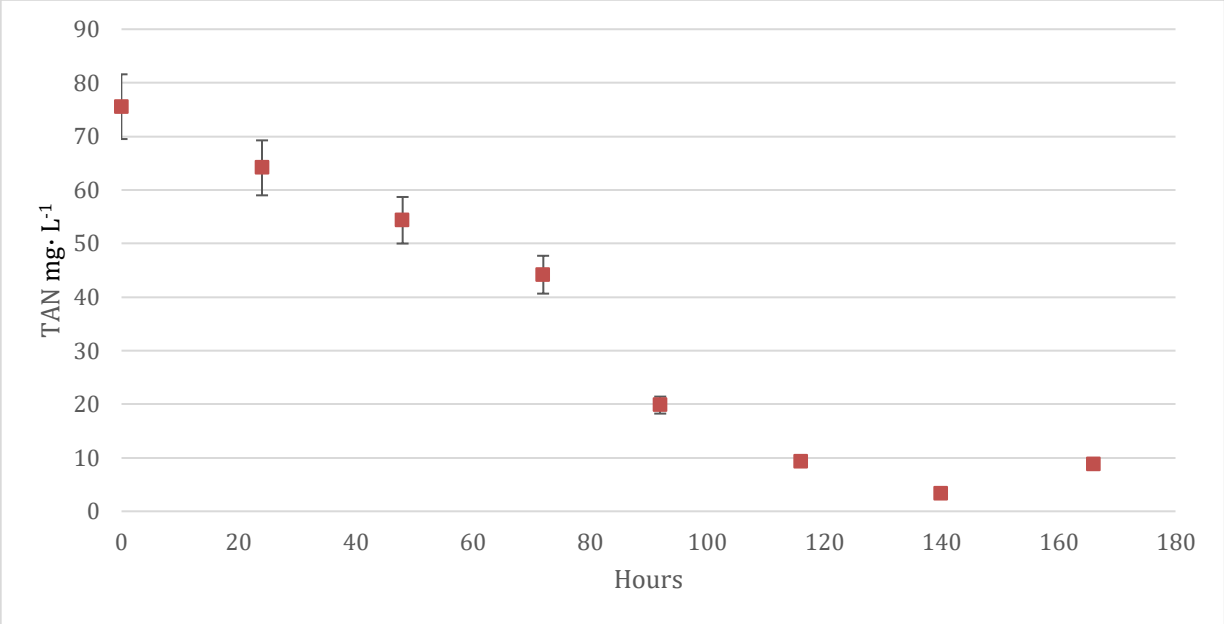


Figure 10. Concentration of total ammoniacal nitrogen (TAN) in media for *H. Pluvialis* grown in BBM with TAN as Nitrogen Source (Squares =TAN Treatment)

Figure 11 presents the removal of phosphorus, which showed considerably less reduction during the growth of *H Pluvialis*. Although there was not a significant reduction in the phosphorus present in the BBM, Kang et al. (2006) showed that phosphate removal from

various dilutions of primary treated swine wastewater was similar on a mass basis to those quantities as observed in this experiment. This suggests that at lower concentrations, *H Pluvialis* could be able to perform more complete recovery of phosphorus species, given that the reduced phosphorous content did not otherwise alter the media stability or limit the growth of the algae. Seemingly, wastewaters would have sufficient phosphorus to not present concern for limiting the growth of the algae when replacing conventional media nutrients, even at these significant dilutions.

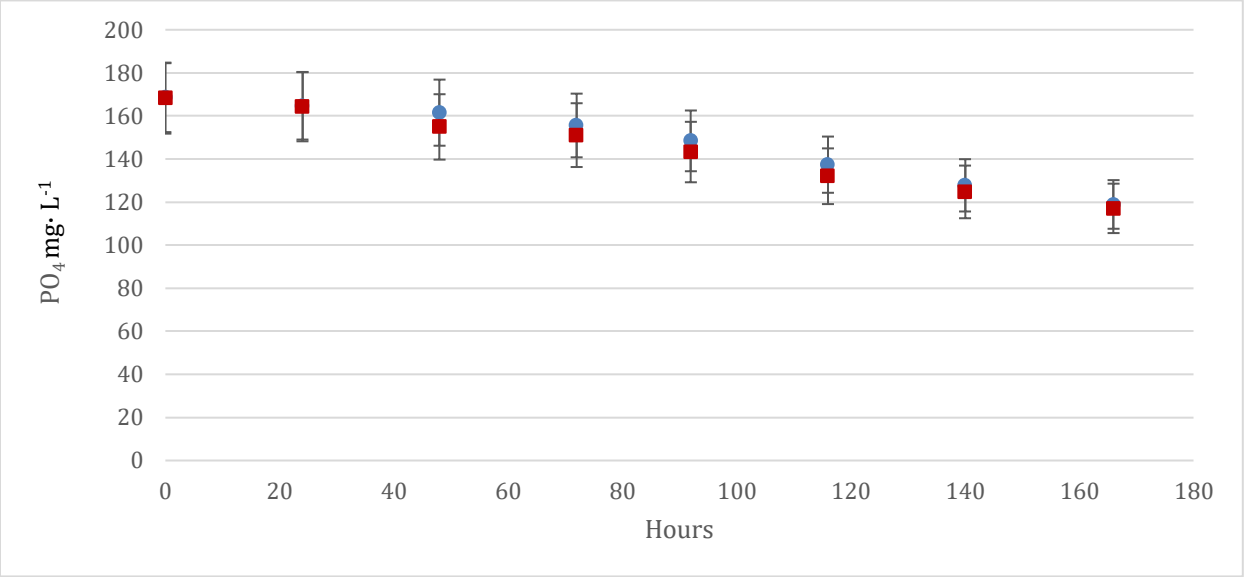


Figure 11. Concentration of Phosphate in media for *H. Pluvialis* grown in Bold's Basal Media comparing Nitrate and TAN as Nitrogen Source (Squares =TAN Treatments; Circles = NO_3 Treatments)

The following experiment and graphs present the growth of *H Pluvialis* in three different concentrations of PHWW (0%, 0.125%, and 0.5%), which was used to replace some of the nitrogen nutrients in BBM. Figure 12 exhibits the growth of the cells as measured by cell counts with a hemocytometer. It is clear that the wastewater nutrients, as a whole, increased the growth of the algae, however, the 0.25% PHWW treatment presented more inhibition than the 0.125% PHWW condition, which resulted in the best growth among the 3 dilutions and nutrient conditions tested. These results are in accordance with Biller et al. (2012), who tested the growth of *Chlorella* and *Scenedesmus* in PHWW augmented media, achieving the highest concentrations of cells at PHWW dilutions below 0.5%.

The enhanced growth of *H Pluvialis* in the PHWW treatments results from additional nutrients present in the PHWW that contribute to heterotrophic and mixotrophic growth of algae. Biller et al. (2012) quantified different nutrients in PHWW produced from HTL of algae biomass and determined the available carbon and nitrogen, as well as some metals and inhibitory compounds, that could affect subsequent algal growth. These constituents that contribute to mixotrophic growth can lead to higher biomass production (Bhatnagar et al., 2011), as also observed in the present study.

