SODIUM BICARBONATE AMENDMENT FOR ENHANCED ASTAXANTHIN PRODUCTION FROM *HAEMATOCOCCUS PLUVIALIS*

by

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DEDICATION

I would like to dedicate my thesis to my parents, Ozden and Turker Erturk, who have been my constant source of inspiration. They have always been role models, teaching me how to tackle a task with enthusiasm and determination; and my sister, Deniz Sinem Erturk Ilhan, who shared her words of advice and encouragement to finish my degree. Without their love and support, none of my success would be possible.

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ABSTRACT

Haematococcus pluvialis is a freshwater green microalga that is widely considered to be the richest natural source of the high value carotenoid astaxanthin. The use of bicarbonate salts as a means of efficiently delivering inorganic carbon in microalgal cultivation is a relatively new concept and its application is continuously growing. Previous studies have largely focused on increasing the lipid content in microalgae via the use of high concentrations of sodium bicarbonate under nitrogen deplete culture conditions. Lipid accumulation is directly related to astaxanthin production as astaxanthin is dissolved and stored in lipid bodies in *H. pluvialis*. Because of this relationship in *H. pluvialis*, the effects of sodium bicarbonate addition on astaxanthin production was investigated in this study. Due to its complex life cycle, *H. pluvialis* is commonly cultivated in two stages called the "green" and "red" stage. Different approaches have been proposed in each stage to increase the astaxanthin production, namely by growing microalgae under nutrient-limited conditions or resuspending the cells into nutrient deplete conditions. In this study, *H. pluvialis* (UTEX 2505) was cultivated in stirred (120 rpm) batch reactors containing MES-Volvox medium with a 12 h:12 h light/dark cycle. Sodium bicarbonate (2.5 mM) was used as an additional inorganic carbon source in the green stage and 50 mM of sodium bicarbonate was used as a trigger mechanism to induce astaxanthin production in the red stage. Following the trigger, the astaxanthin accumulation rate increased from 0.13 mg L^{-1} day^{-1} to 0.64 mg L^{-1} day^{-1} with an astaxanthin concentration of 1.56 ± 0.01 mg L^{-1} and 3.95 ± 1.25 mg L^{-1} respectively. Whereas, an addition of 2.5 mM sodium bicarbonate at the green stage increased the final astaxanthin accumulation rate up to 2.12 mg L^{-1} day^{-1} and the astaxanthin concentration to 11.2 ± 0.56 mg L^{-1}. Increasing biomass in the green stage resulted in higher astaxanthin content at the end of the red stage. In addition to increasing the total astaxanthin content, 2.5 mM of sodium bicarbonate led to faster nitrogen utilization during the green stage. With this faster utilization of nitrogen, the cultures were grown with a one-stage cultivation approach, where the astaxanthin production occurred in continuous mode.

CHAPTER ONE

INTRODUCTION

Project Rationale

The cultivation of photosynthetic, aquatic microalgae requires the input of light, micronutrients and macronutrients. At both industrial and laboratory scales, this cultivation is commonly carried out in liquid environments where carbon dioxide (CO_2) must be dissolved from a gas phase into a bio-accessible form. In commercial production of microalgal by-products, sufficient CO_2 supply may be limited, and sodium bicarbonate ($NaHCO_3$), which has a higher solubility in water compared to CO_2, can be used as an alternative inorganic carbon source. When $NaHCO_3$ dissolves in water, HCO_3^- ions are produced and are readily available as the dissolved inorganic carbon source for microalgae. Recent studies have focused on improving the lipid content in microalgae via the use of high concentrations of $NaHCO_3$ under nitrogen deplete culture conditions (Gardner et al., 2012; Gardner et al., 2013). Adding high concentrations of $NaHCO_3$ at nitrogen deplete conditions has been shown to trigger a metabolic shift from biomass accumulation to lipid accumulation. Further, Gardner et al., 2012 proposed that ceasing cellular replication is the key to promoting triacylglycerol (TAG) accumulation in microalgae, which could act as a sink for the fixed carbon under inhibited cellular replication. Astaxanthin synthesis in *Haematococcus pluvialis* is closely related to TAG accumulation since astaxanthin is stored in lipid bodies under stress conditions (Grunewald et al., 2001). *H. pluvialis* contains

other carotenoids as well, but astaxanthin is the only one which accumulates in TAG-rich lipid droplets (Jin et al., 2017; Peled et al., 2011).

The aim of this study was to quantify effects of targeted bicarbonate addition on the growth of *H. pluvialis* and rate of astaxanthin accumulation. This study focuses on two main objectives:

(1) Improving *H. pluvialis* biomass production using low concentrations of a $NaHCO_3$ amendment as an inorganic carbon source. The rationale is that increased *H. pluvialis* biomass production prior to nitrogen depletion will potentially increase total astaxanthin production.

(2) Enhancing astaxanthin production using high concentrations of a $NaHCO_3$ amendment. The rationale is that high concentrations of $NaHCO_3$ along with combined stress conditions including high pH and nitrogen depletion will cease cellular replication and therefore, will increase astaxanthin production.

To test these hypotheses, the microalga *Haematococcus pluvialis,* which is relevant as it is the highest natural producer of astaxanthin, was used and cultivated with $NaHCO_3$ amendment in two stages “green” and “red” stage.

Algae

Microalgae are photosynthetic aquatic microorganisms that can convert atmospheric CO_2 into valuable products including lipids, proteins, carbohydrates, and other biological molecules (Figure 1.1.).

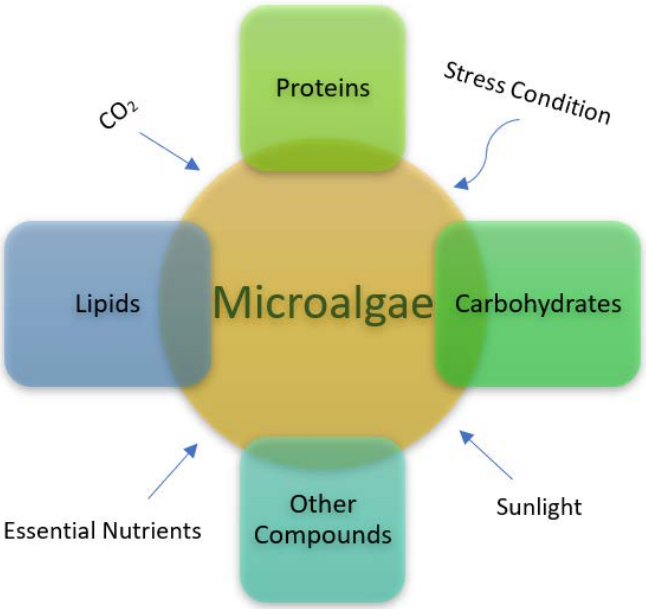

Figure 1.1. Schematic showing that microalgae can convert atmospheric CO_2, light, and essential nutrients into valuable by-products.

Algae naturally exist in fresh or marine aquatic environments under saturated or non-saturated conditions as well as man-made water systems (*e.g.*, wastewater). Algae are categorized according to size as macroalgae and microalgae or color as Rhodophyta (red algae), Xanthophyta (yellow-green algae), Chrysophyta (golden algae), Phaeophyta (brown algae), and Chlorophyta (green algae). Macroalgae, also termed seaweed are visible to the naked eye while individual microalgae are only visible under the microscope. Sunlight, water, CO_2, and some essential nutrients such as phosphorus and nitrogen are the main requirements for algal biomass production. Some advantages inherent in microalgae production include the following: (1) high growth rates and yields compared to other plant crops, (2) ability to fix and sequester atmospheric CO_2, reducing greenhouse gases, (3) ability to grow in diverse aqueous environments (*e.g.* wastewater), (4) do not require arable

land, (5) ability to produce high-value products, such as lipids and antioxidants, and (6) can be used as fertilizers for crops and animal feed (Cai et al., 2013).

With human population growth the demand for global energy has been increased. The increasing use of fossil fuels will eventually lead to their exhaustion because of their nonrenewable nature (Khan et al., 2018). The increasing interest and commercialization of bioenergy has become a potential solution for replacing non-renewable energy sources to meet the huge energy market (Panis et al., 2016). Microalgae have become a promising option in the bioenergy market compared to other crop-derived sources because of their higher growth rates and productivity (Mata et al., 2010). Although, microalgae-based biofuels are not yet economically feasible to replace petroleum-based fuels or compete with other renewable energy technologies.

Microalgae are also widely used in wastewater treatment since wastewater can be a good nutrient (*i.e*. nitrogen and phosphorus) source for microalgae. Growth in wastewater streams results in the exploitation of otherwise unusable nitrogen and phosphorus coupled with valuable biomass production (Markou et al., 2011; Rawat et al., 2011). A significant obstacle for cultivating microalgae in wastewater is the possibility of biomass contamination by native microorganisms which could have detrimental effects on microalgal growth (Markou et al., 2014). Another constraint is that many wastewaters contain high concentrations of inhibitors such as ammonia, nitrite, and heavy metals.

Carotenoids are pigments that are usually colored yellow, orange, and red which are produced by photosynthetic organisms such as microalgae. The common functions of these pigments include light absorption, prevention of photo-oxidative damage related to

excessive light, and quenching the excited state of chlorophyll (Collins et al., 2011). Carotenoids can provide photoprotection by dissipating excess energy in the thylakoids and by forming oil droplets that can function as a sunscreen layer. Besides their role in light harvesting and stability of photosynthetic apparatus, carotenoids take part in other roles in nature including pigments in feathers or skin (*e.g.*, birds and fish).

Haematococcus pluvialis

The class Chlorophyceae includes the most widely used microalgal genera for industrial applications, including *Haematococcus*, *Chlorella*, and *Dunaliella* (Benedetti et al., 2018). *Haematococcus pluvialis (H. pluvialis)* is a freshwater, unicellular green microalga with a complex life cycle. This microorganism is known to be the best natural source for astaxanthin with extraordinary antioxidant capacity (Yuan et al., 2011). *H. pluvialis* has a two-staged culture process, the first stage is called the "green" stage where the cells are green and motile, and the second stage is called the "red" stage where the cells are non-motile and red due to astaxanthin accumulation. The transition between the two stages commonly occurs under an environmental stress condition such as nutrient deprivation, high light, salinity or pH changes.

In industry, *H. pluvialis* is commonly cultured in two-stage systems where the green stage is conducted in closed photobioreactors and the red stage in open outdoor ponds (*e.g.*, Cyanotech, USA) or in closed photobioreactors under stressed conditions (Olaizola, 2000; Shah et al., 2016) Another company, Algatechnologies in Israel, cultivates *H. pluvialis* for astaxanthin production on 10 acres of arid desert land in outdoor tubular photobioreactors, shown in Figure 1.3.

Figure 1.2. Cyanotech corporation microalgae ponds located in Kailua Kona, Hawaii (http://www.cyanotech.com/).

Figure 1.3. Algatech microalgae ponds located in Kibbutz, Ketura (https://www.algatech.com/).

Microalgae Nutrient Requirements

For growth and astaxanthin production *H. pluvialis* requires three main nutrient sources including carbon, nitrogen, and phosphorus.

Carbon

Microalgae are capable of fixing carbon in the inorganic form of CO_2 or bicarbonate (HCO_3^-) via photosynthesis. Carbon can compose up to 65% of dried microalgal biomass (Markou et al., 2014). Limitations in other essential nutrients such as nitrogen and phosphorus can lead to an increase in the carbon content (Kumar et al., 2010; Raven et al., 2014; Sydney et al., 2010). The dissolved inorganic carbon (DIC) level can affect the growth of microalgae significantly (Uusitalo, 1996). The cultivation of microalgae is commonly carried out in aqueous environments where CO_2 must be dissolved before the microalgae can utilize it. When mixed, CO_2 and water forms a weak acid-base buffer system where the equilibrium is shown in Equation 1.

$$CO_{2(g)} \overset{1}{\leftrightarrow} CO_{2(aq)} + H_2O \overset{2}{\leftrightarrow} H_2CO_3 \overset{3}{\leftrightarrow} HCO_3^- + H^+ \overset{4}{\leftrightarrow} CO_3^{2-} + 2H^+ \qquad \text{(Eq. 1)}$$

The pKa of reactions 2, 3, and 4 are 3.6, 6.3, and 10.3 respectively. Reaction 2 is the limiting reaction in this equilibrium which requires high energy for the conversion of CO_2 to carbonic acid. When a gaseous form of CO_2 is supplied to the culture, pH, salinity, pressure, and temperature play an important role in its solubility (Markou et al., 2014). Under alkaline culture conditions, the excess OH^- reacts with CO_2 which forms HCO_3^-, accelerating CO_2 uptake and increasing the total carbon availability (Münkel et al., 2013). The rate of uptake of the gaseous form of CO_2 also depends on the design of different microalgae cultivation systems. The mass transfer of CO_2 into the culture medium strongly

depends on the air-liquid interface area. For example, in the open pond cultivation systems, the solubility of CO_2 occurs passively from the atmospheric air to the medium. Since the air-liquid contact area and the concentration of CO_2 in atmospheric air is low, the mass transfer of CO_2 is also low for microalgal uptake. To replace the assimilated CO_2 in a fast-growing microalgal cultivation systems, the CO_2 can be provided to the system actively (Suh et al., 2003). The CO_2 can be sparged into the culture system via an ambient air pump, or high concentration of CO_2 supply. An alternative source of inorganic carbon for microalgal growth is the use of bicarbonate salts (*e.g.* $NaHCO_3$).

Inorganic carbon is fixed intracellularly via the Calvin cycle through the activity of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) (Price et al., 2008). Carbon uptake in microalgae can be either passively through membrane diffusion or actively by membrane transport mechanisms for CO_2 and HCO_3^- (Moroney et al., 2007). HCO_3^- can only be taken up actively through transporters, but some microalga can convert bicarbonate to CO_2 via carbonic anhydrase (CA), shown in Equation 2 (Markou et al., 2014).

$$HCO_3^- \xrightarrow{CA} CO_2 + OH^- \qquad \text{(Eq. 2)}$$

CA in microalgae is considered to be the part of the carbon concentrating mechanism (CCM) used to elevate the concentration of intracellular CO_2 around Rubisco relative to the extracellular concentration. The main function of the CCM is to actively transport and concentrate the inorganic carbon so that it can be used in the carbon fixation process.

In summary, inorganic carbon supply in the form of CO_2 plays a significant role in microalgae cultivation systems. Since the amount of CO_2 in the atmosphere is as low as 0.04% (400 ppmv) high concentrations of CO_2 are often pumped into the fast-growing

microalgal cultivation systems, resulting in high energy consumption. This increases the costs of commercial microalgae cultivation.

Nitrogen

Nitrogen is essential in microalgal growth for the synthesis of nucleic acids, amino acids, and pigments. One form of nitrogen is nitrate where the assimilation starts when microalgae transport NO_3^- into the cell. Inside the cell, nitrate reductase (NR) catalyzes NO_3^- reduction to NO_2^- which will be transported into the chloroplast. In the chloroplast, the enzyme Nitrite Reductase (NiR) will further catalyze NO_2^- reduction to NH_4^+ (Sanz-Luque et al., 2015). Uptake of NO_3^- causes an increase in pH of the culture medium since it releases1 mol OH^- ions for every 1 mol of NO_3^- that is consumed. The uptake of NO_3^- requires energy, but it does not have toxic effects on the microalgal cells (Markou et al., 2011). When the concentration of NO_3^- increases there is a possibility that the activity of nitrate reductase also increases, which results in higher intracellular NO_2^- (NO_2^-) and NH_4^+. High concentrations of NO_2^- and NH_4^+ can be toxic to the cells (Chen et al., 2009; Jeanfils et al., 1993; Kim et al., 2013).

Ammonia/ammonium is another commonly used nitrogen source in microalgal growth. Although ammonia is a volatile molecule it has a high solubility in water. Ammonia reacts with water via the equilibrium shown in Equation 3.

$$NH_4^+ + OH^- \overset{pKa=9.25}{\longleftrightarrow} NH_3 + H_2O \qquad \text{(Eq. 3)}$$

This equilibrium strongly depends on pH. If the pH goes over 9.25 (25°C), free ammonia will be dominant in the medium and can volatilize rapidly from solution, as shown in Figure 1.4. Although, this equilibrium is also temperature-dependent and the transition

from ammonium to ammonia will occur at lower pH values if the temperature is high. NH_4^+ may cause a pH drop due to formation of H^+ions as a result of its assimilation (Markou et al., 2011).

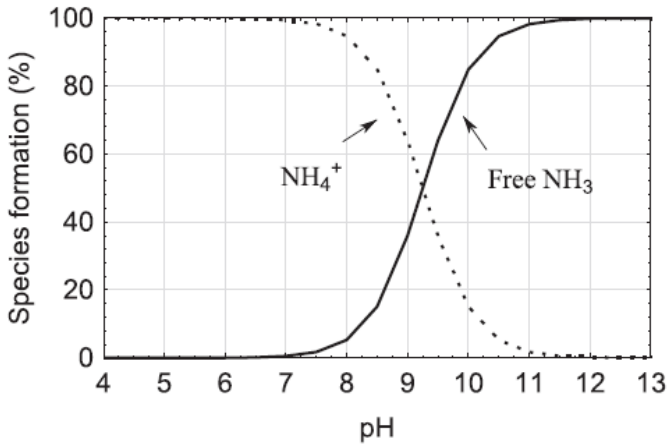

Figure 1.4. pH effects on the ammonium/ammonia equilibrium. When the pKa goes above 9.25 the dominant form will be free ammonia in the culture medium (Markou et al., 2014).

In general, microalgae tend to prefer the most reduced form of nitrogen for uptake and the order of the preference can be listed as $NH_4^+ > NO > NO_2^- > NO_3^-$ (Perez-Garcia et al., 2011). When more than one nitrogen source is present in the culture medium, microalgae tend to prefer NH_4^+ over other nitrogen sources since it requires less energy for uptake. Microalgal cells will usually completely consume the NH_4^+ before switching to other nitrogen sources. Ammonium and ammonia usually repress the uptake of nitrate and nitrite (Boussiba et al., 1991; Fernandez et al., 2007). On the contrary, high concentrations of nitrate have been shown to repress ammonium and ammonia uptake (Florencio et al., 1983). One constraint on using ammonium/ammonia as a nitrogen source is that even at low concentrations it can be toxic to microalgal cells and may negatively impact growth.

Phosphorus

Phosphorus is an essential element for microalgal growth since it is critical to the formation of nucleic acids, membrane phospholipids, and ATP metabolisms (Geider et al., 2002). Phosphorus uptake by microalgae depends on several factors such as light, pH, temperature, salinity, and available ions (K^+, Na^+, Mg^{2+}) (Cembella et al., 1982; Correll, 1998; Rigby et al., 1980). Microalgae can uptake phosphorus via two mechanisms. In the first mechanism, microalgae assimilate phosphorus into the biomass by assembling organic cellular components (*e.g.* phospholipids). The second mechanism is called the luxury uptake (Schmidt et al., 2016). Microalgae and cyanobacteria have the ability to store intracellular phosphorus, which is known as luxury uptake, and as the phosphate gets consumed, they use these phosphorus reserves (Bolsunovskii et al., 2000; Powell et al., 2009).

Potassium

Potassium is another essential nutrient for algal growth. It is a crucial element for the enzymes that are involved in photosynthesis and respiration. Potassium uptake is closely related to the concentration that is present in the medium. Potassium is consumed actively at low concentrations and passively at high concentrations. (Malhotra et al., 1995). Potassium can be included in the microalgal medium in the forms of K_2HPO_4, KH_2PO_4, KNO_3, K_2SO_4, and KCl.

Other Micronutrients

The cultivation medium of microalgae requires micronutrients in addition to the four essential macronutrients (carbon, nitrogen, phosphorus, and potassium). The most

important micronutrients can be listed as Mg, S, Ca, Na, Cl, Fe, Zn, Cu, Mo, Mn, B, and Co (Markou et al., 2014). These micronutrients play various important roles in cellular processes such as being involved in ATP reactions for carbon fixation, components of the photosynthetic apparatus and amino acids that are part of the lipid layer of the membrane, and fundamental enzymatic processes (Melis et al., 2005; Naito et al., 2005).

Under nutrient limited conditions, microalgae tend to balance their biomass composition by triggering the accumulation of carbohydrates, lipids, or pigments (Markou et al., 2014) Recently, there has been increase in the cultivation of microalgae under nutrient deplete conditions to increase the production rate of other valuable products such as lipids and pigments (Khan et al., 2018).

Microalgae Bioproducts

Microalgae have evolved to produce primary and secondary metabolites that are targeted as natural bioproducts. Natural production using microalgae is an effective approach compared to synthetic production of certain bioproducts such as proteins, fatty acids, vitamins, and carotenoids (Mobin et al., 2019).

High-value Products from Microalgae

A previous study suggested that microalgal biomass is a good source for carotenoids (Kathrein et al., 1964). First commercialized carotenoid was β-carotene from *Dunaliella salina* in the 1980s (Ben-Amotz et al., 1989). Astaxanthin from *H. pluvialis* was approved by the US Food and Drug Administration (FDA) in the early 2000's to be used as a color additive in salmons (Lorenz et al., 2000).

The increasing demand for high-value compounds produced by microalgae has led to extensive research on these active metabolites. Bioproducts such as astaxanthin, β-carotene, and omega-3 fatty acids have many health benefits including antioxidant, anti-inflammatory, anticancer, and antiviral properties (Lauritano et al., 2016). Large-scale cultivation of microalgae still has challenges, so an effective biorefinery process should be used to produce these high-value products. Biorefining refers to the integration of numerous products in one system to maximize the benefits and limit the costs (Li et al., 2015) Recently, there has been a rapid increase in the use of microalgal bioproducts for example, the carotenoid market is expected to exceed $1.53 billion USD by 2021 (Barkia et al., 2019; Borowitzka, 2013).

Carotenoids

Carotenoids are yellow, orange, or red accessory pigments that are generally located in the thylakoid membranes (Li et al., 2019) The main function of carotenoids is to absorb light and quench excess energy to protect the photosynthetic apparatus from photodamage (Collins et al., 2011). Carotenoids can be classified into two classes, xanthophylls (oxygenated carotenes) and carotenes (pure hydrocarbons) (Varela et al., 2015). Xanthophylls contain oxygen whereas carotenes are purely hydrocarbons without oxygen. Animals, which do not have the ability to synthesize carotenoids de novo should include them in their diet to benefit from the vitamin, antioxidant, and coloring features. The antioxidants like astaxanthin, *β*-carotene, lutein, and canthaxanthin are the most common carotenoids that can be produced by microalgae. There are two types of carotenoids according to their biological functions: primary and secondary. The primary

carotenoids like lutein function as pigments where the absorbed energy is being transferred to chlorophylls and are essential for survival, whereas the secondary carotenoids like astaxanthin and canthaxanthin serve as a protection mechanism inside lipid globules only under unfavorable stress conditions (Gong et al., 2016; Varela et al., 2015). Secondary carotenoids can protect other molecules and tissues from damage by absorbing the excited energy of singlet oxygen. They are the most common pigments found in nature and are considered potent antioxidants (Gong et al., 2016; Varela et al., 2015).

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CHAPTER TWO

LITERATURE REVIEW

Biology of *Haematococcus pluvialis*

History and Classification

H. pluvialis, also known as *Haematococcus lacustris* or *Sphaerella lacustris,* is a green microalga that belongs to the class Chlorophyceae, order Volvocales, family Haematococcaseae, genus *Haematococcus,* and species *pluvialis* (Hazen et al., 1899). This microalga was first described by J. Von Flotow in 1844 and was later extensively studied by Tracy Elliot Hazen in 1899 (Hazen et al., 1899). *H. pluvialis* is common in a wide range of freshwater habitats (Hazen et al., 1899). This microalga can survive under extreme conditions such as high or low light, temperature, and nutrient deprivation due to its ability to form cysts, which are surrounded by a robust cell wall (Proctor, 1957).

Morphology and Life Cycle

There are four cellular morphologies observed in the life cycle of *H. pluvialis*: macrozooids, microzooids, palmella, and hematocysts (Hazen et al., 1899). The life cycle of *H. pluvialis* cells is shown in Figure 2.1.

