USE OF BICARBONATE SALTS IN ALGAL GROWTH FOR ENHANCEMENT OF LIPID CONTENT

by

Todd Christian Pedersen

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 2016

DEDICATION

I dedicate my thesis to Dick and Linda, whose everlasting inspiration will always determine me; and to my parents Pam and Rob, who have always been unconditionally supportive in my endeavors.

ACKNOWLEDGEMENTS

A special thanks to my committee chair, Dr. Brent Peyton, who helped provide me with the opportunity to complete a M.S. in chemical engineering. He was a patient mentor and an attentive advisor. I would also thank the other members of the committee, Dr. Robin Gerlach and Dr. Robert Gardner, who were also instrumental in providing me with an opportunity to complete a graduate degree. Dr. Gerlach was influential in adeptly educating me regarding the use analytical instruments and their maintenance. Dr. Gardner is an admired mentor, a cherished friend, and largely responsible for ingraining fundamental scientific discipline into my research through his continued close mentorship.

Thanks to Dr. Egan Lohman, who was a pleasure to work with in the early days of my graduate studies and was sincerely missed for the remainder. Graduate students Karen Moll, Muneeb Rathore, and Matthew Jackson, and undergraduate students Ashley Berninghaus and James Crawford all deserve distinguished thanks. Their accommodating help in the lab was refreshing and are all admirable people to work with. Final thanks to the MSU Algal Biofuels Group for setting a high standard of research in our growing interdisciplinary collaborations, and to all the wonderful faculty and staff at the Center for Biofilm Engineering.

TABLE OF CONTENTS

TABLE OF CONTENTS – CONTINUED

TABLE OF CONTENTS – CONTINUED

LIST OF TABLES

LIST OF TABLES – CONTINUED

Table Page

LIST OF FIGURES

LIST OF FIGURES – CONTINUED

Figure Page

ABSTRACT

Production of biofuel from microalgae has thus far been cost prohibitive due in part to expenses associated with providing the necessary nutritional requirements for growth of the algal culture. In particular, inorganic carbon must be supplied in higher concentrations than available atmospherically to achieve high density cultures necessary for biofuel production strategies. Cost of algal biomass, as a feedstock, will be the limiting factor to the realization of algal biofuels moving forward. Prior research has demonstrated bicarbonate to enhance lipid content in select algal cultures grown under stress conditions, such as nitrogen depletion. This phenomenon has come to be known as bicarbonate-induced lipid accumulation, colloquially known as 'bicarbonate triggering', and has unrealized potential in executing economical and productive algal biofuel. Still, this method has only been demonstrated in select microalgal species and relatively little metabolic information is available regarding its use. Here, two species were investigated with the use of bicarbonate salts for algal growth and lipid accumulation. *Nannochloropsis gaditana* is a marine microalga which produces relatively high lipid content under nutrient stressed conditions, and has not been thoroughly studied under use of bicarbonate. This organism was studied under bicarbonate supplementation in batch photobioreactor systems. *Chlorella vulgaris* is a fresh water green alga which has received attention as a biofuel candidate, due to high growth rates and lipid content. This organism was investigated under bicarbonate supplementation during nitrogen depletion with the use of high resolution-magic angle spinning (HR-MAS) nuclear magnetic resonance (NMR) spectroscopy over 38 hours with a 14:10 diel cycle. *N. gaditana* showed best growth rates under pH controlled growth strategies during nitrogen replete conditions, and bicarbonate was seen to increase lipid content following nitrogen depletion when paired with this growth strategy. However, *N. gaditana* may not be an ideal candidate for biofuel production as it has relatively low growth rates compared to other industrially relevant organisms, and demonstrated low productivity in batch systems. Metabolite investigation in *C. vulgaris* revealed large incorporation of inorganic carbon from bicarbonate amendments into biomass, specifically monitored as increases to the biological sucrose pool and subsequent synthesis of fatty acids as carbon storage compounds.

CHAPTER ONE

INTRODUCTION

Project Rationale

Algal research regarding biomass generation for biofuel and bio-product precursors has been under investigation for years, with substantial previous work occurring when the U.S. Department of Energy's Aquatic Species Program was at its peak funding in the mid 1980's. Fundamental studies dating back even further laid the groundwork of some of the media and methods still used today (Allen and Nelson 1910, Provasoli, McLaughlin et al. 1957, Nichols and Bold 1965). Still, commercial production of microalgal biofuels is not today's reality due to the economic and environmental limitations associated with production of these biofuels and the volatile nature of crude oil prices. One or two things must happen for commercialization of algal biomass as an industry to be achieved: new technologies must be developed to increase the practicality of algae biomass as a sustainable energy and product source, or the cost, both economic and environmental, of using fossil fuels must increase drastically. It is unlikely that the latter will happen, even as environmental crises demand change regardless of the price of fossil fuel use. The work described herein contributes to ongoing research aimed at investigating for enhancements to growth and lipid content in microalgae cultures through the use of sodium bicarbonate, as an alternate to CO_2.

Over the course of the last half decade, significant research has been conducted on the use of bicarbonate (HCO_3^-) salts in culturing of microalgae and was culminated in the

technology associated with 'bicarbonate triggering' as described in U.S. Patent No. 9,096,875. The scope of work here focuses on a recent grant entitled "Lipid-Derived Biofuels: Bicarbonate-Induced Microalgae for Biofuel and Specialty Chemicals" with emphasis placed on two tasks:

(1) Optimization of algal culturing and biofuel characteristics using bicarbonate – *Low concentrations of bicarbonate can potentially be used to maximize biomass production prior to lipid accumulation. Increased biomass accumulation prior to nitrogen depletion will ultimately increase total fuel potential after lipid accumulation, especially if paired with bicarbonate enhancement of lipid accumulation. These technologies shall be investigated in an industrially relevant strain Nannochloropsis gaditana.*

(2) NMR based fluxomics for metabolic pathway elucidation under bicarbonate supplementation – *High resolution-magic angle spinning (HR-MAS)* ^{1}H *nuclear magnetic resonance (NMR) coupled with* $H^{13}CO_3^-$ *cellular labeling strategies can allow for real time tracking of metabolic information in live algal cells. Implementation of this technique on algal cells cultured using bicarbonate will elucidate fundamental questions regarding lipid synthesis and carbon metabolism in Chlorella vulgaris.*

Background

Since the dawn of the industrial revolution in the 1750's, global carbon dioxide (CO_2) concentration has risen approximately 43% (USEPA 2016), which is primarily due

to combustion of fossil fuels. The Kyoto Protocol (1997) has largely promoted the reduction of CO_2 and other greenhouse gas (GHG) emissions and emphasized research for replacement energy sources (Sydney, Sturm et al. 2010, Ho, Chen et al. 2011). Yet, in 2014, fossil fuel combustion in the United States accounted for a staggering 93.7% of CO_2 (USEPA 2016). Transportation related emissions accounted for 33.4 % of these emission, which is of primary concern here, because the majority of these emissions are from direct combustion of petroleum in transportation related activities (*e.g.* driving, flying, and shipping). These activities will be more difficult to replace with alternative energy sources which directly create electricity (*e.g.* solar and wind) and will need sustainable alternative replacements in the future. Moreover, the United States Department of Energy is prioritizing the reduction of U.S. dependence on oil. From 2005 to 2012 our reliance on imported fuels decreased from 60% to 40%, with domestic oil production on the rise, but also, partly due to expanded use of renewable fuels (USDOE 2013). Biofuels have received attention for the production of renewable alternatives to petroleum derived transportation fuels (Hill, Nelson et al. 2006, Brennan and Owende 2010). In large, the focus and actual development of biofuels in the past has been on ‘first-generation’ biofuels, which are those produced from crop plants (*i.e.* corn, beets, and oil seeds); however these have received criticism due to their influence on food commodity prices and their impact on valuable crop land (Williams and Laurens 2010). Advanced biofuels or ‘second-generation’ biofuels, are those from agriculture or forestry residues and non-food crops, and are becoming the primary focus for biofuel production because they have potential to alleviate many of the problems associated with first-

generation biofuels (IAE 2008). Microalgae are a renewable source of non-food crop biomass with potential to significantly contribute to the demand for second-generation biofuels. Furthermore, depending on the conversion process applied to the biomass, some consider a fraction of biofuels created from algae by to fall under another category of 'third-generation' biofuels. These 3rd generation biofuels are those which are produced into a drop-in ready fuel (those which can be directly utilized with current engine technology), such as fatty acid methyl esters (FAMEs) created through the transesterification of algal oils.

Algae as a Biomass Alternative

Algae is a broad term, which historically has been used to define anything that grows in water and performs photosynthesis. Further distinctions have been made to categorize these organisms into macroalgae, commonly known as seaweed (multicellular organisms), and microalgae. Many microalgae are single celled, oxygenic phototrophic eukaryotes which are ubiquitous in natural environments. Like other phototrophs, microalgae require light energy, water, CO_2, and a few inorganic nutrients (*e.g.* nitrogen, phosphorus, iron, etc.) which they use to produce biomass with diverse biochemical compositions (Markou, Vandamme et al. 2014). Whilst other options exist for second/third-generation biofuel production from terrestrial biomass, there are several advantages to utilizing microalgae for biofuel production. Advantages of utilizing microalgae for the production of biofuels are: (i) they have increased theoretical photosynthetic efficiency (10-12%) over terrestrial plants (4-6%) and high cell division

rates (1-3 day^{-1}) which leads to overall improved biomass yield per unit area; (ii) they are able to be cultivated continuously year-round further improving their productivity over terrestrial plants; (iii) they have higher lipid content (20-50% wt., >80% wt.) than terrestrial plants (<5% wt.); (iv) they can be grown with brackish, salt, and wastewater sources reducing their demand for freshwater, and in general require less-water than terrestrial plants; (v) they can utilize nitrogen and phosphorus from agricultural, industrial, and municipal wastewaters as low-cost nutrient sources and as a method for remediation of the wastewater; (vi) they can be grown on marginal or non-arable lands reducing the direct competition with land for food crops; and (vii) they can be coupled to CO_2 mitigation/sequestration for production of CO_2 neutral biofuels by utilizing CO_2 emitted from point-sources such as coal or natural gas fired power plants or industrial steel and iron factories (Chisti 2008, Hu, Sommerfeld et al. 2008, Schenk, Thomas-Hall et al. 2008, Amin 2009, Brennan and Owende 2010, Bhateria and Dhaka 2015). These advantages have been **estimated** to decrease the land requirement to replace the current U.S. transportation fuel demand from 61% (terrestrial plants) to only 3% (microalgae) (Chisti 2008).

The conceptualization of algal biofuel production is generally presented in the form of a bio-refinery, whereby the steps necessary for start-to finish production of biofuels are integrated in one location. Similar to a conventional petroleum refinery, a bio-refinery would produce biofuels, secondary co-products and energy from algal biomass. An operation such as this would have a variety of processes, but the main sequences are as follows: cultivation of algae for biomass generation, harvesting of algal

biomass, and extraction/conversion of algal biomass. Significant life cycle analyses and techno-economic analyses will be needed in regard to each step of this process to determine the most advantageous strategy for each phase of the operation (Lardon, Hélias et al. 2009, Davis, Aden et al. 2011).

Cultivation of Algae for Biomass

Primary cultivation systems for the production of algal biomass are through the use of raceway ponds or photobioreactors (Chisti 2007). Raceway ponds are typically large closed-loop ponds, in which microalgal culture is continuously circulated through a designed path, generally with the use of revolving paddle wheels (Chisti 2007). Raceway ponds can suffer from large evaporative losses, poor mass transfer properties for the application of CO_2, and the risk of contamination (Terry and Raymond 1985), but are relatively low-cost to build and operate. Conversely, photobioreactors are collections of small to medium diameter (< 10cm) transparent tubes which are all oriented parallel to each other and usually stacked vertically to increase the reactor volume in a given footprint (Chisti 2007). Primary concerns regarding the use of photobioreactors are the design limitations on tube length, which is dependent on the degree of O_2 production, CO_2 depletion, and pH variation (Brennan and Owende 2010). While photobioreactors can cost more to build, they typically offer a more controlled environment and higher productivity than open raceway ponds (Sheehan, Dunahay et al. 1998).

Harvesting of Algal Biomass

Even dense cultures of microalgae require removal of excess water for downstream processing to some extent, and cost-effective and energy-efficient processes for the removal of water and subsequent harvesting of algal biomass are required for economical production of algal biofuels (Schenk, Thomas-Hall et al. 2008). Methods of microscreening, filtration, centrifugation, and flocculation with sedimentation have been investigated. Microscreening or filtration have been limited in application to larger cells and filamentous strains of algae, and has suffered at large-scale operations from problems such as membrane clogging and high maintenance costs. Commercial centrifuges, especially those with continuous operation ability such as a decanting centrifuges, have been used to increase solids content to that of an algal paste (100-200g·L^{-1}), but further dewatering is considered to cost- and energy-intensive for centrifugation to be utilized as the primary harvesting approach. Flocculation with sedimentation has also been considered as a method for pre-concentrating algal biomass. Recent developments have been focused on promoting self-flocculation in algal cultures, usually as a response to environmental stimuli such as, nitrogen depletion or pH shifts. Flocculation with sedimentation is an attractive process due to its low-cost, but will be limited by time restrictions for large-scale processes and the reliability of flocculation. It is likely that combinations of these processes will be utilized for harvesting and concentration of algal biomass to a higher solids content slurry (~20% wt. solids). Further removal of residual water would require the use of existing processes such as spray-drying, freeze-drying, or thermal-drying for downstream processes requiring completely dried algal biomass.

Extraction and Conversion of Algal Biomass

Conversion of algal biomass can be accomplished through one of two methods, thermochemical conversion or biochemical conversion (Amin 2009, USDOE 2013). In the biochemical conversion pathway, microalgal oils are converted to biodiesel through the process of transesterification. Microalgal oils are primary divided into two categories. The neutral, or extractable, non-membrane bound lipids, and the membrane bound, most commonly phospholipids, which make up cell and organelle membranes. While both can be converted to biofuel, the neutral lipids are of particular interest because they are much easier to extract. The mono-, di-, and triacylglycerol (MAG, DAG, TAG) neutral lipid compounds which are used for carbon storage in microalgae can be readily converted to biodiesel through the process of transesterification. Through the use of heat and an acid or base catalyst, the alkoxy group of ester compound is replaced with an alcohol group producing fatty acid methyl esters (FAME's) and glycerol. These FAME's are crude biodiesel and are similar in composition to those produced from transesterification of vegetable oils. Another biochemical conversion pathway is fermentation of algal slurry to produce ethanol. Fermentation of algae is a less common method for energy production, mostly because of the energy intensive process of separating produced ethanol after the fermentation process, as well as, the relatively low starch content of microalgae compared with other biomass.

As an alternative to biochemical conversion pathways, thermochemical conversion pathways are being heavily researched for their application to microalgal biomass (Biller and Ross 2011, Barreiro, Prins et al. 2013, Li, Liu et al. 2014). Of the

many different techniques for thermochemical conversion, hydrothermal liquefaction and pyrolysis are emerging as the two benchmarks. Although, gasification and hydrogenation will likely play smaller roles in utilizing all products from the conversion process. Hydrothermal liquefaction is a process which uses sub-critical water at moderate temperature and pressure (~300 °C and 10 MPa) to convert wet biomass into liquid fuel, primarily oil. This oil can be separated and purified using organic solvents. Other products from hydrothermal liquefaction, such as the aqueous and gas phases, can be recycled to supply nutrients back for successive algae cultivation, and used in a gasification or hydrogenation processes to produce more products. Pyrolysis is another thermochemical conversion process to produce energy rich compounds from algal biomass such as biofuel, charcoal and gaseous products. Short residence times and high temperatures (500 °C) are used to crack biomass into short chain molecules which can then be rapidly cooled into a liquid phase to produce biofuel. Substrate preparation for pyrolysis does have a high energy input due to the drying requirement for algal biomass. There is still no general consensus on whether biochemical or thermochemical conversion processes will be exclusive solutions, or perhaps a combination of both; however, life cycle analyses and techno-economic analyses considering the entire production of algae to biofuels are being completed with considerations for multiple types of conversion methods. These will assist in determining the proper application, depending on the final use of the product, providing insights to the outcomes for different variables such as cultivation application, location, and scale or needs of the producer.

High pH and Alkaline Systems for Growth of Microalgae

Microalgae are capable of fixing inorganic carbon from both CO_2 and bicarbonate (HCO_3^-) (Kumar, Ergas et al. 2010, Sydney, Sturm et al. 2010, Raven, Beardall et al. 2014). Despite this, the growth and physiology of algae can be affected to different extents by the speciation and abundance of dissolved inorganic carbon (DIC) (Uusitalo 1996, Chen, Zhang et al. 2016). Therefore, it is critical to have an understanding of pH and carbonate alkalinity, and how these change with, and affect, algal growth.

Carbon is mainly absorbed by photosynthetic organisms in the inorganic form of CO_2 but, since microalgae are generally cultured in liquid environments, CO_2 must be dissolved in the aquatic environment. The reaction of CO_2 with water forms a weak acid-base buffer system with the following equilibrium.

$$CO_{2(g)} \overset{1}{\Leftrightarrow} CO_{2(aq)} + H_2O \overset{2}{\Leftrightarrow} H_2CO_3^* \overset{3}{\Leftrightarrow} HCO_3^- + H^+ \overset{4}{\Leftrightarrow} CO_3^{2-} + 2H^+ \qquad \text{(Eq. 1.1)}$$

Reaction 1 is limited by mass transfer effects from temperature, partial pressure of $CO_{2(g)}$, surface area at the air-liquid interface, and concentration of DIC. H_2CO_3* refers to the grouping of carbonic acid, H_2CO_3, and dissolved $CO_{2(aq)}$, although dissolved CO_2 is typically a small fraction of H_2CO_3*. Reactions 3 and 4 are mediated by acid-base chemistry and related to the pH of the solution (Markou, Vandamme et al. 2014), though the two may not be independent of each other (Keymer, Lant et al. 2014). Reaction 2 is the rate limiting step, with significant energy required for the conversion of aqueous CO_2 to carbonic acid. Carbon uptake by microalgae is performed either passively, through diffusion, or actively through transport enzymes for CO_2 and HCO_3^- (Moroney and Ynalvez 2007). Some algae species have developed sophisticated methods for increasing

the levels of intracellular inorganic carbon to several times that of exogenous concentrations (Giordano, Beardall et al. 2005, Raven, Beardall et al. 2014). This is primarily achieved through the use of enzyme symporters and carbonic anhydrases (CA). Carbonic anhydrases catalyze the conversion of HCO_3^- to CO_2, which is ultimately the singular substrate for inorganic carbon fixation by ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) in the Calvin cycle (Markou, Vandamme et al. 2014), as shown below.

$$HCO_3^- \overset{CA}{\Leftrightarrow} CO_2 + OH^- \qquad \text{(Eq. 1.2)}$$

This reaction ultimately drives up the pH due to the release of OH^- from cells due to the necessity to maintain electroneutrality inside the cell (Chen, Zhang et al. 2016), although as shown in Eq. 1.2, this reaction can be reversibly catalyzed by CA as well.