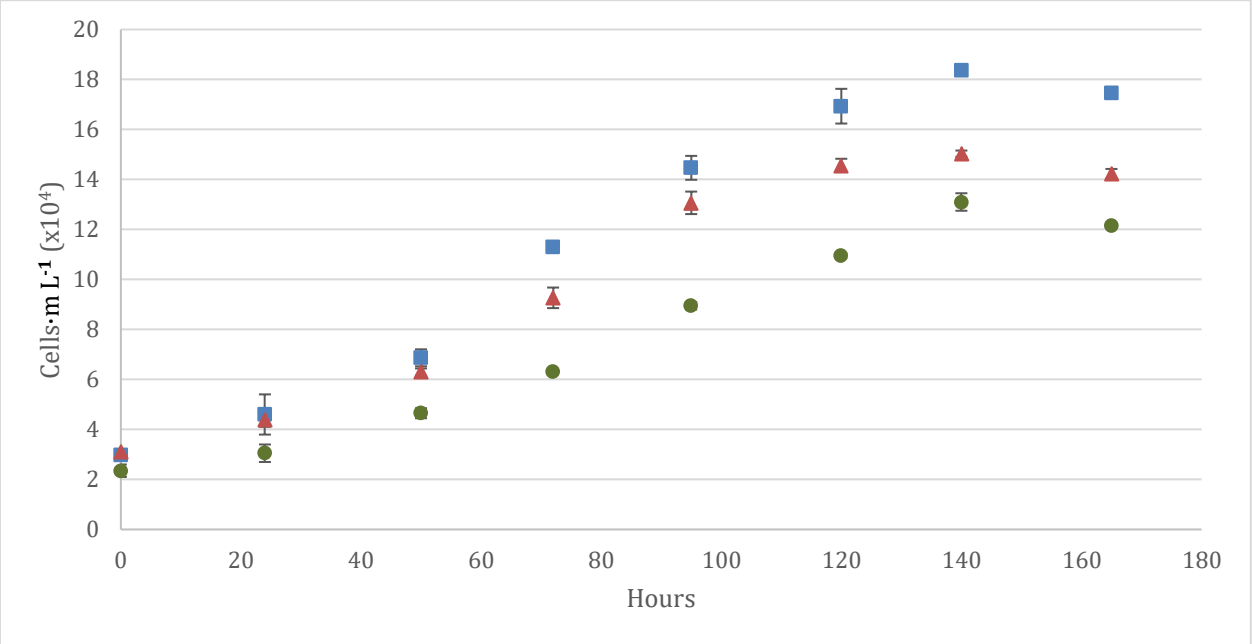


Figure 12. Growth of *H. Pluvialis* grown in Bold's Basal Media augmented with different concentrations of PHWW (Circles = BBM Control; Squares = BBM + 0.125%PHWW; Triangles = BBM + 0.25%PHWW)

Using the statistical functions in the Excel software, a one-way ANOVA was conducted to test the difference in final cell concentration amongst the three conditions. Table 9 shows there was a statistical difference between the three treatment groups at a p value of 0.05. This suggests that the use of PHWW at the 0.125 and 0.25% results in a statistically significant improvement in growth, as compared to the control BBM without any PHWW added.

Table 9. One-way ANOVA for the difference in cell counts between three growth conditions

SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
BBM	2	24.3	12.15	0.245		
mBBM0.125	3	52.4	17.46667	0.083333		
mBBM0.25	3	42.7	14.23333	0.053333		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	36.21667	2	18.10833	174.6785	2.36E-05	5.786135
Within Groups	0.518333	5	0.103667			
Total	36.735	7				

The growth rate of the cells each day, measured as doublings·day^{-1}, is listed in Table 10 below. The arrow colors and direction indicate the increase or decrease in the growth rate between daily measurements of cell density in the cultures. It can be observed that the greatest growth rates were achieved between the 50 and 95 hour mark for the all condition replicates with the greatest rates in the media augmented with the wastewater nutrients. The max growth rate achieved, as an average of 3 replicates of the 0.125% PHWW treatment, was μ=0.201515 doublings·day^{-1}, achieved between the 50 and 72-hour measurement for the PHW 0.125% treatment. Given the concentration of total available nitrogen, these doubling rates are comparable to observations by Orosa et al. (2000), who reported a doubling time of 0.17 doublings·day^{-1} at 0 mg·L^{-1} of nitrate and as much as 0.65 doublings·day^{-1} with 150mg·L^{-1} of nitrate, being more than twice the value measured in the conditions for this experiment.

Table 10. Daily growth rates of *H Pluvialis* grown in different media composition mixtures of BBM and PHWW. Green, yellow, and red arrows indicate change in growth rate as significant increase, stagnant, or significant decrease (* indicates that flask was contaminated)

Flask	Hour 0	24	50	72	95	120	140
BBM*		0.0083	0.0346	0.2	0.0043	-0.08	0.04
BBM		0.0333	0.0846	0.0682	0.1087	0.084	0.105
BBM		0.025	0.0385	0.0818	0.1218	0.076	0.11
BBM_PHWW_0.125		0.1208	0.05	0.1909	0.113	0.112	0.1
BBM_PHWW_0.125		0.0292	0.1308	0.1818	0.1522	0.064	0.095
BBM_PHWW_0.125		0.0542	0.0808	0.2318	0.1478	0.12	0.02
BBM_PHWW_0.25		0.0667	0.0538	0.1636	0.1696	0.02	0.04
BBM_PHWW_0.25		0.05	0.0731	0.1182	0.1696	0.08	0.01
BBM_PHWW_0.25		0.0458	0.0923	0.1227	0.1565	0.08	0.02

As an added component to the counting and study of cells using the hemocytometer, cells were categorized based on their morphology, which was categorized based on cell size and color (Kobayashi, et al., 1997; Hoang et al., 2011; Ohnuki et al., 2013). Figure 13 presents the change in cell morphologies over the course of the growth period, as categorized based on the cell types presented in Table 10. It is evident that as cell concentration increased and nutrients decreased, the cultures shifted from being predominantly macrozoid cells to immotile palmelloid cells, which suggests the end of the growth phase and beginning of maturation with subsequent production of astaxanthin, amongst other carotenogenic compounds. Boussiba and Vonshak (1991) suggested that maturation can be induced by several environmental factors, with nitrogen deprivation and higher light irradiance being among the most effective methods. Across the 3 conditions studied in this experiment, there appears to be little difference in the types of cells during the growth period. This suggests that although the different concentrations of PHWW could improve the total concentration of cells, it did not have a major effect on the maturation process of the cells. In future studies, it could be worthwhile to further increase the light intensity so that more maturation and astaxanthin production is induced earlier on and, then, verify if the alternative nutrients from PHWW and varying nitrogen or inhibitory compounds would affect the maturation rate of *H Pluvialis.*

In addition to the bar graphs for the cell morphology distributions, a set of photos from the Digilab microscope camera are included to better understand how cells were observed and categorized during the experiment. Referring back to Table 11 that includes a scale for sizing of cells using width and length of grids in hemocytometer, Figure 14 provides an example of microscope view of the cells on the hemocytometer grid for cell counting to categorize these cells. Borowitzka et al. (1991) also studied the effect of different nutrients on the prevalence of cells types within a culture, but the authors observed that either temperature or NaCl resulted in notable increases in formation of palmelloid or aplanospore (akinete) cells. In the current study, it was concluded that while the inhibitory phenolic and cyclic nitrogen compounds were able to limit *H Pluvialis* to growth in concentrations of 0.25% PHW, these compounds did not induce the desired carotenoid production in the given environmental conditions.