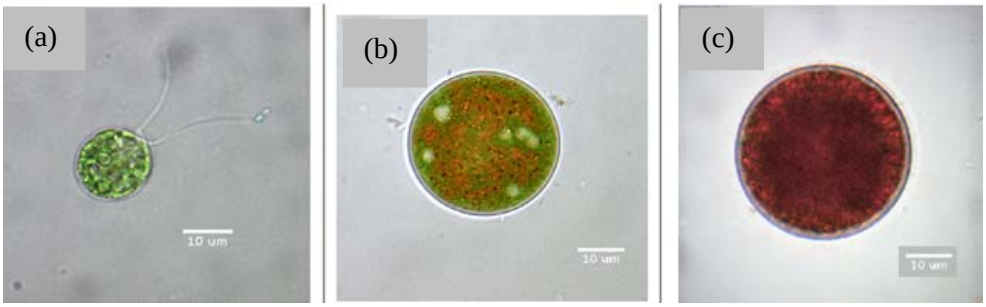

Figure 2.1. The life cycle of *H. pluvialis* cells. (a) Green motile stage. (b) Under stress conditions cells will lose their flagella and transform into resting cells. (c) Red non-motile stage where the astaxanthin accumulation occurs. Photo credit: Berrak Erturk.

Macrozooids, microzooids, and palmella stages are all part of the green stage, while the hematocysts (aplanospores) stage is seen in the red cyst stage. Macrozooids can be spherical, ellipsoidal, or pear-shaped with two flagella surrounded by a gelatinous extracellular matrix. They are most common in early vegetative growth under favorable culture conditions, and their size can vary from 8 to 20 μm (Hagen et al., 2002). Macrozooids can produce 2-32 daughter cells by mitosis (Wayama et al., 2013). When the macrozooid cells encounter unfavorable stress conditions they start to lose their flagella while they increase their cell size and turn into non-motile palmella cells, which can be defined as resting vegetative cells (Hagen et al., 2002). If stress conditions persist, the palmella cells develop into hematocysts and start to accumulate astaxanthin.

Since astaxanthin accumulation is part of the survival mechanism cells produce a thick secondary cell wall to resist extreme stress conditions. As mature hematocysts produce astaxanthin, the cells start to turn bright red. Astaxanthin is produced inside lipid droplets located in the cytoplasm (Wang et al., 2004). If stressed conditions shift into optimal growth conditions the cells germinate to form zoospores and start a new life cycle.

Due to the complex life cycle of *H. pluvialis,* the biochemical composition is distinctively different between its green and red stages. Typically, the cells predominantely consist of proteins, carbohydrates, and carotenoids. Starch is the primary biosynthetic output but will get consumed with prolonged stress exposure (Shah et al., 2016). Two-step cultivation strategies are commonly used in the commercial production of astaxanthin from *H. pluvialis*. The first step is called the green stage where the aim is to promote algal growth and increase biomass production. The second step is called the red stage where the cells are exposed to stress conditions to induce astaxanthin production.

Astaxanthin

Astaxanthin (3,3′-dihydroxy-β, β′-carotene-4,4′-dione) is a red secondary carotenoid with an extraordinary antioxidant activity. Astaxanthin is known to be more powerful than other antioxidants such as vitamin C, β-carotene, canthaxantin, and lutein. (Koller et al., 2014; Pérez-López et al., 2014). Due to this antioxidant activity, it is widely used in the nutraceutical, cosmetics, food and feed industries. Astaxanthin has a value of US$2500-7000 kg^{-1} (Bauer & Minceva, 2019). There are two types of astaxanthin available on the market: synthetic and natural. Natural astaxanthin can be synthesized *de novo* by some microalgae, yeast, and bacteria. The sources of natural astaxanthin are listed in Table 2.1. *H. pluvialis* has been shown to be the richest natural source for astaxanthin in which levels can reach 3 to 6% astaxanthin on a dry weight basis (Lorenz et al., 2000).

Table 2.1. Microorganisms that produce astaxanthin and dry weight percentages.

Sources	Highest Astaxanthin % DW	Reference
Microalgae		
Haematococcus pluvialis	3-6	Chekanov et al., 2017
Chlorococcum	0.2	Zhang et al., 1997
Chlorella zofingiensis	0.001	Wang et al., 2008
Neochloris wimmeri	0.6	Orosa et al., 2000
Enteromorpha intestinalis	0.02	Banerjee et al., 2009
Ulva lactuca	0.01	Banerjee et al., 2009
Catenella repens	0.02	Banerjee et al., 2009
Thraustochytrium sp.	0.2	Yamaoka et al., 2008
Yeast		
Xanthophyllomyces dendrorhous	0.5	Kim et al., 2005
Bacteria		
Agrobacterium aurantiacum	0.01	Yokoyama et al., 1995

Synthetic astaxanthin is made from petrochemicals (Krause et al., 1997). Although synthetic astaxanthin has lower antioxidant activity and has not been proven to be safe for human consumption, it dominates the current market as food colorant and aquaculture feed (Koller et al., 2014; Lorenz et al., 2000). These concerns, coupled with the efficient production of natural astaxanthin by *H. pluvialis*, make this microalga more valuable (Li et al., 2011). So far, *H. pluvialis* has been approved in the USA, Japan, and several European countries to be used as a human dietary-supplement and as a color additive for salmon feeds (Yuan et al., 2011).

Structure and Functions of Astaxanthin

Astaxanthin (3,3′-dihydroxy-ß-carotene-4,4′-dione) is a member of the xanthophyll family of carotenoids with a molecular formula of $C_{40}H_5O_4$ and molecular weight of 596.86 g · mol^{-1}. It has three different stereoisomers, (3S, 3′S); (3R, 3′S), and (3R, 3′R). The 3*S*, 3′*S* stereoisomer, which is naturally found in *H. pluvialis,* is considered to be the most valuable (Yang et al., 2013). The molecular structure of astaxanthin, shown in Figure 2.2, includes a hydroxyl (-OH) and a keto group (C=O) on each of the ionone rings.

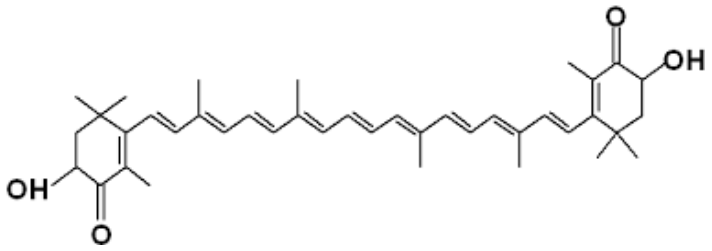

Figure 2.2. Chemical structure of astaxanthin.

The chemical structure of astaxanthin gives this carotenoid the ability to have higher antioxidant activity. Carotenoids have the ability to capture singlet reactive oxygen and polar end groups help astaxanthin to span the lipid membrane bilayer and quench radicals from the surface or the inside of the membrane (Yang et al., 2013).

Applications of Astaxanthin

Free radicals are highly reactive forms of oxygen, that are produced in the body during metabolic reactions and processes. They can be any form of oxygen species with an unpaired electron, namely superoxide anions (O_2^-), peroxides (O_2^{2-}) or hydroxyls (OH).

These free radicals can damage DNA, proteins, and lipid membranes (Guerin et al., 2003). The production of free radicals can increase under stress conditions such as exposure to air pollution, tobacco smoke, various chemicals, and UV light (Guerin et al., 2003). There have been studies that have linked the oxidative damage to aging, atherogenesis, carcinogenesis, and macular degeneration (Guerin et al., 2003). Additionally, research has shown that astaxanthin has therapeutic potential outside of antioxidant activity, such as anti-inflammatory effects, cancer therapies, and improving cardiovascular health (Li et al., 2011; Park et al., 2010). The biological functions of astaxanthin, as well as example literature references that defined the function, are listed in Table 2.2.

Table 2.2. Biological functions of astaxanthin.

Biological Functions	**Reference**
Anti-oxidant activity	Fasano et al., 2014
Protection from UV	Rao et al., 2013
Anti-skin cancer	Rao et al., 2013
Anti-inflammatory	Bhuvaneswari et al., 2014
Anti-gastric activity	Kamath et al., 2008
Anti-diabetes	Chan et al., 2012
Cardiovascular health	Nakao et al., 2010
Immune response	Park et al., 2010
Neuroprotection	Lu et al., 2019

Biosynthesis of Astaxanthin in *H. pluvialis*

The pigment that was produced in *H. pluvialis* was referred to as “haemotochrom” until 1944 when Tisher identified the carotenoid as astaxanthin (Tisher, 1944). The carotenoid fraction varies amongst green-stage and red-stage cells. The green-stage cells contain predominantly lutein (75-80%) and β-carotene (10-20%), whereas the main

carotenoid inside the red-stage cells is astaxanthin (80%) (Lorenz et al., 2000). In the red-stage, cells contain higher concentrations of astaxanthin to protect themselves from photodamage. Red-stage *H. pluvialis* were shown to contain about 5% of free astaxanthin, 10% of astaxanthin diester, 70% astaxanthin monoester and the remaining 15% carotenoids were a mixture of β-carotene, lutein, canthaxanthin and other carotenoids (Collins et al., 2011; Grunewald et al., 2001).

Astaxanthin accumulation is upregulated under stress conditions and is deposited inside cytosolic lipid bodies along with triacylglycerols (TAGs) during the “red” stage (Cheng et al., 2017). Isopentenyl pyrophosphate (IPP) is a major intermediate of carotenoid synthesis and can either be produced through the mevalonate pathway (MVA) in the cytosol or via the non-mevalonate pathway (MEP) in the chloroplast (Lichtenthaler, 1999). According to transcriptomic analysis of astaxanthin biosynthesis in *H. pluvialis*, IPP is predominately synthesized by the MEP pathway. The main reason that *H. pluvialis* cells prefer the MEP over the MVA pathway is that these cells lack the essential enzymes of MVA (Gwak et al., 2014).

IPP that is derived from the MEP pathway is the starting building block of astaxanthin synthesis (Gwak et al., 2014). The next step is IPP isomerization to dimethylallyl diphosphate (DMAPP) (Gwak et al., 2014), which initiates the elongation of the isoprenoid chain and is followed by the line addition of three molecules of IPP. This process is catalyzed by the enzyme geranylgeranyl pyrophosphate synthase (GGPS) and at the end, a C_{20} compound, geranylgeranyl pyrophosphate (GGPP) is formed (Sun et al., 1998). Phytoene, a C_{40} compound is then formed from two GGPP molecules in a catalysis

reaction with the assistance of phytoene synthase. Phytoene has been shown to be as a precursor for astaxanthin production and the phytoene synthase gene has been shown to be up-regulated under the unfavorable conditions where the cells transition from green to red (Gwak et al., 2014; Sun et al., 1998). Four desaturation steps after the formation of phytoene leads to the formation of a highly unsaturated compound called lycopene. The desaturation steps are catalyzed by phytoene desaturases (PDS) and ζ-carotene desaturases (ZDS) (Han et al., 2013). The main purpose of the desaturation reactions is to elongate the conjugated carbon-carbon double bonds and form a colorless molecule ζ-carotene and then convert it to a pink-colored lycopene (Sun et al., 1998; Han et al., 2013). PDS and ZDS enzymes use plastid terminal oxidase (PTOX) and plastoquinone (PQ) as electron acceptors (Han et al., 2013). According to previous studies, increased solar irradiance and nitrogen deprivation both caused an increase in transcripts of PDS and PTOX in *H. pluvialis*. A possible explanation for this increase could be that PDS and PTOX may be responsible for removing excess electrons under stress. Removing excess electrons can prevent over-reduction of the photosynthetic electron transport chain and the formation of excess reactive oxygen species (ROS) (Han et al., 2013).

The next step is cyclization, a branching point of the carotenoid synthesis where α-carotene and β-carotene are formed (Sun et al, 1998). α-carotene is the precursor of lutein, whereas β-carotene is the precursor of other carotenoids such as astaxanthin. According to previous research, high levels of LCY-b is observed under unfavorable environmental conditions in *H. pluvialis* (Lorenz et al., 2000). The final two oxygenation steps that lead to synthesis of astaxanthin are catalyzed by β-carotene ketolase (BKT) and β-carotene

hydroxylase (CrtR-b) (Steinbrenner et al., 2001). The biosynthesis pathway of astaxanthin in *H. pluvialis* is shown in Figure 2.3.

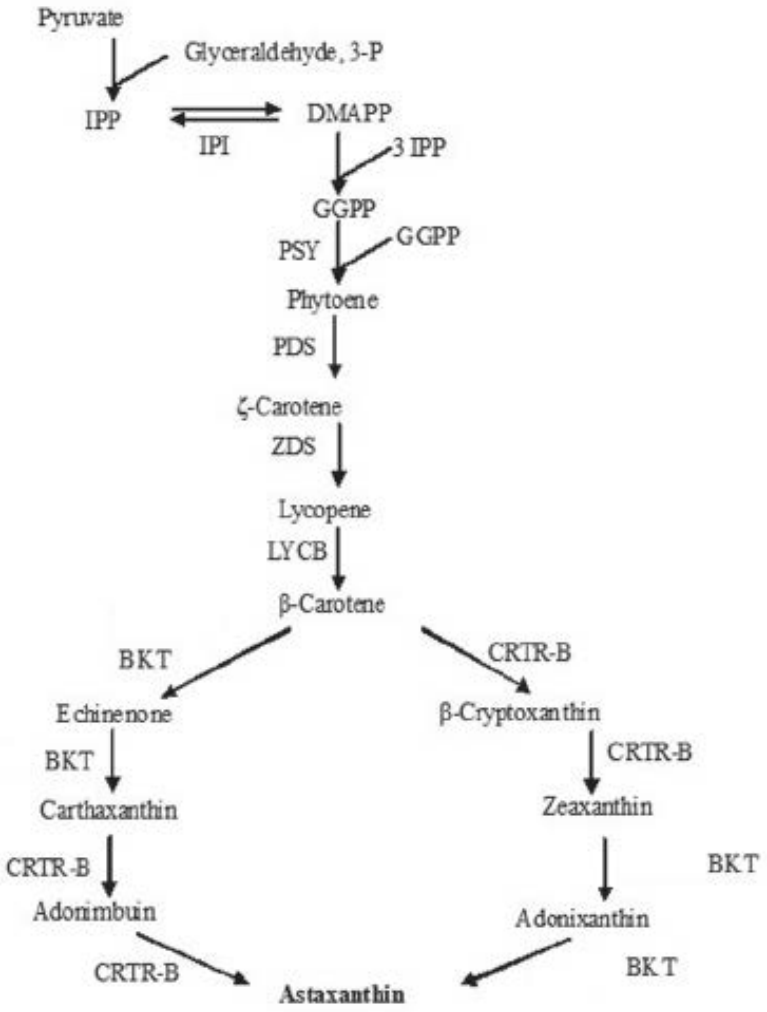

Figure 2.3. Biosynthesis pathway of astaxanthin in *H. pluvialis*. Enzyme abbreviations: IPI, Isopentenyl pyrophosphate isomerase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYB, lycopene β-cyclase; BKT, β-carotene ketolase; CRTRB, β-carotene 3,3′ -hydroxylase.

Cultivation of *H. pluvialis*

Biomass and astaxanthin production from *H. pluvialis* include cultivation, harvesting, and extraction. Choosing appropriate culture parameters such as light, pH, temperature, and growth medium are crucial for maximizing biomass production and astaxanthin yield.

Culture conditions for the green and red stage

Light is the main energy source for photoautotrophic growth. Microalgae have the ability to convert light energy into chemical energy via photosynthesis (Mohsenpour et al., 2012). Microalgae can absorb wavelengths in the range of 400-700 nm (*i.e*. photosynthetically active radiation (PAR)). The type of light source has a high impact on cell growth and productivity, and previous studies have shown that microalgal species have variable responses to different light spectra (Singh et al., 2015). According to a previous study, the maximum growth of *H. pluvialis* was achieved at 260 μmol photons m^{-2} s^{-1} (Evens et al., 2007). The light/dark cycle, photoperiod, is another important factor that affects the growth of microalgae. According to previous studies, the most effective photoperiod was shown to be 12 h: 12 h light/dark during the green stage and continuous illumination during the red stage of *H. pluvialis* (Park et al., 2014; Saha et al., 2013).

The pH can affect microalgal growth especially by changing the distribution of CO_2 species and carbon availability. The pH is closely related to the concentrations of CO_2, HCO_3^-, and CO_3^{2-} in the bicarbonate/carbonate equilibrium. When pH increases above 10.3, HCO_3^- decrease as CO_3^{2-} increases. This is important because CO_3^{2-} cannot be directly

fixed by microalgae (Chen et al., 1994). During exposure to light, photosynthesis, CO_2 fixation, and high microalgal cell densities lead to an overall increase in pH, whereas during the night, respiration leads to a decrease in pH (Bartley et al., 2014; Pires et al., 2017). Previous studies have used CO_2 to maintain the pH at optimal levels for microalgae (Chi et al., 2011; Giordano et al., 2005). According to some studies, the maximum biomass production of *H. pluvialis* was observed at a pH range between 7.00-7.85 (Sarada et al., 2002).

Besides light and pH, microalgal growth also depends on temperature. *H. pluvialis* can execute photosynthesis and cell division over a wide range of temperature between 15-30 °C (Evens et al., 2007). However, the optimal temperature conditions for microalgae has generally been shown to be between 20-25 °C (Khan et al., 2018). At temperatures that exceed the optimal range, there is a decrease in the growth rate of microalgae, potentially due to heat stress (Ras et al., 2013). Most studies have indicated that a temperature between 20 and 28 °C is optimal for the growth of *H. pluvialis* cells, while temperatures above 30 °C started to induce the transition of green cells to red cells. (Fan et al., 1994; Hata et al., 2001).

Cultivation of *H. pluvialis* cultures during the green stage is challenging due to slow growth rates and susceptibility to contamination (Sipauba-Tavares et al., 2015). Various types of growth media have been used to grow *H. pluvialis*, including BG-11 (Fan et al., 1998), BBM (Dominguez-Bocanegra et al., 2004), OHM (Fabregas et al., 2001), KM1-basal medium (Kobayashi et al., 1993), and MES-Volvox (Gwak et al., 2014).

Nitrogen (N) and carbon (C) are two macronutrients that are essential for the growth of microalgae. Nitrate and ammonium were reported as the optimal inorganic nitrogen sources for *H. pluvialis* (Sarada et al., 2002). Many microalgae have been shown to assimilate ammonium more readily than nitrate when both of them are present in the growth medium. A previous study, conducted with six green microalgae, showed that this is not true for all species of microalgae. *H. pluvialis* was shown to assimilate nitrate first and nearly twice as fast when both nitrate and ammonium were present in the medium (Proctor, 1957). CO_2 can be sparged into the culture system via an ambient air pump, or a concentrated CO_2 supply. Microalgae have the ability to fix CO_2 which can help to reduce greenhouse gases (Moreno-Garcia et al., 2017).

Optimal growth conditions are important to achieve high growth rates and biomass during the green stage, and the red stage only starts when the cultures are exposed to a stressor such as nutrient deficiency or high irradiation (Saha et al., 2013). N or P deficiency is a widely used stress condition for astaxanthin production. According to previous research, N-starvation was found to be twice as effective for inducing astaxanthin production compared to P-starvation (Boussiba et al., 1999). C/N ratio is another factor that plays an important role in astaxanthin production process in *H. pluvialis.* When microalgae continue to fix CO_2 under N-deprived conditions, the cellular C/N ratio increases and results in non-nitrogenous material production (*e.g.* TAG), which can serve as a sink for the carbon that is fixed through photosynthesis (Mayzaud et al., 1989). According to a previous study, N depletion increases astaxanthin production more than

light intensity stress partially due to a stronger inhibition of cell division (Fabregas et al., 2003).

Culture Systems

Microalgae are capable of growing in photoautotrophic, heterotrophic, or mixotrophic growth conditions. Under photoautotrophic growth conditions microalgae use light as an energy source and assimilate CO_2 as an inorganic carbon source. Photoautotrophic culture systems are commonly preferred if the biomass is aimed to be used in high-value product synthesis, where there is a lower possibility of contamination and impurities in the culture medium (Moreno-Garcia et al., 2017). Under heterotrophic culture conditions, microalgae can utilize organic substrates as energy and access to inorganic carbon is independent of light (Moreno-Garcia et al., 2017). Many microalgae start to accumulate higher lipid content under heterotrophic culture systems where easily accessible carbon is combined with nitrogen limitation (Liu et al., 2014; Miao et al., 2006). However, unlike other microalgae, *H. pluvialis* exhibits slower growth rates and lower astaxanthin accumulation under heterotrophic culture conditions, so this culture condition is often not utilized in commercial astaxanthin production. Also, the presence of organic carbon substrates in the medium increases the bacterial contamination risk (Hata et al., 2001).

A sequential heterotrophic-photoautotrophic culture system has been shown to be effective in *H. pluvialis* cultivation. According to a previous study, heterotrophic cultivation was used during the green stage to enhance biomass production and photoautotrophic culture conditions were used during the red stage to induce astaxanthin

production (Kang et al., 2005). The red stage was carried out under nitrogen depletion. The final achieved astaxanthin content was 3.4-fold higher compared to the heterotrophic culture systems (Kang et al., 2005).

Under mixotrophic culture conditions, microalgae utilizes both organic compounds and CO_2. *H. pluvialis* can be grown mixotrophically especially by using acetate as a carbon and energy source (Kobayashi et al., 1993). Photoautotrophic growth of microalgae at the commercial scale is more economically feasible compared to the other (*e.g.* heterotrophic or mixotrophic) culture systems in large-scale cultivation systems where sunlight can be used during outdoor cultivation (Shah et al., 2016).

Commercial production of astaxanthin from *H. pluvialis* is commonly accomplished in both open and closed systems. Usually closed systems are employed during the green stage and open systems during the red stage. Open systems are generally open tanks mixed with a paddlewheel and exposed to sunlight and the environment. The main function of the paddlewheels used in open raceway ponds is to suspend the cells uniformly under a constant flow rate and to provide enhanced CO_2 mass transfer. The advantages of open systems include lower initial investment, cheaper maintenance, and easier set-up, whereas the disadvantages include lower productivity, possible bacterial contamination, and the necessity of large amounts of water (Li et al., 2011). The biggest advantage of closed systems, namely photobioreactors (PBR), are that they are more controlled in terms of temperature, light, and nutrient supply (Moreno-Garcia et al., 2017), however PBRs are expensive to build and operate. Academic and industrial researchers are searching for an inexpensive strategy to scale-up the growth of *H. pluvialis*.

Cyanotech corporation is a leading company in the production of astaxanthin from *H. pluvialis* located in Keahole Point, Hawaii (http://www.cyanotech.com/). This company uses closed systems during the green stage, shown in Figure 2.4, and open raceway ponds during the red stage of *H. pluvialis* cultivation as shown in Figure 2.5,

Figure 2.4. 400-liter column closed culture system used in Cyanotech Corporation during the astaxanthin production from *H. pluvialis* (http://www.cyanotech.com/).

Figure 2.5. Final stage of astaxanthin production from *H. pluvialis* carried out in Cyanotech Corporation. 500,000-liter open system ponds (http://www.cyanotech.com/).

The two-step cultivation strategy is commonly used in commercial astaxanthin production since the green and red stage have different optimal environmental and nutritional conditions (Del Rio et al., 2007) . Achieving a high cell density before introducing the stressor is important to obtain higher astaxanthin production. According to previous studies, biomass productivity during the green stage was reported to be in a range between 0.01-0.5 g L^{-1} d^{-1} and astaxanthin accumulation rate ranged between 0.01-4.8 g L^{-1} d^{-1}. Whereas the astaxanthin content varied from 0.8 to 4.8 % of DW (Harker et al., 1996; Olaizola, 2000; Torzillo et al., 2003; Del Rio et al., 2005; Aflalo et al., 2007; Ranjbar et al., 2008; Kang et al., 2010; J. Wang et al., 2013).

Harvesting Techniques

Many researchers have proposed microalgae as a feedstock for biofuel production. However, despite the major advances in the cultivation of microalgae for biofuel

production unless the costs and energy requirements of the cultivation, dewatering, and harvesting technologies are improved it is unlikely that microalgae can complete with the petroleum-based fuel industry. However, the production of high-value products such as astaxanthin may justify the high costs of the cultivation and harvesting process.