As was pointed out by Keymer, Lant et al. (2014), the pH can be predicted by the Henderson-Hasselbach equation as shown below:

$$pH = pKa_{c1} + \log_{10}\left(\frac{[HCO_3^-]}{[H_2CO_3^*]}\right) \qquad \text{(Eq. 1.3)}$$

$$pH = pKa_{c2} + \log_{10}\left(\frac{[CO_3^{2-}]}{[HCO_3^-]}\right) \qquad \text{(Eq. 1.4)}$$

Here, pKa_{c1} and pKa_{c2} are acid-base equilibrium constants for the bicarbonate-carbonate buffer system, and are temperature dependent. It is therefore observable that the pH will influence the relative concentrations of these ions as pH changes. Hence, the pH and total inorganic carbon speciation in algal systems are not totally independent of each other, and temperature and algal growth add further complexity to the system.

In recent years, knowledge of these complex systems has been leveraged by researcher's by utilizing both high pH and high concentrations of bicarbonate to induce lipid accumulation in selected microalgal species with tolerance to high pH and alkaline systems (Gardner, Peters et al. 2010, Gardner, Cooksey et al. 2012, Gardner, Lohman et al. 2013, Gardner, Lohman et al. 2013, White, Pagarette et al. 2013, Lohman, Gardner et al. 2014, Moll, Gardner et al. 2014, Laurens, Cooksey et al. 2015, Pancha, Chokshi et al. 2015). These works summarize a large contribution to the current understanding of the use of bicarbonate in microalgal culturing, with the majority of studies focusing on the effects under nutrient stressed conditions, resulting changes to biomass productivity, lipid, and carbohydrate content. The work described herein further this same field of research, including the study of *Nannochloropsis gaditana,* with increased inorganic carbon and bicarbonate supplementation, and a metabolic approach to further understand lipid accumulation in the freshwater organism, *Chlorella vulgaris.*

CHAPTER TWO

ASSESMENT OF *NANNOCHLOROPSIS GADITANA* GROWTH AND LIPID ACCUMULATION WITH ENHANCED INORGANIC CARBON DELIVERY

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Todd C. Pedersen

Contributions: Carried out growth, pH, chlorophyll, nutrient, and biomass harvest assays, conducted lipid extraction and analysis, participated in study design, study coordination, data analysis/interpretation and drafted the manuscript.

Co-Author: Brent M. Peyton

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

Co-Author: Robert D. Gardner

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

Co-Author: Robin Gerlach

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

Manuscript Information Page

Todd C. Pedersen, Robert D. Gardner, Robin Gerlach, Brent M. Peyton
Journal of Applied Phycology
Status of Manuscript:
_x__ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

Springer Netherlands

Abstract

Algal biomass refineries for sustainable transportation fuels, specifically biodiesel, will benefit from algal strain enhancements to improve biomass and lipid productivity. Specifically, the supply of inorganic carbon to microalgal cultures, which is a pre-requisite for growth, represents an area of great interest due to the potential for improved growth of microalgae and the possibility for incorporation with CO_2 mitigation processes. Combinations of bicarbonate (HCO_3^-) salt addition and application of CO_2 to control pH have shown compelling increases in growth rate and lipid productivity of the fresh water alga *Chlorella vulgaris*. Here, we focus on a marine organism, *Nannochloropsis gaditana* (CCMP 526), to investigate growth and lipid accumulation under various strategies of enhanced inorganic carbon supply.

Three gas application strategies were investigated: continuous sparging of atmospheric air, continuous sparging of 5% CO_2 during light hours until nitrogen depletion, and continuous sparging of atmospheric air with supplementation of 5% CO_2 for pH control between (8.0 to 8.3). These gas sparging schemes were combined with addition of low concentrations (5mM) of sodium bicarbonate at inoculation and high concentration (50mM) sodium bicarbonate amendments just prior to nitrogen depletion. The optimum scenario observed for growth of *N. gaditana* under these inorganic carbon conditions determined controlling pH with 5% CO_2 (v/v) on demand appeared to be sufficient carbon delivery to increase both growth rate and lipid accumulation. Total biodiesel potential (wt. % FAME) reached 40.3% in this condition. FAMEs were primarily comprised of C16:0 (palmitic) and C16:1 (palmitoleic) aliphatic chains.

Additionally, the use of high concentration (50mM) bicarbonate amendments was seen to further improve lipid content (up to 48.6%) under nitrogen deplete conditions when paired with pH controlled strategies.

Introduction

Non-renewable, fossil fuels have contributed to anthropogenic climate change and as a result, use of these energy sources should be significantly slowed to reduce the net emission of greenhouse gases. While electrical power generation from coal or natural gas power plants will likely be replaced with more renewable electrical technologies (*e.g.* solar, wind, nuclear), liquid transportation fuels will still be required for combustion engines used in shipping and aviation.

In 2015, the overall contribution from liquid biofuels as a whole to the US primary energy consumption was ~1% (Brennan and Owende 2010, Williams and Laurens 2010). Extensive use of fossil fuels in the transportation sector is contributing to carbon dioxide (CO_2) emissions which is causing detrimental impacts on the global environment including ocean acidification, rising sea-level, and increasing global temperatures (Yang and Gao 2003, Lam, Lee et al. 2012). Recently, large interest has been placed on the removal or capture of CO_2, either chemically or biologically, though it is generally agreed that enhanced biological processes will be the favored choice due to the high energetic and fiscal cost of chemical processes (Kumar, Ergas et al. 2010, Sydney, Sturm et al. 2010, Ho, Chen et al. 2011).

One appealing method of enhanced biological CO_2 fixation is the use of microalgae (Sawayama, Inoue et al. 1995, Sydney, Sturm et al. 2010). Temporary CO_2 sequestration in biomass is an attractive process because atmospheric carbon is recycled in an active cycle of biofuel production from biomass and subsequent combustion of that biomass, resulting in nominal additional CO_2 production (Kumar, Ergas et al. 2010, Lam, Lee et al. 2012) and the biological conversion of CO_2 has relatively low energy requirements. Biofuels produced from microalgal biomass have potential to contribute to the increasing global demand for liquid fuels (Chisti 2008, Davis, Aden et al. 2011). For biofuel production, microalgae have distinct advantages over terrestrial plants such as increased solar energy efficiency, higher potential as lipid feedstock, and feasible consumption of industrial CO_2 sources (Hu, Sommerfeld et al. 2008, Lardon, Hélias et al. 2009, Bhateria and Dhaka 2015). Similar to crop plant feedstocks, a large assortment of biofuels can be produced from microalgae, including biodiesel, bioethanol, methane, and jet fuel (Schenk, Thomas-Hall et al. 2008, Amin 2009). Primary requirements for growth of microalgal biomass include sunlight, water, inorganic carbon, and inorganic fertilizers (Markou, Vandamme et al. 2014). Next to sunlight and water, which are relatively available if an appropriate location for algal cultivation is chosen, inorganic carbon is the next most important substrate for algal growth. Microalgae are capable of fixing inorganic carbon from atmospheric CO_2, exhaust gases with increased CO_2 concentrations, and in solutions of soluble bicarbonate (Kumar, Ergas et al. 2010, Sydney, Sturm et al. 2010). This ability lends itself to various strategies for supplementing the necessary inorganic carbon, such as co-location with various flue gas

streams (Brennan and Owende 2010, Davis, Aden et al. 2011), or use of bicarbonate solutions produced from absorption of flue gas CO_2 (Chi, O'Fallon et al. 2011, Chi, Xie et al. 2013). Furthermore, microalgae can be grown on non-arable land and low quality water sources which can reduce production costs by 50%, if associated with the co-location of these CO_2 sources (Fields, Hise et al. 2014). In this fashion, microalgae would not compete with terrestrial crop plants for land use, but the use of nitrogen and phosphorus fertilizers will certainly have to be assessed to determine the impact of nutrient utilization from microalgae (Lardon, Hélias et al. 2009). Despite all of the advantages afforded by microalgae, relatively high consumption of energy, water, and nutrients has thus far made algal biofuels economically unfavorable (Williams and Laurens 2010, Markou, Vandamme et al. 2014).

Research focusing on upstream growth and production of algal biomass will be pivotal to the realization of algal biofuels. Strain selection has been highlighted as one of the main areas for improvement (Mata, Martins et al. 2010, Ahmad, Yasin et al. 2011), with specific focus placed on high lipid content strains (Griffiths and Harrison 2009). Optimization of species with industrial relevance, as well as screening of newly identified strains for favorable characteristics, make up a substantial portion of work being done in this field. One such industrially relevant strain is *Nannochloropsis gaditana.* Previous work on *N. gaditana* has provided useful insights for culturing characteristics with regards to enhancements in growth and lipid content. There is a general consensus that lipid accumulation is improved under nitrogen deplete conditions (Simionato, Block et al. 2013, Ren and Ogden 2014, Hallenbeck, Grogger et al. 2015), while other culturing

conditions have been reported to have different effects. One study revealed improved growth using 40mM Tris-HCl for pH control (Rocha, Garcia et al. 2003), indicating that pH controlled strategies may be beneficial, although the use of expensive organic buffers may not be realistic at industrial scale. Another study reported culture densities as high as 15.5 $g \cdot L^{-1}$ with the use of additional CO_2 supplementation (4.5%) under conditions of optimized light and culture inoculum (Hallenbeck, Grogger et al. 2015). Other researchers have demonstrated steady state continuous growth of *N. gaditana* with pH controlled strategies using CO_2 and obtained productivities of 0.42 $g \cdot L^{-1} \cdot d^{-1}$ (Camacho-Rodríguez, Cerón-García et al. 2015). Additionally, the genome of *N. gaditana* was recently published (Radakovits, Jinkerson et al. 2012). While the genome sequence was not used in this research, the work presented here could have potential to lend itself to future work exploiting genetic characterization of *N. gaditana* (*e.g.* transcriptomics or qPCR). Research on *N. gaditana* will likely be reinvigorated as advancements are made in the genetics and physiology of the alga. Other members of the *Nannochloropsis* genus have also been studied by Ma, Wang et al. (2014) who observed that *N. gaditana* obtained similar biomass productivity compared to their top performer, *Nannochloropsis oceanica* (IMET1), but *N. gaditana* demonstrated less overall lipid content, resulting in a lower value feedstock for biofuel production. To date, *N. gaditana* has not been reported to be the most outstanding species with respect to lipid productivity; however, it accurately reflects characteristics of Eustigmatophyceae, and is a model organism to study under conditions of enhanced carbon delivery.

Lipid accumulation under nitrogen deplete conditions has been studied with marine *Nannochloropsis* species for extended periods (Dong, Williams et al. 2013), and in other marine species, enhancements under increased carbon supplementation have been demonstrated (Guihéneuf and Stengel 2013). Additional work has shown compelling results for the use of bicarbonate salts in lipid promoting strategies (Gardner, Cooksey et al. 2012, Gardner, Lohman et al. 2013, White, Pagarette et al. 2013), and a recent study demonstrated enhanced biomass growth in the chlorophyte *Chlorella vulgaris* (Lohman, Gardner et al. 2015). These results have highlighted the need for additional research focused on the effects of enhanced carbon supplementation on the physiology of a larger portfolio of organisms, specifically when these delivery strategies are applied. In this study, inorganic carbon was provided to *N. gaditana* cultures through increased CO_2 concentrations in sparge gas, addition of bicarbonate (HCO_3^-) salt to the culture medium, and combinations of these methods. This served to obtain fundamental observations on the culturing characteristics of *N. gaditana.* Growth was monitored throughout nitrogen replete and deplete conditions, and lipid accumulation was monitored after nitrogen depletion.

Materials and Methods

Strain, Experimental, and Culturing Conditions

Nannochloropsis gaditana strain CCMP526 was obtained from the National Center for Marine Algae and Microbiota and was cultured in ASP_{II} medium (Provasoli, McLaughlin et al. 1957) using varied nitrogen concentrations to test nutrient sensitivity.

For each condition one of three fixed gas supply strategies were used: i) continuous sparge of atmospheric air; ii) continuous sparge of atmospheric air supplemented with 5% CO_2 during light hours until nitrogen depletion; and iii) continuous sparge of atmospheric air supplemented with 5% CO_2 as needed to maintain pH between 8.0 and 8.3. Bicarbonate additions were performed for some treatments and were accomplished in one of two ways: i) addition of 5mM $NaHCO_3$ initially with inoculation, or; ii) addition of 50mM $NaHCO_3$ amendment just prior to nitrogen depletion. The experimental conditions are outlined by their various combinations in Table 2.1.

Table 2.1. Experimental design for assessment of *N. gaditana* growth and lipid accumulation under various carbon application strategies.

	Air/Gas Delivery			**$NaHCO_3$ Amendments**	
	Continuous Atmospheric	Continuous 5% CO_2	pH Controlled (8.0 to 8.3)	Initial [5mM]	Near nitrogen depletion [50mM]
Air-1	x			N	N
Air-2	x			N	Y
Air-3	x			Y	Y
CO_2-1		x		N	N
CO_2-2			x	N	N
CO_2/HCO_3-1			x	N	Y
CO_2/HCO_3-2/3			x	Y	Y
CO_2/HCO_3-4		x		Y	N
CO_2/HCO_3-5		x		N	Y

While 12 conditions would have fulfilled the full factorial design with the fixed effects discussed above, here, a select few conditions are absent. Conditions which were omitted from the experimental design can partially be elicited from data of other conditions and combined for interpretive conclusions to be drawn.

Experiments were conducted in triplicate with the exception of the continuous air only control, which was run in duplicate after nitrogen depletion. Batch reactors consisting of 70 x 500 mm glass tubes containing 1 L of medium were temperature controlled at 23°C ± 1°C by submersion in circulated water baths. Banks of T5 tubes were used to provide light (approximately 250 µmol photons$\cdot m^{-2} \cdot s^{-1}$) maintained on a 14h:10h (L/D) cycle. In experimental conditions utilizing sodium bicarbonate, analytical grade $NaHCO_3$ was used (Fisher Chemical, USA). Sparge gas (0.4 L$\cdot min^{-1}$) was humidified by bubbling through Nalgene bottles containing ultrapure (18.2Ω) deionized water (diH_2O) water and controlled using individual rotameters for each condition with gas supply outlined as above.

Analysis of Culture and Media Components

Cell numbers were determined using an optical hemocytometer with a minimum of 400 cells counted per sample for statistical reliability. A standard benchtop pH meter was used to measure sample pH. Nitrate was measured using the colorimetric assay based on the Szechrome NAS reagent (Polysciences Inc., USA). For each sample, 20 µL of 0.2µm filtered culture was combined in a 96-well plate with 200 µL of Szechrome reagent and thoroughly mixed. After 30 minutes of incubation at room temperature the absorbance was read at 570 nm and nitrate concentrations were determined using a six-point calibration curve.

Chlorophyll Measurements

Chlorophylls and carotenoids were determined using a heat based methanol extraction method, modified from Ördög, Stirk et al. (2012). Briefly, 1 mL of culture was centrifuged at 6,000×g for 10 min and the supernatant separated. 1 mL of methanol was added to the remaining pellet and the extract was sonicated for 15 seconds. Immediately after, the mixture was exposed to 70°C for 5 min and then centrifuged again at the same conditions as above. Absorbance of the supernatant was read at 666, 653, and 470 nm. Chlorophyll calculations were carried out according to equations described by Ördög, Stirk et al. (2012).

Harvesting

Samples were collected just prior to nitrogen depletion and at the commencement of each experiment. For each sample, 50 mL of culture was centrifuged (Thermo Scientific, Sorvall Legend XTR, Waltham, MA) at 4,700 rpm (4,816×g) for 10 min at 4°C. Cultures were re-suspended in 35 mL of diH_2O to rinse the cultures of medium salts and bicarbonate. Samples were again centrifuged as before, the residual water removed, and promptly frozen. The resulting algal pellets were lyophilized (Labonco lyophilzer, Kansas City, MO) until remaining water was removed.

Transesterification for Fatty Acid
Methyl Ester (FAME) Analysis

Samples were transesterified using a previously described protocol (Lohman, Gardner et al. 2013) with minor modifications. Approximately 30 mg of lyophilized algal biomass was transferred to a 15-mL Pyrex test tube with a Teflon lined screw cap

(Kimble-Chase, Vineland, NJ). 1 mL of toluene and 2 mL of sodium methoxide (Fisher Scientific, Pittsburgh PA) were added to each sample along with 10 μL of a 10 mg·mL^{-1} standard mixture (C11:0 and C17:0 TAG) to monitor transesterification efficiency of the TAG into FAME. Samples were heated in an oven for 30 min at 90°C and vortexed every 10 min. After cooling to room temperature, 2 mL of 14% boron tri-fluoride in methanol (Sigma-Aldrich, St. Louis, MO) was added to each sample, and samples were heated for 30 mins at 90°C, vortexing every 10 min. Samples were again allowed to cool before 10 μL of a 10 mg·mL^{-1} of C23:0 FAME was added to assess the FAME partitioning into the organic phase. Additionally, 0.8 mL of hexane and 0.8 mL of a saturated salt water solution (NaCl in diH_2O) were added. To facilitate FAME partitioning, samples were heated for 10 min at 90°C, vortexed for 10 s and centrifuged at 1200×g for 2 min to enhance phase separation. 1 mL of the organic phase was removed from the top layer using a gas tight syringe and transferred to a 2-mL GC vial for gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS Analysis

GC–MS analysis was performed as previously described (Lohman, Gardner et al. 2013). Briefly, 1-μL split (2:1) injections were performed using an autosampler into a GC–MS (Agilent 6890 N GC and Agilent 5973 Networked MSD) equipped with a 30 m × 0.25 mm × 0.15 mm Agilent DB-23 column (0.25 μm phase thickness). The injector temperature was 240°C, and the detector temperature was 150 °C. The initial column temperature of 50°C was held for 1 min, increased to 175 °C at a rate of 25°C·min^{-1}, immediately followed by a ramp at 4°C·min^{-1} to a final temperature of 230°C which was

held for 10 min before termination of the run. The carrier gas was ultra-high purity helium and column flow was held at 0.5 mL·min^{-1}. FAME's were determined by quantifying each response peak with the nearest eluting calibration standard based on retention time, using MSD ChemStation software (Ver. D.02.00.275), with additional analyses performed using a custom MATLAB function programmed to align retention times of unknown peaks to standards, as well as discard peaks below the minimum quantification limit. A 28-component FAME standard prepared in methylene chloride (NLEA FAME mixture; Restek, Bellefonte, PA) was used for GC-MS retention time identification and response curve generation ($R^2 > 0.99$).

Calculations

Equation 2.1 was used to calculate specific growth (μ) rate during the time interval estimated for uninhibited exponential growth:

$$\mu = \frac{(\ln(X_2) - \ln(X_1))}{(t_2 - t_1)} \quad t_2 > t_1 \qquad \text{(Eq. 2.1)}$$

Biomass productivity (P) was calculated with Equation 2.2 over the duration of the experiment:

$$P = \frac{(X_2 - X_1)}{(t_2 - t_1)} \quad t_2 > t_1 \qquad \text{(Eq. 2.2)}$$

where X_1 and X_2 refer to biomass concentrations (cells·mL^{-1} or g·L^{-1}) at time points t_1 and t_2, respectively.