Table 11. Description of different *H Pluvialis* cell morphologies for categorization during cell counts (Boussiba and Vonshak., 1991; Boussiba et al., 1999; Orosa et al., 2005)

Cell Type	Cell Size	Cell Mass	ATX· cell^{-1}	ATX Production Rate	Mobility
Macrozoid	**10µm ± 5**	**2.5 ng**	**45pg**	**0.0244· hr^{-1}**	**Y**
Palmelloid	**30µm ± 10**	**8.25 ng**	**20pg**	**0.0243· hr^{-1}**	**N**
Akinete	**45µm ± 5**	**>10 ng**	**150pg**	**0.5833· hr^{-1}**	**N**

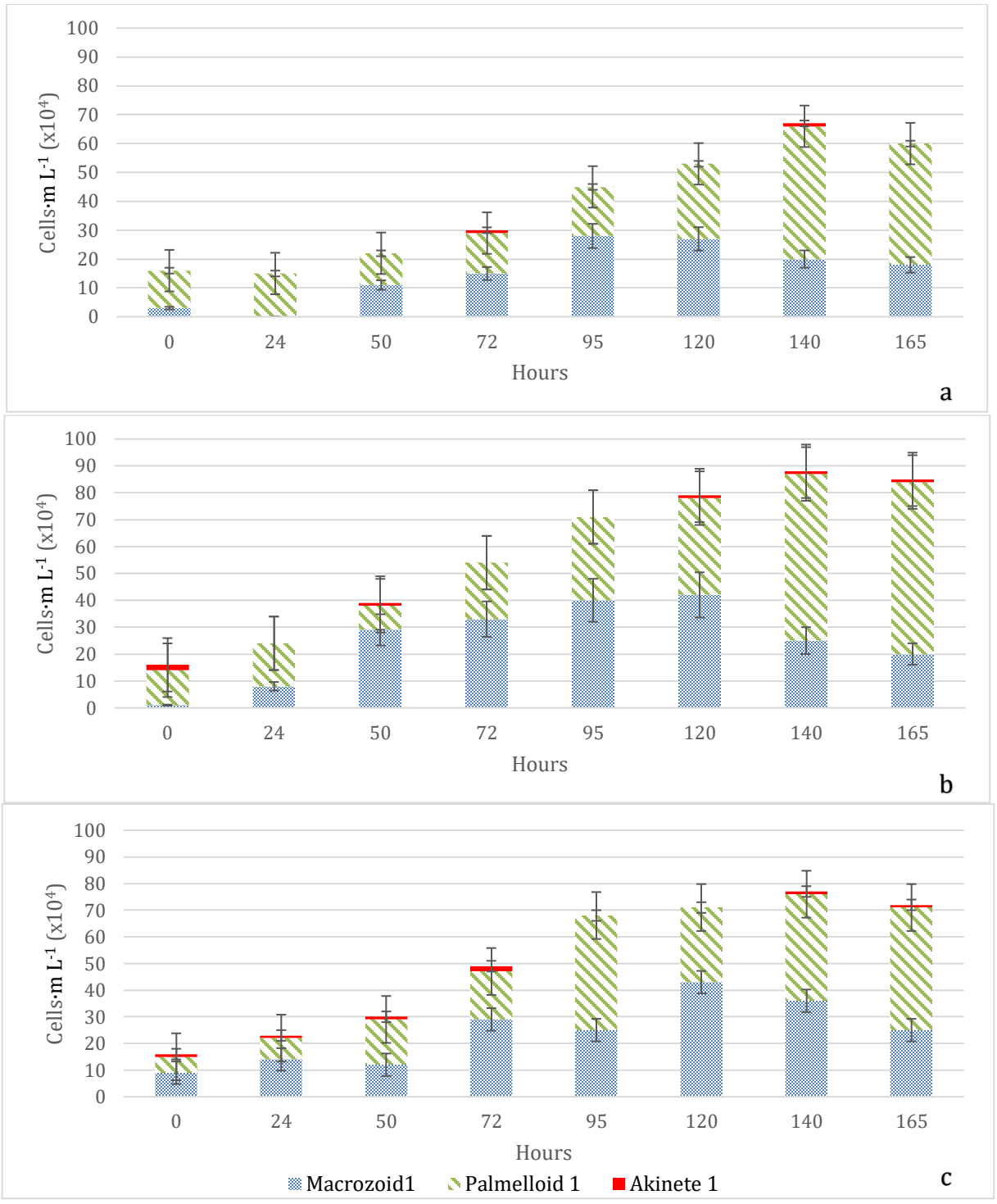


Figure 13: Distribution of *H Pluvialis* cell morphologies grown in (a) BBM control treatment; (b) BBM+0.125% PHWW treatment; (c) BBM+0.25% PHWW treatment (Macrozoid = Blue; Palmelloid = Green Stripes; Akinete = Solid Red)

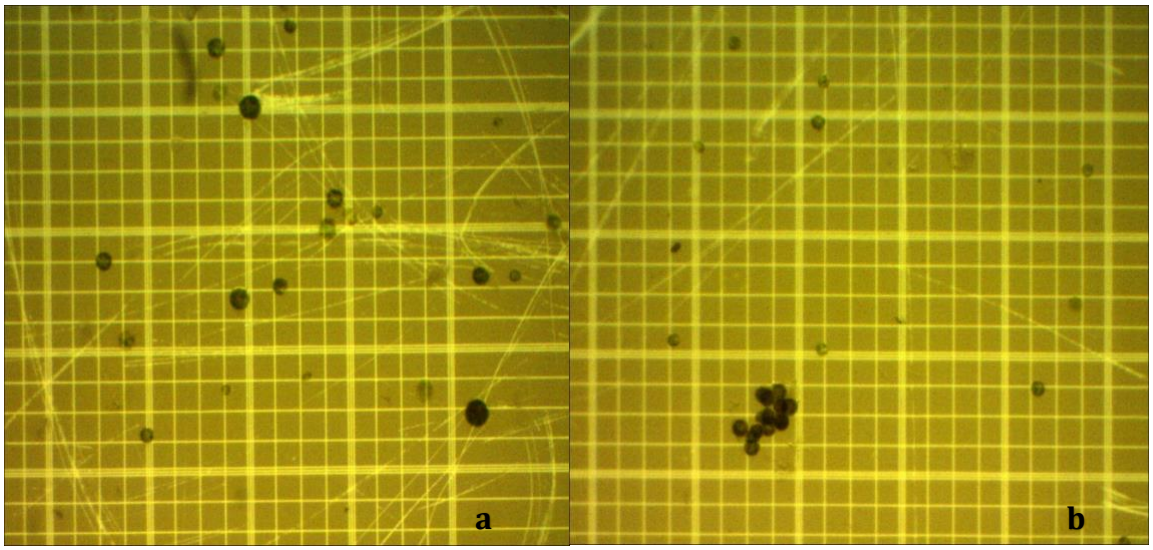


Figure 14: (a) Picture of cells in hemocytometer from BBM on Day 6 under 100X magnification; (b) Picture of cells in hemocytometer from BBM on Day 7 under 100X magnification

Figure 15 and 16 show the removal of the nitrate and total ammoniacal nitrogen from the varying media compositions. The total inorganic nitrogen (NO_3^- + TAN) was kept constant for all three media formulation at approximately 75mg·L^{-1} across all conditions. As shown in the Figure 15, there was between an 80-90% reduction of nitrate after 140h across all the conditions and replicates. This finding corroborates the high nitrate removal reported by Kang et al. (2006) who grew *H Pluvialis* on wastewater from diluted primary-treated piggery effluent collected from a wastewater disposal plant employing a membrane bioreactor with a four-stage Bardenpho system and intermediary clarifier. However, the previous study reported complete removal of nitrate species at higher concentrations in the same number of days during the growth phase. This finding can be explained by two factors. First, there was a significantly higher number of cells used for the inoculum of the experiment, which suggests that the higher reported number of cells could assimilate nutrients in greater quantities. Secondly, and in conjunction with the first reason, the higher light incidence would enhance cell growth and the uptake of the nitrate species.