Harvesting of *H. pluvialis* refers to the recovery of microalgal biomass. Harvesting is generally one of the greatest cost-contributors in large-scale astaxanthin production since it is energy-intensive and contributes significantly to the operating cost (Shah et al., 2016). Conventional harvesting techniques are centrifugation, chemical flocculation, electro-flocculation, membrane filtration, and ultrasonic separation. Centrifugation is the most common harvesting method for large-scale production of high-value products (Singh et al., 2015). The main idea of centrifugation is to separate the cells from water by gravitational settling (Han et al., 2013). Flotation and disk-stack centrifugation are some other alternative harvesting techniques each having a 95% biomass recovery efficiency (Panis et al., 2016).

Extraction of Astaxanthin

Extraction and purification methods are both extremely important in maximizing astaxanthin yield. *H. pluvialis* cells form thick cell walls under stress conditions. After the cell wall is disrupted and the biomass is dried, the cells can go through the extraction process for astaxanthin recovery. Astaxanthin is a lipophilic compound and so it is often dissolved in solvents and oils for extraction (Shah et al., 2016). There are two types of common extraction techniques: the use of solvents and the use of supercritical carbon dioxide (SC-CO_2). The three main reasons that make SC-CO_2 method more favorable are

that it is non-toxic, non-flammable, and has shorter extraction time. Even though SC-CO_2 seems to be the best option for large-scale production it still requires further development before commercial use.

Major Challenges in Astaxanthin Production Industry

Microalgae represent a potential source for many commercial applications, although many are still under investigation. β-carotene from *Dunaliella salina* and astaxanthin from *H. pluvialis* are the most common high-value products being used today (Leu et al., 2014). Other microalgal strains can also produce high concentrations of lipids and pigments under stress conditions. However, a significant amount of work still must be done on strain development and cultivation technologies to decrease the high production costs and improve the efficiency of the process before they can compete with the synthetic products used in food, cosmetics, aquaculture, and agriculture. Especially in *H. pluvialis*, where astaxanthin is stored inside the cytosolic lipid bodies along with triacylglycerols (TAGs) under stress conditions, the key to increasing astaxanthin concentration might be through a better understanding of the metabolic regulation of TAG biosynthesis (Gwak et al., 2014). This understanding can also provide better knowledge for physiological or genetic manipulation of this microorganism to enhance the astaxanthin and lipid production.

There are many challenges related to large-scale production of astaxanthin from *H. pluvialis* including slow cell growth rates, high costs of cultivation and biorefinery processes, and unavailability of genetically modified *H. pluvialis* strains (Shah et al., 2016). Development of biorefinery approaches including cultivation, harvesting and product

extraction of high-value products are the main challenges of the microalgal bio-based product industry. Improving these approaches and testing for various products and microalgal strains will help to reduce the costs of the production processes.

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CHAPTER THREE

SODIUM BICARBONATE AMENDMENT FOR ENHANCED ASTAXANTHIN PRODUCTION FROM *HAEMATOCOCCUS PLUVIALIS*

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Berrak Erturk

Contributions: Carried out growth, pH, chlorophyll, and nutrient assays. Conducted astaxanthin extraction and analysis. Participated in study design, data analysis/interpretation, and manuscript preparation.

Co-Author: Christian Lewis

Contributions: Assisted with growth, pH, chlorophyll, nutrient assays and astaxanthin extraction.

Co-Author: Brent M. Peyton

Contributions: Participated in study design, study coordination, data analysis/interpretation, and manuscript preparation.

Manuscript Information

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Abstract

Haematococcus pluvialis is a freshwater green microalga that is widely considered to be the richest natural source of the high value carotenoid astaxanthin. The use of bicarbonate salts as a means of efficiently delivering inorganic carbon in microalgal cultivation is a relatively new concept and its application is continuously growing. Previous studies have largely focused on increasing the lipid content in microalgae via the use of high concentrations of sodium bicarbonate under nitrogen deplete culture conditions. Lipid accumulation is directly related to astaxanthin production as astaxanthin is dissolved and stored in lipid bodies in *H. pluvialis*. Because of this relationship in *H. pluvialis*, the effects of sodium bicarbonate addition on astaxanthin production was investigated in this study. Due to its complex life cycle, *H. pluvialis* is commonly cultivated in two stages called the "green" and "red" stage. Different approaches have been proposed in each stage to increase the astaxanthin production, namely by growing microalgae under nutrient-limited conditions or resuspending the cells into nutrient deplete conditions. In this study, *H. pluvialis* (UTEX 2505) was cultivated in stirred (120 rpm) batch reactors containing MES-Volvox medium with a 12 h:12 h light/dark cycle. Sodium bicarbonate (2.5 mM) was used as an additional inorganic carbon source in the green stage and 50 mM of sodium bicarbonate was used as a trigger mechanism to induce astaxanthin production in the red stage. Following the trigger, the astaxanthin accumulation rate increased from 0.13 mg L^{-1} day^{-1} to 0.64 mg L^{-1} day^{-1} with an astaxanthin concentration of 1.56 ± 0.01 mg L^{-1} and 3.95 ± 1.25 mg L^{-1} respectively. Whereas, an addition of 2.5 mM sodium bicarbonate at the green stage increased the final astaxanthin accumulation rate up to 2.12 mg L^{-1} day^{-1} and the astaxanthin concentration to 11.2 ± 0.56 mg L^{-1}. Increasing biomass in the green stage resulted in higher astaxanthin content at the end of the red stage. In addition to increasing the total astaxanthin content, 2.5 mM of sodium bicarbonate led to faster nitrogen utilization during the green stage. With this faster utilization of nitrogen, the cultures were grown with a one-stage cultivation approach, where the astaxanthin production occurred in continuous mode.

Introduction

Astaxanthin is a secondary carotenoid that can be produced by microalgae through photosynthesis. It is known to be the strongest and most expensive antioxidant in the market with a price between US$2500-7000 kg^{-1} (Bauer et al., 2019). The strong antioxidant activity has been suggested to have the ability to reduce free radicals and oxidative stress to promote human health (Hussein et al., 2006; Rao et al., 2010). Due to these and other antioxidant qualities, astaxanthin has shown increasing commercial success in industries such as nutraceuticals, pharmaceuticals, food, and aquaculture (Higuera-Ciapara et al., 2006). Apart from its antioxidant properties, it is a well-known natural food colorant, giving the pinkish-red hue to salmonids, shrimp, lobsters, and crayfish (Lorenz et al., 2000).

Currently, astaxanthin can be produced either naturally from microalgae or synthetically from petrochemicals. Recently, the demand for commercial astaxanthin production has been rapidly growing, but due to lower prices and the technical problems related to large-scale cultivation of microalgae, synthetic (petroleum-based) astaxanthin dominates the market. Because of a rising consumer demand for natural ingredients and health concerns related to the consumption of synthetic astaxanthin, the production of natural astaxanthin has recently increased significantly (Koller et al., 2014; Pérez-López et al., 2014). Natural astaxanthin is produced by various microorganisms, but *Haematococcus pluvialis* is acknowledged to be the richest natural source (Wan et al., 2014). Thus, this freshwater green microalga has become very popular for the production of astaxanthin.

H. pluvialis has a complex life cycle that makes the cultivation process challenging, thus, two-stage culture systems of *H. pluvialis* are commonly used to produce astaxanthin. The first stage is called the “green” motile stage, where cell growth and biomass cultivation are targeted under favorable culture conditions (*e.g.* nitrogen-replete). After the cultures have reached high cell density, the cultures are exposed to stress conditions (*e.g.* nitrogen depletion, intense light, pH, temperature, or high salinity) to induce astaxanthin accumulation. This second stage is called the “red” non-motile stage where the cultures start to accumulate by-products such as astaxanthin and lipids as secondary metabolites. (Kobayashi et al., 1991; Harker et al., 1996; Olaizola, 2000). As the cells enter the red stage the cellular replication stops and the cells start to build a thick cell wall as a survival mechanism (Boussiba, 2000; Hagen et al., 2002).

Photoautotrophic growth of microalgae strongly depends on the presence of inorganic carbon such as CO_2. The atmospheric concentration of CO_2 is approximately 0.04% and the cultivation of microalgae requires active aeration, resulting in high energy inputs. Many studies have focused on improving the productivity of microalgal cultivation by sparging high concentrations of CO_2 into the culture systems (Cheng et al., 2016; Chekanov et al., 2017). High costs related to the capture, compression, and transportation of CO_2 have resulted in a demand for an alternative inorganic carbon source.

Microalgae are capable of fixing carbon in the form of CO_2 or bicarbonate (HCO_3^-) via photosynthesis (Kumar et al., 2010; Raven et al., 2014; Sydney et al., 2010). The cultivation of microalgae is commonly carried out in liquid environments where CO_2 must be dissolved from a gas phase into the aqueous medium. The relationship between

dissolved CO_2 and water forms a weak acid-base buffer system where the equilibrium is shown in Equation. 1.

$$CO_{2(g)} \overset{1}{\leftrightarrow} CO_{2(aq)} + H_2O \overset{2}{\leftrightarrow} H_2CO_3 \overset{3}{\leftrightarrow} HCO_3^- + H^+ \overset{4}{\leftrightarrow} CO_3^{2-} + 2H^+ \qquad \text{(Eq. 1)}$$

pKa_2, pKa_3, pKa_4 are 3.6, 6.3, and 10.3 respectively. When pH values are between 6.5 and 10, bicarbonate (HCO_3^-) is the dominant form, whereas at pH values above 10.3, CO_3^{2-} is predominant. The rate of uptake of the gaseous form of CO_2 also depends on the design of different microalgae cultivation systems as the mass transfer of CO_2 into the culture medium strongly depends on the air-liquid interface. For example, in open pond cultivation systems, the dissolution of CO_2 occurs passively from the atmospheric air into the medium. Since the air-liquid contact area and the concentration of CO_2 in atmospheric air is low, the mass transfer of CO_2 is also low in the cultivation systems. To replace the assimilated CO_2 in a fast-growing microalgal cultivation systems, the CO_2 can be provided to the system actively (Suh et al., 2003). The CO_2 can be sparged into the culture system via an ambient air pump, or high concentration of CO_2 supply. Pumping CO_2 into the system directly results in a greater air-water interface than in open pond systems. As an alternative to CO_2, bicarbonate salts (*e.g.* $NaHCO_3$) can be used as a carbon source that has a higher solubility in water compared to CO_2. This source of inorganic carbon can be a good alternative for CO_2 in commercial production of microalgal by-products and could alleviate costs associated with CO_2 sparging.

Recent studies have focused on improving the lipid content in microalgae via the use of high concentrations of sodium bicarbonate under nitrogen deplete culture conditions (Gardner et al., 2012; Gardner et al., 2013). This concept is based on adding high

concentrations of sodium bicarbonate at nitrogen deplete conditions to trigger a metabolic shift from biomass accumulation to lipid accumulation. A previous study proposes that ceasing cellular replication is the key to promote triacylglycerol (TAG) accumulation in microalgae, which could act as a sink for the fixed carbon under inhibited cell cycle conditions (Lohman et al., 2015). Astaxanthin synthesis in *H. pluvialis* is closely related to TAG accumulation as astaxanthin is stored in lipid bodies under stress conditions (Grunewald et al., 2001). *H. pluvialis* contains other carotenoids as well, but astaxanthin is the only one which accumulates in TAG-rich lipid droplets (Jin et al., 2017; Peled et al., 2011).

The aim of this study was to quantify effects of targeted bicarbonate addition on the growth of *H. pluvialis* and the rate of astaxanthin accumulation. This study focuses on two main objectives: (1) Improving *H. pluvialis* biomass production using low concentrations of $NaHCO_3$ amendment as an inorganic carbon source. The rationale is that increased *H. pluvialis* biomass production prior to nitrogen depletion will potentially increase total astaxanthin production. (2) Enhancing astaxanthin production using high concentrations of $NaHCO_3$ amendment. The rationale is that a high concentration $NaHCO_3$ amendment along with combined stress conditions including high pH and nitrogen depletion will stop cellular replication, provide large amounts of bioavailable carbon and therefore increase astaxanthin production. To reach these objectives, the microalga *H. pluvialis,* the highest natural producer of astaxanthin, was cultivated with targeted inorganic carbon amendments coupled with the exposure to multiple stressors in a two-stage culturing system.

Materials and Methods

Microalgal Strain and Medium Composition

Haematococcus pluvialis strain UTEX 2505 was obtained from the Algae Culture Collection at the University of Texas, Austin, TX, USA. Cultures were grown in a modified MES-Volvox medium with a pH of 6.7. MES-Volvox medium contains 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.16 mM $MgSO_4 \cdot 7H_2O$, 0.16 mM Na_2glycerophosphate$\cdot 5H_2O$, 0.67 mM of KCl, 10 mM of MES, 1 mM of NH_4Cl, 1.02 x 10^{-5} mM of Biotin, 1.11 x 10^{-6} mM of Vitamin B_{12}, and P-IV Metal Solution consisted of 2 mM of $Na_2EDTA \cdot 2H_2O$, 0.36 mM of $FeCl_3 \cdot 6H_2O$, 0.21 mM of $MnCl_2 \cdot 4H_2O$, 0.037 mM of $ZnCl_2$, 0.0084 mM of $CoCl_2 \cdot 6H_2O$, 0.017 mM of $Na_2MoO_4 \cdot 2H_2O$. In all four experimental phases, cultures were grown under optimal conditions (*i.e.* without any stress) during the green stage and were "triggered" with 50 mM of sodium bicarbonate near nitrogen depletion. The astaxanthin content was measured every day for five days during the red stage. The growth conditions of each phase during the green and red stage is listed in Table 3.1.

Table 3.1. Growth conditions of each experimental phase during *H. pluvialis* green and red stage.

Stages	**"Green" Stage**				**"Red" Stage**			
Conditions	$NaHCO_3^-$ (mM)	Stress	Light Cycle (L/D)	pH Control	$NaHCO_3^-$ (mM)	Stress	Light Cycle (L/D)	pH Control
Phase I	Ø	Ø	12 h:12 h	Ø	50	ND	14 h:10 h	Ø
Phase II	2.5	Ø	12 h:12 h	Ø	50	ND	14 h:10 h	Ø
Phase III	2.5	Ø	12 h:12 h	Y	50	ND	14 h:10 h	Ø
Phase IV	2.5	Ø	12 h:12 h	Y	50	ND + L	24 h	Ø

* ND refers to nitrogen depletion and L to light.

Shake Flask Photobioreactors

Phase I and II were conducted in shaking flask photobioreactors. Cells were cultured in 1L batch flasks containing 600 ml MES-Volvox medium continuously shaken at 120 rpm with a 12 h:12 h light/dark cycle during the green stage and 14 h:10 h light/dark during the red stage. All flasks were aerated continuously with humidified ambient air (0.04% CO_2 v/v) at a rate of 400 mL min^{-1} measured and controlled by needle-valve rotameters (Cole-Parmer, USA). The pH was set initially to 6.7, but not controlled during these experiments, and the aforementioned two-stage culturing approach was used. The cultures were transferred into a nitrogen-free medium near nitrogen depletion. Immediately after resuspension, 50 mM of analytical grade sodium bicarbonate (Sigma-Aldrich, USA)

was used as a “trigger” mechanism for astaxanthin production. Experiments were conducted at room temperature (24 ± 1 °C), and astaxanthin concentration was measured daily for 5 days.

Tubular Photobioreactors

Phase III and IV were conducted in tubular photobioreactors. Cultures were grown in 70 x 500 mm cylindrical glass tubes (1.25 L) sealed at the top with a rubber stopper with ports for sampling, aeration, and a pH probe attached to a pH controller (Milwaukee, SMS-122). *H. pluvialis* cultures were grown in 1L MES-Volvox medium. Cultures were sparged continuously with humidified ambient air and were supplemented with 5% CO_2 (v/v) on demand to keep the pH around 7.5. The cultures were amended with low concentrations (2.5 mM) of sodium bicarbonate in the green stage and were triggered with 50 mM of sodium bicarbonate in the red stage. To test the effects of a combination of multiple stressors, 24 h light stress was tested in addition to nitrogen depletion and pH stress in Phase IV. All the other conditions such as the light cycle, temperature, and stirring speed were kept the same as the Phase I and II shake flasks. One issue with using tubular photobioreactors was that under high pH conditions *H. pluvialis* cultures started to form a biofilm at the top of the growth medium (air-water interface). To prevent this issue “algae scrubbers”, which are like small magnetic aquarium cleaning devices, were designed and 3-D printed to control the *H. pluvialis* biofilms that were formed at the air-water interface under high alkalinity conditions in the red stage.

Cell Concentrations

Cell counts were determined using a Reichert hemocytometer under a light microscope and calculations were based on 400 cells counted per sample for statistical reliability (Andersen 2005). The specific growth rate was calculated using Equation 4.

$$\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0) \quad \text{(Eq. 4)}$$

where N_1 is the cell concentration at time t_1 and N_0 is the cell concentration at t_0.

In addition to the hemocytometer technique, culture growth was measured using dry cell weight (DCW), where 10 ml of sample was filtered through pre-weighed 0.7 µm glass fiber filters. After filtration, the filters were dried at 80°C for 24 h and then stored in a desiccator for another 24 h until a constant weight was obtained. DCW [g biomass L^{-1}] was calculated by subtracting the weight of the clean filter without biomass from the desiccated filter with biomass and dividing by the volume of the collected sample.

Nitrogen Measurements

The nitrate and ammonium concentrations were measured every 48h using colorimetric assays. For nitrate, Szechrome NAS reagent (Polysciences Inc., USA) was used and for ammonium, 2-phenylphenol (2PP) assays were used. In brief, 1 ml of culture was centrifuged, and the supernatant was collected for nitrogen quantification. For the NAS assay, 20µl of the sample was mixed with 200µl of NAS reagent and incubated at room temperature for 30 min. The absorbance was read on a spectrophotometer (Synergy H1 hybrid reader, BioTek) at 570 nm and the nitrate concentration was calculated by using a standard curve. For ammonium, 25 µl of sample was mixed with 175 µl citrate reagent and after 1 min it was mixed with 50 µl of 2-phenylphenol-nitroprusside reagent and 25 µl of

buffered hypochlorite reagent. The mixture was put into a 37 °C incubator for 30 seconds and the absorbance was read at 660 nm.

Astaxanthin Extraction and Quantification

The astaxanthin concentration was measured with a spectrophotometric method described by Wan et al. (2014). The red stage cells were collected and concentrated by centrifugation at 6,000xg for 15 min. The remaining pellet was dissolved in 5% KOH and 30% (v/v) methanol at 65 °C to destroy chlorophyll and then washed two times with water. 5 ml of dimethyl sulfoxide (DMSO) and acetone at a ratio of 4:1 (v/v) were added to break the cell walls, and a probe sonicator (QSonica, XL-2000) was used for extraction. The process was repeated two or three times until there was no change observed in the color of the pellet. The absorbance of the extract was read in a spectrophotometer (Genesys 10-S, Thermo Electron Corporation) at 490 nm. The astaxanthin concentration was calculated using Equation 5. Derived from the Beer-Lambert equation using an astaxanthin standard with a known concentration.

$$C = 4.5 \cdot OD_{490} \cdot \left(\frac{V_a}{V_b}\right) \cdot f \qquad \text{(Eq. 5)}$$

C represents the astaxanthin concentration (mg/L), V_a the volume of the extract (mL), V_b the volume of the collected sample (mL), and f the dilution ratio of the measured absorbance.

Chlorophyll Measurements

Chlorophyll was determined using a hot methanol method modified from Ordog et al. (2012). In brief, 1 ml of sample was centrifuged at 6,000xg for 10 min and the supernatant discarded. The remaining pellet was combined with 1 ml of methanol and

sonicated until it was homogenized. Immediately after homogenizing, the mixture was heated at 70°C for 5 min and centrifuged again at 6,000xg for 10 min. The absorbance of the collected supernatant was measured at 666, 653, and 470 nm. Chlorophyll calculations were carried out using Equations 6, 7, and 8 (Nemeth, 1998).

$$\text{Chl a}\left(\frac{\text{mg}}{\text{L}}\right) = 17.12 \cdot \text{A}_{666} - 8.86 \cdot \text{A}_{653} \quad \text{(Eq. 6)}$$

$$\text{Chl b}\left(\frac{\text{mg}}{\text{L}}\right) = 32.23 \cdot \text{A}_{653} - 14.55 \cdot \text{A}_{666} \quad \text{(Eq. 7)}$$

$$\text{Chl a + b}\left(\frac{\text{mg}}{\text{L}}\right) = 2.57 \cdot \text{A}_{666} + 23.6 \cdot \text{A}_{653} \quad \text{(Eq. 8)}$$

Results

Phase I

In the first set of experiments, high concentration (50 mM) amendments of sodium bicarbonate were used as a trigger mechanism to increase astaxanthin concentration. Figure 3.1. shows cell growth where the cells exhibited a maximum growth rate of 0.21 ± 0.03 d^{-1} during the green stage. As the cultures were resuspended into new medium and triggered with sodium bicarbonate, the green motile cells changed into non-motile spherical red cells called cysts and started to accumulate astaxanthin. Although, as shown in Figure 3.1, it was unclear whether bicarbonate addition resulted in the arrest of cellular replication. The production of lipids, pigments, and carbohydrates under stress caused the DCW to increase significantly immediately after entering the red stage (Data provided in Appendix B).

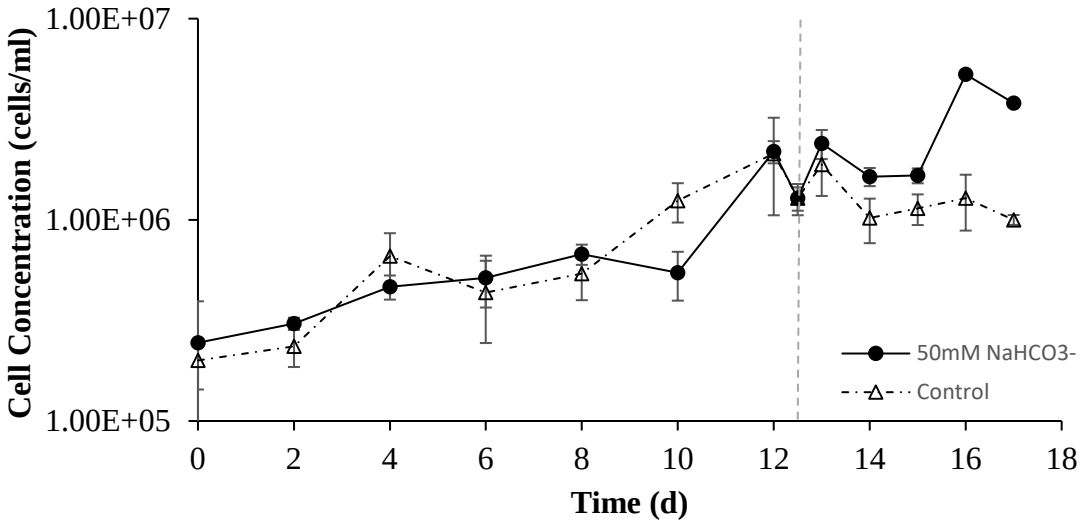

Figure 3.1. Average cellular concentrations with standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparged with ambient air only and were triggered with 50 mM of sodium bicarbonate. Control condition represents the untriggered cultures in the red stage. The grey dashed line, right before the new light cycle, represents the resuspension and the trigger time (t=12.5 d).

The cultivation medium contained two nitrogen sources, nitrate and ammonium to balance the pH throughout the experiment. Figure 3.2. shows the pH measured over the course of the experiment. The nitrogen preference of *H. pluvialis* is shown in Figure 3.2., where the cultures tended to prefer nitrate over ammonium. Ammonium and nitrate concentrations were measured at the end of the light cycle every 48 h with colorimetric assays. Ammonium was not consumed for the first four days and unexpectedly showed a slight increase until day four. By day 12, all the nitrate was utilized, although the cultures showed a slower ammonium consumption than anticipated, which prolonged the cultivation time. Also, it can be seen that the pH correlated with nitrogen consumption, shown in Figure 3.3, where the pH increased as *H. pluvialis* consumed nitrate and decreased as the culture switched to ammonium uptake. The maximum pH observed during

the green stage was 8.52. At day 12, where the resuspension in nitrogen-free media occurred, the pH was 7.55. The cultures were harvested on day 17 and the pH of cultures with sodium bicarbonate was 9.95, whereas the unamended culture pH was 7.16. All cultures grown in Phase I were re-suspended in nitrogen-free medium just before starting a new light cycle referred as t=12.5 d, which is before day 13 light cycle. The dashed grey line in Figures 3.2. and 3.3. represents day 12.5 where the resuspension and sodium bicarbonate trigger occurred.