Statistics

Results are expressed as data means ± 95% confidence interval (CI) assuming a student's t-distribution (n=3) and were calculated using Excel 2013 and the CONFIDENCE.T function. Multiple comparison tests were performed using Minitab® (v 17.3.1) using two-sided confidence intervals with a 95% confidence level ($\alpha = 0.05$). Tukey comparison for statistical grouping was performed assuming equal variances with the error rate for comparisons set to 5. Data means that do not share a letter are significantly different.

Results and Discussion

Cell Growth and Culture Density for Different Carbon Supplementation Strategies

Increased inorganic carbon delivery (~2% CO_2) has been utilized for growth of the marine organism *N. gaditana* (Radakovits, Jinkerson et al. 2012, Ma, Wang et al. 2014), but a comprehensive study investigating various carbon delivery strategies and additional carbon supplementation (*i.e.* bicarbonate salts) is lacking. Additionally, recent findings using the freshwater microalga *C. vulgaris* demonstrated enhanced growth with low concentration bicarbonate supplementation (Lohman, Gardner et al. 2015). Therefore, experiments were conducted to test the efficacy of this method on a marine species. Initially, experiments were designed to investigate carbon delivery strategies of air, CO_2, and HCO_3^- supplementation, each individually, and characterize the resulting effects on cell concentration [cells·mL^{-1}], culture pH, CDW [g·L^{-1}], chlorophyll content [mg·L^{-1}], and biomass productivity [g·L^{-1}·d^{-1}]. Nitrate was also monitored for timing of

bicarbonate amendment in conditions investigating this carbon delivery method. To investigate the use of atmospheric air for gas delivery, three conditions were investigated: (Air-1), cultures sparged with atmospheric air only; (Air-2,) cultures sparged with atmospheric air and supplemented with 50mM $NaHCO_3$ just prior to nitrogen depletion; and (Air-3), cultures sparged with atmospheric air, supplemented with 5mM $NaHCO_3$ at inoculation, and additionally supplemented with 50mM $NaHCO_3$ just prior to nitrogen depletion. Culture and environmental data from *N. gaditana* cultures grown with atmospheric air supply can be seen in Figure 2.1 and Table 2.2 and 2.3.

Cultures sparged with atmospheric air only (Air-1 and Air-2) experienced a statistically different grouping (e) for growth rate (μ) when compared to cultures supplemented with an initial 5mM HCO_3 amendment (Air-3), which was grouped statistically different (c and d). The same result was not true of biomass productivity. Air-2 had the highest productivity (0.08 ± 0.04 $g \cdot L^{-1} \cdot d^{-}1$) of these conditions, while both Air-1 and Air-3 were slightly lower (0.06 ± 0.00 and 0.06 ± 0.01 $g \cdot L^{-1} \cdot d^{-1}$), but could not be placed in a different statistical grouping. All of these conditions achieved similar CDW when compared at nitrate depletion, and were grouped together at this point (a and b); however, when compared at harvest, Air-3 was grouped statistically different than Air-2 (a vs. b). Air-3 shared one statistical group with Air-1 (b), but a lower CDW was observed. The lengthy time requirement for growth with atmospheric air alone (~20 days) was not conducive to high productivity in a batch system. Maximum specific growth rate was improved with initial supplementation of bicarbonate, however, initial carbon supplementation is thought to have been consumed before late exponential growth and

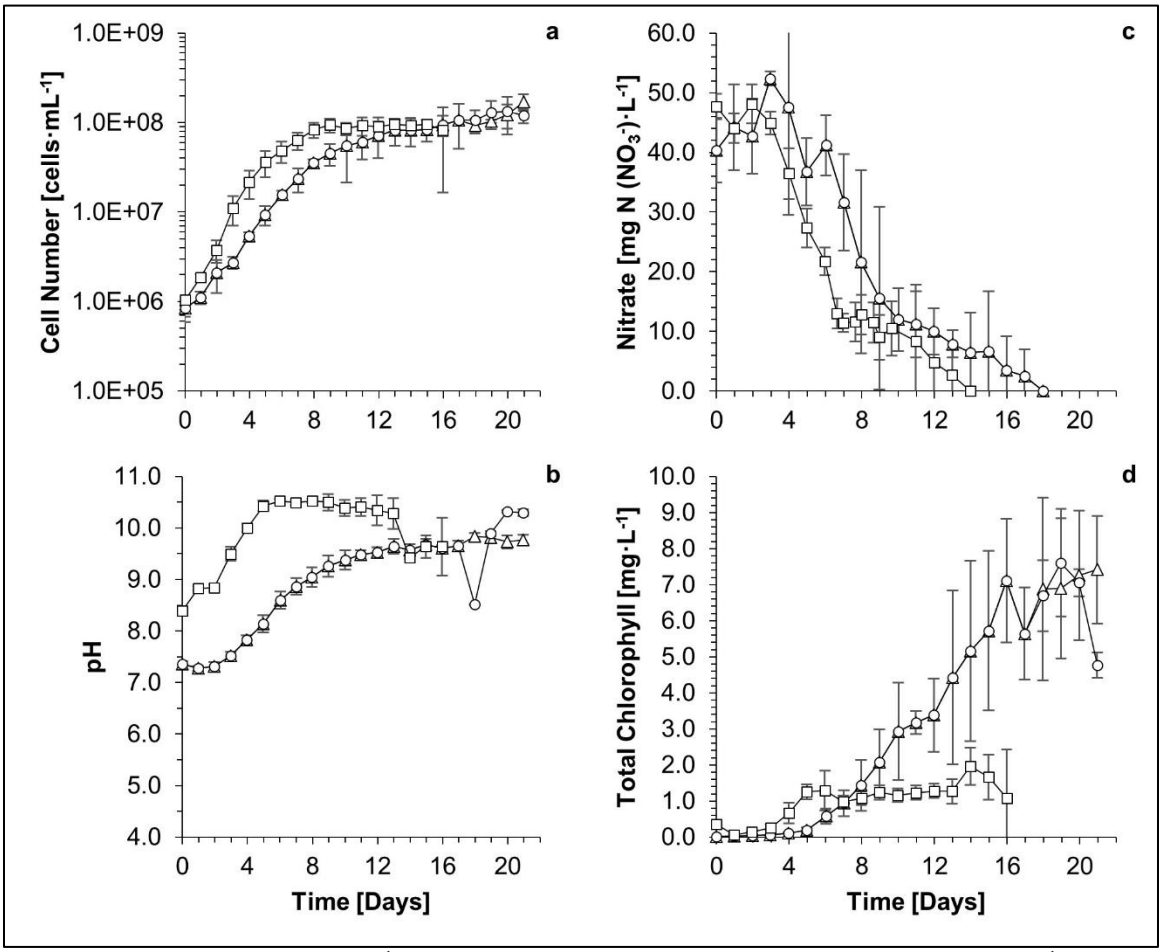

Figure 2.1. Growth [cells mL^{-1}] (a), pH (b), nitrate concentration [mg N (NO_3^-) L^{-1}] (c), and total chlorophyll [mg L^{-1}] (d) of cultures of *N. gaditana* grown using atmospheric air sparge. Air-1: Continuous sparge of atmospheric air (△). Air-2: Continuous sparge of atmospheric air and 50mM $NaHCO_3$ just prior to nitrogen depletion (○). Air-3: Continuous sparge of atmospheric air with 5mM initial $NaHCO_3$ and an additional 50mM $NaHCO_3$ just prior to nitrogen depletion (□). Time of nitrate depletion is indicated in Table 2.2. Error bars represent ± 95% CI (n=3).

cultures then became carbon limited. As with other conditions supplemented with atmospheric air only (0.04% CO_2), this possible carbon limitation slowed nitrogen consumption.

Table 2.2. *N. gaditana* growth and culturing characteristics when grown utilizing various carbon delivery methods.

Test	Aeration	Time of NO_3^- depletion [day]	Specific Growth Rate μ_{max} [day^{-1}]	Biomass Productivity [$g\ L^{-1}\ day^{-1}$; CDW[a]]
Air - 1	Air	18	0.42 ± 0.08^{e}	0.06 ± 0.01^{c}
Air - 2	Air → HCO_3^-	18	0.42 ± 0.08^{e}	$0.08 \pm 0.05^{b,c}$
Air - 3	Air + HCO_3^- → HCO_3^-	13	$0.56 \pm 0.09^{c,d}$	0.06 ± 0.02^{c}
CO_2 - 1	5% CO_2 → Air	14	$0.49 \pm 0.08^{d,e}$	0.07 ± 0.01^{c}
CO_2 - 2	Air + 5% CO_2 on demand (pH Controlled)	6.6	0.77 ± 0.03^{a}	0.13 ± 0.00^{a}
CO_2/HCO_3^- -1	pH Controlled → pH Controlled + 50mM HCO_3^-	10	$0.68 \pm 0.09^{a,b}$	$0.10 \pm 0.01^{a,b}$
CO_2/HCO_3^- - 2	pH Controlled + 5mM HCO_3^- → pH Controlled + 50mM HCO_3^-	10	$0.63 \pm 0.16^{b,c}$	$0.09 \pm 0.06^{b,c}$
CO_2/HCO_3^- - 3	pH Controlled + 5mM HCO_3^- → pH Controlled + 50mM HCO_3^-	6.6	$0.72 \pm 0.07^{a,b}$	0.13 ± 0.04^{a}
CO_2/HCO_3^- - 4	5% CO_2 + 5mM HCO_3^- → Air	7	0.67 ± 0.09^{b}	0.13 ± 0.01^{a}
CO_2/HCO_3^- - 5	5% CO_2 + → Air + 50mM HCO_3^-	7.6	$0.66 \pm 0.04^{b,c}$	$0.12 \pm 0.01^{a,b}$

All values reported as average ± 95% CI (n=3). All specific growth rates were calculated for uninhibited growth during exponential phase and all productivity values were calculated for the duration of the experiment (*i.e.* from inoculation until final harvest). Time of nitrate depletion is concomitant with 50mM bicarbonate amendments for those respective treatments as well as for CO_2 shut-off.
Cell Dry Weight (CDW) was determined gravimetrically from lyophilized biomass
Different superscripts indicate differences between the treatments (ANOVA, Tukey's range test, α=0.05)
Data means that do not share a letter are significantly different

Table 2.3. *N. gaditana* total chlorophyll content [mg L^{-1}] and cell dry weight (CDW) [g L^{-1}] at both nitrogen depletion and harvest (72 hours post depletion) when grown utilizing various carbon delivery methods.

Test	Total Chlorophyll [mg·L^{-1}]	Cell Dry Weight [g·L^{-1}]	Total Chlorophyll [mg·L^{-1}]	Cell Dry Weight [g·L^{-1}]
	Nitrate Depletion		Harvest	
Air - 1	6.70 ± 1.21^{a}	$0.75 \pm 0.18^{a,b}$	7.41 ± 2.11^{a}	$1.26 \pm 0.14^{a,b}$
Air - 2	6.70 ± 1.21^{a}	$0.75 \pm 0.18^{a,b}$	4.77 ± 0.43^{b}	1.62 ± 1.15^{a}
Air - 3	1.27 ± 0.42^{c}	$0.83 \pm 0.20^{a,b}$	1.08 ± 1.66^{d}	0.90 ± 0.36^{b}
CO_2 - 1	3.44 ± 1.95^{b}	-	$2.93 \pm 2.56^{b,c,d}$	$1.17 \pm 0.27^{a,b}$
CO_2 - 2	$4.78 \pm 0.60^{b,c}$	0.65 ± 0.07^{b}	$2.22 \pm 0.52^{c,d}$	$1.25 \pm 0.02^{a,b}$
CO_2/HCO_3 - 1	6.74 ± 2.72^{a}	0.93 ± 0.12^{a}	$4.04 \pm 2.63^{b,c}$	$1.36 \pm 0.16^{a,b}$
CO_2/HCO_3 - 2	6.12 ± 2.84^{a}	$0.80 \pm 0.44^{a,b}$	$2.45 \pm 4.23^{b,c,d}$	$1.11 \pm 0.81^{a,b}$
CO_2/HCO_3 - 3	4.35 ± 1.04^{b}	0.61 ± 0.09^{b}	$2.41 \pm 0.50^{c,d}$	$1.26 \pm 0.43^{a,b}$
CO_2/HCO_3 - 4	$3.11 \pm 1.11^{b,c}$	$0.76 \pm 0.10^{a,b}$	$2.22 \pm 0.26^{c,d}$	$1.28 \pm 0.13^{a,b}$
CO_2/HCO_3 - 5	3.21 ± 0.51^{b}	0.68 ± 0.16^{b}	1.68 ± 0.26^{d}	$1.22 \pm 0.07^{a,b}$

All values reported as average ± 95% CI (n=3)
Cell Dry Weight (CDW) was determined gravimetrically from lyophilized biomass
Different superscripts indicate differences between the treatments (ANOVA, Tukey's range test, α=0.05)
Data means that do not share a letter are significantly different

While atmospheric air was sufficient for growth, it was found that additional carbon delivery can improve growth rates, and in some cases productivity. Common strategies for increasing inorganic carbon delivery are supplementation with CO_2 in varying concentrations, either continuously or on demand (pH control), or supplementation with bicarbonate salts (Gardner, Cooksey et al. 2012, Gardner, Lohman et al. 2013, Pancha, Chokshi et al. 2015). To investigate carbon delivery strategies incorporating only CO_2 delivery, two conditions with a fixed concentration of CO_2 were investigated: (CO_2-1), cultures sparged with atmospheric air supplemented with 5% CO_2 (v/v) continuously during daylight hours until nitrogen depletion; and (CO_2-2), cultures sparged with atmospheric air continuously and supplemented with 5% CO_2 (v/v) as needed to control the pH between 8.0 and 8.3. Culture and environmental data from *N. gaditana* cultures grown with increased CO_2 supplementation are summarized in Figure 2.2 and Table 2.2 and 2.3.

Cultures supplemented continuously with CO_2 (CO_2-1) had a statistically different growth rate (d and e) relative to cultures supplemented with 5% CO_2 for pH control (CO_2-2), which were in group (a). The same result was true of biomass productivity, CO_2-1 averaging 0.07 ± 0.01 $g \cdot L^{-1} \cdot day^{-1}$ (c), while CO_2-2 averaged 0.13 ± 0.00 $g \cdot L^{-1} \cdot day^{-1}$ (a). Meanwhile, CDW for both cultures at harvest were not statistically different, with CO_2-1 and CO_2-2 being grouped together at harvest. However, the increase in CDW between nitrogen depletion and harvest (72 hours) for CO_2-2 was from 0.65 ± 0.07 $g \cdot L^{-1}$ to 1.25 ± 0.02 $g \cdot L^{-1}$, indicating that pH control after nitrogen depletion enhanced biomass generation.

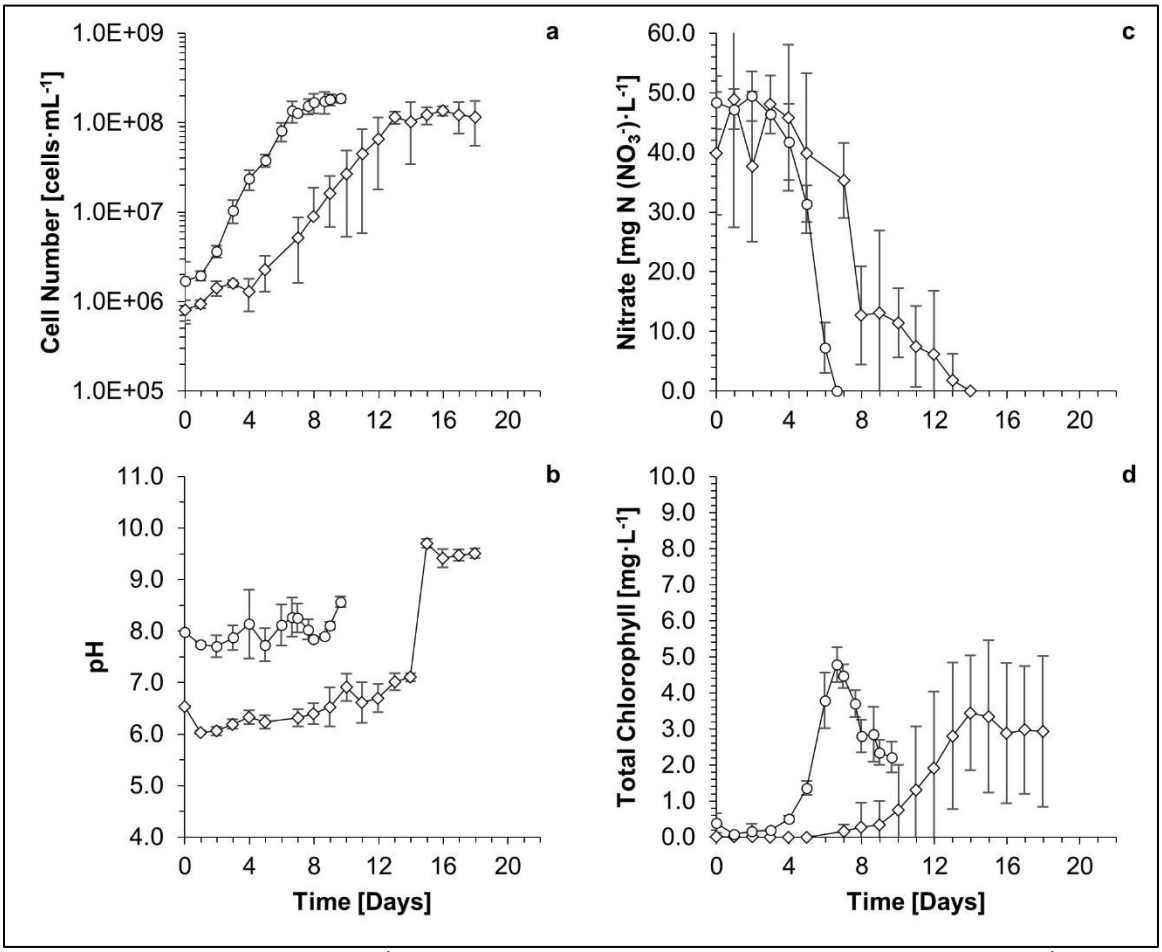

Figure 2.2. Growth [cells mL^{-1}] (a), pH (b), nitrate concentration [mg N (NO_3^-) L^{-1}] (c), and total chlorophyll [mg L^{-1}] (d) of cultures of *N. gaditana* cultured with CO_2. CO_2-1: Continuous sparge of atmospheric air supplemented with 5% CO_2 (v/v) during daylight hours until nitrogen depletion (◇). CO_2-2: Continuous sparge of atmospheric air supplemented periodically with 5% CO_2 (v/v) to control pH (8.0 to 8.3) (○). Time of nitrate depletion is indicated in Table 2.2. Error bars represent ± 95% CI (n=3).