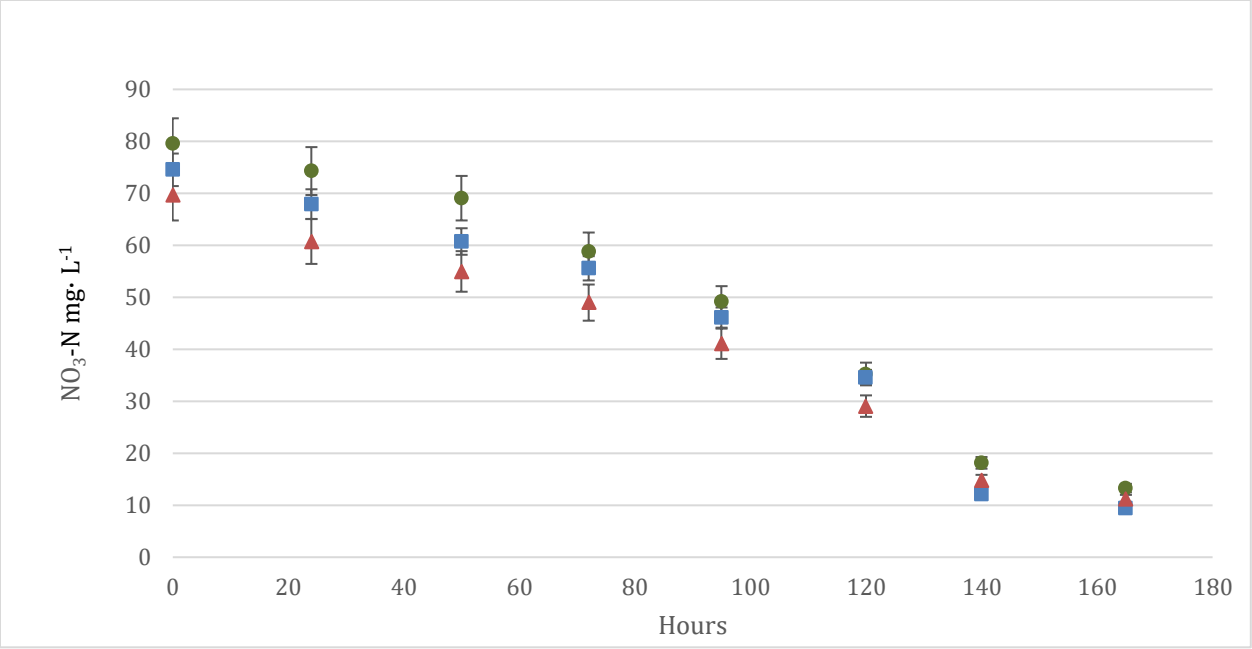


Figure 15. Concentration of nitrate in media during *H. Pluvialis* cultivation in BBM and different concentrations of PHWW (Circles = BBM Control; Squares = BBM +0.125% PHWW; Triangles = BBM + 0.25%PHWW)

The TAN species, contributed in different concentration by the different amounts of PHWW used, were reduced from the wastewater amended media but increased slightly in the BBM only condition. This increase in TAN at the end of the growth phase could suggest that ammonia was being excreted by cells, as suggested by Dong et al. (2007). While no akinete cells were found in the cell counts, it appears that the rate of ammonia expulsion was already detected, and this could also indicate that the maturation phase had begun, even though the cells had not matured enough to be classified as akinete cells at the respective levels of nitrogen in the various conditions.

Observing that the TAN concentrations seem to approach a similar final value between 1-4 mg·L^{-1}, it could be recommended that the use of this algae for removal of wastewater nutrients would need to be monitored more closely at the transition between growth and maturation to avoid release of TAN. These values of ammonia in the wastewater are above the typical discharge limits of 1 mg·L^{-1} (Sedlak, 1991). Given that this organism can assimilate notable quantities of either nitrogen source, it could be used to recover these nutrients at higher concentration of TAN and then be followed by additional treatment steps to remove residual species for discharge. Alternatively, algae system effluent could be

recycled to the head of the plant for dilution and retreatment with the main stream wastewater influent. Recycling is commonly practiced with wastewater side streams that have elevated nutrient levels.

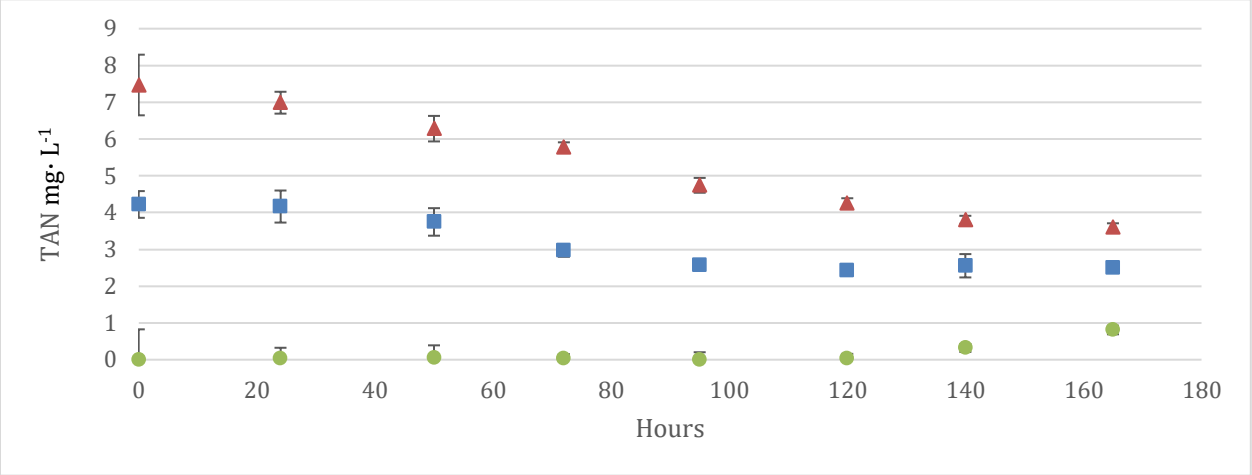


Figure 16. Concentration of TAN in media during *H. Pluvialis* cultivation in BBM and different concentrations of PHWW (Circles = BBM Control; Squares = BBM +0.125% PHWW; Triangles = BBM + 0.25%PHWW)

Figure 17 presents the concentration of phosphorus across the different PHWW cultivation conditions. This experiment also showed considerably less reduction than nitrogen across the varying wastewater concentrations during growth of *H. Pluvialis*. As suggested before, *H Pluvialis* could be able to recover a higher percentage of phosphorus if the concentration was lower in the media or only provided from the wastewater. While phosphorus can be included in value and nutrient recovery, this study was focused primarily on the difference in growth when using wastewater and the recovery of nitrogen at tolerable concentrations of PHWW for the algae.

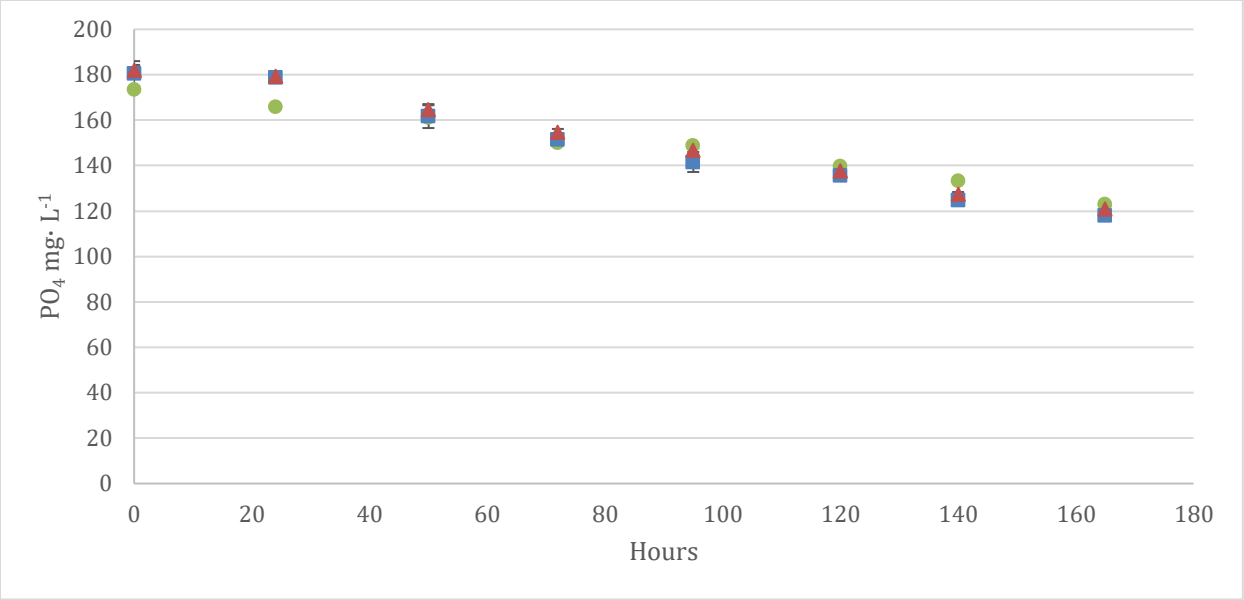


Figure 17. Concentration of Phosphate in media during *H. Pluvialis* cultivation in BBM and different concentrations of PHWW (Circles = BBM Control; Squares = BBM +0.125% PHWW; Triangles = BBM + 0.25%PHWW)