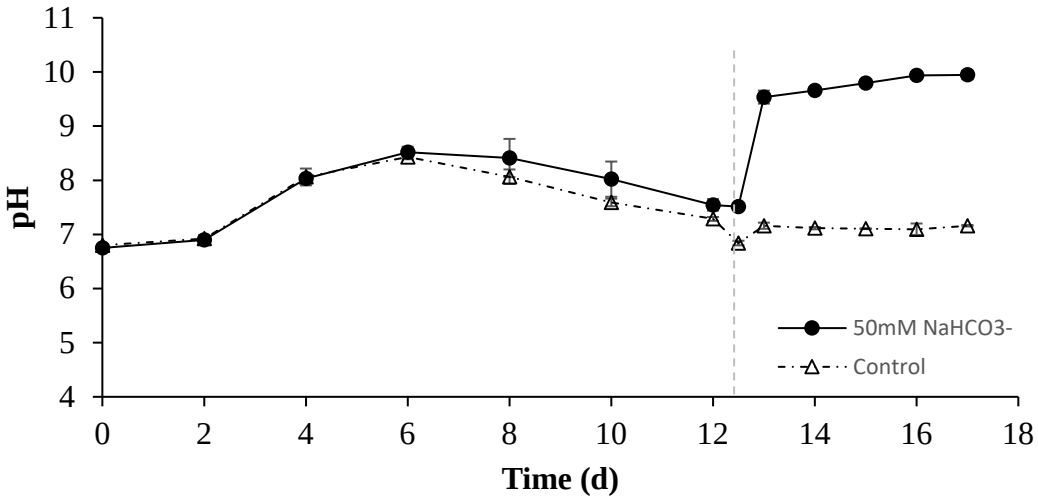

Figure 3.2. Average pH values with standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparged with ambient air only and were triggered with 50mM of sodium bicarbonate. Control condition represents the untriggered cultures in the red stage. The grey dashed line represents the resuspension step in N-free medium and the trigger time (t=12.5 d).

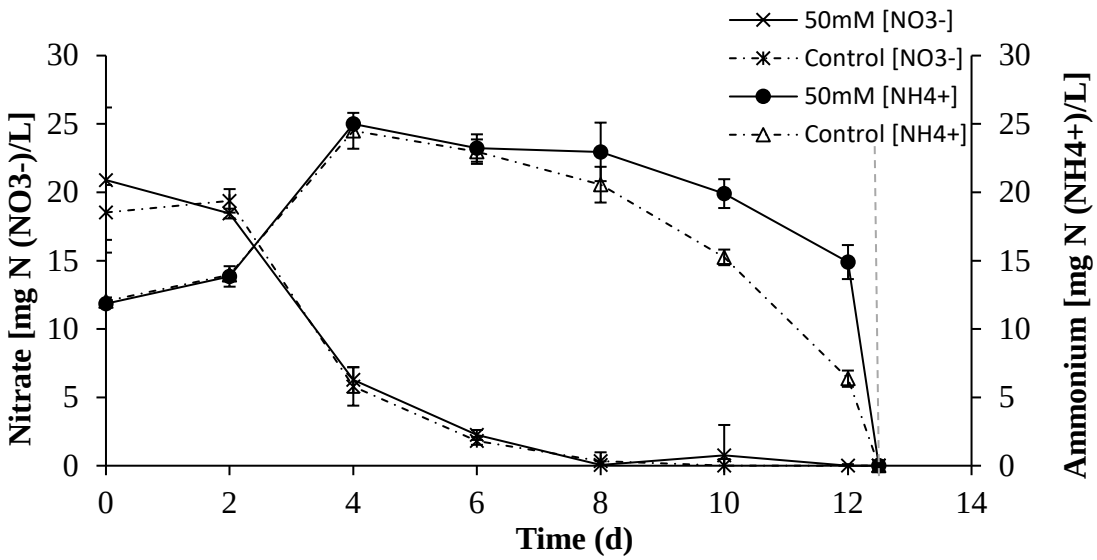

Figure 3.3. Average nitrogen concentrations with standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparged with ambient air only. Control condition represents the untriggered cultures in the red stage. The primary y-axis shows the nitrate concentration whereas the secondary y-axis shows the ammonium concentration of the cultures during the green stage. The grey dashed line, right before the new light cycle, represents the resuspension step and the trigger time (t=12.5 d).

An exponential increase in astaxanthin concentration was observed at day 12 immediately after the 50 mM sodium bicarbonate trigger (Figure 3.4). The unamended control also showed an increase in astaxanthin concentration, but it was significantly lower. Cells were harvested after five days of the red stage on day 17. The astaxanthin accumulation rate was calculated only based on the red stage. Results of Phase I showed that the astaxanthin accumulation rate of the controls and the sodium bicarbonate amended cultures increased from 0.13 mg L^{-1} day^{-1} to 0.64 mg L^{-1} day^{-1} relative to the controls with a maximum astaxanthin concentration of 1.56 ± 0.01 mg L^{-1} and 3.95 ± 1.25 mg L^{-1} respectively. The maximum astaxanthin as a fraction of dry weight in Phase I was calculated as 1.01 ± 0.16 %.

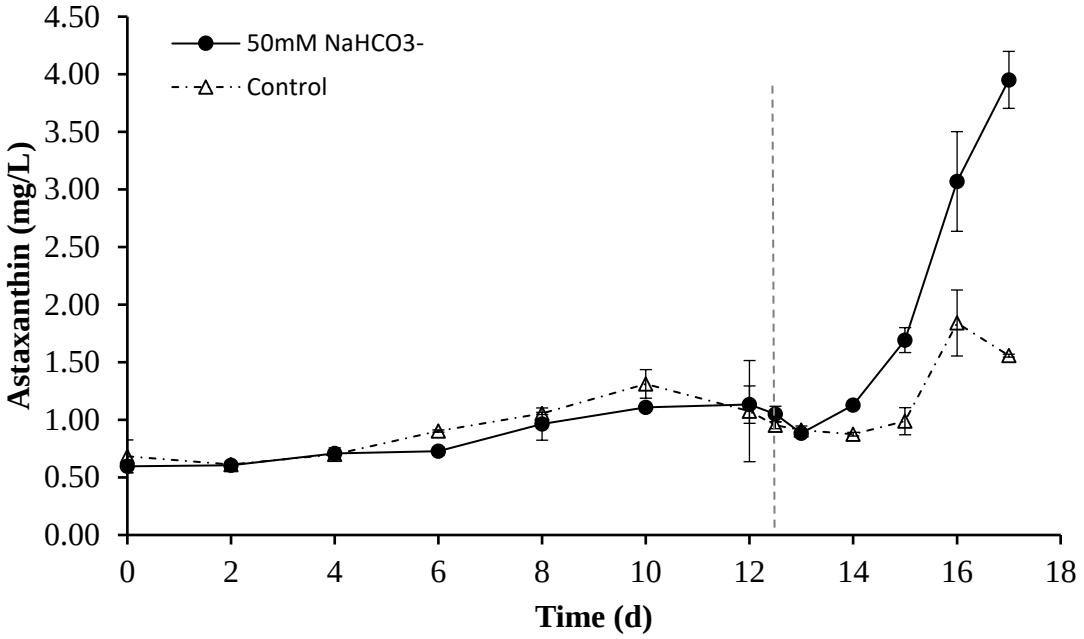

Figure 3.4. Average astaxanthin concentration with standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparged with ambient air only and were triggered with 50 mM of sodium bicarbonate. Control condition represents the untriggered cultures in the red stage. The grey dashed line, right before the new light cycle, represents the resuspension step and the trigger time (t=12.5 d).

Phase II

According to the calculated growth rates and doubling times in Phase I, it was hypothesized that the cultures may not have had a sufficient amount of DIC to grow efficiently. With the goal of producing more astaxanthin at a higher rate, sodium bicarbonate was added to the cultures at the beginning of the green stage to increase biomass production. In this study, as shown in Table 2.1, Phase II subsequent phases used 2.5 mM sodium bicarbonate as an inorganic carbon source during the green stage.

As shown in Figure 3.5, cultures grown with a 2.5 mM sodium bicarbonate amendment from the beginning of the green stage had a pH that was substantially higher

than the control. After the last light cycle, before the resuspension step, the pH was 10.67 and reached at a maximum of 10.92 during the green stage. The cultures with pH values above 9.3 (pKa of ammonium) may have experienced conversion of ammonium to ammonia, which can be toxic to the cultures and may off-gas from the solution. The results of nitrogen measurements, shown in Figure 3.6, also supported the off-gas scenario where the ammonium started to decrease immediately after bicarbonate amendment. The cultures reached nitrogen depletion at day 10 and were harvested on day 15.

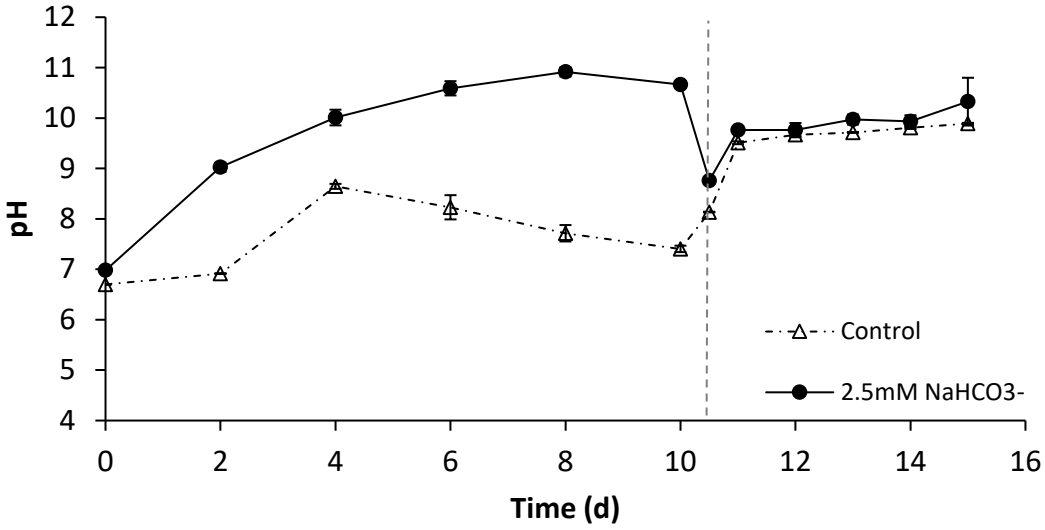

Figure 3.5. Average pH values with standard deviation in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air and were triggered with 50 mM of sodium bicarbonate. Control condition represents the unamended cultures in the green stage. The grey dashed line, right before the new light cycle, represents the resuspension step and the trigger time (t=10.5 d).

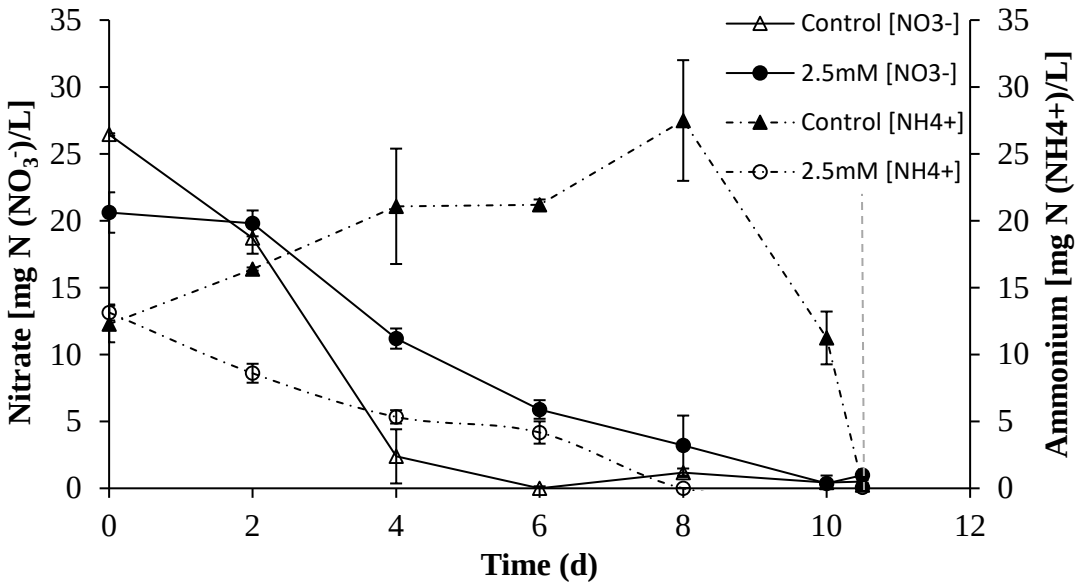

Figure 3.6. Average nitrogen concentrations with standard deviation in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air. Control condition represents the unamended cultures in the green stage. The grey dashed line, right before the new light cycle, represents the resuspension step and the trigger time (t=10.5 d).

The astaxanthin accumulation rate of the controls versus the 2.5 mM sodium bicarbonate amended cultures increased from 0.18 mg L^{-1} day^{-1} to 0.71 mg L^{-1} day^{-1} and the astaxanthin concentrations reached to 2.25 ± 0.23 mg L^{-1} and 6.70 ± 0.89 mg L^{-1} respectively (Data provided in Appendix B). The cell concentrations of the 2.5 mM sodium bicarbonate amended cultures increased to 4.17 ± 0.11 E+06 cells/ml whereas the control condition stayed at 1.60 ± 0.13 E+06 cells/ml indicating that the sodium bicarbonate amendment increased cell growth.

Phase III

To avoid the pH related issues observed in experimental Phase II, cultures were grown in tubular photobioreactors with pH control in Phase III. The pH was maintained at

7.5 by sparging with 5% CO_2 (v/v) on demand. Controlling pH allowed for both ammonium and nitrate to be utilized without promoting excessive volatilization of ammonia gas, allowing for greater biomass cultivation during the green stage. One issue with the tubular photobioreactors was that in higher alkaline conditions *H. pluvialis* cultures tended to produce biofilms on the side of the tube reactors at the air-water interface, resulting in biomass loss. To prevent this issue in experimental Phase III, sterilized 3-D printed magnetic scrubbers were used to resuspend the biomass attached to the sides of the reactor back into the medium. This biofilm formation was not observed when the cultures were grown under pH-controlled conditions, but *H. pluvialis* biofilms were significant during sodium bicarbonate triggering in the red stage, where the pH-control was shut off (Photo provided in Appendix C).

In Phase III, cultures were amended with 2.5 mM sodium bicarbonate and pH was controlled via CO_2 sparging during the green stage. Cultures were then triggered with 50 mM of sodium bicarbonate at the beginning of the red stage to initiate astaxanthin accumulation. It was observed that cultures amended with sodium bicarbonate under controlled pH conditions tended to utilize nitrogen faster than unamended cultures. As shown in Figure 3.7, 2.5 mM of sodium bicarbonate decreased the time required to reach nitrogen depletion (day six). Since the cultures utilized all the nitrogen by day six, one-stage cultivation was used in Phase III, where no transfer to new nitrogen free medium was required.

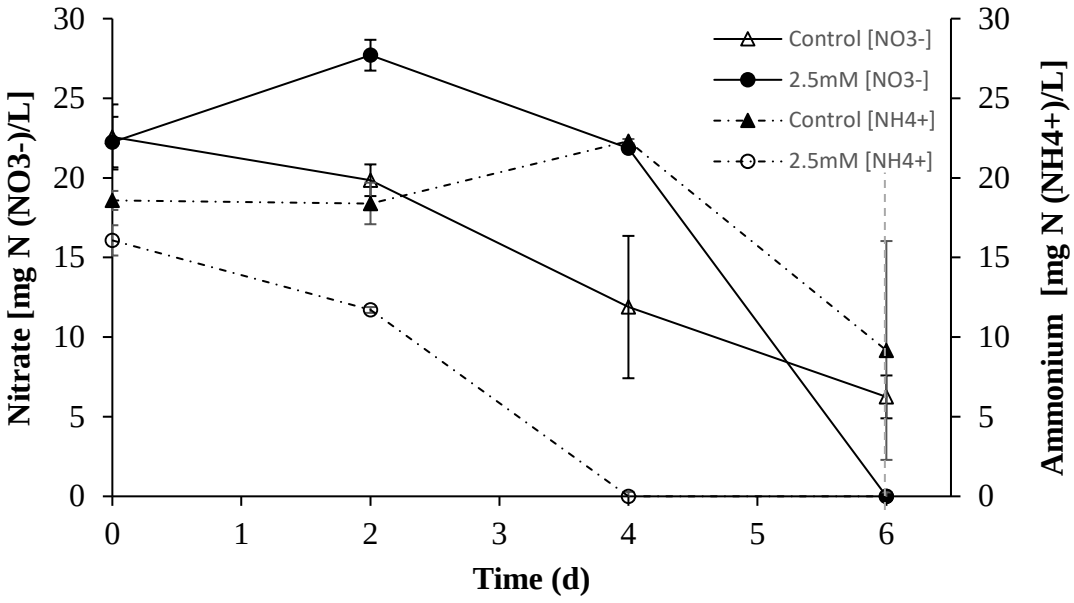

Figure 3.7. Average nitrogen concentrations with standard deviation in *H. pluvialis* cultures in Phase III. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air. Control condition represents the unamended cultures in the green stage. The primary y-axis shows the nitrate concentration whereas the secondary y-axis shows the ammonium concentration of the cultures during the green stage. The grey dashed line, right before the new light cycle, represents the resuspension step and the trigger time (t=6.5 d).

In Phase III, the red stage took seven days in total, however, the astaxanthin accumulation rate increased to 1.10 mg L^{-1} day^{-1} with an astaxanthin concentration of 9.14 ± 0.15 mg L^{-1}. Contrast this with phase II, where the red stage took 5 days in total (Data provided in Appendix B).

Phase IV

In experimental phase IV, 24 h light stress during the red stage was used to address the issues that arose during phase III. In addition to light stress, Phase IV cultures were exposed to nitrogen deplete conditions and an increase in pH immediately after the 50 mM sodium bicarbonate trigger. The stress conditions were introduced gradually as to avoid

over-stressing the cultures. The cultures reached nitrogen depletion on day eight at a pH of 7.55. The pH control was shut-off one day earlier than the trigger day, meaning that the cells were exposed to nitrogen deplete conditions first, pH stress second, and sodium bicarbonate trigger last. The green stage of Phase IV was same as Phase III, however during the red stage, the cultures were exposed to an additional stress of 24h light.

The sodium bicarbonate amended cultures in the red stage showed an increase in astaxanthin accumulation rate and in final astaxanthin concentration. The astaxanthin accumulation rate increased to 2.12 mg L^{-1} day^{-1} with an astaxanthin concentration of 11.2 ± 0.56 mg L^{-1} and 2.36 ± 0.08 % (w/w).

Total chlorophyll concentration [$mg \cdot L^{-1}$] in all conditions were also monitored and is presented in Table 3.3. As the culture transitioned from the green stage to the red stage the chlorophyll content decreased. Table 3.2 shows the chlorophyll concentration at nitrogen depletion and at harvest time. Cultures that received a bicarbonate amendment during the green stage and prior to nitrogen depletion experienced a more drastic decrease in chlorophyll concentrations between nitrogen depletion and harvest.

Table 3.2. Total chlorophyll concentration during the green and red stage in each phase along with the nitrogen depletion and harvest time.

	Green Stage at Nitrogen Depletion		Red stage at Harvest	
Phase	**Total Chlorophyll [mg · L^{-1}]**	**Nitrogen Depletion Time (d)**	**Total Chlorophyll [mg · L^{-1}]**	**Harvest Time (d)**
I	4.96 ± 0.51	12	2.79 ± 0.27	17
II	8.20 ± 0.65	10	4.29 ± 0.63	15
III	11.20 ± 2.11	6	4.36 ± 0.82	13
IV	10.01 ± 0.83	8	3.72 ± 0.56	13

Discussion

The purpose of this study was to evaluate the effects of sodium bicarbonate addition to improve growth and astaxanthin production in *H. pluvialis*. Increasing the cell density of *H. pluvialis* in the green stage was likely important for maximizing the astaxanthin production in red stage. Different approaches have been proposed to increase astaxanthin production particularly by growing microalgae under nutrient-limited conditions or resuspending the cells into nutrient deplete conditions after reaching optimal growth.

Inorganic carbon is crucial for growth in photoautotrophic microalgae and the most common technique to provide it is to sparge cultures with CO_2. Most studies have focused on increasing CO_2 concentration that was sparged into the culture systems. Even though

microalgae can and will grow under atmospheric CO_2, to increase the high-value biomass yield the cultures need to be sparged with more CO_2, so as an alternative option sodium bicarbonate ($NaHCO_3$) with higher solubility in water compared to CO_2 can be used as an inorganic carbon source. When $NaHCO_3$ dissolves in water it produces HCO_3^- ions which are readily available as the dissolved inorganic carbon source for microalgae. Using $NaHCO_3$ during the green stage can also cut down the costs related to the capture, compression, and transportation of high purity CO_2 (Hsueh et al. 2007).

Sodium bicarbonate is being widely tested as a "trigger" mechanism in lipid production (Gardner et al., 2013; Lohman et al., 2015). Since carotenoids are hydrophobic, pigments are being dissolved and stored inside fatty acids resulting in an increase in pigment content as the lipid production increases (Boussiba, 2000). *H. pluvialis* is the microalga known to produce the highest amount of astaxanthin and the reason may have to do with the efficient deposition of astaxanthin into lipid globules as esters (Bidigare et al., 1993), so fatty acid metabolism maybe correlated with pigment accumulation which can be a key factor in the future to improve astaxanthin biosynthesis in this microalga. In this study, low concentrations of $NaHCO_3$ (2.5 mM) were used during the "green" stage (the growth stage) to promote biomass production and high concentrations of $NaHCO_3$ (50 mM) were used during the "red" stage as a trigger mechanism for astaxanthin production.

Light/Dark Cycle Effects on *H. pluvialis* Growth

Choosing appropriate culture parameters such as light, pH, and appropriate growth medium is crucial in improving biomass production and astaxanthin yield. The light/dark cycle (photoperiod) is important for algal growth and biomass production as frequencies

of photoperiods have essential impacts on productivity and photosynthesis efficiency (Grobbelaar, 2009). Different microalgae may have variable responses to adjusting the photoperiod. Studies on *H. pluvialis* cultivation have reported that a 12 h:12 h light/dark cycle during green stage leads to the greatest biomass production, which was supported by this study (Saha et al., 2013). In this study the red stage was conducted under 14 h:10 h light/dark cycle and during Phase IV the cultures were exposed to an additional 24 h light stress in the red stage. Astaxanthin production seemed to be higher under continuous light conditions.

Sodium Bicarbonate Effects on pH and *H. pluvialis* Growth

H. pluvialis grows best in a medium with a neutral pH (Proctor, 1957). In Phase I, the cultures were grown in MES-Volvox medium which contained nitrate and ammonium. Uptake of NO_3^- causes an increase in pH of the culture medium since it releases 1 mol OH^- ions for every mol NO_3^- that is consumed and ammonium will release 1 mol H^+ ions for every mol NH_4^+ that is consumed which will result in a pH decrease. So, even though the pH was not controlled in Phase I the two nitrogen sources managed to balance the pH during active growth in the green stage. As the cultures started to fix carbon and accumulate more cells, the pH, which was measured at the end of every light cycle, started to increase until all the nitrate was consumed. As the cultures started to switch to ammonium consumption the pH started to decrease until resuspension in a nitrogen free medium. The maximum pH was 8.52, which was measured on day six. As cultures switched to ammonium utilization the pH started to decrease until day 12 when the red stage was initiated.