Building on the first set of experiments, combinations of carbon delivery strategies were tested in an effort to further improve growth rate and productivity. Four combinations of enhanced carbon delivery were tested and are outlined in Table 2.1; results are presented in Figure 2.3 and Table 2.2 and 2.3. The conditions of CO_2/HCO_3-2 and CO_2/HCO_3-3 were the same, however they were run independently to replicate experimental conditions. Of the four treatments tested, all of them shared at least one

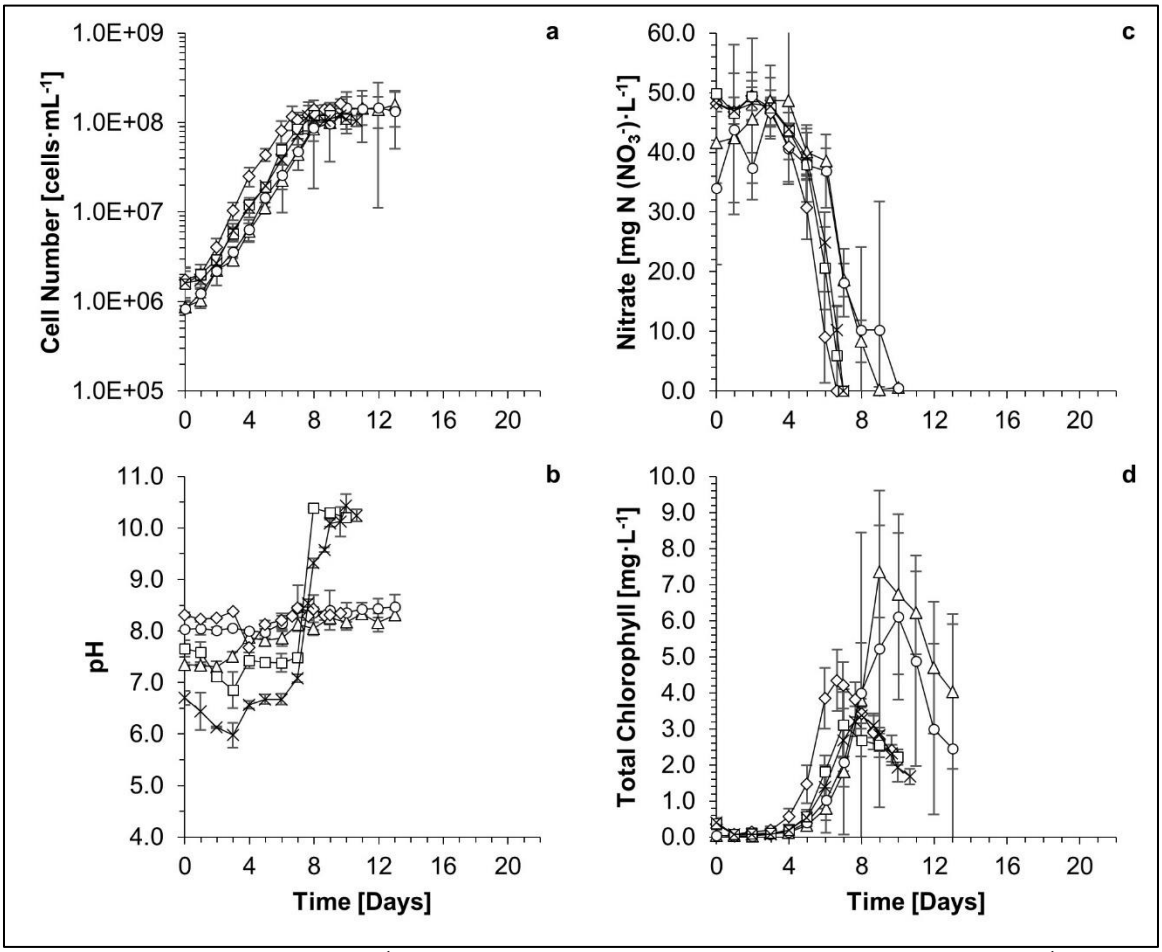

Figure 2.3. Growth [cells mL^{-1}] (a), pH (b), nitrate concentration [mg N (NO_3^-) L^{-1}] (c), and total chlorophyll [mg L^{-1}] (d) of cultures of *N. gaditana* cultured with CO_2 and bicarbonate. CO_2/HCO_3-1: pH control (8.0 to 8.3) and supplemented with 50mM $NaHCO_3$ just prior to nitrogen depletion (△). CO_2/HCO_3-2 and -3: pH control (8.0 to 8.3) with 5mM initial $NaHCO_3$ and an additional 50mM $NaHCO_3$ just prior to nitrogen depletion (○ and ◇). CO_2/HCO_3-4: 5% CO_2 (v/v) during daylight hours until nitrogen depletion and supplemented with 5mM initial $NaHCO_3$ (□). CO_2/HCO_3-5: 5% CO_2 (v/v) during daylight hours until nitrogen depletion and supplemented with 50mM $NaHCO_3$ just prior to nitrogen depletion (×). Error bars represent ± 95% CI (n=3).

statistical grouping (b) for growth rate. Further, conditions CO_2-2, CO_2/HCO_3-1 and CO_2/HCO_3-3 (grown with pH control) shared a statistical grouping (a), demonstrating that additional supplementation of bicarbonate at inoculum proved no better than pH controlled conditions alone for improving the growth rate. Final cell density of cultures at

harvest shared statistical groupings (a and b), but were not all similar at nitrate depletion. At nitrate depletion, CO_2/HCO_3-1, 2, and 4 shared a statistical grouping (a), while CO_2/HCO_3-2 and -4 also shared a different statistical grouping (b). These observations showed that in fact, CO_2/HCO_3-1 could only be shown to be statistically different from CO_2/HCO_3-3 and -5 at nitrate depletion. Biomass productivities were also observed to vary amongst the combination conditions. Statistical grouping of biomass productivity spanned three groups (a-c), with CO_2/HCO_3-3, and CO_2/HCO_3-4 grouping highest (a) with productivities of 0.13 ± 0.04, and 0.13 ± 0.01 $g \cdot L^{-1} \cdot day^{-1}$, respectively. CO_2/HCO_3-2 was the lowest productivity in these treatments, 0.09 ± 0.05 $g \cdot L^{-1} \cdot day^{-1}$, and was the only combination condition to share grouping (c). Since final CDW at harvest could not be statistically separated amongst the top performing treatments (CO_2-2 and CO_2/HCO_3-1, -2, -3, -4, and -5) it is suggested that consideration be placed on improvements to growth rate to result in higher biomass generation at nutrient depletion. Treatments which utilized pH control demonstrated the most improvement to growth rate over carbon supplementation from air, thus, pH control should be sufficient by itself to improve the nutrient replete culturing of *N. gaditana.*

Total Chlorophyll
Accumulation and Degradation
<u>with Varying Carbon Supplementation</u>

Total chlorophyll concentration [$mg \cdot L^{-1}$] in all conditions was also monitored and is presented in Figures 2.1-2.3 and Table 2.3. Air-1 and Air-2, were grouped statistically different (a) at nitrate depletion when compared to Air-3 (c). Cell concentration and CDW in all three conditions reached similar amounts at nitrogen depletion, but

significant differences in pH were encountered between cultures supplemented with 5mM initial $NaHCO_3$ and those grown only with atmospheric air. Within the first 6 days of culturing Air-3, the pH had increased to almost 10.5 from photosynthetic driven pH rise, whereas Air-1 and Air-2 never increased past pH 10 before nitrogen depletion. Accordingly, after day 6, Air-3 did not continue to accumulate chlorophyll until nitrogen depletion as Air-1 and Air-2 did. The addition of 5mM $NaHCO_3$ at inoculation appears to be responsible for the initial pH increase in Air-3 due to the large portion of HCO_3^- in the inorganic carbon pool. Use of HCO_3^- as the primary carbon source results in a build-up of OH^- in the cell, which is neutralized by H^+ uptake (Chi, O'Fallon et al. 2011). Air-1 and Air-2 cultures received their carbon through the in-gassing of CO_2 from atmospheric air and thus the pH increase was smaller. This appeared to maintain cellular ability to accumulate chlorophyll whereas cultures in Air-3 seemingly halted additional accumulation of chlorophyll. Both cultures receiving bicarbonate amendment just prior to nitrogen depletion (Air-2 and Air-3) experienced decreases in chlorophyll concentrations between nitrate depletion and harvest. Cultures with no additional bicarbonate (Air-1) experienced an increase in chlorophyll concentration during this same period.

CO_2 gas sparging was effective at inducing chlorophyll accumulation, with CO_2-1 reaching 3.44 ± 1.59 $mg \cdot L^{-1}$ at nitrogen depletion and CO_2-2 reaching 4.78 ± 0.49 $mg \cdot L^{-1}$ at nitrogen depletion. While these shared a statistical grouping (b), there appeared to be improvements to chlorophyll content through pH control, being that this strategy accumulated more chlorophyll in less time than observed with continuous CO_2 supply (CO_2-1). Alternatively, there was statistical decrease in total chlorophyll at harvest when

compared to nitrogen depletion for CO_2-2, from 4.78 ± 0.49 mg·L^{-1} to 2.22 ± 0.52 mg·L^{-1}. As with Air-2 and -3, the presence of increased inorganic carbon under nitrogen deplete conditions caused a degradation of chlorophyll.

Total chlorophyll content at nitrate depletion in cultures from the combination conditions varied, likely owing to the variation in CDW at this point. Interestingly, CO_2/HCO_3-4 only accumulated 3.11 ± 0.91 mg·L^{-1}, and was the only treatment from the combination conditions to be included in the lowest statistical grouping (c) at nitrate depletion. This test implemented 5mM initial $NaHCO_3$ and continuous sparge of CO_2 during light hours. As with Air-3, it appears here that the addition of initial $NaHCO_3$ may have had unfavorable effects on the accumulation of chlorophyll under nitrogen replete conditions. However, CO_2/HCO_3-4 was also the only combination condition to not receive additional carbon supplementation post nitrate depletion, and correspondingly, experienced the least extent of chlorophyll degradation, from only 3.11 ± 0.91 mg·L^{-1} to 2.22 ± 0.26 mg·L^{-1}. All other combination conditions appeared to suffer more consequential losses to chlorophyll content.

Fatty Acid Methyl Ester (FAME) Content

Both bicarbonate addition after nitrogen depletion or continued pH control with CO_2 after nitrogen depletion led to increased fatty acid content in *N. gaditana*, but the method of carbon supplementation may significantly impact lipid accumulation. Table 2.4 shows FAME profiles at nitrogen depletion for all combinations of treatments, while Table 2.5 shows FAME profiles at harvest (72 hours post nitrogen depletion). In all experiments of this study, cultures grown under pH controlled CO_2 sparging schemes

demonstrated the highest lipid content at harvest, reaching similar levels whether supplemented with additional bicarbonate after nitrogen depletion or not. All results in Table 2.4 and 2.5 are reported on a weight FAME per weight dry biomass percentage basis after direct transesterification. As increased growth rates were observed in conditions supplemented with additional inorganic carbon under nitrogen replete conditions, increased lipid accumulation was observed in these same cultures during nitrogen deplete conditions. Meanwhile, cultures grown with atmospheric air only generally experienced no change in lipid content under nitrogen deplete conditions, whether supplemented with additional bicarbonate or not. Cultures grown with atmospheric air only (Air-1 and Air-2) or even with an initial 5mM $NaHCO_3$ supplementation (Air-3), did not perform well as potential candidates for lipid accumulation. Air-1 and Air-2 accumulated 16.0% ± 2.1 % (b and c) FAMEs at nitrogen depletion while Air-3 accumulated 20.0% ± 3.1% (a, b, and c). These values remained in the lower statistical groupings at harvest, supporting that cultures grown without additional carbon supplementation resulted in lower lipid accumulation. The pH of the medium in these conditions remained high after nitrogen depletion (pH>10). These high pH conditions observed in atmospheric air cultures were likely responsible for the lower lipid accumulation in these treatments.

For cultures grown with CO_2 supplemented air, lipid accumulation was greater for pH controlled conditions (CO_2-2) as opposed to continuous supply of CO_2 (CO_2-1). At nitrogen depletion, the lipid content for CO_2-2 was relatively low, 15.7% ± 1.5% (b and c), yet it dramatically improved at harvest, reaching 40.3% ± 2.4% (a and b). Cultures

Table 2.4. *N. gaditana* FAME profiles for cultures grown under various carbon application strategies at nitrogen depletion when grown under different carbon delivery methods.

Treatment	% FAME					Total biodiesel potential (%)
	C16:0	C16:1	C18:1	C20:5	Residual	
Air - 1	$4.1 \pm 0.6^{c,d}$	$4.9 \pm 0.6^{b,c}$	$1.5 \pm 0.2^{a,b}$	3.8 ± 0.6^{d}	$1.7 \pm 0.2^{b,c}$	$16.0 \pm 2.1^{b,c}$
Air - 2	$4.1 \pm 0.6^{c,d}$	$4.9 \pm 0.6^{b,c}$	$1.5 \pm 0.2^{a,b}$	3.8 ± 0.6^{d}	$1.7 \pm 0.2^{b,c}$	$16.0 \pm 2.1^{b,c}$
Air -3	$6.3 \pm 1.0^{b,c,d}$	$5.7 \pm 1.0^{a,b,c}$	2.2 ± 0.2^{a}	3.1 ± 0.4^{e}	$2.6 \pm 0.5^{a,b}$	$20.0 \pm 3.1^{a,b,c}$
CO_2 - 1	-	-	-	-	-	-
CO_2 - 2	$4.4 \pm 0.7^{c,d}$	$3.9 \pm 0.3^{b,c}$	0.1 ± 0.6^{c}	5.6 ± 0.3^{a}	$1.6 \pm 0.3^{b,c}$	$15.7 \pm 1.5^{b,c}$
CO_2/HCO_3^- - 1	10.5 ± 2.2^{a}	8.6 ± 1.9^{a}	$1.5 \pm 0.5^{a,b}$	$5.3 \pm 0.6^{a,b}$	2.8 ± 0.8^{a}	28.6 ± 6.1^{a}
CO_2/HCO_3^- - 2	$8.9 \pm 9.2^{a,b}$	$7.3 \pm 7.4^{a,b}$	$1.1 \pm 2.3^{b,c}$	$4.8 \pm 0.7^{b,c}$	$2.5 \pm 1.8^{a,b}$	$24.6 \pm 21.3^{a,b}$
CO_2/HCO_3^- - 3	3.5 ± 2.6^{d}	3.3 ± 2.0^{c}	0.1 ± 0.6^{c}	$5.4 \pm 0.5^{a,b}$	1.3 ± 1.2^{c}	13.6 ± 6.7^{c}
CO_2/HCO_3^- - 4	$8.0 \pm 3.9^{a,b,c}$	$6.7 \pm 3.3^{a,b,c}$	$0.5 \pm 0.2^{b,c}$	$4.4 \pm 0.4^{c,d}$	$1.7 \pm 1.0^{b,c}$	$21.2 \pm 8.8^{a,b,c}$
CO_2/HCO_3^- - 5	$5.6 \pm 2.3^{b,c,d}$	$5.1 \pm 1.8^{b,c}$	0.3 ± 0.6^{c}	4.6 ± 0.6^{c}	1.5 ± 0.8^{c}	$17.1 \pm 5.1^{b,c}$

All values reported as average ± 95% CI (n=3).
Values reported as weight % [g FAME/g biomass]
Total FAMEs (sum of all quantified FAMEs) [wt. % FAMEs]
Different superscripts indicate differences between the treatments (ANOVA, Tukey's range test, α=0.05)
Data means that do not share a letter are significantly different

Table 2.5. *N. gaditana* FAME profiles for cultures grown under various carbon application strategies at harvest (72 hours post nitrogen depletion).

Treatment	% FAME					Total biodiesel potential (%)
	C16:0	C16:1	C18:1	C20:5	Residual	
Air - 1	$5.5 \pm 3.3^{e,f}$	$5.7 \pm 2.3^{d,e}$	$2.2 \pm 1.2^{b,c}$	3.8 ± 0.4^{a}	$2.2 \pm 0.4^{b,c}$	$19.3 \pm 6.8^{d,e}$
Air - 2	$5.7 \pm 2.6^{e,f}$	5.5 ± 2.5^{e}	$1.8 \pm 1.0^{b,c}$	$2.7 \pm 0.7^{a,b,c}$	1.7 ± 0.8^{c}	17.4 ± 7.6^{e}
Air -3	4.8 ± 7.4^{f}	4.3 ± 6.9^{e}	1.1 ± 1.6^{c}	1.7 ± 2.3^{c}	1.5 ± 2.2^{c}	13.5 ± 20.4^{e}
CO_2 - 1	$10.6 \pm 8.1^{c,d,e}$	$8.7 \pm 6.5^{b,c,d,e}$	$2.1 \pm 1.6^{b,c}$	$2.3 \pm 0.6^{b,c}$	$2.5 \pm 1.3^{b,c}$	$26.1 \pm 17.0^{b,c,d,e}$
CO_2 - 2	$16.7 \pm 0.5^{a,b}$	$13.7 \pm 0.6^{a,b}$	$2.5 \pm 0.5^{b,c}$	3.8 ± 0.5^{a}	$3.6 \pm 0.4^{a,b}$	$40.3 \pm 2.4^{a,b}$
CO_2/HCO_3^- - 1	19.8 ± 3.2^{a}	16.6 ± 2.7^{a}	4.5 ± 0.8^{a}	3.6 ± 0.7^{a}	4.1 ± 1.0^{a}	48.6 ± 8.4^{a}
CO_2/HCO_3^- - 2	$16.0 \pm 5.5^{a,b,c}$	$12.7 \pm 6.0^{a,b}$	$3.1 \pm 2.8^{a,b}$	$2.7 \pm 1.7^{a,b,c}$	$3.5 \pm 0.4^{a,b}$	$38.0 \pm 16.4^{a,b}$
CO_2/HCO_3^- - 3	$14.5 \pm 4.5^{a,b,c,d}$	$12.3 \pm 4.5^{a,b,c}$	$2.1 \pm 1.5^{b,c}$	$3.4 \pm 0.8^{a,b}$	$3.2 \pm 1.1^{a,b}$	$35.5 \pm 12.2^{a,b,c}$
CO_2/HCO_3^- - 4	$9.0 \pm 1.7^{d,e,f}$	$7.3 \pm 1.7^{c,d,e}$	1.3 ± 0.2^{c}	$2.6 \pm 0.5^{a,b,c}$	1.7 ± 0.3^{c}	$21.9 \pm 4.5^{c,d,e}$
CO_2/HCO_3^- - 5	$13.5 \pm 4.7^{b,c,d}$	$11.3 \pm 4.4^{b,c,d}$	$2.2 \pm 1.5^{b,c}$	$2.8 \pm 0.4^{a,b,c}$	$2.7 \pm 1.1^{b,c}$	$32.4 \pm 12.1^{b,c,d}$

All values reported as average ± 95% CI (n=3).
Values reported as weight % [g FAME/g biomass]
Total FAMEs (sum of all quantified FAMEs) [wt. % FAMEs]
Different superscripts indicate differences between the treatments (ANOVA, Tukey's range test, α=0.05)
Data means that do not share a letter are significantly different

grown continuously with CO_2 (CO_2-1) accumulated less lipid at harvest, and demonstrated wide variability, being included into four statistical groupings (b, c, d, and e), suggesting that the lack of carbon supplementation after nitrogen depletion had negative impacts on lipid accumulation. In both conditions, it was observed that fatty acid methyl ester chains of C16:0 (Palmitic) and C16:1 (Palmitoleic) were the dominant speciation; C18 (Octadecanoic) and C20 (Eicosanoic) unsaturated chains were present, albeit at substantially lower percentages.