After the growth phase of 7 days (168 hours), the cells were exposed to greater light incidence, nitrogen deprivation, and resuspension in 2.5% saltwater (w/v) conditions to induce carotenoid production. Using DMF as the solvent and the equations provided by Lichtenthaler et al. (1987), the concentrations of chlorophyll and carotenoids were calculated to determine the degree of encystment. Figure 18 shows pictures of the cells on day 0 and day ten of the encystment phase. Each one of the 16 smaller square units per box on the hemocytometer has a width and length of 50μm, as presented with a red scalar for measurement of cell diameter based on the hemocytometer grid. It is clear that the size and red coloration of the akinete cells was an indicator of this encystment and carotenoid production.

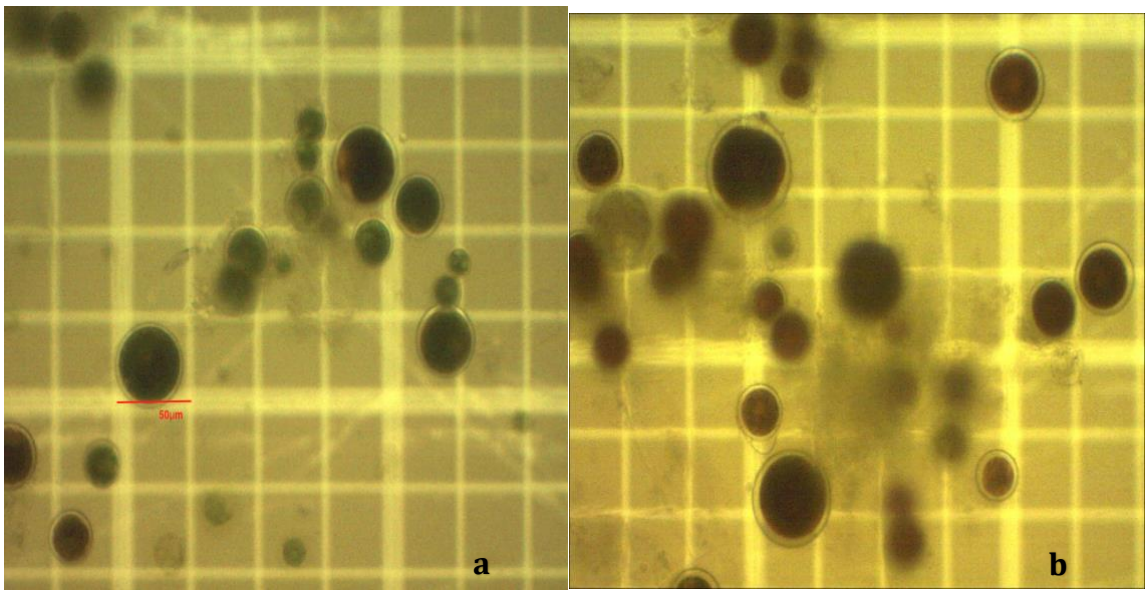


Figure 18 (a). Picture of cells in hemocytometer from day 0 of encystment under 400X magnification; (b) Picture of cells in hemocytometer from day 10 of encystment under 400X magnification

Figure 19 presents the chlorophyll:carotenoid ratio for the cell extracts during the maturation phase for all treatments, and the measured data follows an exponential decrease in the concentration of chlorophyll during the production of carotenoids in the cells. Orosa et al. (2000) reported that a chlorophyll/carotenoids value of greater than 4 indicates sufficient nitrogen in the media for further growth or lack of environmental stress otherwise. The total cells harvested from the cultivation state exhibited no signs of stress, as indicated by the lack of akinete cells and the high chlorophyll:carotenoid ratio number, however, these values dropped over the 300 hours that they were measured for this ratio during the maturation phase. The results from this study match the previous observations over 12 days of encystment, where the chlorophyll/carotenoids ratios fell below 3 and in most cases, below 2 (Orosa et al., 2000). Ultimately, the control condition had the lowest ratio, and the wastewater conditions were more similar to each other. However, this ratio and the total carotenoids measurement does not specify which carotenogenic compounds was prominent in the cells after maturation. Thus, HPLC was used to quantify astaxanthin concentration and determine the presence of other carotenoids.

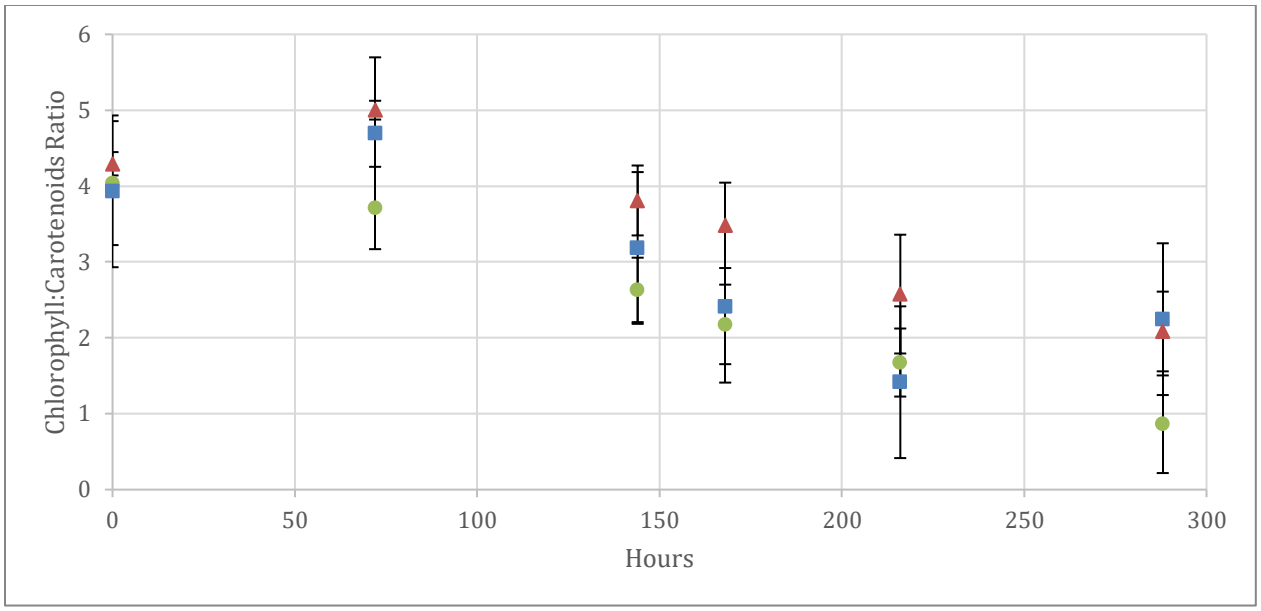


Figure 19. Ratio of total chlorophyll to total carotenoids for biomass harvest and introduced into stress conditions (Circles = BBM Control; Squares = BBM +0.125%PHWW; Triangles = BBM + 0.25%PHWW)

The methods developed by Sarada et al. (2006) were used to extract the pigments from the encysted cells. Figure 20 presents photos from the microscopic analysis of each step of the extraction process. It can be seen that the encysted cells in photo A contained the carotenoid pigments, as indicated by the large size and color. After treatment in 2M HCl at 70˚C for ten minutes, the membranes of the cells appear to leak the inner contents of the cells, making them more available for extraction. As described in the methodology, acetone was used after centrifuging out the biomass to separate the pigments from the cellular debris for measurement using HPLC.