In Phase II, a low concentration of sodium bicarbonate (2.5 mM) was used as an additional inorganic carbon source during the green stage. The maximum measured pH of the sodium bicarbonate amended cultures was 10.92 and for the cultures with only ambient air was 8.65. The pKa of the bicarbonate/carbonate equilibrium is pH 10.3 and at 10.92 the majority of the inorganic carbon in the system is in the form of carbonate. Carbonate is potentially unavailable for carbon fixation. The sodium bicarbonate amended cultures' specific growth rate was calculated as $0.16 \pm 0.06\ d^{-1}$ whereas the cultures grown with ambient air were $0.17 \pm 0.02\ d^{-1}$. Indicating that the oncreased pH was sufficient to temporarily slow the growth of *H. pluvialis.* Also, at high pH values the ammonium deprotonates to form ammonia which could have been toxic to the cultures and inhibited growth (pKa ~ 9.3) (Proctor, 1957).

In Phase III and IV, the cultures were sparged continuously with ambient air and supplemented with 5% CO_2 (v/v) as needed to keep the pH below 7.5 in an effort to prevent growth inhibition. The specific growth rate of the sodium bicarbonate amended cultures in Phase III was calculated as $0.39 \pm 0.01\ d^{-1}$ and in Phase IV $0.87 \pm 0.28\ d^{-1}$ the cultures with only ambient air in Phase III stayed at $0.29 \pm 0.06\ d^{-1}$ and in Phase IV $0.36 \pm 0.1\ d^{-1}$. Increasing the inorganic carbon concentrations and controlling the pH during the green stage increased the growth rates throughout the phases., which resulted in higher astaxanthin production. Although, unlike in the study conducted by Gardner et al. (2012) it was difficult to determine whether the high concentrations of sodium bicarbonate added before the red stage ceased cellular replication (Figure 3.1).

Sodium Bicarbonate Effects on Nitrogen Utilization

Nitrogen (N) is essential for the growth of microalgae. Nitrate and ammonium were reported as the optimal inorganic nitrogen sources for *H. pluvialis* (Sarada et al., 2002). Although many microalgae would assimilate ammonium more readily than nitrate since it requires less energy for uptake, a study that was conducted on six green microalgae by Proctor in 1957 showed that this is not true for all species. *H. pluvialis* was a part of this study and it was proven that this microalga assimilated nitrate first and nearly twice as fast whenever the two were present in the medium (Proctor, 1957). This preference of nitrate over ammonium was also observed in this study, Figure 3.3.

In Phase I, nitrate was preferred over ammonium and was entirely consumed by day 12. On day 12, the cultures were resuspended in nitrogen-free medium at the end of that light cycle and were triggered with 50 mM sodium bicarbonate. The nitrogen preference showed a consistent pattern in each phase under conditions that did not include a sodium bicarbonate amendment during the green stage. Additionally, there was an increase in ammonium concentration in the cultures that were not amended with sodium bicarbonate in all phases. To investigate potential factors leading to increasing ammonium concentrations, 16S SSU rRNA gene sequencing was conducted to identify the algal microbiome present in samples that were collected at the end of the green stage in Phase III. According to the 16S data (Appendix B.26. and B.27.), the predominant bacteria found in all conditions was *Rhizobium*, which belongs to the class alphaproteobacteria. In the literature, *Rhizobium* is known to be a enhance the growth of green algae and has the ability to produce ammonium (Kim et al., 2014; Lian et al., 2018). The presence of *Rhizobium* in these cultures could explain the observed increase in ammonium concentration. Although,

further research has to be done on this matter. Often coexisting microorganisms have been considered to be contaminants in algal cultures, but recent studies have shown that symbiotic interactions between algae and bacteria promotes algal growth compared to axenic cultures (Chevanton et al., 2013).

During experimental Phase II, the cultures which were amended with 2.5 mM sodium bicarbonate and ammonium concentration started decrease first even though nitrate was also present in the medium. Figure 3.6. shows the nitrogen concentration in Phase II. Ammonium starts to decrease immediately as the pH increases. Since the pKa of ammonium is 9.3, it was difficult to determine whether there was an off-gassing, or the cultures consumed both N sources at the same time. Although, in Phase III where there was pH control to reduce the concern of off-gassing, ammonium was still observed to be preferentially used, showing a decrease in the first four days. The microalga might be giving priority to the carbon metabolism over nitrogen metabolism under limited carbon conditions. This is supported by the finding in this study that under higher inorganic carbon levels, (i.e $NaHCO_3$ amended cultures), the microalga reached nitrogen depletion faster compared to the control conditions.

pH control coupled with the use of 2.5 mM of sodium bicarbonate as an inorganic carbon source had a strong influence on nitrogen utilization. Nitrogen consumption occurred rapidly in cultures that were amended with sodium bicarbonate during the green stage, as shown in Figure 3.7. In Phase II, sodium bicarbonate amendment decreased the time required to reach nitrogen depletion to 10 days and in Phase III with pH control, the cultures utilized all of the nitrogen by day six, which shortened the green stage

significantly. Since the medium was nitrogen deplete after six days, shown in Figure 3.7, one-stage cultivation was tested and the transition of the green stage to red stage was carried out with a 50 mM sodium bicarbonate trigger without changing the medium. The one-stage culture system may have an advantage for the growth of *H. pluvialis* for astaxanthin production since it simplifies the cultivation process leading to one-stage production and may reduce costs.

In Phase IV, an additional stress of 24 h light during the red stage was added to accelerate the transition of green motile cells to red stage cells. The combination of multiple stress conditions increased the astaxanthin accumulation rate as well as the final astaxanthin concentration.

Sodium Bicarbonate Effects on Chlorophyll Content and Astaxanthin Production

The main purpose of astaxanthin accumulation is to protect the cells from photodamage. If the cells cannot perform optimal photosynthesis under nutrient-limited conditions the chlorophyll will rise to an excited level due to excess light and start to produce singlet oxygen. Singlet oxygen production will lead to photo-oxidation which can damage the cells (Krieger-Liszkay, 2005). As shown in Table 3.2. the chlorophyll content started to decrease as the cells began producing astaxanthin.

Carbon/nitrogen (C/N) ratio is a key factor that contributes to astaxanthin accumulation. High C/N ratio, which can be induced by the sodium bicarbonate trigger, results in faster and greater astaxanthin accumulation (Kang et al., 2005; Kobayashi et al., 1993). In this study, a combination of nitrogen deficiency and high concentrations (50 mM) of sodium bicarbonate showed a significant increase in astaxanthin accumulation rate and

final astaxanthin concentration, shown in Figure 3.4. The hypothesis was that the astaxanthin accumulation was enhanced by the increase in carbon assimilation.

Overall, in Phase I, high concentrations (50 mM) of sodium bicarbonate addition near nitrogen depletion increased the demand for nitrogen to be able to synthesize cellular components, which resulted in higher astaxanthin production. Biomass production during the green stage was improved with 2.5 mM of sodium bicarbonate amendment, which promoted carbon fixation in Phases II, III and IV and increased astaxanthin production under nitrogen deplete conditions. This improvement in astaxanthin production can clearly be seen in Table 3.3.

Table 3.3. Accumulation rates and final astaxanthin concentrations of each experimental phase.

Phase	**Astaxanthin Accumulation Rate**	**Astaxanthin Concentration**
I	0.64 mg · L^{-1} day^{-1}	3.95 ± 1.25 mg · L^{-1}
II	0.71 mg · L^{-1} day^{-1}	6.70 ± 0.89 mg · L^{-1}
III	1.10 mg · L^{-1} day^{-1}	9.14 ± 0.15 mg · L^{-1}
IV	2.12 mg · L^{-1} day^{-1}	11.2 ± 0.56 mg · L^{-1}

In summary, sodium bicarbonate was an effective inorganic carbon amendment in both the green and red phases, increasing both growth rate in the green stage and astaxanthin production and extent in the red stage. The addition of a high concentration of sodium bicarbonate (50 mM) had a strong effect on the cellular accumulation of astaxanthin. The increased concentrations of astaxanthin also indicated that even under a high concentration of sodium bicarbonate a base level of inorganic carbon fixation was

occurring, where the cells were possibly storing carbon and energy for recovery after the stress conditions. Furthermore, sodium bicarbonate amendment can significantly affect the nitrogen depletion time, which in this study reduced the time needed to reach nitrogen depletion and overall reduced the production time of astaxanthin.

Conclusions

In this study, the effects of low concentrations of sodium bicarbonate amendment in the green stage and high concentrations of sodium bicarbonate amendment under nitrogen deplete conditions in the red stage were studied. The cultures amended with 2.5 mM of sodium bicarbonate during the green stage showed an increase in the culture density compared to cultures that were grown under only atmospheric air. The increase in pH during the amendment was controlled at 7.5 to prevent culture inhibition. After reaching nitrogen depletion the cultures were triggered with 50 mM of sodium bicarbonate which resulted in an increase in astaxanthin production. Improving the growth resulted in higher astaxanthin content at the end of the red stage. The final astaxanthin dry weight in Phase IV was calculated as 2.36 ± 0.08 % (w/w), which is under the reported value in commercial production. However, implementing the proposed strategies in this study to commercial astaxanthin production could improve the whole cultivation process.

These findings indicate that the inclusion of sodium bicarbonate resulted in higher concentrations of astaxanthin. Recently, bicarbonate has been started to be used as an alternative approach because of its advantages including higher solubility in water, storage, and transport compared to the gaseous form of CO_2. However, the use of bicarbonate to

increase the microalga biomass and by-products is relatively new, so it is important to understand the effects of bicarbonate amendment on microalgal physiology.

Using bicarbonate salts as a trigger mechanism to boost production of the high-value product astaxanthin, still requires a more detailed understanding of the metabolic responses of *H. pluvialis* under different stress conditions.

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CHAPTER FOUR

CONCLUSIONS

Project Outcomes

The use of bicarbonate salts in microalgal cultivation is a relatively new concept and its application is continuously growing. Previous studies have mainly focused on increasing the lipid content in microalgae via the use of high concentrations of sodium bicarbonate under nitrogen deplete culture conditions (Gardner et al, 2012). Since lipid accumulation is closely related to astaxanthin synthesis in *H. pluvialis*, the effects of $NaHCO_3$ amendment on astaxanthin production from this microalga was investigated in this study.

This study advanced the understanding of bicarbonate induced astaxanthin production and provided future questions for successive work. The aims of the project here were to:

(1) Improve *H. pluvialis* biomass production using low concentrations of $NaHCO_3$ amendment as an inorganic carbon source. The rationale was that increased *H. pluvialis* biomass production prior to nitrogen depletion would potentially increase total astaxanthin production.

(2) Enhance astaxanthin production using high concentrations of $NaHCO_3$ amendment which can be used to cease cellular replication especially when coupled with high pH under nitrogen deplete conditions. The rationale was that the high concentration of $NaHCO_3$

amendment along with combined stress conditions would cease cellular replication, therefore, increase astaxanthin production.

Bicarbonate Effects on
the Green and Red Stage

H. pluvialis cultures that were amended with $NaHCO_3$ showed limited growth due to rapid pH increase, so the pH had to be controlled at 7.5 with 5% CO_2 on demand. Under pH-controlled conditions, the cultures that were amended with 2.5 mM of sodium bicarbonate during the green stage showed an increase in growth compared to the cultures that were grown under only atmospheric air.

Additionally, in this study $NaHCO_3$ amendment reduced the time needed to reach nitrogen depletion, which decreased the overall production time of astaxanthin. Faster nitrogen utilization also provided an opportunity to test one-stage cultivation, so the transition to red stage was carried out with sodium bicarbonate amendment without changing the medium or reactor. The one-stage culture system may be an advantage for the commercial growth of *H. pluvialis* since it simplifies the cultivation process and may reduce the production costs.

In the red stage of *H. pluvialis* cultivation, high concentrations (50 mM) of $NaHCO_3$ amendment under nitrogen depletion conditions was an effective trigger for astaxanthin accumulation when the cultures had been grown previously under nitrogen replete conditions with the use of lower concentrations of $NaHCO_3$ as an inorganic carbon source. The combination of low concentrations of $NaHCO_3$ amendment under pH-controlled conditions with high concentrations of $NaHCO_3$ amendment near nitrogen depletion was

observed to produce the highest astaxanthin content at harvest. At harvest the astaxanthin accumulation rate was 2.12 mg L^{-1} day^{-1} with an astaxanthin concentration of 11.2 ± 0.56 mg L^{-1} and 2.36 ± 0.08 % (w/w). The final achieved astaxanthin concentrations in the sodium bicarbonate amended cultures were 7.2-fold higher compared to the unamended cultures.

Concluding Remarks

The purpose of this study was to evaluate the effects of sodium bicarbonate addition on growth and astaxanthin production in *H. pluvialis*. Different approaches have been proposed to improve the green and red stage to increase astaxanthin production particularly by growing microalgae under nutrient-limited conditions or resuspending the cells into nutrient deplete conditions after reaching optimal growth.

Using bicarbonate salts as a trigger mechanism can improve the microalgae derived bio-product industry to be able to increase the production of high-value products such as astaxanthin, but to be able to optimize the production of astaxanthin a better understanding of the metabolic responses of *H. pluvialis* is necessary. The results obtained from this study have been prepared for submission to a scientific journal. The strategies used in this study can reference for future studies.

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CHAPTER FIVE

FUTURE WORK

Impact of *Haematococcus pluvialis-Rhizobium* Symbiosis on *H. pluvialis* Growth and Astaxanthin Production

In the microalgae biofuel and bioproducts industry, cultures are generally treated as though they are axenic, or only containing a single type of organism. While most cultures are unialgal, the reality is that the majority of microalgae coexist with a variety of microorganisms (Lian et al., 2018). These coexisting microorganisms, predominantly bacteria, have often been referred to as contaminants in algal research. The "phycosphere" is the mucus layer surrounding a microalgal cell and is often inhabited by bacterial counterparts (Amin et al., 2012). When axenic cultures are compared to those with an active phycosphere, often the axenic cultures have poorer growth and productivity (Lian et al., 2018). The most common bacteria that have been shown to be beneficial to microalgae include *Rhizobium, Sphingomonas, Bacillus, Flavobacterium,* and *Hyphomonas* (Lian et al., 2018). Numerous studies have focused on the mutualistic interaction of *Azosprillum brasilense* and *Rhizobium* which are nitrogen-fixing alphaproteobacteria, with *Chlorella* sp. The presence of these two bacteria in *Chlorella* sp. cultures promotes the accumulation of lipids, pigments, and starch in addition to increasing cell density of the microalga (Chevanton et al., 2013; Cho et al., 2016; Kim et al., 2014).

In this study, *H. pluvialis* was grown in MES-Volvox medium that contained both nitrate (NO_3^-) and ammonium (NH_4^+). Throughout the experiments an increase in NH_4^+

concentration was observed during the green stage with the cultures that were not amended with sodium bicarbonate ($NaHCO_3$). These cultures were grown under the same conditions to observe the time needed to reach nitrogen depletion. The cultures depleted NO_3^- by day 12, but even at day 17, the $NH_4^+$was not consumed. The uptake of NH_4^+ was also slow compared to NO_3^- in both the control and in the $NaHCO_3$ samples. The increase in NH_4^+ concentration was not observed when the cultures were amended with 2.5 mM of $NaHCO_3$ during the green stage.

The presence of bacteria, especially those which can partially or completely reduce NO_3^- to NO_2^-or NH_4^+respectively, may explain the increase in NH_4^+ concentration in the controls. To have a better understanding of the potential activity of the phycosphere microbiome and investigate the reason causing the increase in NH_4^+ concentration, total DNA extraction and 16S SSU rRNA sequencing was conducted (Data provided in Appendix B). All samples were collected by centrifugation at the end of the green stage. The most common and predominant bacterium class was alphaproteobacteria, the most common genus was *Rhizobium* throughout all samples. *Rhizobium* species have been described to fix NO_3^- into NH_4^+, which can increase ammonium concentrations and could delay nitrogen starvation (Lian et al., 2018). The increase in NH_4^+ concentration was only observed in the control condition where the cells were not amended with $NaHCO_3$. The microalga might be giving priority to the carbon metabolism over nitrogen metabolism under limited carbon conditions. This observation along with the described one suggests the findings in this study where under higher carbon levels, (*i.e.* $NaHCO_3$ amended

cultures), the microalga consumed nitrogen and reached nitrogen depletion faster compared to the control conditions.

To elucidate the impact of interactions in the phycosphere further, the productivity and microalgal physiology of xenic cultures should be compared to that of axenic cultures. There are not many researches that has been conducted on obtaining axenic *Haematococcus* cultures. Although previous research was able to obtain axenic *Haematococcus* cultures using antibiotics, many antibiotics have adverse effects on this microalga (Joo et al., 2007; Lee et al., 2019). *H. pluvialis* has been reported to be resistant to four antibiotics including cephalosporin, griseofulvin, ampicillin, and amphotericin B. In nature, microalgae are often in an interaction with bacteria especially with symbiotic, plant growth-promoting bacteria, which promote growth of microalga using nitrogen and phosphorus metabolism (Lee et al., 2019). Although *H. pluvialis* is of particular interest in the high-value pigment-producing market, the cultivation of this microalga with symbiotic bacteria has not been studied previously, and interest in xenic culture productivity is growing (Lee et al., 2019).

The phycosphere community profile of *H. pluvialis* has not been extensively studied. Given that this microalga has a broad industrial application, an important area of research would be to use xenic cultivation conditions to improve the growth and astaxanthin production. Addition of specific beneficial bacterial cultures could significantly enhance astaxanthin production rate. A better understanding of large-scale algal-bacterial cocultivation could be a key factor in the algae industry since there is a high expectation for microalgal growth to produce bio-based products.

Integration of Astaxanthin and Lipid Production from *Haematococcus pluvialis*

H. pluvialis is a freshwater green microalga that goes through morphological and biochemical changes to survive stress conditions. As the green motile cells turn into red non-motile cells, they start to accumulate lipids and carotenoids. Lipids that are produced from microalgae are classified into two groups based on their carbon numbers. Fatty acids with 14-20 carbon are mainly used for biodiesel production, whereas the polyunsaturated fatty acids with more than 20 carbon atoms are used as food supplements in the form of for instance docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Sun at al., 2018). Carotenoids are hydrophilic compounds that are the most common pigments found in nature (Gong et al., 2016). There are two types of carotenoids: primary and secondary. The function of primary carotenoids is closely related to structure and function, whereas secondary carotenoids are crucial for cell protective mechanisms and are stored in oil droplets (Gong et al., 2016). Since the carotenoids are hydrophilic, it has been hypothesized that the lipids provide accommodation for carotenoids under unfavorable stress conditions (Boussiba, 2000).

Previous research on *H. pluvialis* has indicated that the production of triacylglycerols (TAGs) is essential for astaxanthin production. It was reported that oleic acid (18:1), palmitic (16:0), and linoleic (18:2) acids are the most common lipids produced in *H. pluvialis* and it is reported that this microalga can produce up to 60-70% of total fatty acids (Liang et al., 2015; Saha et al., 2013; Zhekisheva et al., 2002). High lipid content

along with the antioxidant properties of astaxanthin can increase the value of the various food, cosmetics, and nutraceutical products. Also, high levels of saturated and mono-unsaturated fatty acids make microalgal lipids suitable for biodiesel. Since there is a correlation between the astaxanthin and lipid production, additional research into *H. pluvialis* should be conducted as it is a good candidate for the simultaneous commercial production of these co-products. After refining, the high-value product (*e.g.*, astaxanthin) from *H. pluvialis*, the remaining biomass could be used as a feedstock for biofuel production (Saha et al., 2013), as well as a salmon fish food additive.

Although biofuel production from microalgae is still energy intensive and not economically feasible, the idea of combining the production of high value bioproducts and biofuels can be promising way to maximize the economic value of the biofuel production process. To develop more efficient co-products, it is important to increase *H. pluvialis* biomass yields. This study partly focuses on increasing the biomass of *H. pluvialis* during the green stage with $NaHCO_3$ amendment. Further research should be done on these cultures, where some of the biomass can be transesterified and used in lipid analysis. Also, a better understanding of lipid metabolism may be a key to higher astaxanthin accumulation.

In summary, integrating lipid and astaxanthin production may play an important role in making biofuel production more economically feasible by taking advantage of all the products that are produced by a single microalga.

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APPENDICES

APPENDIX A

TABULATED DATA

Table A. 1. Phase I. Only ambient air (*i.e*. without bicarbonate amendment) average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	6.80	0.01	2.00E+05	5.66E+04	18.53	2.01	12.04	0.27	0.05	0.01	3.12	0.63	0.68	0.14
2	6.92	0.07	2.35E+05	4.95E+04	19.37	0.86	13.97	0.09	0.11	0.01	2.82	0.07	0.61	0.02
4	8.06	0.16	6.60E+05	1.98E+05	5.80	1.40	24.50	1.31	0.15	0.05	3.80	0.16	0.70	0.04
6	8.43	0.04	4.35E+05	1.91E+05	1.82	0.29	22.97	0.89	0.13	0.02	3.94	0.33	0.90	0.01
8	8.07	0.13	5.40E+05	1.41E+05	0.33	0.66	20.56	1.31	0.26	0.01	4.90	0.22	1.06	0.01
10	7.59	0.07	1.25E+06	2.76E+05	0.00	0.49	15.24	0.57	0.20	0.02	5.80	0.54	1.31	0.12
12	7.29	0.04	2.14E+06	1.08E+06	0.00	0.02	6.37	0.59	0.24	0.06	6.60	0.78	1.08	0.44
12.5	6.84	0.04	1.28E+06	2.26E+05	n/d	n/d	n/d	n/d	0.14	0.01	4.79	0.27	0.95	0.03
13	7.16	0.06	1.88E+06	5.66E+05	n/d	n/d	n/d	n/d	0.19	0.01	4.87	0.50	0.91	0.04
14	7.12	0.02	1.02E+06	2.55E+05	n/d	n/d	n/d	n/d	0.21	0.04	4.40	0.04	0.88	0.02
15	7.11	0.01	1.14E+06	1.98E+05	n/d	n/d	n/d	n/d	0.20	0.04	6.06	0.49	0.99	0.12
16	7.10	0.11	1.28E+06	3.96E+05	n/d	n/d	n/d	n/d	0.17	0.01	5.22	0.21	1.84	0.29
17	7.16	0.01	1.00E+06	5.66E+04	n/d	n/d	n/d	n/d	0.15	0.05	3.95	0.03	1.56	0.01

Table A.2. Phase I. 50 mM sodium bicarbonate amendment average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	6.75	0.03	2.45E+05	1.48E+05	20.90	5.31	11.86	0.07	0.10	0.00	3.20	0.14	0.60	0.05
2	6.90	0.01	3.05E+05	2.12E+04	18.44	0.36	13.85	0.75	0.10	0.00	3.17	0.20	0.61	0.03
4	8.04	0.04	4.65E+05	6.36E+04	6.27	0.94	25.00	0.07	0.13	0.02	3.57	0.57	0.71	0.05
6	8.52	0.10	5.15E+05	1.48E+05	2.25	0.36	23.24	1.00	0.18	0.05	3.27	0.02	0.73	0.04
8	8.41	0.35	6.75E+05	7.78E+04	0.05	0.27	22.96	2.14	0.11	0.02	3.82	0.83	0.96	0.14
10	8.02	0.33	5.45E+05	1.48E+05	0.76	2.22	19.90	1.05	0.19	0.01	3.87	1.04	1.11	0.00
12	7.55	0.11	2.19E+06	2.76E+05	n/d	n/d	14.90	1.24	0.21	0.04	4.96	0.51	1.13	0.16
12.5	7.51	0.06	1.28E+06	1.70E+05	n/d	n/d	n/d	n/d	0.22	0.04	5.16	0.16	1.05	0.07
13	9.54	0.12	2.40E+06	3.96E+05	n/d	n/d	n/d	n/d	0.21	0.05	4.83	0.20	0.88	0.00
14	9.66	0.04	1.64E+06	1.70E+05	n/d	n/d	n/d	n/d	0.27	0.01	3.00	0.17	1.13	0.01
15	9.80	0.01	1.66E+06	1.41E+05	n/d	n/d	n/d	n/d	0.33	0.06	2.80	0.11	1.69	0.11
16	9.94	0.01	5.28E+06	1.13E+05	n/d	n/d	n/d	n/d	0.33	0.02	3.07	0.48	3.07	0.43
17	9.95	0.08	3.80E+06	1.13E+05	n/d	n/d	n/d	n/d	0.39	0.06	2.79	0.27	3.95	0.25