Combinations of carbon delivery strategies (CO_2/HCO_3-1, -2, -3, -4, and -5) showed results in congruence with the findings from individual supplementation strategies. In all conditions receiving additional carbon supplementation after nitrogen depletion, a higher FAME content was observed, the highest observed being 48.6% ± 8.4% in CO_2/HCO_3-1. This treatment was the only one to be placed exclusively in the top statistical grouping (a). Other pH controlled conditions demonstrated similar improvements to that of CO_2/HCO_3-1 from bicarbonate amendments. CO_2/HCO_3-2 achieved a lipid content of 38.0% ± 16.4% at harvest (a and b), and CO_2/HCO_3-3 achieved 35.5% ± 12.2% at harvest (a, b, and c). Furthermore, CO_2/HCO_3-5 accumulated 32.4% ± 9.9% at harvest (b, c, and d), indicating that continuous carbon supply could be turned off at nitrogen depletion and replaced with a 50mM $NaHCO_3$ amendment. These significantly higher total FAME contents demonstrate that enhanced carbon supplementation during nitrogen deplete conditions, be it from CO_2 or HCO_3^-, is beneficial for fatty acid production in *N. gaditana.* While pH controlled conditions alone (CO_2-2) also induced accumulation of substantial total FAME, reaching 40.3% ± 2.4% (a

and b) content at harvest, suggesting that bicarbonate amendment in concert with nitrogen depletion may be a redundant form of carbon delivery, consider CO_2/HCO_3-4. This was the only combination condition that did not have additional carbon supplementation after nitrogen depletion, and accordingly showed an inappreciable increase in FAME content between nitrogen depletion and harvest. Meanwhile, CO_2/HCO_3-5 having 50mM $NaHCO_3$ present after nitrogen depletion, when CO_2 was shut-off, increased FAME content from 17.1% ± 5.1% at nitrogen depletion to 32.4% ± 12.1% at harvest. It is difficult to say with statistical certainty if the 50mM $NaHCO_3$ addition to CO_2/HCO_3-5 increased the FAME content, due to the multiple overlap in statistical groups of CO_2/HCO_3-4 at both nitrogen depletion and harvest; yet, CO_2/HCO_3-5 increased FAME content by an average of 15.3% during nitrogen deplete conditions, while CO_2/HCO_3-4 only increased FAME content by an average of 0.7% during the same time. Bicarbonate amendment at nitrogen depletion was shown to significantly increase lipid accumulation, and fatty acid chains of C16:0 and C16:1 were dominant, with C18 and C20 unsaturated chains also present, but at substantially lower percentages.

Within the limits of this experimental design, these data suggest that pH controlled sparging schemes encouraged high lipid productivity in *N. gaditana*, and the timely addition of bicarbonate may also be beneficial for improving lipid accumulation. The use of CO_2 for pH control maintains optimal growth of biomass during nitrogen replete conditions, but also supplies enough inorganic carbon for lipid accumulation once nitrogen deplete conditions are reached. These results show that given a large pool of inorganic carbon in concert with nitrogen depletion, substantial lipid accumulation was

observed. Additionally, bicarbonate salt supplementation is still largely appealing for improvements in lipid content under nitrogen deplete conditions, especially when no additional carbon is available during this period.

Conclusions

Cultures of *N. gaditana* were studied for various carbon supplementation strategies to provide direction for improvements with relation to growth and lipid accumulation. The enhanced carbon supplementation strategies studied here demonstrate ways to improve both growth and lipid accumulation in cultures of *N. gaditana* and may prove useful to the implementation of full scale biofuel production or integrated carbon capture processes.

Specific growth rate and culture density generally increased for cultures which received some form of increased inorganic carbon delivery from CO_2 (*i.e.* 5% CO_2 continuous or on demand), and 5mM initial $NaHCO_3$ additions showed potential for enhancements to cultures grown with atmospheric air. High pH conditions yielded unfavorable results and would have to be mitigated, otherwise culture productivity can be inhibited. In all conditions, CDW was observed to increase at harvest when compared to nitrogen depletion. Initial 5mM $NaHCO_3$ additions were not observed to be effective for CDW enhancements of *N. gaditana* cultures for treatments in which additional carbon was already supplemented during nitrogen replete conditions, and could possibly be detrimental to CDW when paired with 50mM $NaHCO_3$ amendments at nitrogen depletion.

Total chlorophyll accumulation was observed to be faster in pH controlled cultures during nitrogen replete conditions than continuous CO_2 supply or culturing with atmospheric air. Total chlorophyll was generally observed to reach peak concentration near nitrogen depletion, while increased inorganic carbon delivery during nitrogen deplete conditions was observed to induce chlorophyll degradation, which will certainly become a relevant consideration for the optimization of photosynthetic efficiency during lipid accumulation. Under nitrogen deplete conditions, elevated levels of inorganic carbon was recently shown to increase cellular C:N ratios for *N. salina* (Nunez and Quigg 2016). The decreases in chlorophyll observed here may be correlated to these researchers' findings, which suggest that as the carbon content in cells increases, the nitrogen content must remain relatively unchanged for increased C:N ratios to be observed. Under conditions where cellular nitrogen content is fixed, it is feasible that cells could be reallocating nitrogen from chlorophyll during nitrogen deplete conditions. Here, under nitrogen deplete conditions with elevated levels of inorganic carbon, it is thought that the increased uptake of carbon (*i.e.* increased C:N ratios) could have increased the rate of chlorophyll degradation.

Cultures with pH control largely experienced the most positive effects on lipid content both at harvest and nitrogen depletion, while continuous supply of CO_2 experienced marginally better FAME accumulation than cultures grown with atmospheric air. Enhanced carbon delivery to cultures, either in the form of CO_2 gas or HCO_3^- salts, was shown to have significant effects on growth and lipid accumulation for *N. gaditana.* The best growth scenario observed here was supplementation of 5% CO_2 on demand to

control pH. This improved both growth rate and chlorophyll accumulation when compared to cultures grown with atmospheric air or continuous CO_2 sparging. With bicarbonate addition, total biodiesel potential was observed as high as 48.6% ± 3.1%, which could be beneficial if production of liquid biofuels is the primary focus. However, due to its marine nature, *N. gaditana* may not be an ideal candidate for alkaline pH conditions, especially under nitrogen deplete conditions when increased inorganic carbon is present. As shown here, excess inorganic carbon at higher pH resulted in increased lipid accumulation, and appeared to correlate with substantial degradation of chlorophyll likely leading to decreased photosynthetic efficiency. While the relatively high abundance of C16:0 (palmitic) and C16:1(palmitoleic) fatty acid chains lend themselves toward biodiesel production, concurrent accumulation of C18:1 (vaccenic) and C20:5 (eicosapentaenoic-EPA) fatty acid chains could be favorable in nutraceutical applications. Future advancements will certainly have to be made regarding *N. gaditana* beyond the observations here, especially as its genome begins to provide more fundamental knowledge. The results reported here provide more structure to the dynamic field revolving around algal biofuels, with advancements being made concerning everything from growth and production parameters, to economic and environmental modeling.

Competing Interests

A patent entitled "Bicarbonate Trigger for Inducing Lipid Accumulation in Algal Systems" (Pat. No 9,096,875) was co-authored by contributing authors Robert D. Gardner and Brent M. Peyton.

Acknowledgements

A portion of this research was supported by the U.S. Department of Energy (DOE) Office of Energy Efficiency and Renewable Energy (EERE) Biomass Program under Contract No. DE-EE0005993. Support for TCP was also provided by Church and Dwight Co., Inc. Instrumental support was provided through the Environmental and Biofilm Mass Spectrometry Facility at the College of Engineering (COE), and the Center for Biofilm Engineering (CBE), at Montana State University (MSU). A special thanks all members of the MSU Algal Biofuels Group for their introspective discourse on algal biofuel related topics.

References

Ahmad, A. L., N. H. M. Yasin, C. J. C. Derek and J. K. Lim (2011). "Microalgae as a sustainable energy source for biodiesel production: A review." Renewable and Sustainable Energy Reviews **15**(1): 584-593.

Amin, S. (2009). "Review on biofuel oil and gas production processes from microalgae." Energy Conversion and Management **50**(7): 1834-1840.

Bhateria, R. and R. Dhaka (2015). "Algae as biofuel." Biofuels **5**(6): 607-631.

Brennan, L. and P. Owende (2010). "Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products." Renewable and Sustainable Energy Reviews **14**(2): 557-577.

Camacho-Rodríguez, J., M. C. Cerón-García, J. M. Fernández-Sevilla and E. Molina-Grima (2015). "The influence of culture conditions on biomass and high value product generation by Nannochloropsis gaditana in aquaculture." Algal Research **11**: 63-73.

Chi, Z., J. V. O'Fallon and S. Chen (2011). "Bicarbonate produced from carbon capture for algae culture." Trends in Biotechnology **29**(11): 537-541.

Chi, Z., Y. Xie, F. Elloy, Y. Zheng, Y. Hu and S. Chen (2013). "Bicarbonate-based Integrated Carbon Capture and Algae Production System with alkalihalophilic cyanobacterium." Bioresource Technology **133**: 513-521.

Chisti, Y. (2008). "Biodiesel from microalgae beats bioethanol." Trends in Biotechnology **26**(3): 126-131.

Davis, R., A. Aden and P. T. Pienkos (2011). "Techno-economic analysis of autotrophic microalgae for fuel production." Applied Energy **88**(10): 3524-3531.

Dong, H.-P., E. Williams, D.-Z. Wang, Z.-X. Xie, R.-C. Hsia, A. Jenck, R. Halden, J. Li, F. Chen and A. R. Place (2013). "Responses of Nannochloropsis oceanica IMET1 to Long-Term Nitrogen Starvation and Recovery." Plant physiology **162**(2): 1110.

Fields, M. W., A. Hise, E. J. Lohman, T. Bell, R. D. Gardner, L. Corredor, K. Moll, B. M. Peyton, G. W. Characklis and R. Gerlach (2014). "Sources and resources: importance of nutrients, resource allocation, and ecology in microalgal cultivation for lipid accumulation." Applied Microbiology and Biotechnology **98**(11): 4805-4816.

Gardner, R. D., K. E. Cooksey, F. Mus, R. Macur, K. Moll, E. Eustance, R. P. Carlson, R. Gerlach, M. W. Fields and B. M. Peyton (2012). "Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte Scenedesmus sp and the diatom Phaeodactylum tricornutum." Journal of Applied Phycology **24**(5): 1311-1320.

Gardner, R. D., E. Lohman, R. Gerlach, K. E. Cooksey and B. M. Peyton (2013). "Comparison of CO(2) and bicarbonate as inorganic carbon sources for triacylglycerol and starch accumulation in Chlamydomonas reinhardtii." Biotechnol Bioeng **110**(1): 87-96.

Gardner, R. D., E. J. Lohman, K. E. Cooksey, R. Gerlach and B. M. Peyton (2013). "Cellular Cycling, Carbon Utilization, and Photosynthetic Oxygen Production during Bicarbonate-Induced Triacylglycerol Accumulation in a Scenedesmus sp." Energies **6**(11): 6060-6076.

Griffiths, M. J. and S. T. L. Harrison (2009). "Lipid productivity as a key characteristic for choosing algal species for biodiesel production." Journal of Applied Phycology **21**(5): 493-507.

Guihéneuf, F. and D. B. Stengel (2013). "LC-PUFA-Enriched Oil Production by Microalgae: Accumulation of Lipid and Triacylglycerols Containing n-3 LC-PUFA Is Triggered by Nitrogen Limitation and Inorganic Carbon Availability in the Marine Haptophyte Pavlova lutheri." Marine Drugs **11**(11): 4246-4266.

Hallenbeck, P. C., M. Grogger, M. Mraz and D. Veverka (2015). "The use of Design of Experiments and Response Surface Methodology to optimize biomass and lipid production by the oleaginous marine green alga, Nannochloropsis gaditana in response to light intensity, inoculum size and CO2." Bioresource Technology **184**: 161-168.

Ho, S.-H., C.-Y. Chen, D.-J. Lee and J.-S. Chang (2011). "Perspectives on microalgal CO2-emission mitigation systems — A review." Biotechnology Advances **29**(2): 189-198.

Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert and A. Darzins (2008). "Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances." The Plant Journal **54**(4): 621-639.

Keymer, P. C., P. A. Lant and S. Pratt (2014). "Modelling microalgal activity as a function of inorganic carbon concentration: accounting for the impact of pH on the bicarbonate system." Journal of Applied Phycology **26**(3): 1343-1350.

Kumar, A., S. Ergas, X. Yuan, A. Sahu, Q. Zhang, J. Dewulf, F. X. Malcata and H. van Langenhove (2010). "Enhanced CO2 fixation and biofuel production via microalgae: recent developments and future directions." Trends in Biotechnology **28**(7): 371-380.

Lam, M. K., K. T. Lee and A. R. Mohamed (2012). "Current status and challenges on microalgae-based carbon capture." International Journal of Greenhouse Gas Control **10**: 456-469.

Lardon, L., A. Hélias, B. Sialve, J.-P. Steyer and O. Bernard (2009). "Life-cycle assessment of biodiesel production from microalgae." Environmental science & technology **43**(17): 6475.

Lohman, E. J., R. D. Gardner, L. Halverson, R. E. Macur, B. M. Peyton and R. Gerlach (2013). "An efficient and scalable extraction and quantification method for algal derived biofuel." J Microbiol Methods **94**(3): 235-244.

Lohman, E. J., R. D. Gardner, T. Pedersen, B. M. Peyton, K. E. Cooksey and R. Gerlach (2015). "Optimized inorganic carbon regime for enhanced growth and lipid accumulation in Chlorella vulgaris." Biotechnol Biofuels **8**: 82.

Ma, Y., Z. Wang, C. Yu, Y. Yin and G. Zhou (2014). "Evaluation of the potential of 9 Nannochloropsis strains for biodiesel production." Bioresour Technol **167**: 503-509.

Markou, G., D. Vandamme and K. Muylaert (2014). "Microalgal and cyanobacterial cultivation: the supply of nutrients." Water Res **65**: 186-202.

Mata, T. M., A. A. Martins and N. S. Caetano (2010). "Microalgae for biodiesel production and other applications: A review." Renewable and Sustainable Energy Reviews **14**(1): 217-232.

Nunez, M. and A. Quigg (2016). "Changes in growth and composition of the marine microalgae Phaeodactylum tricornutum and Nannochloropsis salina in response to changing sodium bicarbonate concentrations." Journal of Applied Phycology **28**(4): 2123-2138.

Ördög, V., W. Stirk, P. Bálint, J. Staden and C. Lovász (2012). "Changes in lipid, protein and pigment concentrations in nitrogen-stressed Chlorella minutissima cultures." Journal of Applied Phycology **24**(4): 907-914.

Pancha, I., K. Chokshi, T. Ghosh, C. Paliwal, R. Maurya and S. Mishra (2015). "Bicarbonate supplementation enhanced biofuel production potential as well as nutritional stress mitigation in the microalgae Scenedesmus sp. CCNM 1077." Bioresour Technol **193**: 315-323.

Provasoli, L., J. J. A. McLaughlin and M. R. Droop (1957). "The development of artificial media for marine algae." Archiv für Mikrobiologie **25**(4): 392-428.

Radakovits, R., R. E. Jinkerson, S. I. Fuerstenberg, H. Tae, R. E. Settlage, J. L. Boore and M. C. Posewitz (2012). "Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropis gaditana." Nat Commun **3**: 686.

Ren, M. and K. Ogden (2014). "Cultivation of Nannochloropsis gaditana on mixtures of nitrogen sources." Environmental Progress & Sustainable Energy **33**(2): 551-555.

Rocha, J. M., J. E. Garcia and M. H. Henriques (2003). "Growth aspects of the marine microalga Nannochloropsis gaditana." Biomol Eng **20**(4-6): 237-242.

Sawayama, S., S. Inoue, Y. Dote and S.-Y. Yokoyama (1995). "CO2 fixation and oil production through microalga." Energy Conversion and Management **36**(6–9): 729-731.

Schenk, P. M., S. R. Thomas-Hall, E. Stephens, U. C. Marx, J. H. Mussgnug, C. Posten, O. Kruse and B. Hankamer (2008). "Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production." BioEnergy Research **1**(1): 20-43.

Simionato, D., M. A. Block, N. La Rocca, J. Jouhet, E. Maréchal, G. Finazzi and T. Morosinotto (2013). "The Response of Nannochloropsis gaditana to Nitrogen Starvation Includes De Novo Biosynthesis of Triacylglycerols, a Decrease of Chloroplast Galactolipids, and Reorganization of the Photosynthetic Apparatus." Eukaryotic Cell **12**(5): 665-676.

Sydney, E. B., W. Sturm, J. C. de Carvalho, V. Thomaz-Soccol, C. Larroche, A. Pandey and C. R. Soccol (2010). "Potential carbon dioxide fixation by industrially important microalgae." Bioresource Technology **101**(15): 5892-5896.

White, D., A. Pagarette, P. Rooks and S. Ali (2013). "The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgae cultures." Journal of Applied Phycology **25**(1): 153-165.

Williams, P. J. l. B. and L. M. L. Laurens (2010). "Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics." Energy & Environmental Science **3**(5): 554.

Yang, Y. and K. Gao (2003). "Effects of CO2 concentrations on the freshwater microalgae, Chlamydomonas reinhardtii, Chlorella pyrenoidosa and Scenedesmus obliquus (Chlorophyta)." Journal of Applied Phycology **15**(5): 379-389.

CHAPTER THREE

MONITORING *CHLORELLA VULGARIS* METABOLISM DURING BICARBONATE INDUCED LIPID ACCUMULATION USING ^{1}H HIGH-RESOLUTION MAGIC ANGLE SPINNING (HR-MAS) NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Todd C. Pedersen

Contributions: Assisted in sampling of reactors and loading of samples in $NaH^{13}CO_3$ labeling experiments. Performed data analysis for molecular ion follow-up, and executed all physical work for growth comparison. Participated in study design, study coordination, data analysis/interpretation and drafted the manuscript.

Co-Author: Robin Gerlach

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

Co-Author: Brent M. Peyton

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

Co-Author: Gregory L. Helms

Contributions: Operated NMR instruments during $NaH^{13}CO_3$ labeling experiments, performed subsequent data analysis of NMR data, participated in data interpretation, and manuscript preparation.

Co-Author: Robert D. Gardner

Contributions: Assisted in sampling of reactors and loading of samples in $NaH^{13}CO_3$ labeling experiments. Collaborated on study design and data interpretation of molecular ion follow-up, as well as study design and data interpretation of growth comparison. Participated in study design, study coordination, data analysis/interpretation, and manuscript preparation.