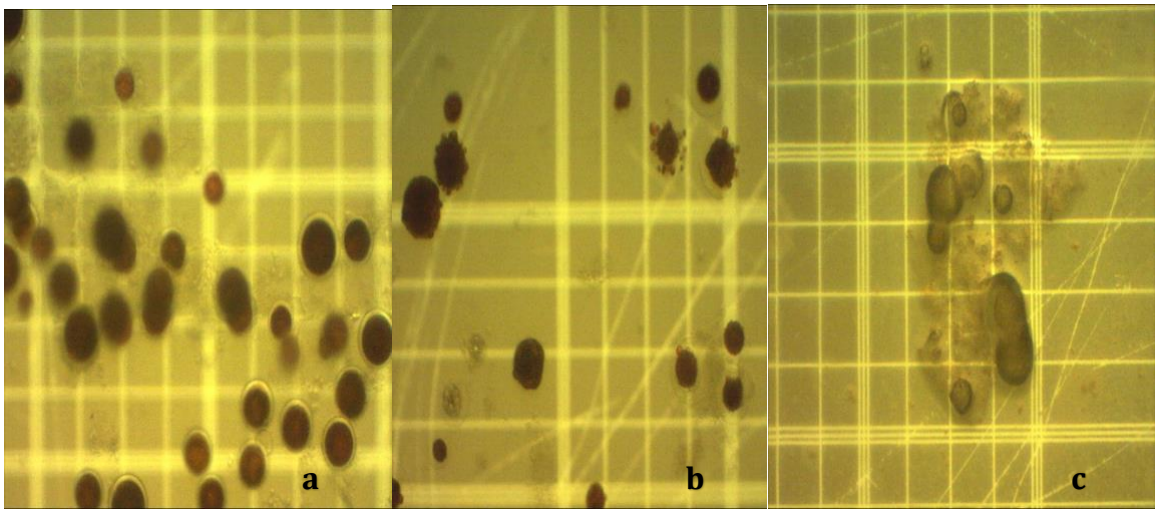


Figure 20. Stress induced cells (a) without treatment, (b) after 2M HCl at 70˚C for 10 min, (c) after acetone extraction

As described in the materials and methods section, a calibration of the astaxanthin using the HPLC was performed with a high-purity analytical standard in order to reliably quantify the concentration of astaxanthin. This calibration curve, presented as Figure 21, was based on peak height values of the HPLC with measured pure astaxanthin samples (Sigma Aldrich SML0982), resulting in an R^2 =0.98678. The calibration gave reasonable certainty that the measured peak height absorbance values were reliable for calculating concentration of astaxanthin in the final extract samples.

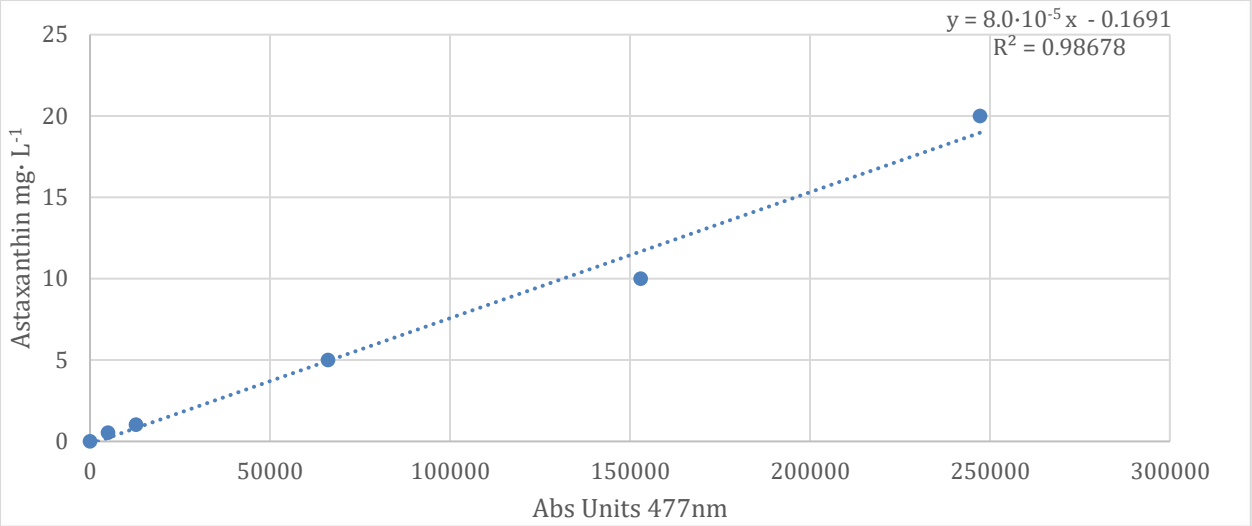


Figure 21. Calibration curves for astaxanthin peak height absorbance units as measured with HPLC, accompanied by corresponding R^2 values and regression equations.

Figure 22 presents the HPLC chromatograms from each cultivation for the extracts, as well as a standard peak generated from the purified sample used in different concentrations to build the calibration curve (Figure 22a). Figure 22 b-d present results for carotenoid extracts from each of the media compositions, 0, 0.125, and 0.25%, respectively. The BBM only condition presented the most easily identified set of peaks, suggesting that the desired trans-astaxanthin product was produced without interference from other isomeric forms or alternative carotenoids. However, the encysted *H Pluvialis* cells grown in media with PHWW nutrients produced various other peaks at 477nm. All astaxanthin extracts had an average retention time of 5.4724±0.015 minutes when passing through the HPLC under the HPLC operational conditions, as described earlier. Additional chromatograms, including the negative control with only PHWW run through the HPLC, are provided in Appendix A.

Additional peaks were also observed by various other authors studying carotenoid and astaxanthin production in algae and fungus (Del Campo et al., 2003; Holtin et al., 2009; Lu et al., 2010), who observed the astaxanthin esters and other carotenoid species in similar chromatographic analyses. Ranga et al., 2009, working with *H. Pluvialis* identified these peaks with purified standards and reported the existence of several astaxanthin esters, identified as the wider peaks appearing after the purified trans astaxanthin species. This is logical because ester groups that bound to astaxanthin molecules would make its total size

larger, and, therefore, in most chromatograph operating conditions, it would require more time to pass through the column in HPLC identification. In this study, identification of other compounds in the HPLC chromatograms was not attempted. However, these molecules could certainly be identified using known standards or coupling chromatography with a mass spectroscopy (MS) and an appropriate library to define the mass of these extract constituents.