Table A.3. Phase II. Only ambient air (*i.e.* without bicarbonate amendment) average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	6.70	0.00	2.49E+05	1.15E+05	26.45	5.31	12.28	1.37	0.09	0.01	3.68	0.03	NA	NA
2	6.92	0.01	9.60E+05	8.49E+04	18.71	1.18	16.39	0.12	0.18	0.06	4.10	0.06	NA	NA
4	8.65	0.05	9.45E+05	2.05E+05	2.39	2.02	21.08	4.31	0.33	0.03	4.68	0.59	NA	NA
6	8.23	0.24	1.34E+06	3.32E+05	0.00	0.15	21.19	0.40	0.40	0.04	5.87	0.44	NA	NA
8	7.72	0.16	2.07E+06	1.63E+05	1.17	0.32	27.49	4.51	0.52	0.08	8.09	0.42	NA	NA
10	7.41	0.06	1.91E+06	1.27E+05	0.43	0.03	11.24	1.97	0.52	0.23	9.29	0.70	NA	NA
10.5	8.14	0.01	1.71E+06	4.95E+04	0.51	0.10	0.25	0.01	0.53	0.02	8.11	0.20	1.44	0.47
11	9.51	0.03	2.61E+06	7.07E+03	n/d	n/d	n/d	n/d	0.70	0.00	5.37	0.23	1.61	0.53
12	9.67	0.03	7.95E+05	1.41E+04	n/d	n/d	n/d	n/d	0.75	0.05	4.13	0.01	1.72	0.08
13	9.72	0.01	1.21E+06	1.56E+05	n/d	n/d	n/d	n/d	0.86	0.25	3.74	0.07	1.84	0.26
14	9.81	0.02	1.81E+06	6.36E+05	n/d	n/d	n/d	n/d	0.52	0.01	3.41	0.13	2.17	0.30
15	9.89	0.01	1.60E+06	2.69E+05	n/d	n/d	n/d	n/d	0.84	0.10	3.24	0.15	2.25	0.23

Table A.4. Phase II. 2.5 mM sodium bicarbonate amendment average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	6.98	0.01	6.40E+05	0.00E+00	20.90	0.09	11.86	0.07	0.09	0.01	3.65	0.18	NA	NA
2	9.03	0.09	1.33E+06	4.95E+04	18.44	0.36	13.85	0.75	0.21	0.08	3.66	0.16	NA	NA
4	10.01	0.16	1.68E+06	2.83E+05	6.27	0.94	25.00	0.07	0.36	0.03	4.31	0.01	NA	NA
6	10.59	0.14	2.14E+06	1.20E+05	2.25	0.36	23.24	1.00	0.51	0.06	5.81	0.18	NA	NA
8	10.92	0.09	2.95E+06	1.98E+05	0.05	0.27	22.96	2.14	0.64	0.10	7.78	0.48	NA	NA
10	10.67	0.06	2.92E+06	1.13E+05	0.76	2.22	19.90	1.05	0.88	0.16	8.20	0.65	NA	NA
10.5	8.76	0.01	3.00E+06	9.19E+04	n/d	n/d	14.90	1.24	0.88	0.01	6.67	0.74	3.49	0.64
11	9.77	0.04	3.33E+06	7.07E+04	n/d	n/d	n/d	n/d	1.46	0.49	7.90	0.27	3.36	0.29
12	9.76	0.14	1.77E+06	1.13E+06	n/d	n/d	n/d	n/d	1.85	0.22	9.02	2.38	3.72	0.91
13	9.97	0.10	1.88E+06	2.83E+05	n/d	n/d	n/d	n/d	1.31	0.10	6.17	0.32	4.26	1.02
14	9.94	0.12	3.25E+06	4.67E+05	n/d	n/d	n/d	n/d	1.36	0.01	4.18	0.54	5.77	0.23
15	10.33	0.47	4.17E+06	2.97E+05	n/d	n/d	n/d	n/d	1.41	0.12	4.29	0.63	6.70	0.89

Table A.5. Phase III. pH controlled. Only ambient air (*i.e.* without bicarbonate amendment) average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	6.56	0.04	5.75E+04	3.54E+03	22.57	2.04	18.58	0.60	0.01	0.04	4.47	1.84	NA	NA
2	7.20	0.07	1.26E+05	1.77E+03	19.85	0.99	18.39	1.30	0.00	0.00	5.53	0.15	NA	NA
4	7.61	0.03	1.53E+05	3.54E+03	11.89	4.47	22.31	0.13	0.06	0.01	4.94	0.93	NA	NA
6	7.23	0.27	2.74E+05	2.65E+04	6.25	1.35	9.16	6.87	0.19	0.09	8.17	1.80	NA	NA
6.5	8.42	0.03	6.03E+05	6.72E+04	n/d	n/d	n/d	n/d	0.13	0.03	9.00	1.02	1.29	0.04
7	9.22	0.01	2.13E+06	2.12E+05	n/d	n/d	n/d	n/d	0.18	0.01	5.09	1.74	1.41	0.15
8	9.32	0.04	2.02E+06	5.66E+04	n/d	n/d	n/d	n/d	0.16	0.03	2.95	0.01	1.41	0.19
9	9.40	0.06	1.90E+06	4.60E+05	n/d	n/d	n/d	n/d	0.22	0.00	2.96	0.21	0.66	0.07
10	9.49	0.01	9.00E+05	1.41E+05	n/d	n/d	n/d	n/d	0.17	0.13	2.92	0.02	1.72	0.10
11	9.63	0.01	3.58E+05	2.47E+04	n/d	n/d	n/d	n/d	0.26	0.03	3.25	0.45	2.46	0.12
12	9.78	0.04	3.05E+05	7.78E+04	n/d	n/d	n/d	n/d	0.26	0.03	3.36	0.59	2.49	0.65
13	9.74	0.01	2.73E+05	1.06E+04	n/d	n/d	n/d	n/d	0.45	0.01	2.82	0.16	4.35	0.26

Table A.6. Phase III. pH controlled. 2.5 mM sodium bicarbonate amendment average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	7.19	0.06	6.50E+04	3.18E+04	20.90	5.31	11.86	0.07	0.02	0.02	4.55	1.52	NA	NA
2	7.68	0.04	1.13E+05	0.00E+00	18.44	0.36	13.85	0.75	0.13	0.00	3.45	0.41	NA	NA
4	7.27	0.21	3.04E+05	5.13E+04	6.27	0.94	25.00	0.07	0.19	0.02	5.78	0.11	NA	NA
6	7.65	0.25	5.40E+05	3.18E+04	2.25	0.36	23.24	1.00	0.35	0.04	10.10	3.22	NA	NA
6.5	8.44	0.04	8.78E+05	5.30E+04	0.05	0.27	22.96	2.14	0.22	0.04	11.20	2.11	1.99	0.06
7	9.54	0.02	3.70E+06	6.01E+05	0.76	2.22	19.90	1.05	0.48	0.04	9.81	0.20	3.46	0.30
8	9.53	0.01	2.36E+06	2.65E+05	n/d	n/d	14.90	1.24	0.45	0.10	5.90	1.49	4.57	1.42
9	9.63	0.02	2.25E+06	1.41E+05	n/d	n/d	n/d	n/d	0.50	0.09	7.39	2.09	1.47	0.37
10	10.05	0.19	2.19E+06	3.71E+05	n/d	n/d	n/d	n/d	0.48	0.09	5.83	0.42	4.26	0.50
11	10.65	0.32	1.60E+06	2.12E+04	n/d	n/d	n/d	n/d	0.65	0.21	6.56	1.46	5.70	1.17
12	10.52	0.08	1.93E+06	9.90E+04	n/d	n/d	n/d	n/d	0.67	0.06	5.22	1.28	5.99	0.97
13	10.58	0.14	9.30E+05	1.98E+05	n/d	n/d	n/d	n/d	0.74	0.11	4.36	0.82	9.14	0.15

Table A.7. Phase IV. pH controlled. Only ambient air (*i.e.* without bicarbonate amendment) average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	6.69	0.08	1.15E+05	7.07E+03	24.72	5.60	14.16	2.05	0.06	0.03	4.31	1.02	0.60	0.05
2	6.91	0.04	2.93E+05	1.10E+05	19.34	2.47	19.68	5.23	0.09	0.06	2.93	0.02	0.61	0.03
4	7.14	0.06	4.40E+05	1.56E+05	14.62	0.89	20.67	0.48	0.13	0.04	3.22	0.03	0.71	0.05
6	6.98	0.10	8.85E+05	1.34E+05	11.93	0.07	13.50	0.82	0.17	0.17	4.79	0.37	0.73	0.04
8	6.75	0.24	1.03E+06	1.77E+05	3.28	0.88	7.16	0.95	0.24	0.07	8.50	0.35	0.76	0.14
8.5	8.58	0.08	8.90E+05	9.90E+04	0.86	0.24	n/d	n/d	0.10	0.01	7.24	0.24	0.81	0.01
9	9.50	0.18	8.65E+05	1.77E+05	n/d	n/d	n/d	n/d	0.22	0.05	7.74	2.50	0.96	0.11
10	10.10	0.33	1.76E+06	3.82E+05	n/d	n/d	n/d	n/d	0.19	0.01	5.50	0.11	1.07	0.03
11	10.46	0.21	2.81E+06	9.26E+05	n/d	n/d	n/d	n/d	0.27	0.06	5.50	1.79	1.07	0.03
12	11.00	0.15	3.62E+06	1.50E+06	n/d	n/d	n/d	n/d	0.28	0.04	4.80	0.38	2.42	0.06
13	10.92	0.01	3.76E+06	1.13E+06	n/d	n/d	n/d	n/d	0.35	0.01	3.85	0.58	3.52	0.07

Table A.8. Phase IV. pH controlled. 2.5 mM sodium bicarbonate amendment average value ± standard deviation for pH, cell density [cells·mL^{-1}], nitrate concentration [mg N (NO_3^-)·L^{-1}], ammonium concentration [mg N (NH_4^+)·L^{-1}], dry cell weight [mg·L^{-1}], total chlorophyll [mg Chl·L^{-1}], and astaxanthin concentration [mg·L^{-1}].

Time [days]	pH		Cell Counts [cells·mL^{-1}]		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Ammonium Concentration [mg N (NH_4^+)·L^{-1}]		DCW [mg·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]		Astaxanthin [mg·L^{-1}]	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
0	7.05	0.01	1.30E+05	1.41E+04	24.91	1.25	15.69	1.56	0.03	0.00	2.95	0.06	0.65	0.06
2	7.35	0.02	1.15E+06	4.95E+04	21.95	0.51	10.48	0.85	0.05	0.04	2.89	0.04	0.66	0.04
4	7.33	0.04	1.73E+06	2.83E+04	13.41	2.26	2.58	0.05	0.14	0.06	4.18	0.35	0.73	0.05
6	7.55	0.06	2.13E+06	3.54E+04	8.19	0.35	n/d	n/d	0.34	0.06	5.99	0.22	0.77	0.04
8	7.55	0.02	2.80E+06	2.12E+04	n/d	n/d	n/d	n/d	0.37	0.02	10.01	0.83	0.81	0.15
8.5	8.62	0.04	1.01E+06	5.66E+04	n/d	n/d	n/d	n/d	0.24	0.15	8.63	0.80	1.66	0.07
9	9.41	0.07	1.21E+06	5.66E+04	n/d	n/d	n/d	n/d	0.22	0.14	7.72	0.66	1.96	0.03
10	9.85	0.05	2.63E+06	4.10E+05	n/d	n/d	n/d	n/d	0.36	0.18	5.46	1.28	4.51	0.90
11	10.13	0.26	4.02E+06	4.24E+05	n/d	n/d	n/d	n/d	0.38	0.11	4.60	0.56	5.09	0.04
12	10.71	0.06	4.02E+06	4.81E+05	n/d	n/d	n/d	n/d	0.40	0.08	3.76	0.85	9.66	0.48
13	10.70	0.09	5.36E+06	1.87E+06	n/d	n/d	n/d	n/d	0.48	0.01	3.72	0.56	11.20	0.56

APPENDIX B

SUPPLEMENTAL DATA

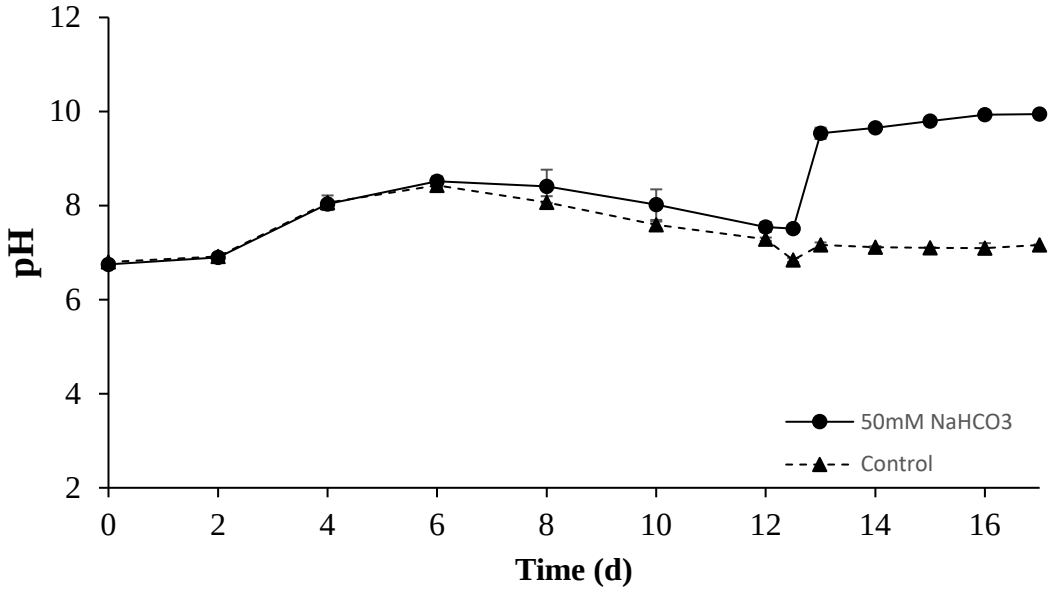

Figure B.1. Average pH value ± standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparges with ambient air only during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=12.5 d). Control condition represents the untriggered cultures in the red stage.

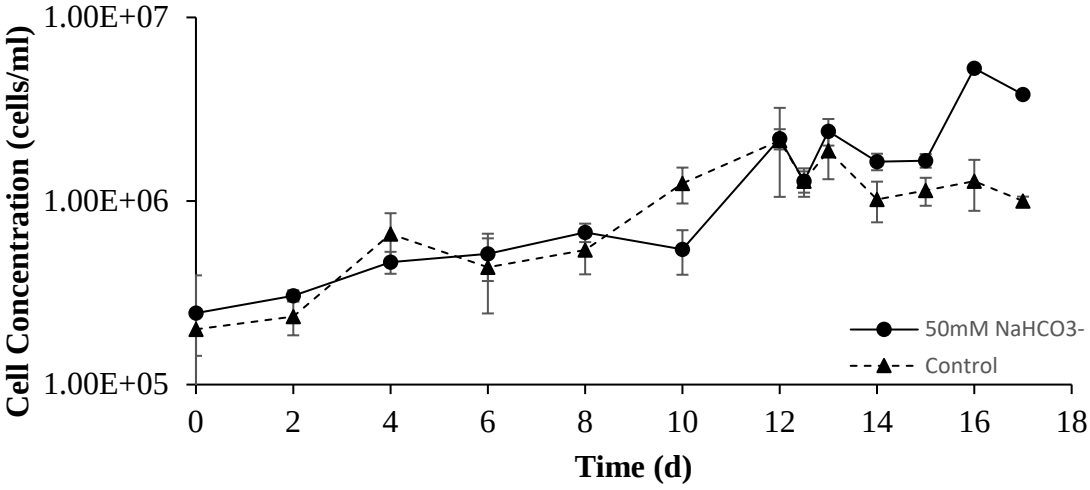

Figure B.2. Average cell density [cells·mL^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparges with ambient air only during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=12.5 d). Control condition represents the untriggered cultures in the red stage.

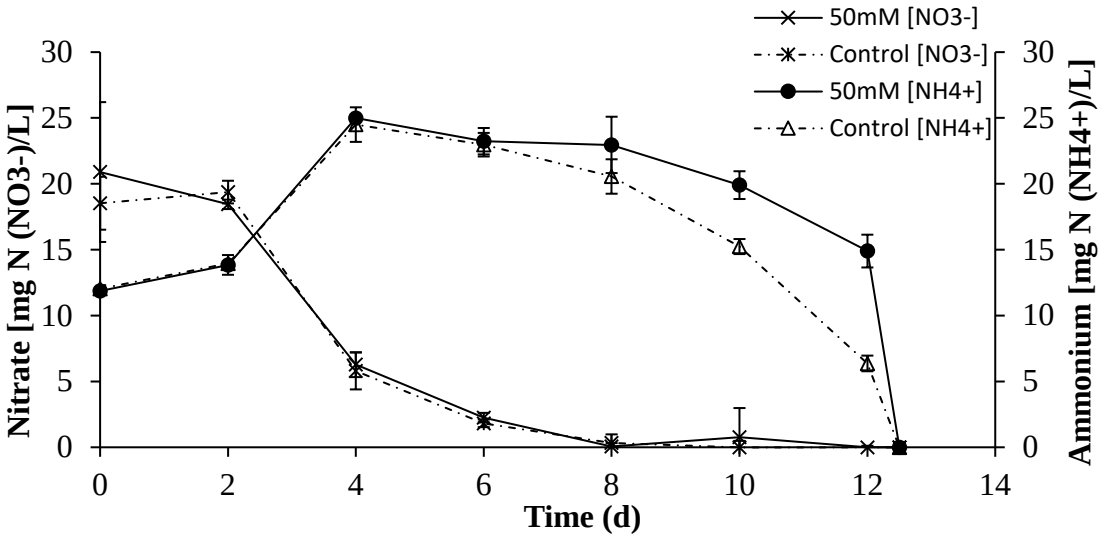

Figure B. 3. Average nitrate concentration [mg N $(NO_3^-)\cdot L^{-1}$] and ammonium concentration [mg N $(NH_4^+)\cdot L^{-1}$] value ± standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparges with ambient air only during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=12.5 d). Control condition represents the untriggered cultures in the red stage. The primary y-axis shows the nitrate concentration whereas the secondary y-axis shows the ammonium concentration.

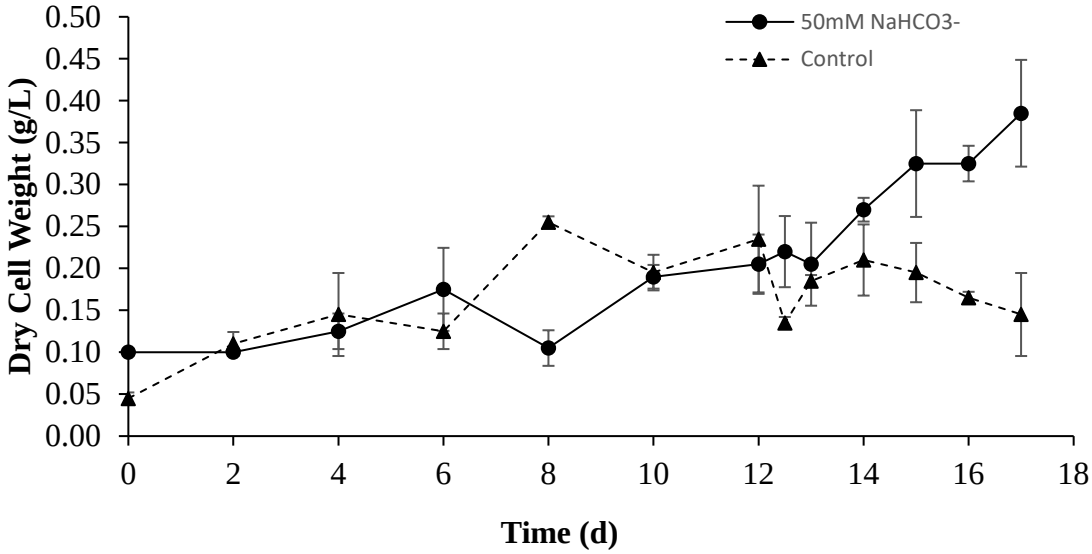

Figure B.4. Average dry cell weight [$mg\cdot L^{-1}$] value ± standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparges with ambient air only during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=12.5 d). Control condition represents the untriggered cultures in the red stage.

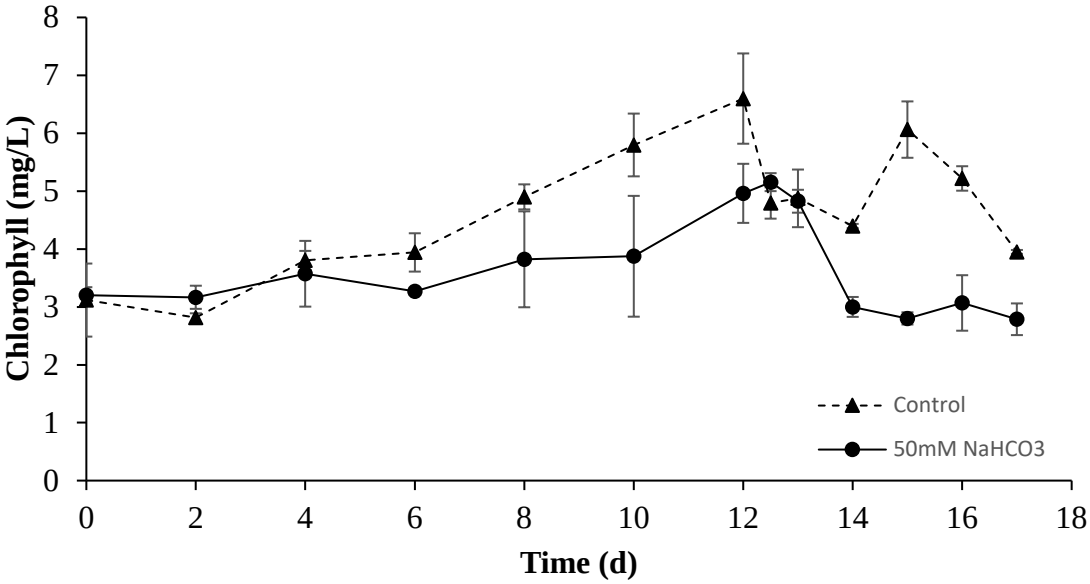

Figure B.5. Average total chlorophyll [mg Chl·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase I. Cultures were sparges with ambient air only during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=12.5 d). Control condition represents the untriggered cultures in the red stage.

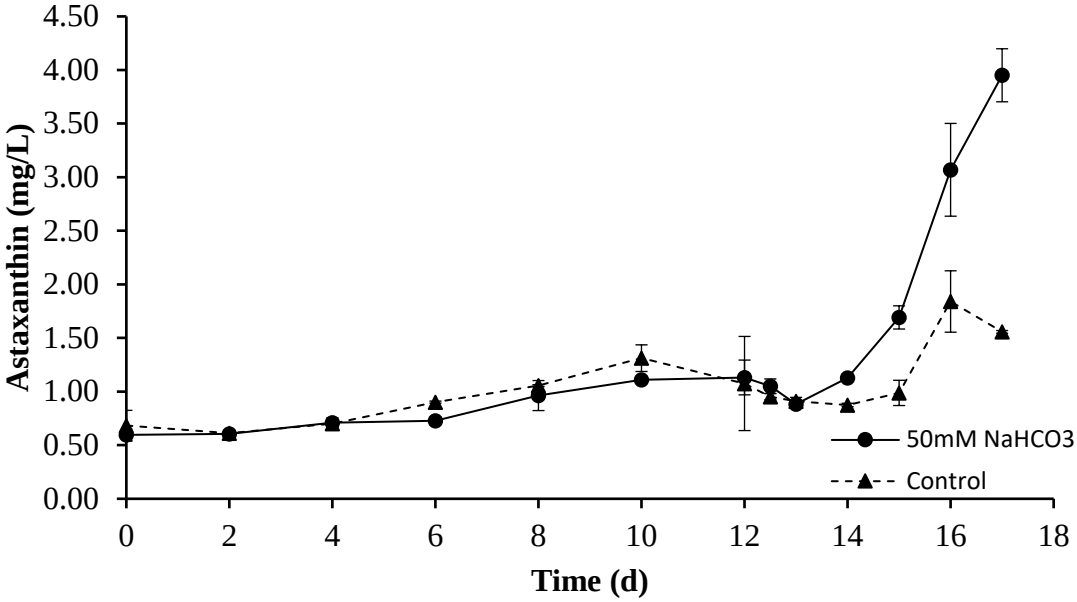

Figure B. 6. Average astaxanthin concentration [mg·L^{-1}] value ± standard deviation of in *H. pluvialis* cultures in Phase I. Cultures were sparges with ambient air only during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=12.5 d). Control condition represents the untriggered cultures in the red stage.