Manuscript Information Page

Todd C. Pedersen, Robin Gerlach, Brent M. Peyton, Gregory L. Helms, Robert D. Gardner
Algal Research
Status of Manuscript: (Put an x in one of the options below)
_x__ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

Elsevier

Abstract

The use of bicarbonate (HCO_3^-) salts to stimulate lipid production in algal biomass is a well-documented phenomenon; however, the intrinsic characteristics of algal product accumulation and shifts in metabolism are relatively understudied. Fundamental research exploring the progression of inorganic carbon incorporation into algal biomass from bicarbonate illuminates the biogenesis of lipids after an exogenous stress is introduced (*e.g.* bicarbonate addition in concert with nitrogen depletion), and may reveal other substantial biochemical shifts. This work describes efforts to uncover these metabolic patterns in microalgae cultured in the presence of bicarbonate salts until near nitrogen depletion and subjected to sufficient bicarbonate concentrations (25mM) to arrest the cell cycle and induce lipid accumulation. To detect and quantitate metabolites in live algal cells, ^{1}H high resolution-magic angle spinning (HR-MAS) nuclear magnetic resonance (NMR) spectroscopy was utilized. Cultures of the chlorophyte *Chlorella vulgaris* (UTEX 395) were grown under a diel cycle (14:10 L/D) utilizing ^{12}C unlabeled or ^{13}C labeled $NaHCO_3$ as the sole inorganic carbon source and then subjected to an increased bicarbonate concentration concurrently with nitrogen depletion. NMR metabolite signals for sucrose, bulk lipid (CH_2), allylic, double allylic, and omega-3 metabolites were investigated over a period of 38 hours post bicarbonate addition, during which time cultures continued to experience the diel cycle. Using forward (^{13}C) and reverse (^{12}C) conditions, de novo lipid accumulation from bicarbonate as well as cellular recycling of carbon was observed. One significant finding from this research was the stabilization of algal metabolites during the dark cycle for cultures after bicarbonate

addition. These findings have implications for the accumulation and preservation of valuable lipid rich biomass during circadian growth.

Introduction

Rising concern over the social, environmental, and economic impacts associated with the continued use of petroleum fossil-fuels has prospered research for finding sustainable alternatives, with considerable focus placed on production of biofuels (Chisti 2008, Brennan and Owende 2010, Williams and Laurens 2010). Microalgae have received a proportionately large amount of interest due because of the higher areal biomass and lipid productivity they offer over terrestrial plants (Hu, Sommerfeld et al. 2008, Schenk, Thomas-Hall et al. 2008). Still, current technologies offered for production of biofuels from microalgae are hindered by high economic and environmental costs which are impeding the large-scale production of microalgal biodiesel (Lardon, Hélias et al. 2009, Davis, Aden et al. 2011). The selection of species and improvements to growth stages of the biofuel production process, with regard to both enhanced biomass and lipid productivity, have been recommended as the crucial areas for improvement moving forward (Griffiths and Harrison 2009).

A novel method for improving the lipid content in selected microalgal species has been developed in recent years for a variety of species via the use of high concentration bicarbonate to induce lipid accumulation in nitrogen starved microalgal cultures (Gardner, Cooksey et al. 2012, Gardner, Lohman et al. 2013, Gardner, Lohman et al. 2013). This method was further elaborated on by Lohman, Gardner et al. (2015), who

implemented the strategy to induce lipid accumulation in the green alga *Chlorella vulgaris*, after having demonstrated an enhanced carbon regime for improved growth of the organism. These studies generally recognize that, under nitrogen deplete conditions the addition of exogenous dissolved inorganic carbon (DIC) (*e.g.* $NaHCO_3$) induces a metabolic shift from biomass accumulation to lipid accumulation. A hypothesis for the initial events in microalgal triacylglycerol (TAG) synthesis under these conditions was recently proposed (Cooksey 2015), in which the author suggests that by the arresting of the cell cycle, neutral lipid accumulation is promoted. Cooksey also iterates the scarcity of information on algal intermediary metabolism, especially under conditions when new cell biomass and replication activity is shifted to lipid accumulation. However, he also suggests a common theme exists that medium nitrogen depletion precludes TAG accumulation in microalgae cells and furthermore, that TAG accumulation may be a sink for newly fixed carbon when the cell cycle is inhibited.

Primary metabolic investigations for microalgae under nitrogen deplete conditions have been completed previously with the use of transcriptomic investigations (Guarnieri, Nag et al. 2011, Valenzuela, Mazurie et al. 2012, Mus, Toussaint et al. 2013) and live monitoring with the use of nuclear magnetic resonance (NMR) spectroscopy (Davey, Hiscox et al. 2012, Wensel, Helms et al. 2014). Valenzuela, Mazurie et al. (2012) reported that carbon dioxide reduction was continued by *P. tricornutum* when population growth was arrested, and that phosphate depletion could be an initial trigger for lipid synthesis which could be magnified by nitrogen depletion. Mus, Toussaint et al. (2013) found similar results and further studied the enhancements to lipid accumulation provided

by increased inorganic carbon (15mM HCO_3^-), indicating the central metabolism pathways associated with bicarbonate transport and carbonic anhydrases were important in lipid accumulation. Mus, Toussaint et al. (2013) also suggested that re-purposing of phospholipids may play a role in lipid accumulation. These results are useful in understanding lipid synthesis in microalgae, particularly the diatom *P. tricornutum*, but lack quantitative monitoring of lipid synthesis in microalgae and specifically the question of *de novo* synthesis vs. carbon recycling in lipid accumulation. Meanwhile, Davey, Hiscox et al. (2012) developed a method to allow for non-invasive quantification of the TAG content within live cultures using ^{1}H-NMR which was further correlated to direct fatty acid methyl ester-gas chromatography (FAME-GC) analysis of algae lipids. Wensel, Helms et al. (2014) used this method to characterize lipid content in two oleaginous micro-algae in a two-stage cultivation scheme where lipid accumulation is induced in a secondary open-pond culture with high levels of $NaHCO_3$ and pH, and reported lipid content of 39.8%. Both quantitative investigations of lipid synthesis and fundamental transcriptomic investigations will be necessary to further answer questions of algal lipid synthesis, specifically with respect to how and when TAG synthesis is initiated, and to what extent de novo synthesis and recycled contributions play a role.

Here, *C. vulgaris* (UTEX 395) was selected as a model green-alga for the monitoring of lipid synthesis and accumulation under conditions of bicarbonate induced lipid accumulation. Experiments were designed to elucidate fundamental questions on to what extent TAG accumulation in microalgal cells is the result of *de novo* synthesis of neutral lipids from exogenous DIC sources and to what extent recycling of cellular

constituents accumulated prior to nitrogen depletion plays a role. Sodium bicarbonate ($NaHCO_3$) was used as the exclusive inorganic carbon source to grow cultures with either unlabeled (^{12}C) or labeled (^{13}C) biomass, and then subjected to high concentrations (25mM) of either labeled or unlabeled bicarbonate amendments just prior to depletion of nitrogen to induce lipid accumulation. Algal growth was monitored through traditional characterization techniques, while lipid accumulation and other carbon metabolism was monitored through the use of high-resolution magic angle spinning (HR-MAS) ^{1}H-NMR. Gas chromatography-mass spectrometry (GC-MS) was used to determine isotopomer patterns for FAMEs derived from neutral lipid extracts.

Materials and Methods

Experimental Conditions

In all experiments, cultures were grown until near nitrogen depletion, harvested via centrifugation at 6000xg at the end of their last nitrogen replete light cycle, re-suspended in new medium deplete of nitrogen but replete of all other constituents at the beginning of the 10-hour dark period, and finally re-introduced into a circadian light environment. For cultures grown with bicarbonate, 25mM $NaHCO_3$ was added just before the first light cycle after re-suspension. Two different ^{13}C labeling experiments were conducted. In the “forward” experiment, cultures were grown using naturally labeled bicarbonate ($H^{12}CO_3^-$) and amended with labeled bicarbonate ($H^{13}CO_3^-$). In the “reverse” experiment, cultures were grown using labeled bicarbonate and amended with naturally labeled bicarbonate. This allowed for the quantitative monitoring of *de novo*

lipid synthesis from exogenous ^{13}C sources in the forward experiment and from recycled pools of ^{13}C in the reverse experiment.

An additional study was performed to monitor growth, where conditions to that of the forward and reverse labeling experiments were replicated, but the use of ^{13}C was omitted. During these growth studies, a second condition investigating the use of CO_2 as an inorganic carbon substrate was also included.

Strain and Culturing Conditions

Chlorella vulgaris strain UTEX 395 was obtained from the University of Texas at Austin culture collection and was cultured on Bold's Basal Medium with $0.5 g \cdot L^{-1}$ $NaNO_3$ adjusted to pH 8.7 prior to autoclaving. For the characterization of growth of *C. vulgaris*, two different inorganic carbon sources were compared. Cultures supplemented with carbon dioxide (CO_2) as the inorganic carbon source were grown with humidified atmospheric air continuously supplied ($0.4 L \cdot min^{-1}$) and supplemented with 5% (v/v) CO_2 during daylight hours until the conclusion of the experiment. Cultures supplemented with bicarbonate (HCO_3^-) as the inorganic carbon source were grown with humidified supplemented gas supply (98% N_2 and 2% O_2) to minimize atmospheric CO_2 contributions to algal biomass, initially supplemented with 5mM $NaHCO_3$, and periodically monitored for DIC concentration to ensure cultures were not carbon limited, which was accomplished through additions of $NaHCO_3$ as necessary. HCO_3^- supplemented cultures were grown identically in the growth comparison and the labeling studies, with the exception of ^{13}C use as described above. Experiments were conducted in triplicate batch cultures containing 1.25L of medium illuminated (400 µmol photons·m$^-$

$^{2}\cdot s^{-1}$) by a light bank containing fluorescent tubes maintained on a 14:10 L/D cycle. Reactor design and temperature control was identical to a previous study (Lohman et al. 2015). $NaH^{13}CO_3$ (^{13}C, 99%) was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). All other $NaHCO_3$ used was ACS grade (Sigma-Aldrich).

Analysis of Culture and Media Components

Cell numbers were determined using an optical hemocytometer with a minimum of 400 cells counted per sample for statistical reliability. A standard benchtop pH meter was used to measure sample pH. Nitrate was measured using the colorimetric assay based on the Szechrome NAS reagent (Polysciences Inc., USA). For each sample, 20 µL of 0.2µm filtered culture was combined in a 96-well plate with 200 µL of Szechrome reagent and thoroughly mixed. After 30 minutes of incubation at room temperature the absorbance was read at 570 nm and nitrate concentrations were determined using a six-point calibration curve.

Dissolved Inorganic Carbon Analysis

Dissolved inorganic carbon (DIC, sum of dissolved carbon dioxide, bicarbonate, and carbonate) in the medium was measured using previously described protocols (Gardner, Lohman et al. 2013). Briefly, 8 mL of culture were filtered, 0.2 µm pore size, and the supernatant was analyzed using a Skalar Formacs TOC/TN analyzer. DIC concentrations were quantified by correlating peak area to a standard curve constructed from bicarbonate and carbonate mixtures (Sigma-Aldrich).

Chlorophyll Measurements

Chlorophylls and carotenoids were determined using a heat based methanol extraction described previously by Lohman, Gardner et al. (2015). Briefly, 1 mL of culture was centrifuged at 16,000×g for 5 min and the supernatant separated. 1 mL of methanol was added to the remaining pellet and the extract was sonicated for 15 seconds. The mixture was heated at 70°C for 5 min and re-centrifuged, again at the same conditions as above. Absorbance of the supernatant was read at 666, 653, and 470 nm. Chlorophyll and carotenoid ($mg \cdot L^{-1}$) calculations were carried out according to equations described by Ördög, Stirk et al. (2012).

Harvesting and Centrifugation

In each experiment, cultures were aseptically transferred from their growth medium to nitrogen deplete medium during the experiment. Algal cultures for each set of experimental conditions were mixed together in sterile Pyrex bottles prior to centrifugation to minimize variability of the replicates. The cells were pelleted (Thermo Scientific, Sorvall Legend XTR, Waltham, MA) at 6,000×g for 10 min at 4°C in a fixed angle rotor using sterile 250mL Nalgene bottles. The pelleted biomass was evenly distributed into three separate Nalgene bottles for each condition, which were re-suspended with fresh medium and mixed directly in corresponding reactors with additional fresh medium back to 1.25L.

Samples for cell dry weight were obtained for analysis over the 48-hour period following re-suspension. Sample were centrifuged (Thermo Scientific, Sorvall Legend XTR, Waltham, MA) at 4,700 rpm (4,816×g) for 10 min at 4°C. Pellets were re-

suspended in diH_2O to remove medium salts and excess bicarbonate. Samples were again centrifuged as before, the residual water decanted, and promptly frozen. Algae pellets were finally lyophilized (Labonco lyophilzer, Kansas City, MO) to remove residual water.

^{1}H HR-MAS NMR Spectroscopy

^{1}H HR-MAS NMR spectra were recorded as previously described (Davey et al. 2012) either with a Varian Inova 500 MHz spectrometer (499.84 MHz ^{1}H frequency) or a Varian VNMRS 600 MHz spectrometer (599.69 MHz ^{1}H frequency) using the acquisition parameters described in Davey, Hiscox et al. (2012). To achieve lock, 4µl of D_2O was added to a 30µl sample of algae culture, which was then placed into a 4mm glass microcell rotor (Varian-Agilent part number 0099259100). Water suppression was done using presaturation of the water signal with a 2.5 s irradiation using a 50 Hz B1 field. A spectral width of 8,000 Hz was used with an acquisition time of 1.5 s. A 90° read pulse was used and a total of 64 transients were signal averaged. The resulting FID was apodized with 0.2 Hz of exponential line broadening prior to Fourier Transformation.

Extraction of Lipids from Dry
Biomass Using Bead Beating

Extraction of neutral lipids were conducted as previously described by Lohman, Gardner et al. (2013). Approximately 30 mg of dried biomass was combined with 1 mL of chloroform in a 1.5-mL stainless steel microvial with a silicone cap (BioSpec Products, Bartlesville, OK). Three types of beads (0.6 g of 0.1 mm zirconium/silica beads, 0.4 g of 1.0 mm glass beads, two 2.7 mm glass beads) were added to each vial

before capping. A FastPrep bead beater (Thermo Savant, Carlsbad, CA) was used to agitate the vials for six 20 s pulses at power level 6.5 followed by a 1 min cool down period between pulses. Total bead beating time was 2 min. The mixture of solvent, residual biomass, and beads was then transferred to a 15-mL Pyrex test tube with a Teflon-lined screw cap (Kimble-Chase, Vineland, NJ), and the steel microvial was rinsed twice with 1 mL of chloroform, which was also added to the test tube, bringing the total solvent volume to 3mL. 1mL of 15 % NaCl (v/v) in water was added to enhance phase separation. Samples were then centrifuged at 1200×g for 2 min to separate the residual biomass. Of the organic phase, 0.5mL was removed from the bottom of the test tube using a gas tight syringe and transferred to a 15-mL Pyrex test tube, the chloroform was evaporated under a stream of nitrogen, and the residue used for transesterification of neutral lipid extracts for GC-MS investigation.

Transesterification for GC-MS Analysis

Samples were transesterified using a previously described protocol from Lohman, Gardner et al. (2013) with minor modifications. The neutral lipid extract residue from the previous section was transesterified as follows. 1 mL of toluene and 2 mL of sodium methoxide, (Fisher Scientific, Pittsburgh PA), were added to each sample along with 10 μL of a 10 $mg \cdot mL^{-1}$ standard mixture (C11:0 and C17:0 TAG) to monitor transesterification efficiency. Samples were heated in an oven for 30 min at 90°C and vortexed every 10 min. After cooling to room temperature, 2 mL of 14% boron tri-fluoride in methanol (Sigma-Aldrich, St. Louis, MO) was added to each sample, and samples were then heated for 30 mins at 90°C, vortexing every 10 min. Samples were

again allowed to cool before 10 μL of a 10 mg·mL^{-1} of C23:0 FAME was added to assess the completeness of FAME partitioning into the organic phase. Additionally, 0.8 mL of hexane and 0.8 mL of a saturated salt water solution (NaCl in diH_2O) were added. To facilitate FAME partitioning, samples were heated for 10 min at 90°C, vortexed for 10 s and centrifuged at 1200×g for 2 min to enhance phase separation. 1 mL of the organic phase was removed from the top layer using a glass syringe and transferred to a 2-mL GC vial for gas-chromatography mass-spectrometry (GC-MS) analysis.

GC-MS Analysis

GC–MS analysis was performed as previously described by Lohman, Gardner et al. (2013). Briefly, 1-μL split (2:1) injections were performed using an autosampler into a GC–MS (Agilent 6890 N GC and Agilent 5973 Networked MSD) equipped with a 30 m × 0.25 mm × 0.15 mm Agilent DB-23 column (0.25 μm phase thickness). The injector temperature was 240°C, and the detector temperature was 150 °C. The initial column temperature of 50°C was held for 1 min, increased to 175 °C at a rate of 25°C·min^{-1}, immediately followed by a ramp at 4°C·min^{-1} to a final temperature of 230°C, which was held for 10 min before termination of the run. The carrier gas was ultra-high purity helium and column flow was held at 0.5 mL·min^{-1}. FAMEs were identified by manual assignment of each chromatographic peak with the nearest eluting calibration standard based on retention time, using MSD ChemStation software (Ver. D.02.00.275). A 28-component FAME standard prepared in methylene chloride (NLEA FAME mixture; Restek, Bellefonte, PA) was used for GC-MS retention time identification.

Determination of isotopomer distribution of fatty acid chains was carried out as previously described by Jennings and Matthews (2005). Unlabeled mass spectra for known standards were extracted at peak total ion current (TIC) intensity from the chromatograms generated from the NLEA FAME mixture and exported for use in the molecular ion study. These mass spectra were used to generate matrices for ion intensities of the various isotopomers as described by Jennings and Matthews (2005), accounting for contributions from natural abundance ^{13}C to the M+1 and M+2 peaks. Mass spectra ion intensities from the isotopomer mixtures (*i.e.* transesterified samples) were also extracted at peak TIC intensity and used to solve for fractional abundances of each isotopomer in the mixture. C16:0, C18:2, and C18:3 fatty acid chains were investigated for isotopomer distributions using biomass from both the forward and reverse experiments.

Results and Discussion

Growth Characterization of *C. vulgaris* Using Different Inorganic Carbon Sources

A growth study was performed analogously to the ^{13}C-labeled carbon-amendment studies described later. The study provided physiological data for the culturing of *C. vulgaris* on two different inorganic carbon sources. Cultures were grown until near nitrogen depletion either in the presence of low concentrations (5mM) of $NaHCO_3$ or a 5% CO_2 gas sparge during the light hours (14:10 L/D). The use of $NaH^{13}CO_3$ was omitted here because NMR and MS studies were performed in the subsequent experiment. All cultures grown in these experiments were re-suspended in medium deplete of nitrogen and replete of all other constituents at the end of the last light cycle in

which they contained sufficient remaining nitrogen. Simultaneously, 25mM HCO_3^- was added to the cultures grown using $NaHCO_3$, while cultures grown on 5% CO_2 continued to receive 5% CO_2 during the light hours under nitrogen deplete conditions.