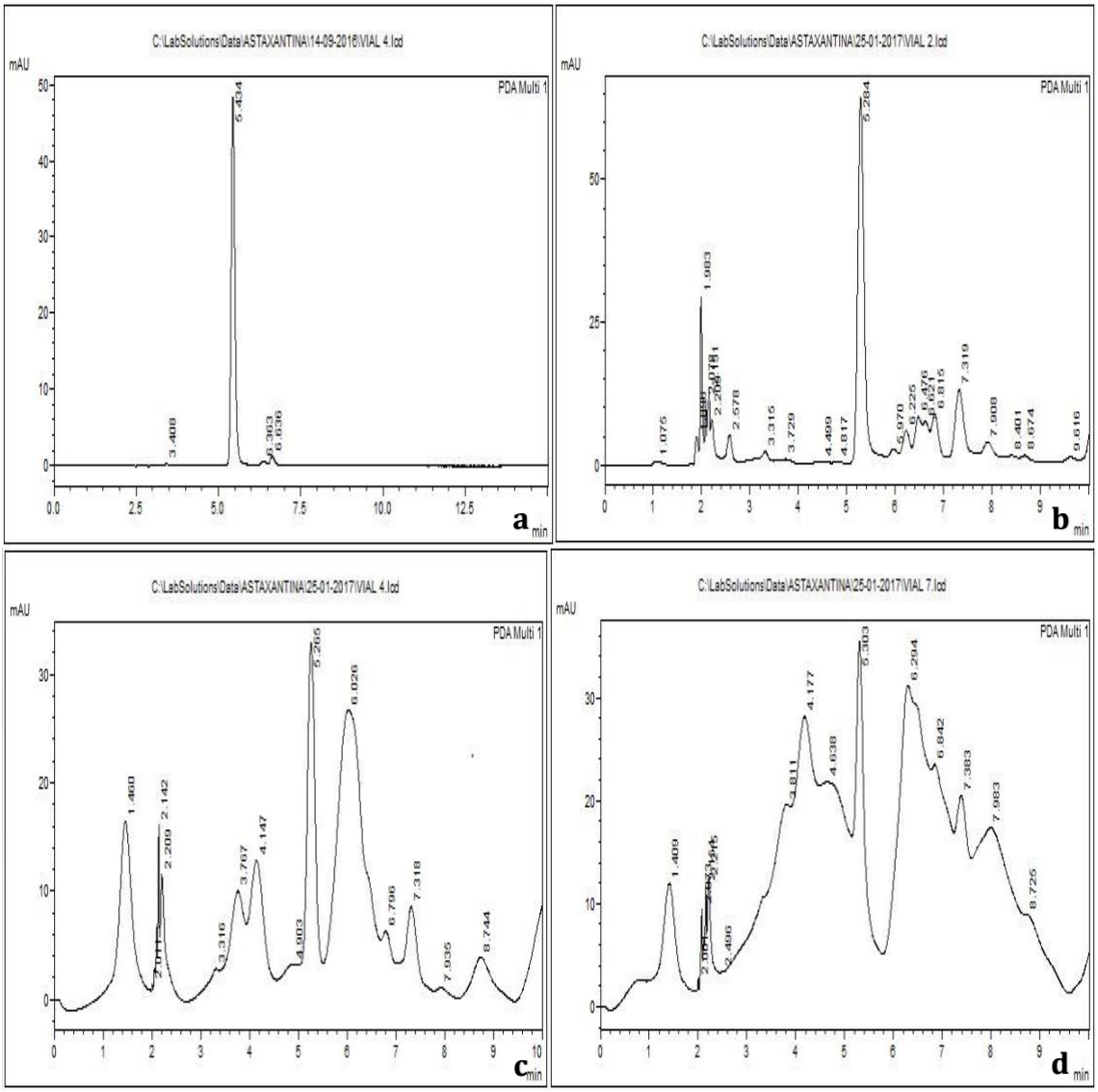


Figure 22. HPLC Chromatograms for (a) Trans-Astaxanthin Analytical Standard; (b) BBM biomass extract; (c) 0.125%PHWW biomass extract; (d) 0.25%PHWW biomass extract

The final astaxanthin contents of the cells grown on the various media compositions are provided in Figure 23 below. These concentrations values were reported based on peak height, which is more typically used for this HPLC method. The peak heights identify that the BBM condition (0% PHWW) resulted in the greatest astaxanthin concentration, but it also presented lesser quantities of other carotenoids in the extract. The peak heights of astaxanthin peaks correspond to concentrations between 2 and 5 $mg \cdot L^{-1}$ in the final biomass extracts. Overall, we see that the control condition resulted in the greatest concentration of astaxanthin, given its peak height. Other HPLC peaks suggested that additional carotenogenic compounds were present in considerable quantities in the biomass extracts from the PHWW wastewater conditions.

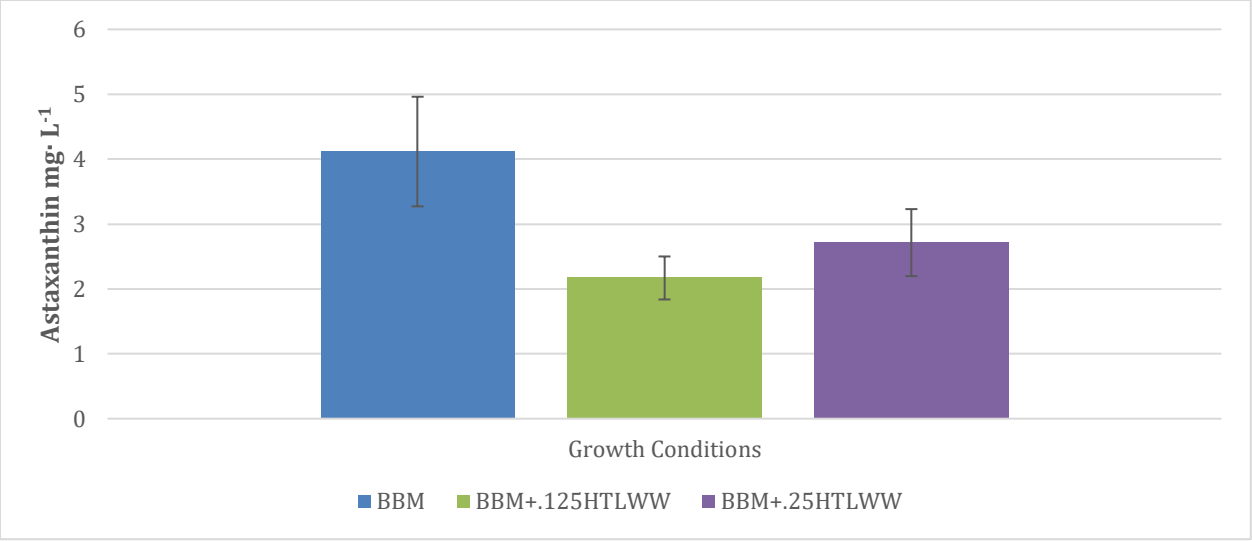


Figure 23. Comparison of astaxanthin concentration in final *H Pluvialis* extract between different media compositions, based on peak height in HPLC

Using the Excel statistical software, a one-way ANOVA was conducted to test the difference in final astaxanthin production amongst the three conditions. Table 12 shows there was a statistical difference between groups at a p value of 0.05. Although there was a more complex carotenoid profile in the wastewater samples, as shown by the chromatograms, the concentrations for trans-astaxanthin product was statistically greater in the control as compared to the other PHWW growth conditions.

Table 12. One-way ANOVA for the difference in astaxanthin production between three growth conditions, based on height

SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
BBM	2	8.23352	4.11676	1.4263605		
mBBM0.125	3	6.505612	2.16853733	0.1647684		
mBBM0.25	3	8.139836	2.71327867	0.40001222		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.65783457	2	2.32891728	4.55592449	0.07472488	5.78613504
Within Groups	2.55592173	5	0.51118435			
Total	7.2137563	7				

CHAPTER 5: CONCLUSIONS

H Pluvialis demonstrated near equivalent growth in terms of cell counts when using only nitrate or only ammoniacal nitrogen as the nitrogen source in BBM. This evidenced that the ammoniacal nitrogen in the PHWW was not the inhibitory compound limiting the growth of the algae on PHWW. In the 0.125% and 0.25% PHWW conditions that were determined to allow for algal growth in the inhibition assay, the growth of *H Pluvialis* increased by as much as 44% in terms of cell counts, noting that the additional nutrients from the wastewater resulted in mixotrophic growth. The balanced inorganic nitrogen, in the forms of nitrate or TAN, presented a removal of 80-90% for the cultivation conditions utilized. The harvested cells were treated with increased 2.5% salt concentration (w/v) and light intensity, in addition to nutrient deprivation, which resulted in the chlorophyll/carotenoid concentration dropping from above 3 after vegetative growth to below 2 for all treatments exposed to these encystment conditions over 300 hours. Astaxanthin production, quantified using HPLC with a purified standard, showed that it was reduced by 47.3% and 34.1% in the 0.125% and 0.25% PHWW augmented cultures, respectively. However, it was observed that the growth with wastewater may have induced production of a more complex carotenogenic profile, as observed in the chromatographs. This study determined that wastewater is a viable source of nutrients for *H Pluvialis* when making sure that inhibitory compounds from PHWW do not limit its growth and also reports that use of the wastewater may result in an alternative carotenogenic profile.