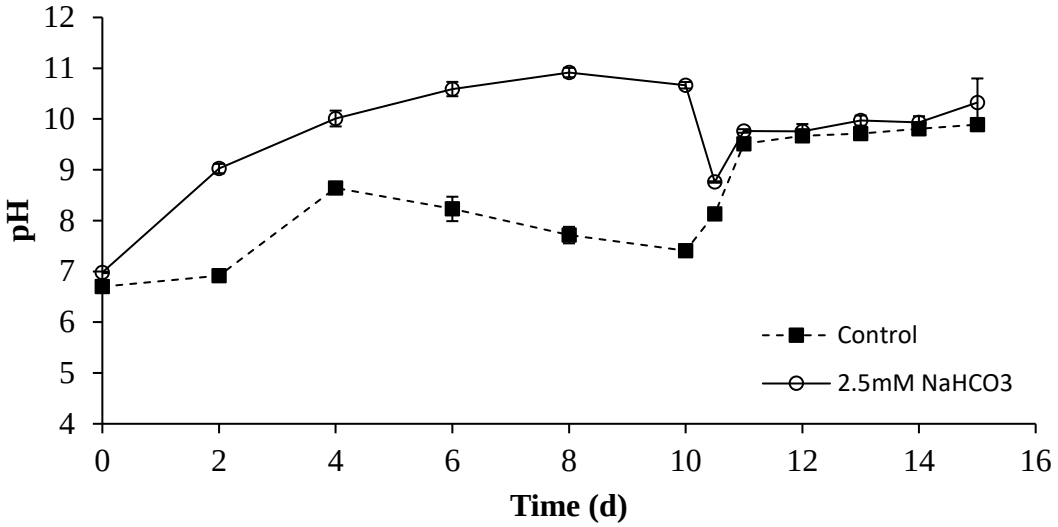

Figure B.7. Average pH value ± standard deviation in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=10.5 d). Control condition represents the unamended cultures in the green stage.

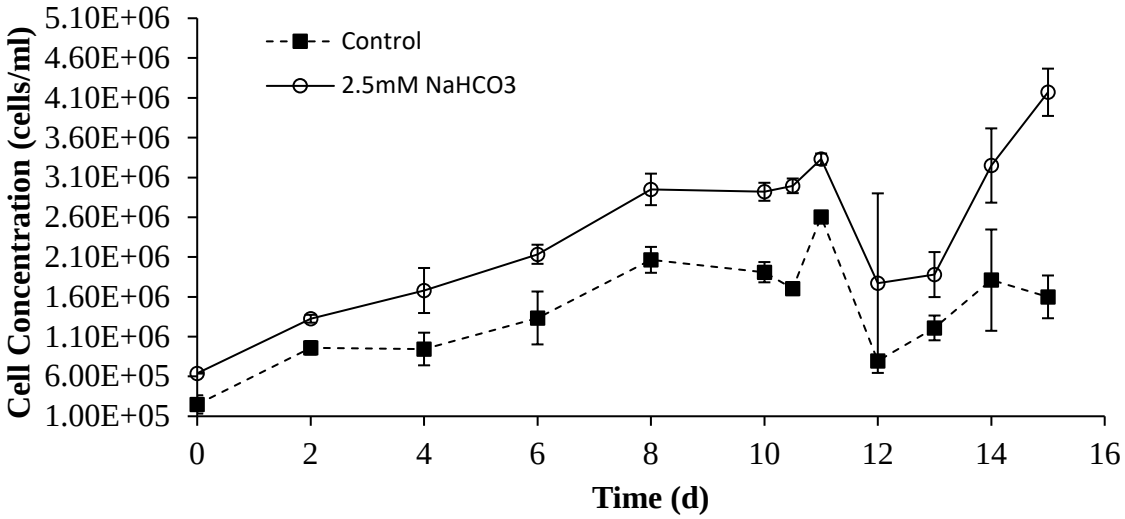

Figure B.8. Average cell density [cells·mL^{-1}] value ± standard deviation of in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=10.5 d). Control condition represents the unamended cultures in the green stage.

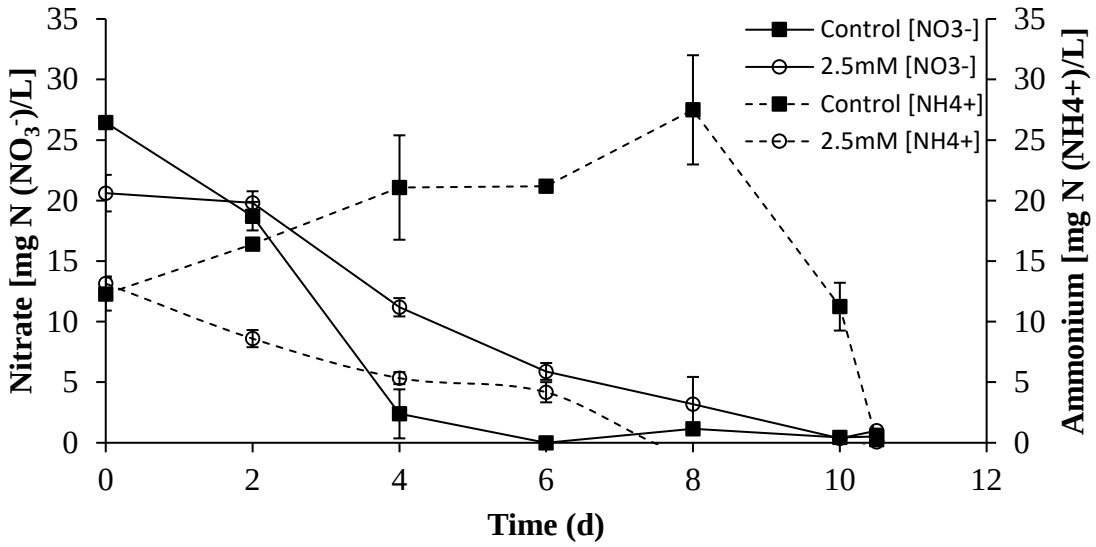

Figure B.9. Average nitrate concentration [mg N (NO_3^-)·L^{-1}] and ammonium concentration [mg N (NH_4^+)·L^{-1}] value ± standard deviation of in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=10.5 d). Control condition represents the unamended cultures in the green stage. The primary y-axis shows the nitrate concentration whereas the secondary y-axis shows the ammonium concentration.

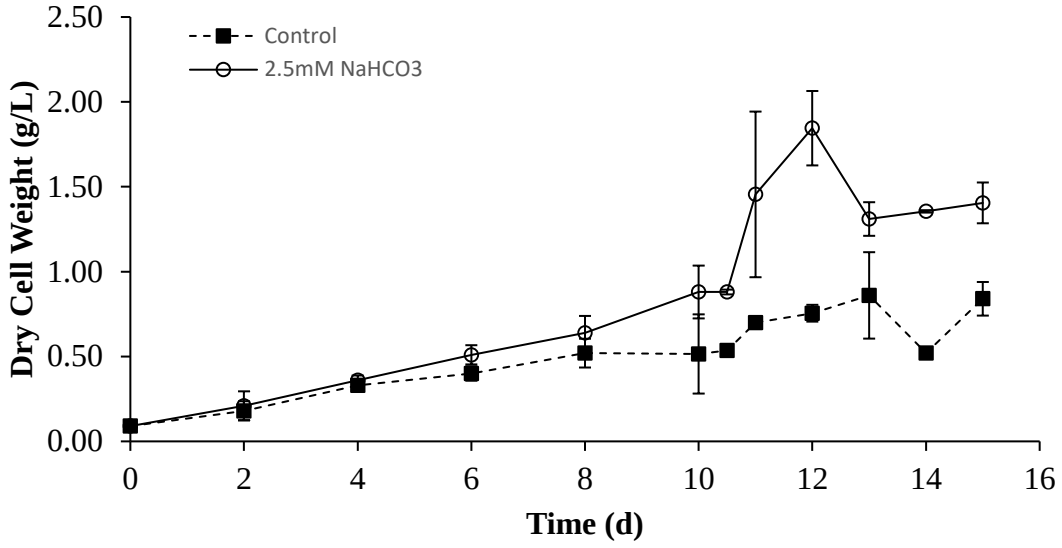

Figure B.10. Average dry cell weight [mg·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=10.5 d). Control condition represents the unamended cultures in the green stage.

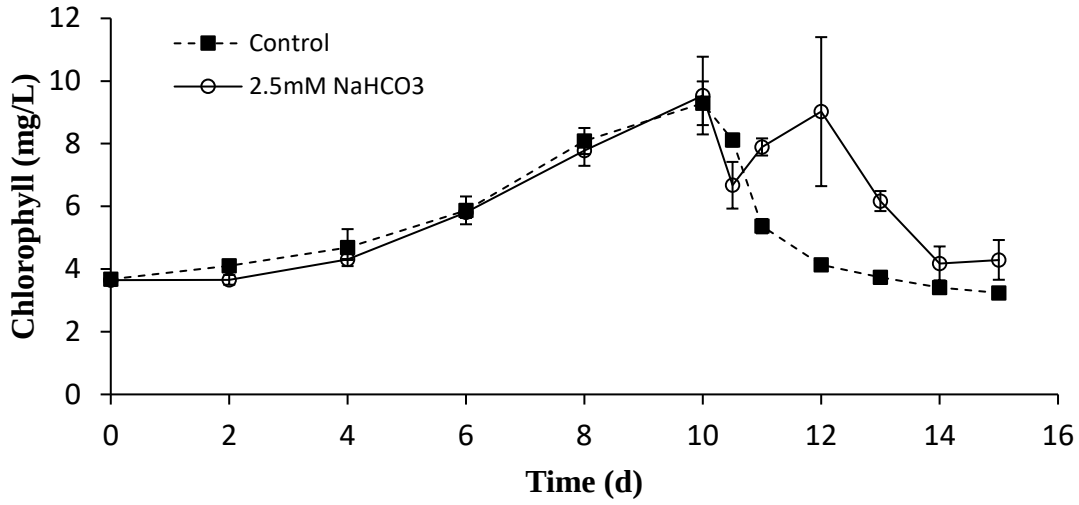

Figure B.11. Average total chlorophyll [mg Chl·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=10.5 d). Control condition represents the unamended cultures in the green stage.

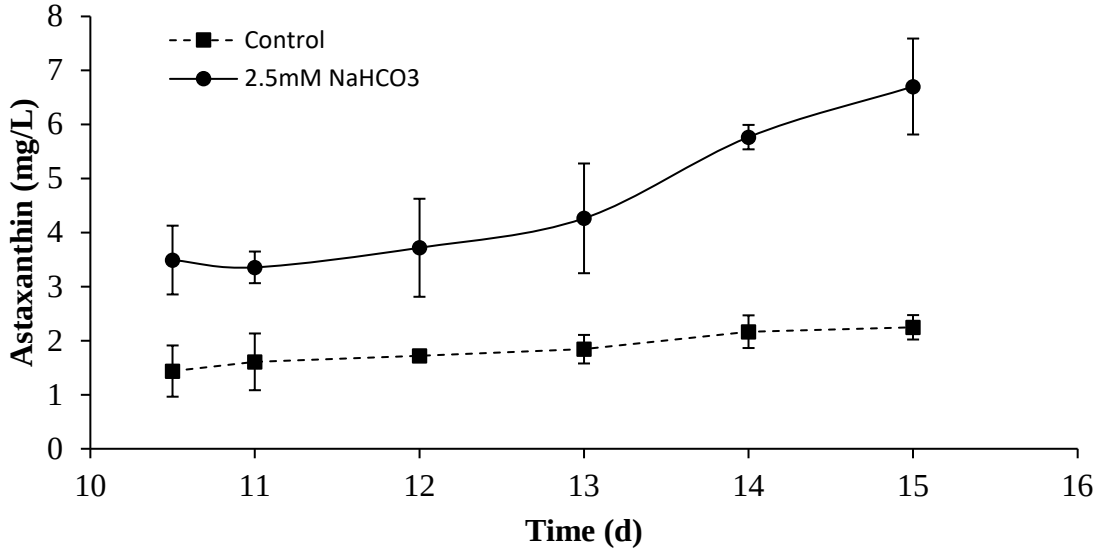

Figure B.12. Average astaxanthin concentration [mg·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase II. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=10.5 d). Control condition represents the unamended cultures in the green stage.

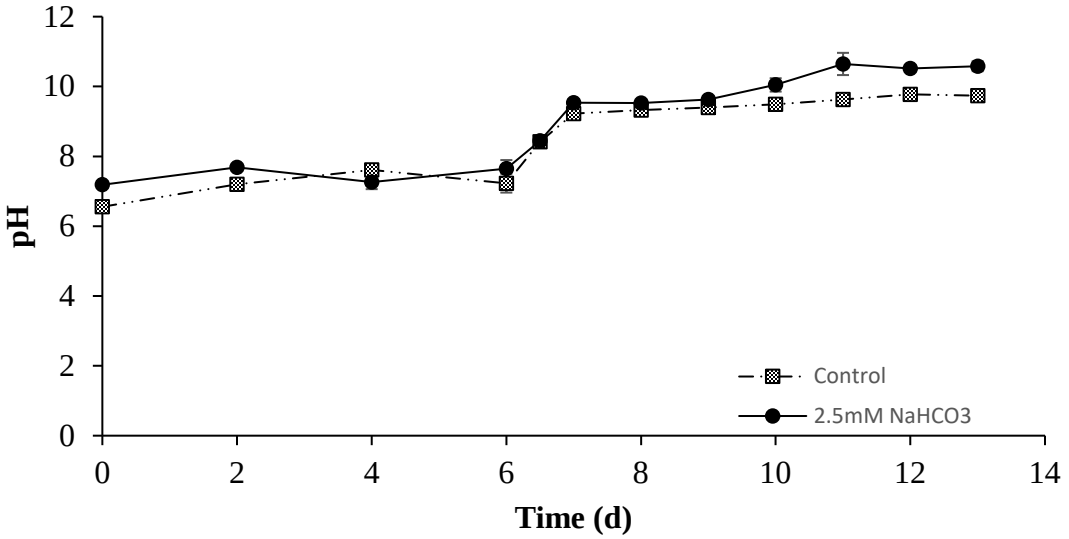

Figure B.13. Average pH value ± standard deviation in *H. pluvialis* cultures in Phase III. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=6.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. Control condition represents the unamended cultures in the green stage.

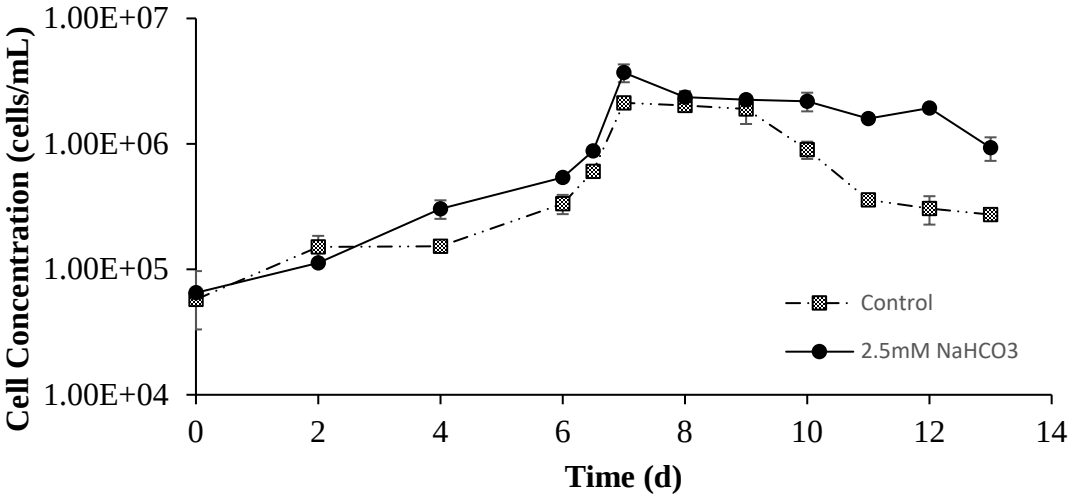

Figure B.14. Average cell density [cells·mL^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase III. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=6.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. Control condition represents the unamended cultures in the green stage.

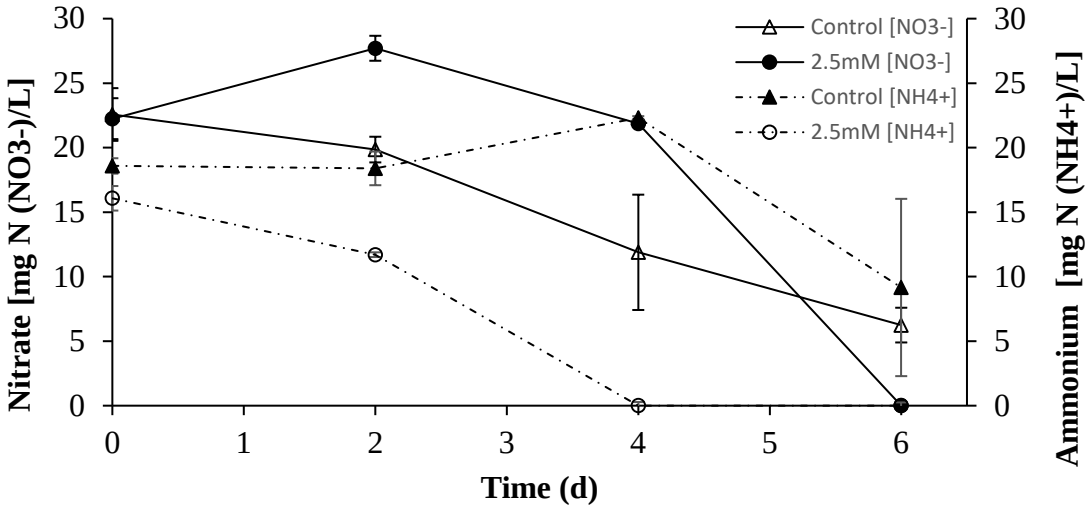

Figure B.15. Average nitrate concentration [mg N $(NO_3^-)\cdot L^{-1}$] and ammonium concentration [mg N $(NH_4^+)\cdot L^{-1}$] value ± standard deviation in *H. pluvialis* cultures in Phase III. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=6.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. The primary y-axis shows the nitrate concentration whereas the secondary y-axis shows the ammonium concentration. Control condition represents the unamended cultures in the green stage.

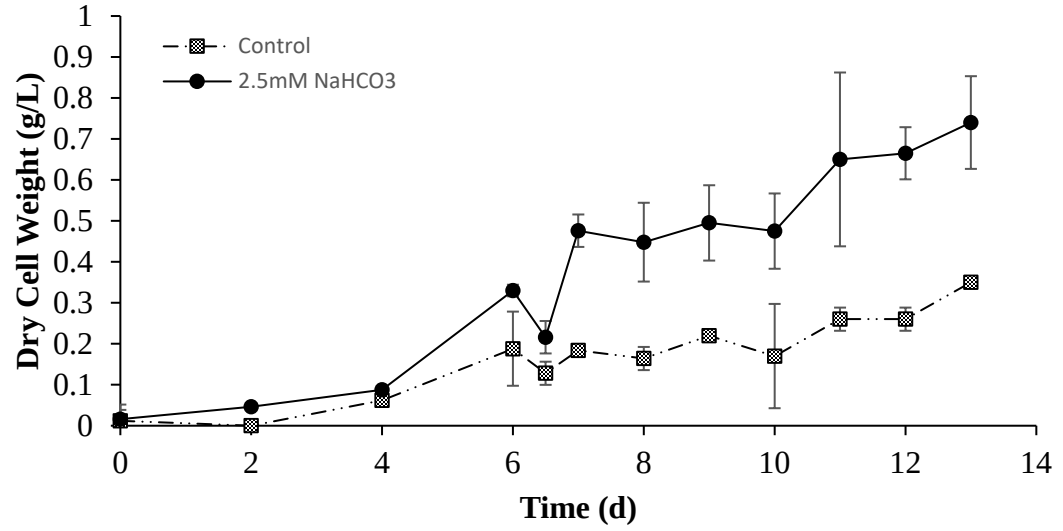

Figure B.16. Average dry cell weight [$mg\cdot L^{-1}$] value ± standard deviation in *H. pluvialis* cultures in Phase III. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=6.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. Control condition represents the unamended cultures in the green stage.

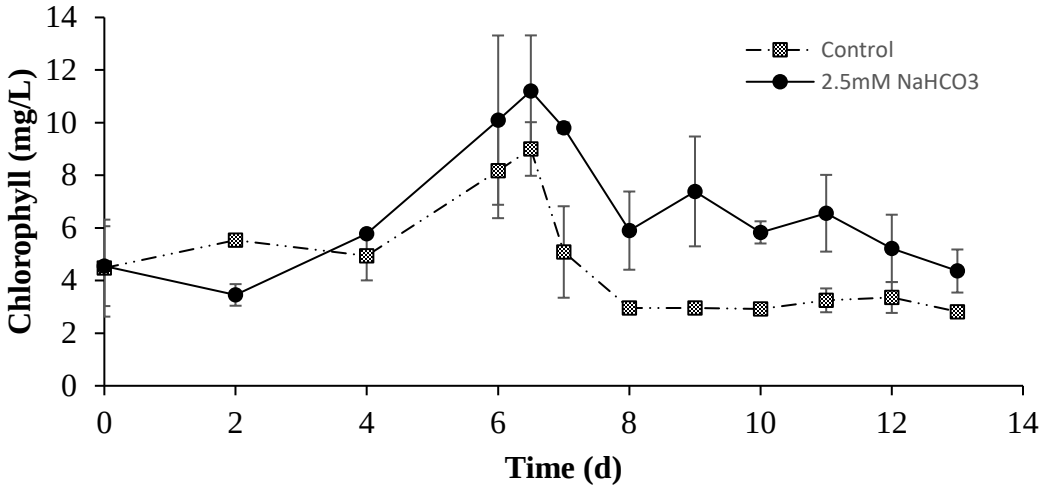

Figure B.17. Average total chlorophyll [mg Chl·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase III. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=6.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. Control condition represents the unamended cultures in the green stage.

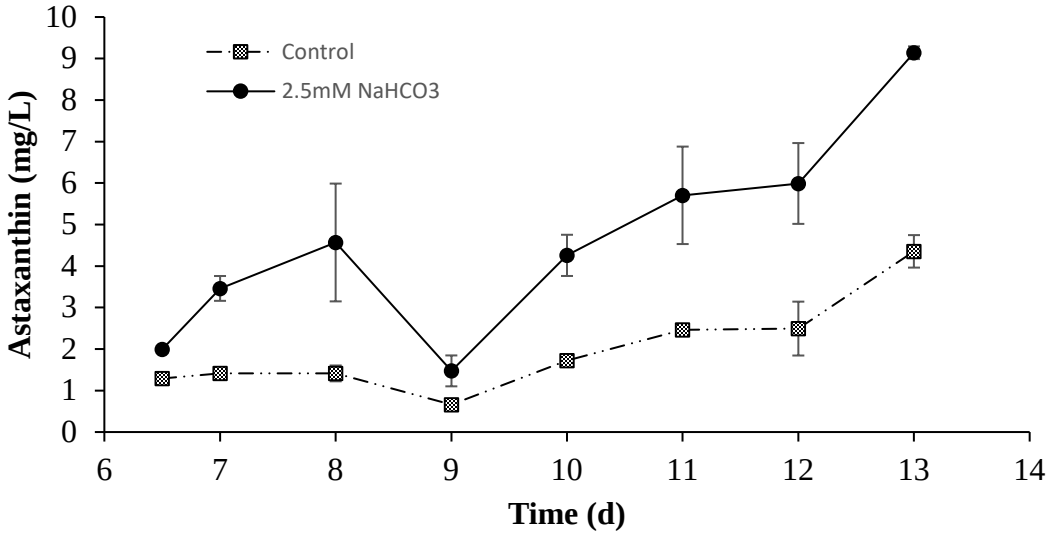

Figure B.18. Average astaxanthin concentration [mg·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase III. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=6.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. Control condition represents the unamended cultures in the green stage.