Figure 3.1 shows cell concentrations [cells·mL^{-1}], pH, nitrate concentrations [mg N (NO_3^-)·L^{-1}], and dissolved inorganic carbon [mmol C·L^{-1}] for cultures of *C. vulgaris* grown in the presence of either $NaHCO_3$ or CO_2 prior to re-suspension in nitrogen free-medium. Cultures grown with CO_2 grew faster (μ=1.37 day^{-1}) and reached nitrogen depletion earlier than cultures grown with HCO_3^- (μ=0.95 day^{-1}). Initially, both conditions were growing similarly until day 3. Afterwards, the CO_2 sparged cultures began to demonstrate faster growth compared to HCO_3^- cultures. Medium pH in the bicarbonate supplemented treatment increased during the light hours because cultures were not buffered against pH increases by the bicarbonate alone. The combination of these data provides insight to some of the underlying factors affecting the growth of the different conditions.

The increased pH observed in 5mM HCO_3^- cultures was enough to temporarily slow growth until the pH could be manually lowered. In an effort to keep performance of these cultures high, manual pH adjustments were performed during light hours when high pH values were observed. 1 N HCl was added incrementally and the pH was subsequently checked. Typical adjustments involved adding 1-4 mL of HCl to lower the pH from 9.4-10.0 to 8.2-8.6. Additionally, during these pH checks, the dissolved inorganic carbon concentration was checked and supplemental HCO_3^- was added to return concentrations to near 5mM. Carbon concentrations before $NaHCO_3$ additions

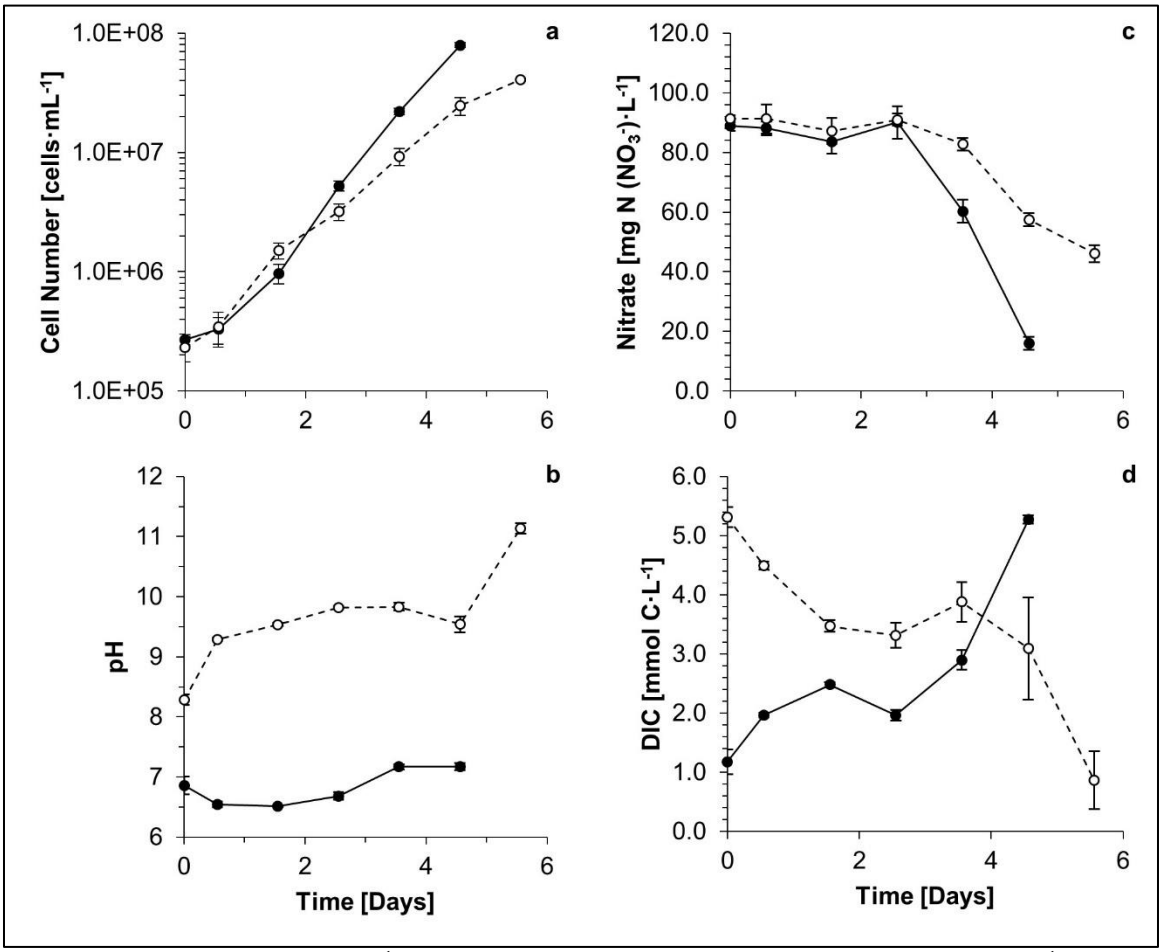

Figure 3.1. Growth [cells mL^{-1}] (a), pH (b), nitrate concentration [mg N (NO_3^-) L^{-1}] (c), and dissolved inorganic carbon (DIC) [mmol C L^{-1}] (d) of cultures of *C. vulgaris* grown in the presence of either CO_2 or $NaHCO_3$ under nitrogen replete conditions. Continuous sparge of atmospheric air supplemented with 5% CO_2 (v/v) during light hours (14:10) L/D (●) and continuous sparge of supplemented mixed gas (98% N_2 and 2% O_2) with 5mM $NaHCO_3$ (○). Cultures were re-suspended in nitrogen free medium at the conclusion of these growth studies, end of day 5 for 5% CO_2 and end of day 6 for 5mM $NaHCO_3$. Error bars represent ±95% CI (n=3).

were typically in the range of 1.5-3.0mM carbon, with additions bringing concentrations back to near 5mM. This maintained bicarbonate cultures such that they were not carbon limited. For cultures grown on CO_2, a slightly higher cell density was reached prior to re-suspension evidenced by the consumption of more nitrate before transfer to nitrogen free media.

The medium pH highlighted a substantial difference in environmental conditions of the two cultures. Cultures grown with a 5% CO_2 sparge remained at reasonably low pH values (6.5-7) during growth and never exceeded pH values of ~7.5. As cultures started to accumulate more cells and thus fix more carbon, the pH (measured at the end of every light cycle) slowly increased, until the cultures were re-suspended in new medium. For cultures grown with HCO_3^-, the pH was substantially higher, initially starting around 8.3 (as expected for a bicarbonate dominated system), and then increased to between pH 9.4-10.0 near the end of the light cycles. After the last light cycle before re-suspension a pH value of above 11 was observed, which was the result of limited pH adjustments during the final light cycle before re-suspension. Culture density was likely high enough to result in a pH increase that caused inhibition. Manual adjustments were not completed frequently enough to mitigate this effect. This appears to agree with the data shown in Figure 3.1c, where the nitrate consumption rate actually decreased, despite higher cell concentrations.

Nitrate consumption in CO_2 cultures occurred rapidly after the third day, until the end of day 5 when culture re-suspension commenced, just prior to nitrogen depletion. Bicarbonate cultures were re-suspended with substantial nitrogen still available in growth medium primarily due to appearance of a foam produced from algal cultures. Previous observations of *C. vulgaris* under high pH conditions have periodically revealed a substantial foam layer at the top of the tubular photobioreactors. This foam can ‘trap’ biomass at the top of the growth medium. To keep cultures from losing significant

amounts of biomass to the foam they were re-suspended well before nitrogen depletion and amended with 25mM $NaHCO_3$.

At the end of their last respective light cycles, cultures were harvested and re-suspended in nitrogen free medium replete of all others components. Growth parameters (cell concentration [cells·mL^{-1}], pH, and dissolved inorganic carbon [mmol C·L^{-1}]) were monitored under nitrogen replete conditions over a 48-hour period at light-dark transitions. In addition, total chlorophyll [mg·L^{-1}] and cell dry weight [g DW·L^{-1}] were also monitored for both growth schemes during this time, and the combined results are shown in Table 3.1. In the subsequent investigation under nitrogen deplete conditions, CO_2 cultures showed almost a full cell doubling in the first 10-hour dark period, after having shown negligible changes during the previous 14 hours of light. After another diel cycle, these cultures showed no additional signs of cell doublings. The HCO_3^- cultures did not experience additional cell divisions after bicarbonate amendment, a result which was expected as the cell cycle is typically reported to be arrested for bicarbonate induced lipid accumulation; however, in both growth scenarios, the trends for cell numbers did not necessarily reflect the observations for cell dry weight (CDW).

During the first light cycle after re-suspension, CDW weight increased for both conditions. Cultures supplemented with CO_2 increased from 0.73 ± 0.15 to 1.32 ± 0.03 g·L^{-1}, while cultures supplemented with 25mM $NaHCO_3$ increased from 0.42 ± 0.04 to 0.71 ± 0.04 g·L^{-1}. Both conditions experienced losses in CDW over the first successive dark cycle, and then again, increased biomass production in the second light cycle. Interestingly, the cultures supplemented with CO_2 lost a substantial amount of biomass

Table 3.1. Growth [cells mL^{-1}], pH, total chlorophyll [mg L^{-1}], dissolved inorganic carbon (DIC) [mmol C L^{-1}], and cell dry weight [g DW L^{-1}] of *C. vulgaris* cultures at light-dark transitions after nitrogen depletion over two successive diurnal cycles. Variation is represented as ± 95% CI (n=3). Circadian growth was carried out on a 14:10 L/D diel cycle with the initial light cycle starting at 0 hrs.

Cell number [cells·mL^{-1}]					
Condition	**0 hrs**	**14 hrs**	**24 hrs**	**38 hrs**	**48 hrs**
5% (v/v) CO_2 14:10 L/D	**1.76E+08 ± 3.09E+07**	**1.78E+08 ± 8.78E+06**	**3.14E+08 ± 7.70E+07**	**3.24E+08 ± 9.62E+07**	**2.90E+08 ± 4.98E+07**
98% N_2 2% O_2 with 25mM HCO_3^-	8.08E+07 ± 9.99E+06	8.26E+07 ± 2.94E+07	8.88E+07 ± 1.37E+07	8.96E+07 ± 1.55E+07	8.29E+07 ± 2.56E+07
pH					
Condition	**0 hrs**	**14 hrs**	**24 hrs**	**38 hrs**	**48 hrs**
5% (v/v) CO_2 14:10 L/D	6.67 ± 0.30	6.23 ± 0.05	7.22 ± 0.06	6.38 ± 0.13	6.98 ± 0.08
98% N_2 2% O_2 with 25mM HCO_3^-	8.66 ± 0.07	9.75 ± 0.04	9.70 ± 0.06	10.64 ± 0.06	10.42 ± 0.05
Total Chlorophyll [mg·L^{-1}]					
Condition	**0 hrs**	**14 hrs**	**24 hrs**	**38 hrs**	**48 hrs**
5% (v/v) CO_2 14:10 L/D	16.5 ± 3.2	16.6 ± 1.8	16.4 ± 0.7	16.5 ± 0.2	16.5 ± 0.7
98% N_2 2% O_2 with 25mM HCO_3^-	9.6 ± 0.3	9.4 ± 0.2	9.2 ± 1.8	8.8 ± 1.1	8.5 ± 1.7
Dissolved inorganic carbon [mmol C·L^{-1}]					
Condition	**0 hrs**	**14 hrs**	**24 hrs**	**38 hrs**	**48 hrs**
5% (v/v) CO_2 14:10 L/D	1.11 ± 0.40	1.69 ± 0.14	0.64 ± 0.00	0.13 ± 0.02	0.91 ± 0.03
98% N_2 2% O_2 with 25mM HCO_3^-	19.0 ± 1.0	25.3 ± 1.1	27.8 ± 2.5	19.8 ± 1.5	26.3 ± 0.8
Cell dry weight [g DW·L^{-1}]					
Condition	**0 hrs**	**14 hrs**	**24 hrs**	**38 hrs**	**48 hrs**
5% (v/v) CO_2 14:10 L/D	0.73 ± 0.15	1.32 ± 0.03	1.16 ± 0.06	1.42 ± 0.33	0.58 ± 0.13
98% N_2 2% O_2 with 25mM HCO_3^-	0.42 ± 0.04	0.71 ± 0.04	0.60 ± 0.08	0.80 ± 0.09	0.75 ± 0.12

over the second dark cycle, while 25mM $NaHCO_3$ supplemented cultures retained the majority of their biomass over the second dark cycle. Dissolved inorganic carbon and pH lend helpful insight to this observation.

During light hours when CO_2 was sparged, the pH decreased as CO_2 dissolved in the algal medium, but as off-gassing occurred during the dark hours, the pH increased again. Even though CO_2 cultures did not appear to be carbon limited during the first light cycle after re-suspension, after the second light cycle, DIC concentrations appeared to decrease to a point where they might not have been sufficient to satisfy the photosynthetic demand and where cultures were possibly carbon limited. It was perhaps this carbon limitation which caused cells to reallocate previously fixed carbon for cellular maintenance processes, and might have subsequently been responsible for the loss of biomass during the dark hours.

After nitrogen depletion, the inorganic carbon pool in the bicarbonate amended systems was substantially higher compared to the cultures sparged with 5% CO_2 during light hours. In cultures supplemented with additional HCO_3^-, an initial spike in DIC concentration after re-suspension was observed. Even though, the target of 25mM bicarbonate might not have been reached exactly, the bicarbonate addition was sufficient to arrest the cell cycle. Additional supplementation was provided during the first light cycle after re-suspension (10mM addition after the first 10 hours) to reach the appropriate level. During the second light cycle after re-suspension, a decrease in DIC concentrations was observed, indicating that cells indeed utilized bicarbonate during light hours.

Cultures sparged with 5% CO_2 reached total chlorophyll concentrations of over 16 mg·L^{-1} at the time of re-suspension and did not appear to degrade chlorophyll during nitrogen deplete conditions. Meanwhile, HCO_3^- supplemented cultures accumulated a chlorophyll content of slightly over 9 mg·L^{-1} at re-suspension. Slight degradation of chlorophyll was observed after nitrogen depletion, occurring steadily during light and dark cycles. While chlorophyll degradation was observed, there appeared to be sufficient chlorophyll for photosynthetic reactions, and with abundant carbon, the photosynthetic rate likely remained high under these conditions. Cultures sparged with 5% CO_2 mostly maintained chlorophyll content; however, with limited carbon supply they may have been unable to fix inorganic carbon at their peak potential. While chlorophyll concentrations do not directly correlate with rates of photosynthesis, they can provide an indication of culture health and overall photosynthetic potential.

^{1}H HR-MAS NMR
Monitoring of In Vivo Metabolites

Selected metabolites in *C. vulgaris* were monitored over the initial 38 hours after re-suspension in nitrogen deplete medium and simultaneous amendment with 25mM $NaHCO_3$. For these studies, cultures were grown identically to the growth study, with $NaHCO_3$ supplied as the exclusive carbon source. This was to ensure carbon incorporation in metabolites from recycled biomass and bicarbonate addition at nitrogen depletion could be distinguished through the selective use of $H^{13}CO_3^-$ without other interference, such as contributions from atmospheric CO_2. Following dark incubation after re-suspension, 25mM $NaHCO_3$ was supplemented, followed by the beginning of a

diel cycle (14:10 L/D), which was carried out over a 38-hour investigation. Samples were collected for NMR analysis at hours 0 (initial light), 1, 2, 4, 8, 14, 16, 24, 28, and 38. Transition between light to dark occurred at 14 hours and the transition back to light hours for the second light cycle occurred at 24 hours.

NMR signals from the forward and reverse study were quantified (μM C) to give carbon source contributions, characterized as *de novo* vs. recycled contributions, and discussed below. In figures showing quantified NMR data, the forward experiment was used to gain information on metabolites synthesized *de novo* from the labeled bicarbonate amendment supplied at nitrogen depletion. Meanwhile, the reverse experiment measurements were used to gain information from metabolites recycled after nitrogen depletion, which were synthesized from labeled bicarbonate supplied during exponential growth under nitrogen replete conditions. Figure 3.2 shows measurements determined from the chemical shift for bulk CH_2 during the time course in the diel investigation. These measurements are indicative of saturated fatty acid chains, and are distinguished from signals associated with allylic (monounsaturated fatty acids) and double allylic (polyunsaturated fatty acids), which have different characteristic chemical shifts and are discussed later.

Incorporation of labeled bicarbonate into saturated fatty acids in the forward experiment was observed at 8 hours, indicating that *do novo* synthesis of saturated lipid begun to occur at least after this, during the initial light cycle after bicarbonate amendment. This synthesis continued to occur between 8-14 hours, with increased carbon being *de novo* synthesized to saturated lipids over the remaining period of the light cycle.

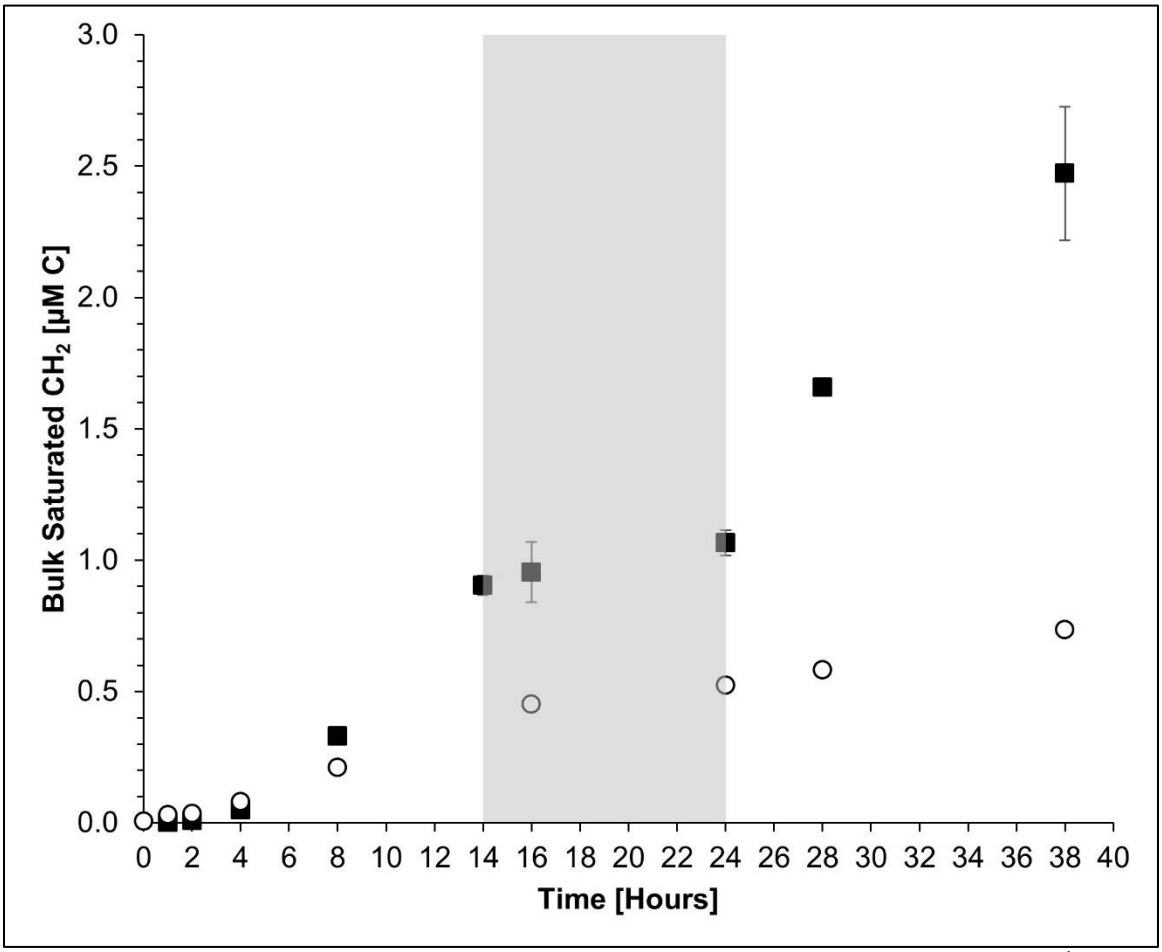

Figure 3.2. Saturated fatty acid chain accumulation monitored in *C. vulgaris* using ^{1}H HR-MAS NMR and $H^{13}CO_3^-$ labeling. Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.