CHAPTER 6: FUTURE WORK

Acclimation and adaptation of *H Pluvialis* to PHWW were not studied but is highly recommended for studying the improved growth of this algae on wastewater over time. This could especially help to mitigate inhibitory effects of the PHWW observed even at very low levels. Other wastewater nutrient sources can also be considered for enhanced and more sustainable production of algal biomass and its derivative metabolites. With respect to the astaxanthin production, further work is needed to understand the mechanisms by which the carotenoids are produced in this species during stress, as well as how nutrient sourcing or inhibitory compounds might affect these processes.

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APPENDIX A: HPLC CHROMATOGRAMS

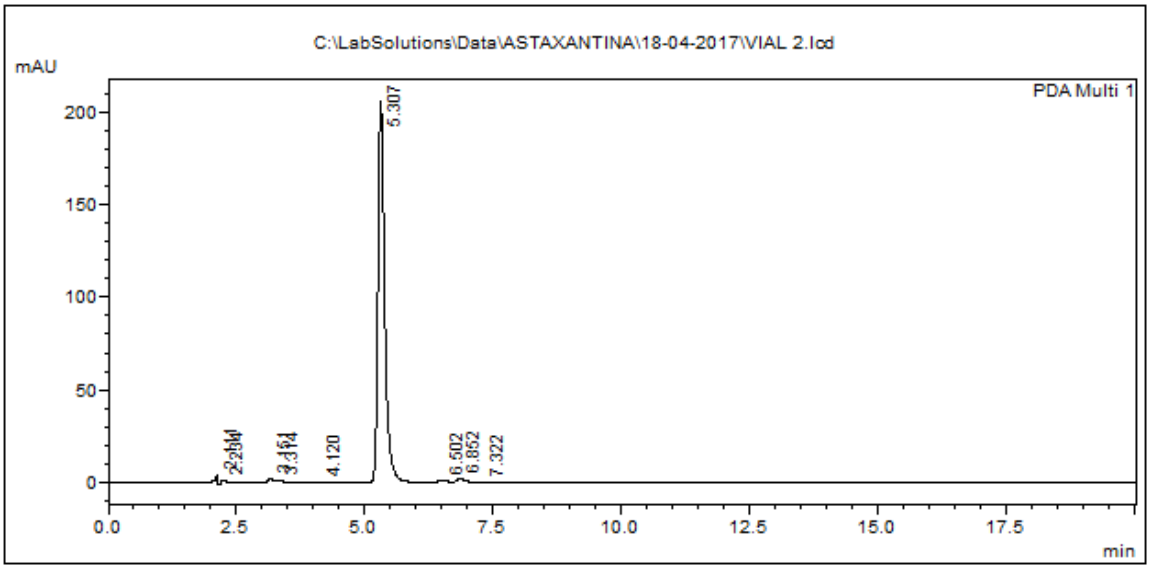

Figure 24. Astaxanthin Standard with 0.25% PHWW

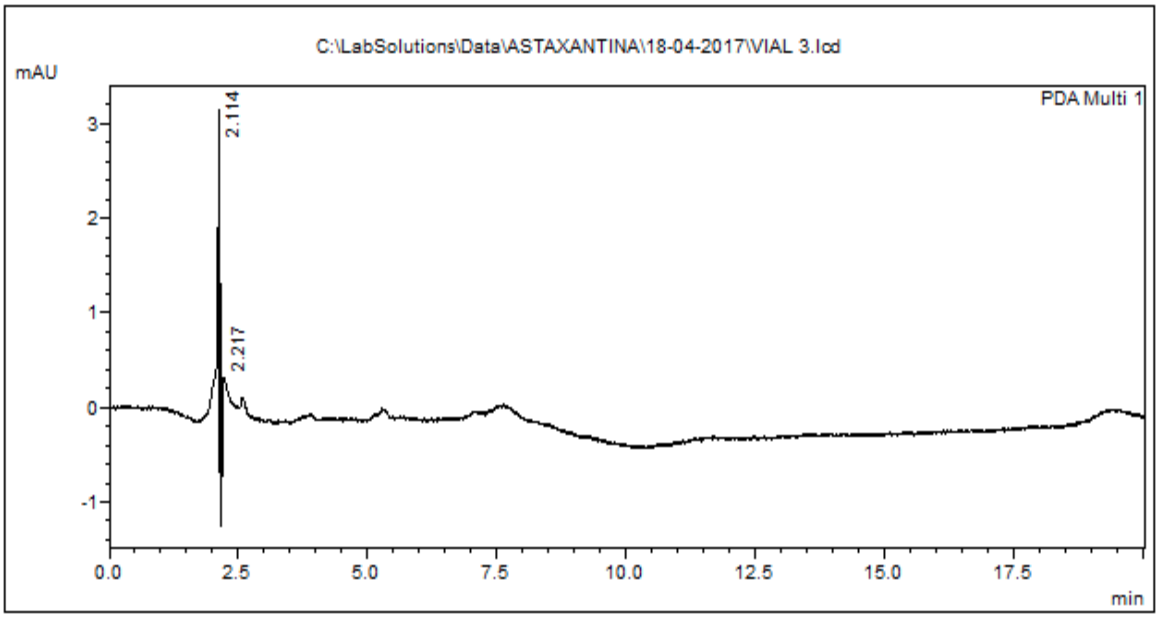

Figure 25. PHWW alone used as negative control

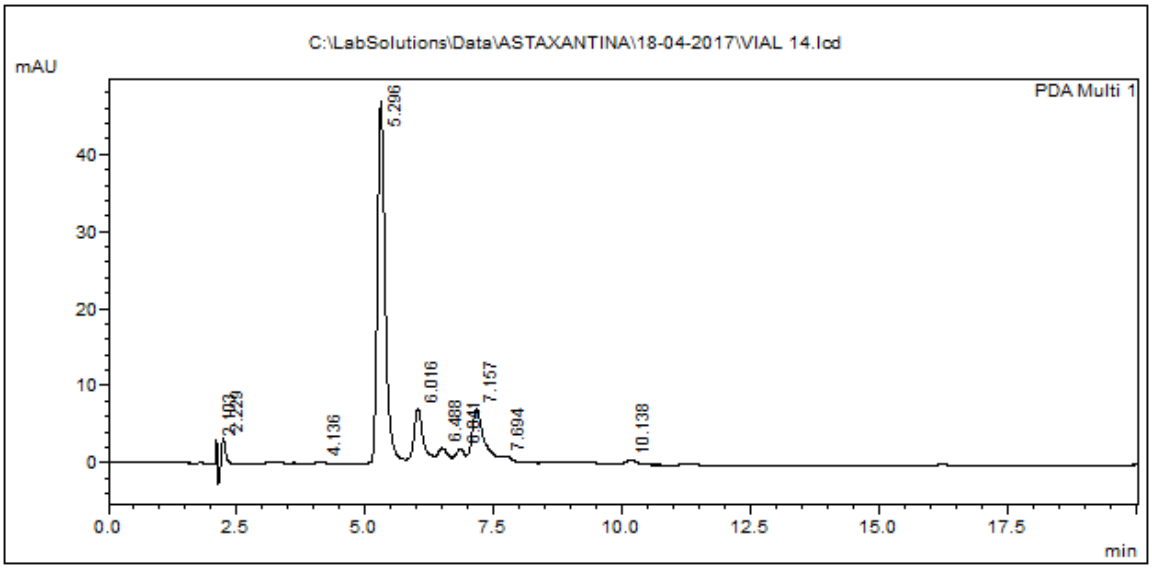

Figure 26. *H Pluvialis* Extract after hydrolysis in 2M HCl at 70C for 10 min