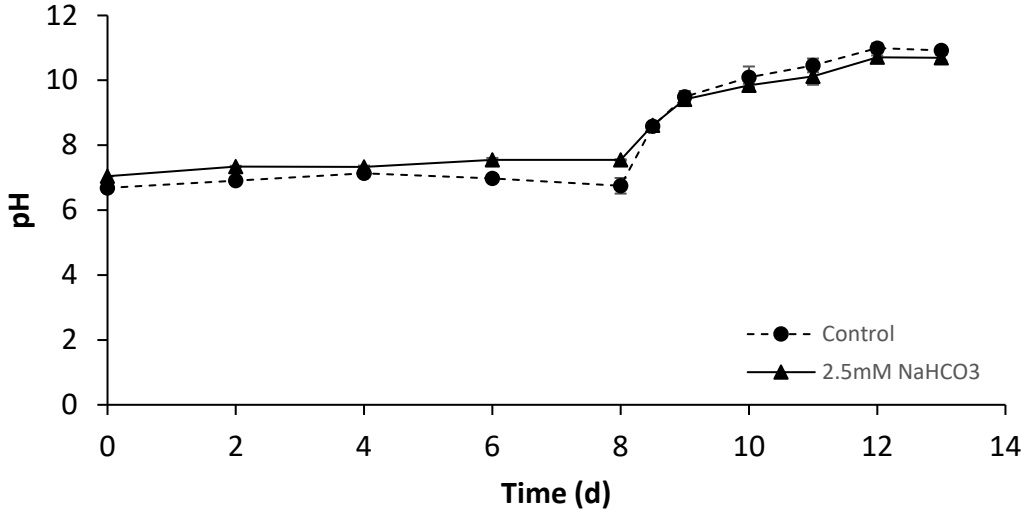

Figure B.19. Average pH value ± standard deviation in *H. pluvialis* cultures in Phase IV. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=8.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. The cultures were exposed to an additional 24h light stress during the red stage. Control condition represents the unamended cultures in the green stage.

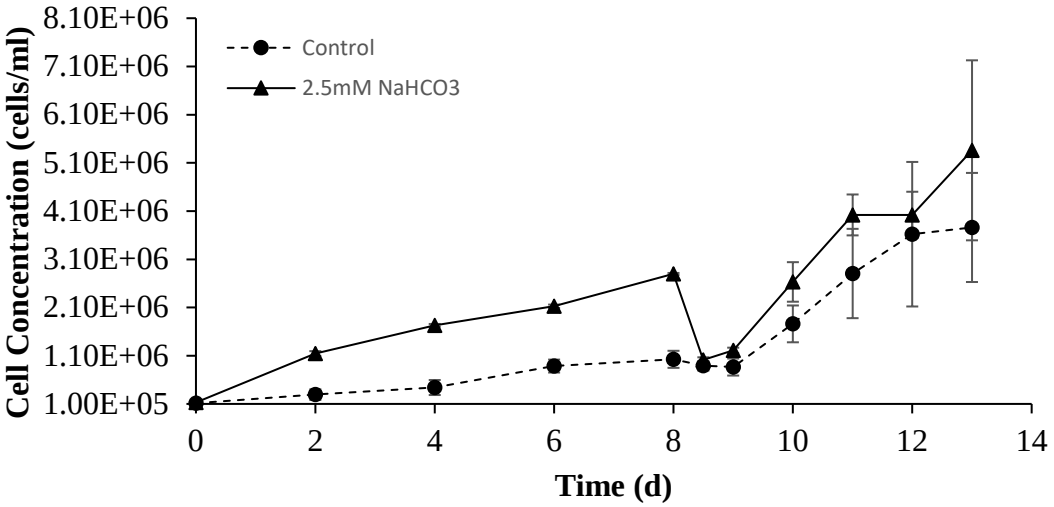

Figure B.20. Average cell density [cells·mL^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase IV. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=8.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. The cultures were exposed to an additional 24h light stress during the red stage. Control condition represents the unamended cultures in the green stage.

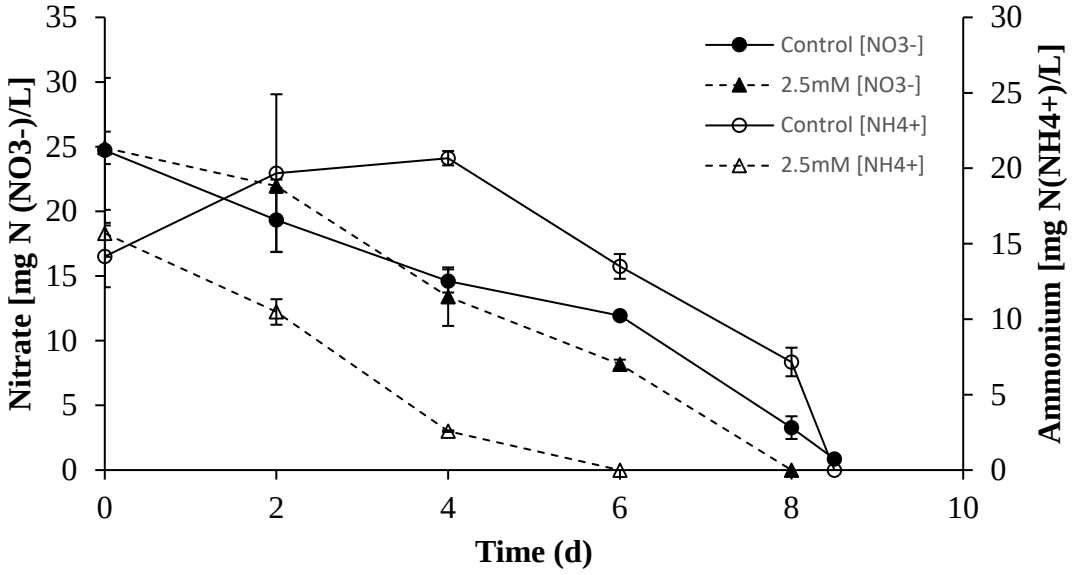

Figure B.21. Average nitrate concentration [mg N (NO_3^-)·L^{-1}] and ammonium concentration [mg N (NH_4^+)·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase IV. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=8.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. The cultures were exposed to an additional 24h light stress during the red stage. Control condition represents the unamended cultures in the green stage. The primary y-axis shows the nitrate concentration whereas the secondary y-axis shows the ammonium concentration.

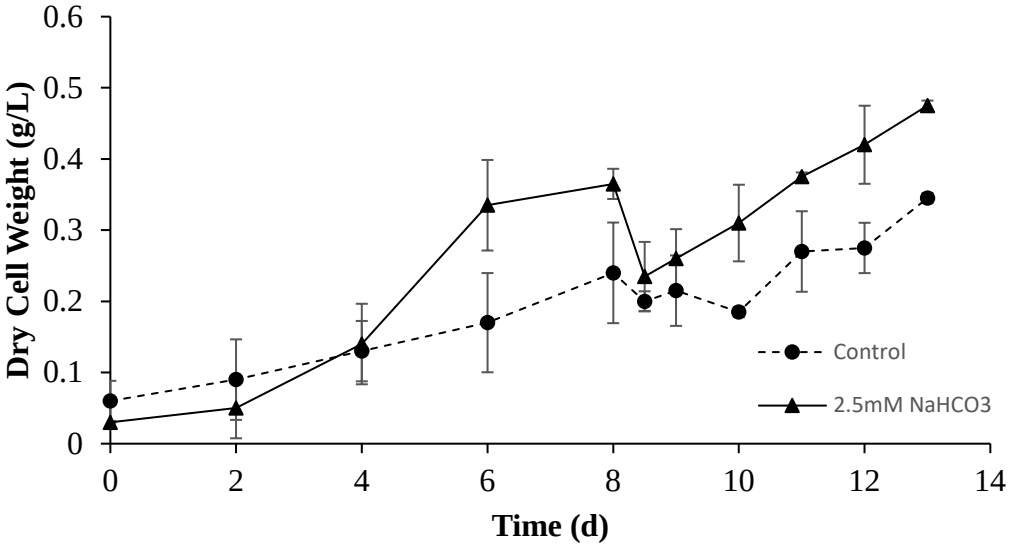

Figure B.22. Average dry cell weight [mg·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase IV. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=8.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. The cultures were exposed to an additional 24h light stress during the red stage. Control condition represents the unamended cultures in the green stage.

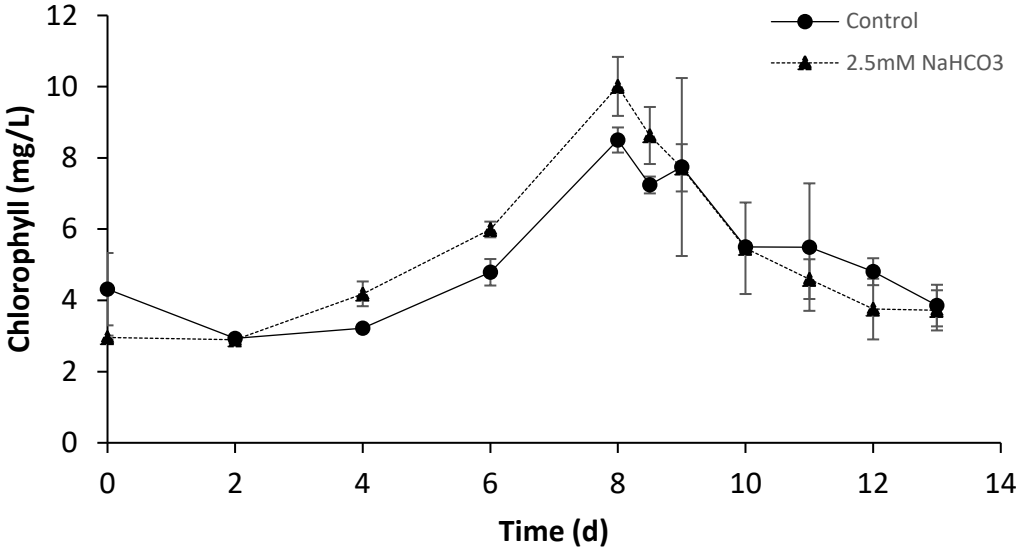

Figure B.23. Average total chlorophyll [mg Chl·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase IV. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=8.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. The cultures were exposed to an additional 24h light stress during the red stage. Control condition represents the unamended cultures in the green stage.

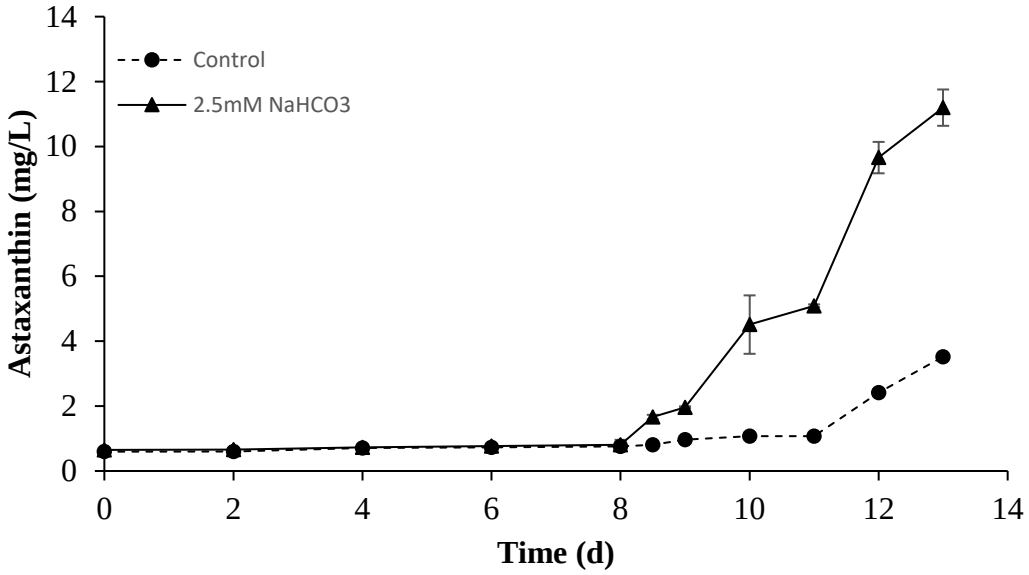

Figure B.24. Average astaxanthin concentration [mg·L^{-1}] value ± standard deviation in *H. pluvialis* cultures in Phase IV. Cultures were amended with 2.5 mM of sodium bicarbonate in addition to ambient air during the green stage and were triggered with 50 mM of sodium bicarbonate during the red stage (t=8.5 d). pH was controlled at 7.5 with 5% CO_2 (v/v) on demand during the green stage. The cultures were exposed to an additional 24h light stress during the red stage. Control condition represents the unamended cultures in the green stage.

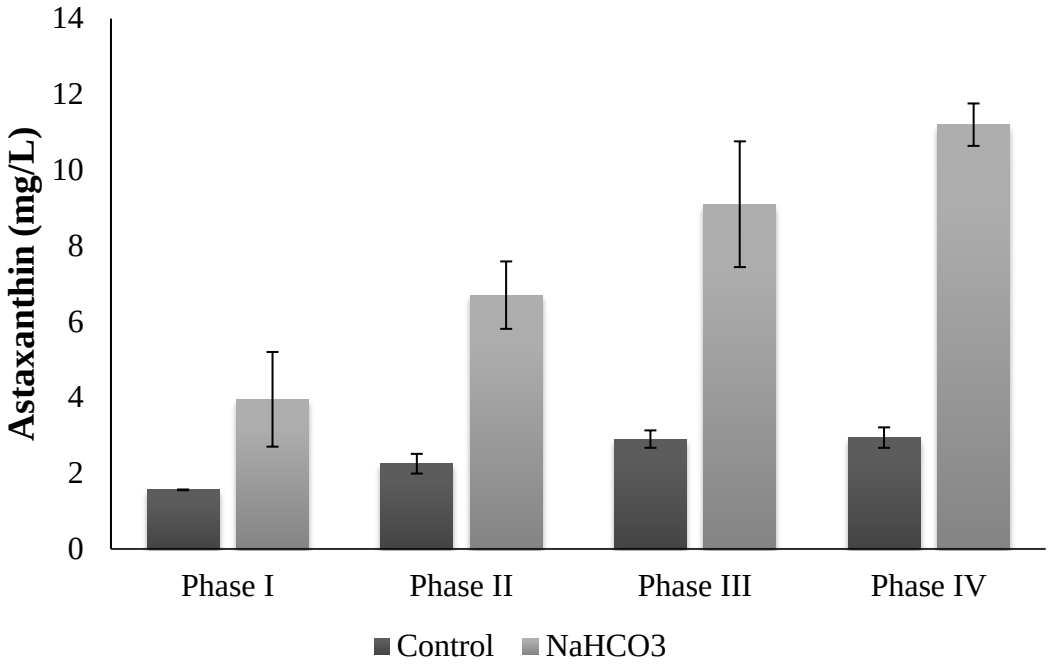

Figure B.25. Average value ± standard deviation of final astaxanthin concentration comparison of each phase. Blue bars represent the control condition and the red bars represent the sodium bicarbonate amended conditions.

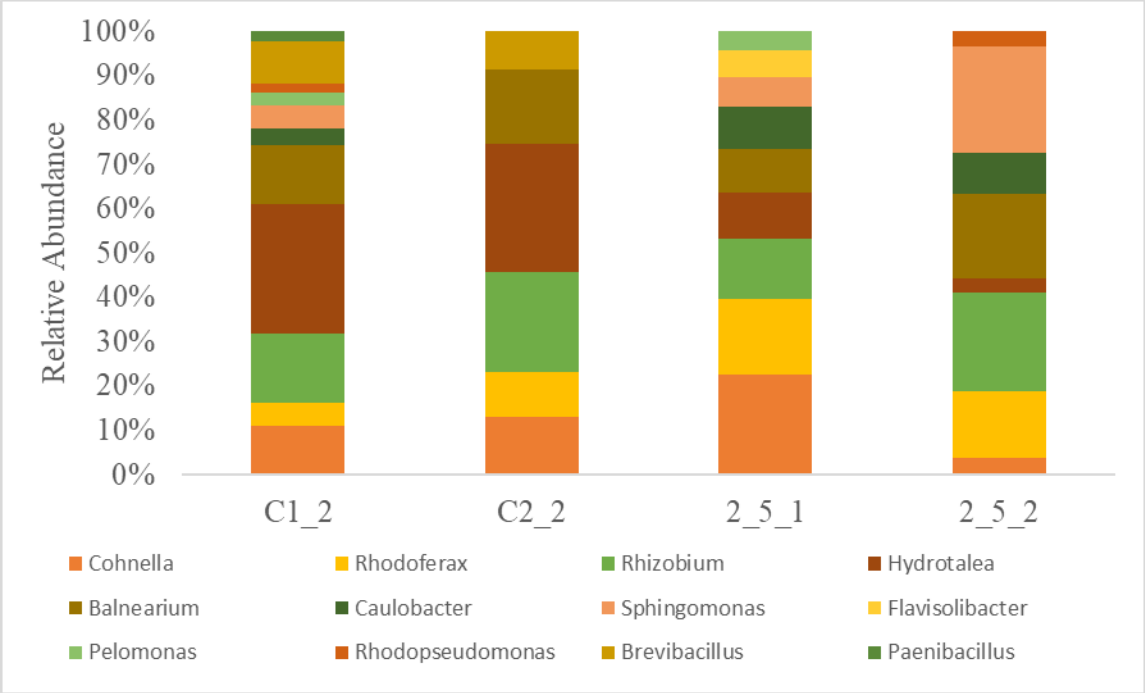

Figure B.26. Dominant bacterial OTU percentages based on the genus of the bacterium. The samples were collected at the end of the green stage in Phase III. C represents the control conditions and 2.5 represents the sodium bicarbonate (2.5 mM) amended conditions. C_1 and C_2 are the duplicates of the control condition. 2.5_1 and 2.5_2 are the duplicates of 2.5 mM sodium bicarbonate amended condition.

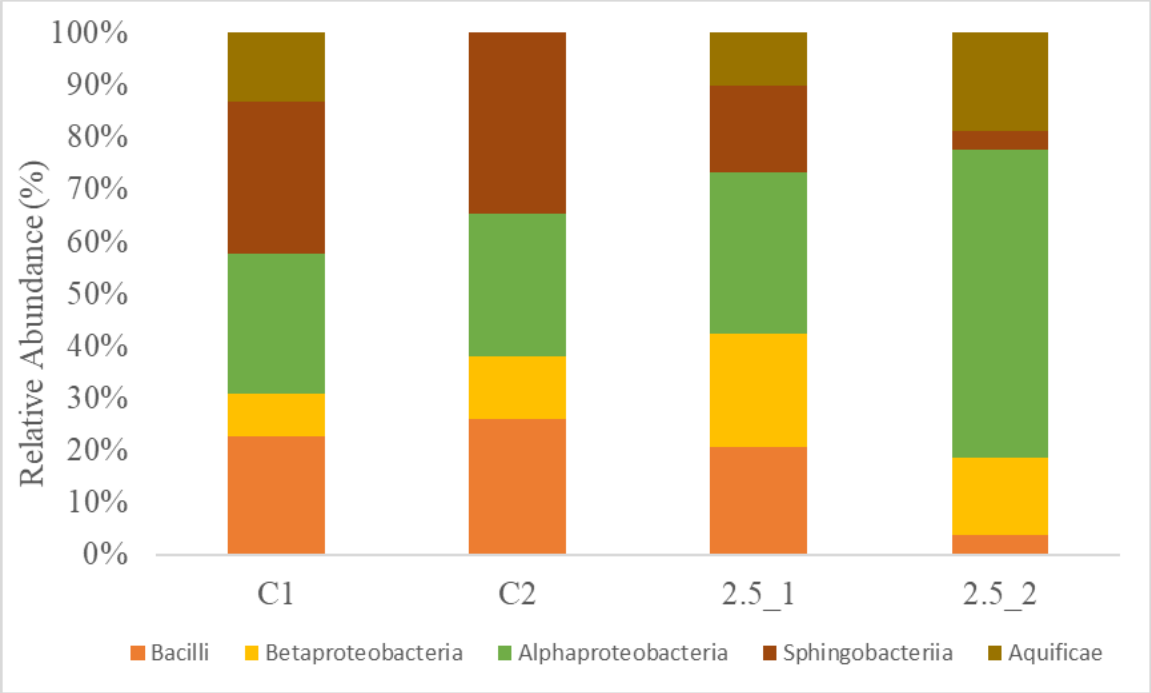

Figure B.27. Dominant bacterial OTU percentages based on the class of the bacterium. The samples were collected at the end of the green stage in Phase III. C represents the control conditions and 2_5 represents the sodium bicarbonate (2.5 mM) amended conditions. C_1 and C_2 are the duplicates of the control condition. 2.5_1 and 2.5_2 are the duplicates of 2.5 mM sodium bicarbonate amended condition.

APPENDIX C

CULTIVATION SYSTEM PHOTOS

Figure C.1. Shake flask photobioreactor used in Phase I and II.

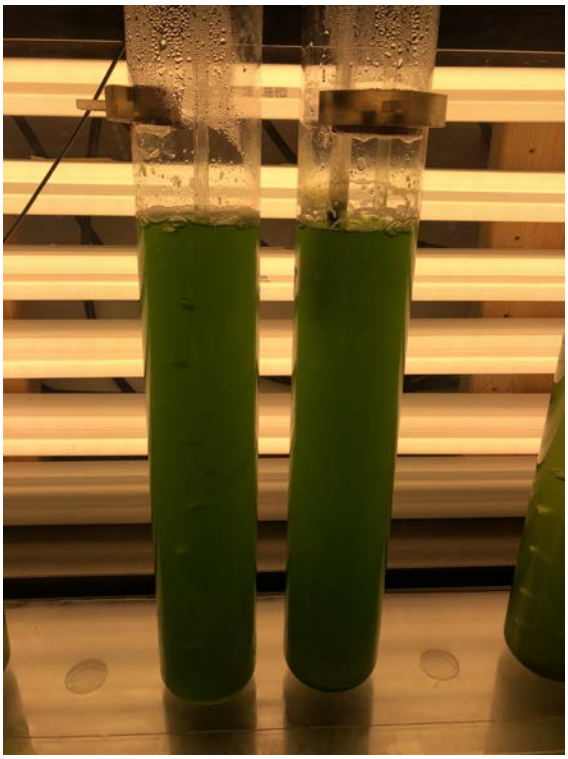

Figure C.2. Tube reactors used in Phase III and IV.

Figure C.3. Problem seen with the tube reactors (*e.g.* biomass loss).

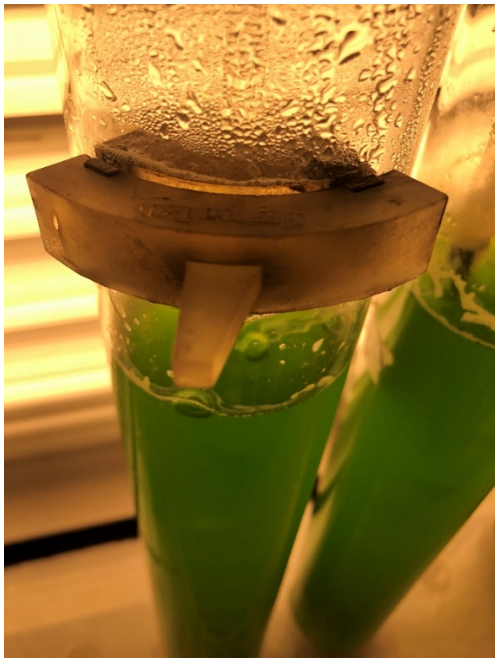

Figure C.4. 3-D printed algae scrubbers that was to resuspend the biomass that was lost back into the medium.