Interestingly, during the dark cycle, *de novo* synthesis of saturated fatty acids remained relatively stagnant, both at 2 hours into the dark cycle (16 hours), and at the end of the dark cycle (24 hours). Over the second light cycle, contributions from *de novo* synthesis of saturated fatty acids further increased until the end of the light cycle, 38 hours after bicarbonate addition. It was also observed, that the recycled contributions to saturated lipids were not negligible.

Incorporation of recycled carbon was observed for saturated fatty acid synthesis after 8 hours, following a similar time line to *de novo* synthesis of these lipids. Incorporation of recycled carbon continued over the remaining light hours, but to a lesser extent than the *de novo* synthesis. Again, there appeared to be minor incorporation of recycled carbon into saturated fatty acids during the dark period, but curiously, there also appeared to be no degradation of these previously accumulated lipids. This same result was true of the *de novo* synthesized saturated fatty acids. This is in stark contrast to previous work, which has shown rapid accumulation of lipids during light cycles, followed by degradation of lipids during dark cycles (Bigelow, Hardin et al. 2011, Gardner, Cooksey et al. 2012).

Quantified NMR signals associated with the chemical shift indicative of double allylic groups (poly-unsaturated fatty acids – PUFAs) are shown in Figure 3.3. Results from these measurements displayed an opposite trend to that of saturates. *De novo* synthesis of PUFAs occurred after 8 hours, similar to the saturates; however, recycled contributions to PUFAs was observed after 4 hours, and recycling of carbon fixed during growth continued quickly after this until the end of the first light cycle. *De novo* synthesis did appear to continue after 8 hours until the end of the first light cycle, but as a smaller portion compared to recycled contributions. Dark cycle stabilization of PUFAs was observed for the *de novo* incorporation of carbon, but recycled contributions were seen to increase over the dark cycle. This accumulation of PUFAs from recycled carbon contributions increased throughout the dark cycle and continued into the second light cycle. During the second light cycle, *de novo* synthesis of PUFAs resumed and was

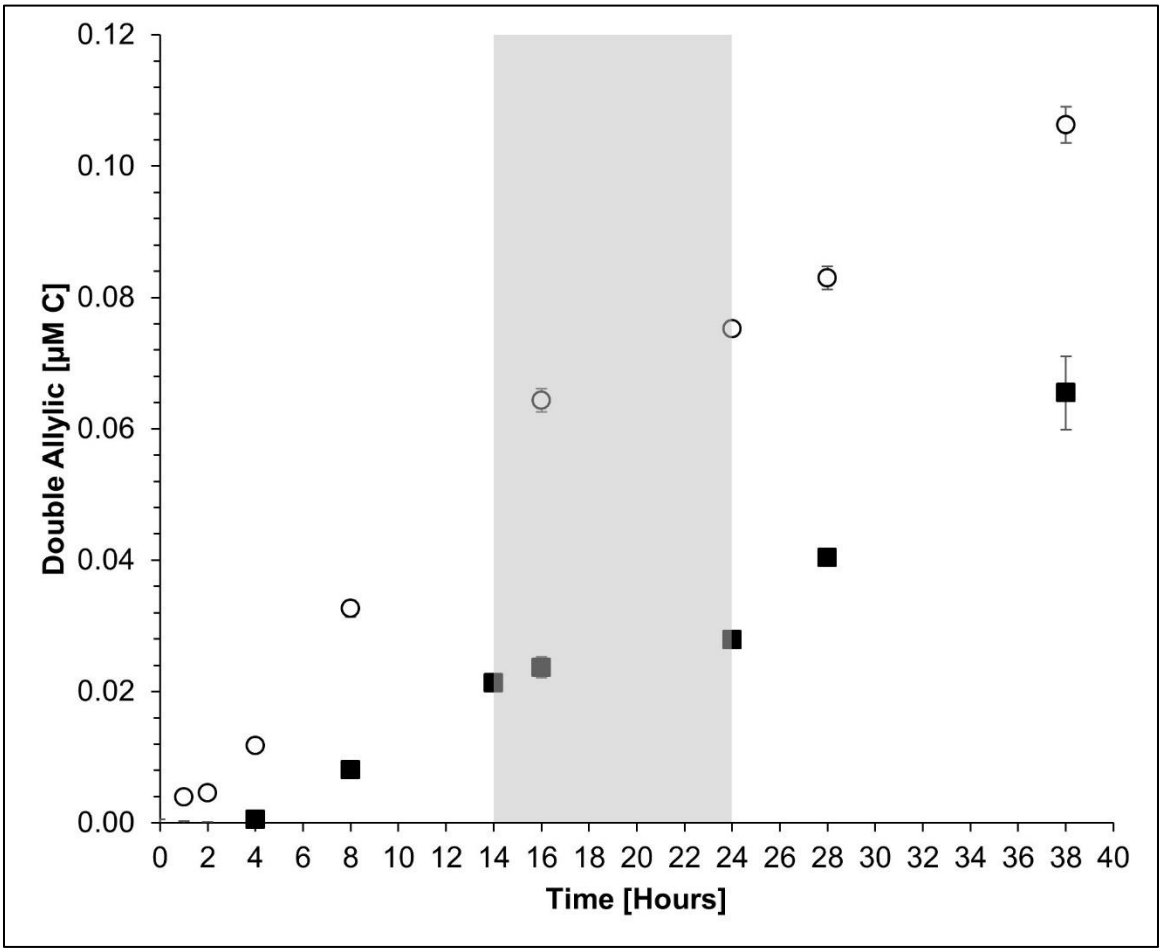

Figure 3.3. Polyunsaturated fatty acid (PUFA) chain accumulation monitored in *C. vulgaris* using ^{1}H HR-MAS NMR and $H^{13}CO_3^-$ labeling. Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.

maintained over the following 14 hours. These data suggest that there is preferential allocation of recycled carbon to unsaturates, while saturates appear to be newly synthesized from bicarbonate supplied during nitrogen deplete conditions.

NMR results for the sucrose signals are shown in Figure 3.4. Within the first hour after bicarbonate supplementation, there were noticeable *de novo* contributions to the sucrose pool from the bicarbonate amendment, and the contributions continued to

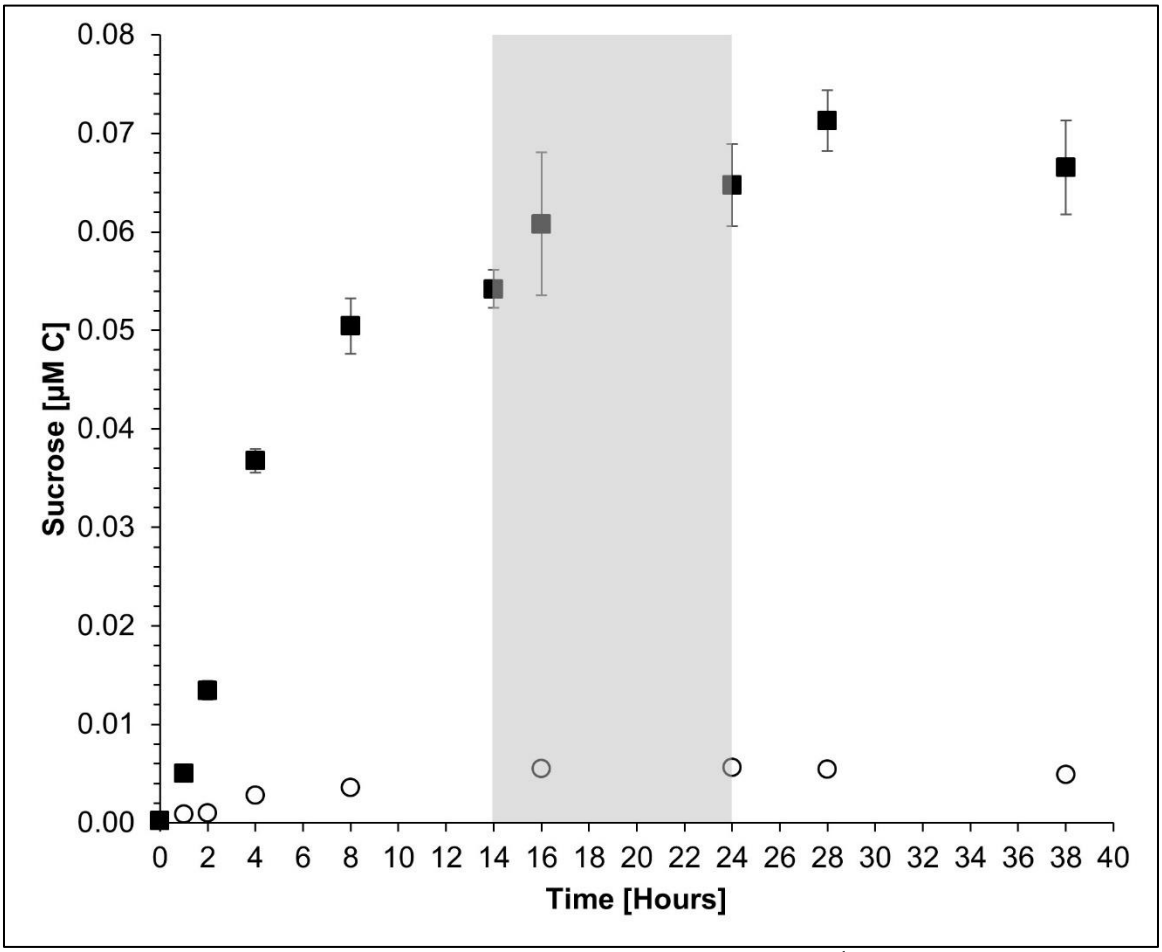

Figure 3.4. Sucrose accumulation monitored in *C. vulgaris* using ^{1}H HR-MAS NMR and $H^{13}CO_3^-$ labeling. Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.

increase until 8 hours. On the other hand, contributions from recycled biomass stayed consistently low. After the first 8 hours the sucrose pool appeared to reach a biological steady-state. The results demonstrate that the sucrose pool did not vary greatly over the remainder of the first light cycle. A slight increase was observed over the consecutive dark cycle, suggesting that energy for cellular maintenance processes may be derived from carbohydrate carbon storage compounds (*e.g.* starch). This would also serve to

explain why lipid compounds are not being preferentially degraded during the dark cycles, as cells must get energy from some form of carbon when light is not available to maintain energy reserves. Over the second light cycle no further substantial accumulation was observed, though the cells did appear to maintain this sucrose pool. This also added insight to the results from the incorporation of bulk CH_2 during the initial hours after re-suspension. Observation of little to no incorporation of carbon into saturated fatty acids at 4 hours was likely due to the shuttling of exogenous bicarbonate into the metabolic sucrose pool during this time. However, once the sucrose pool reached biological steady state, increased accumulation of saturated fatty acids towards the end of the first light cycle was observed, demonstrating the fixation of inorganic carbon to sugars and then to fatty acids.

Allylic signal measurements (mono-unsaturated fatty acids – MUFAs) and omega-3 signal measurements were also analyzed and are included as supplementary information in Appendix B, Figure B.1 and B.2. The trends observed for MUFAs were observed to have primary accumulation after 8 hours, with contributions from recycled carbon and *de novo* incorporation being closely aligned during the first light cycle. As with the other discussed metabolites, dark cycle accumulation was minimal, although did appear to increase slightly over the 10-hour period. During the second light cycle, *de novo* synthesis appeared to become the preferred method for accumulation of MUFAs, although recycled incorporation did increase until the end of the second light cycle over this 14-hour period as well, 38 hours after bicarbonate addition. Measurements of the omega-3 signal revealed high initial and continued incorporation of recycled biomass

occurring after 4 hours, similar to the PUFA signals shown in Figure 3.3. Marginal *de novo* synthesis from incorporation of bicarbonate between 8 to 14 hours was observed, but remained low relative to recycled carbon contributions over the initial 24 hours. *De novo* synthesis of omega-3's started to occur during the second light cycle, which matched the trend for recycling during this same period. A large majority of the results presented here are closely aligned with previous findings under similar conditions (Gardner et al. – Unpublished). The primary contrast in these works, is the elucidation of dark cycle stabilization for lipid compounds under bicarbonate supplemented conditions, as compared to previous research which has utilized CO_2 sparging and shown preferential degradation of these compounds during dark cycles.

^{13}C FAME Isotopomer Distribution

During the course of this experiment, biomass was harvested at 24 and 38 hours after re-suspension in nitrogen free medium for investigation of isotopomer labeling patterns by mass spectrometry. Isotopomers are molecules with the same structure but differ by the presence of a combination of isotopically labeled atoms (Jennings and Matthews 2005). Samples were taken from both the forward and reverse experiments, again so contributions from *de novo* synthesis and recycled biomass could be monitored. Neutral lipids from dried biomass of the harvested samples were extracted and subsequently transesterified for FAME analysis. This was done as (opposed to whole cell biomass transesterification) to investigate the speciation of the neutral lipids only, since the NMR method monitors only these lipids which have relatively unrestricted mobility in cells, as opposed to the polar lipid bodies found in membrane structures which are

highly restricted. Mass spectra for three FAME isotopomer mixtures (C16:0, C18:2, C18:3) were extracted at max peak intensity (TIC) and used for molecular ion investigation. Isotopomer distributions are displayed as fractional abundance with respect to the number of additional ^{13}C labels on each fatty acid chain.

NMR results for the forward labeling experiment suggested high incorporation of bicarbonate supplemented at nitrogen depletion for the *de novo* synthesis of saturated fatty acids (Bulk CH_2 signals). Complimentary data from the MS study of saturated fatty acids are shown as isotopomer distributions for highly abundant C16:0 chains in Figure 3.5. The data from the MS studies coincided with this finding, with most observed fatty acid chains being labeled with greater than 10 ^{13}C's. However, at 24 hours there was still ~43% of completely unlabeled C16:0 chains, which suggests incorporation of from lipids accumulated during growth. The results from the reverse experiment complimented this, as there was evidence of highly labeled (>14 ^{13}C's) chains at 24 hours. At 38 hours the abundance of unlabeled chains in the forward experiment decreased while the abundance of labeled chains increased, suggesting that most lipids accumulated during this time were synthesized from the bicarbonate amendment.

MS investigations were also performed for PUFA and omega-3 fatty acid chains and are included as supplementary information in Appendix B, Figure B.3 and B.4. PUFA signals (double allylic) during NMR studies demonstrated less incorporation of newly synthesized fatty acids and more from recycled contributions. The data shown in Figures B.3 indicated similar findings. Forward results from 24 hours showed a distribution of abundances for low labeled (0-4 ^{13}C's) fatty acid chains, but also similar

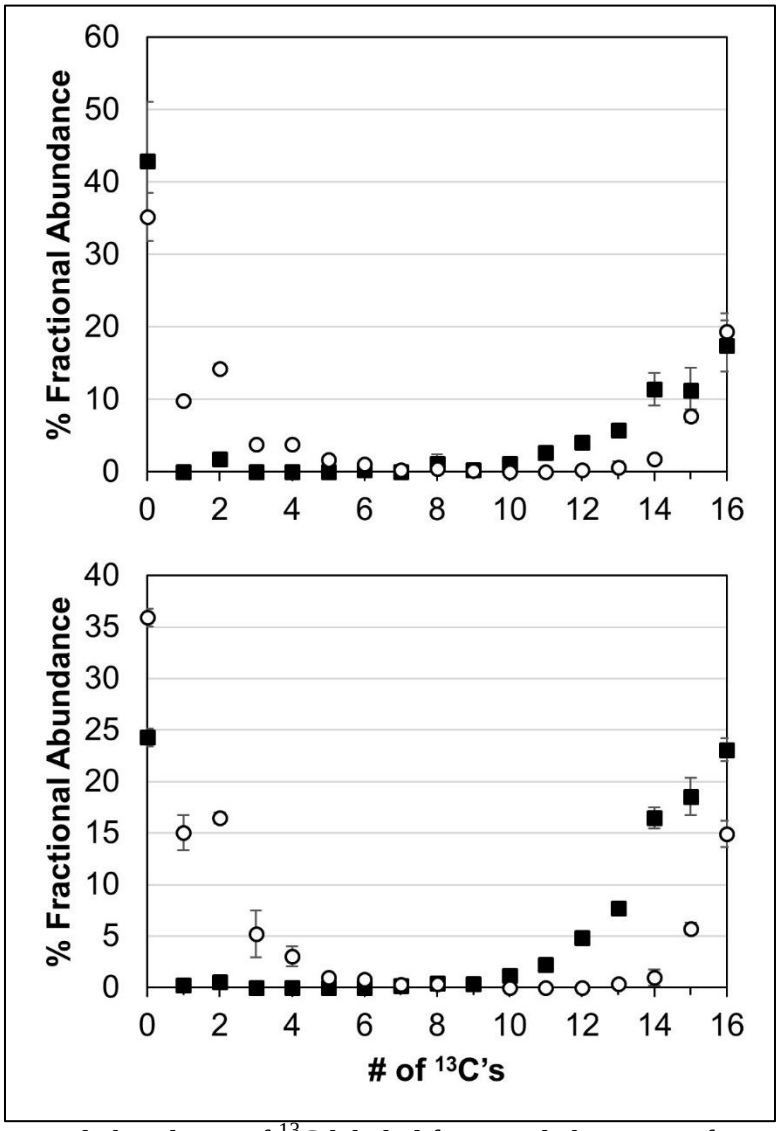

Figure 3.5. Fractional abundance of ^{13}C labeled fatty acid chains as a function of number of added ^{13}C labels for saturated (C16:0) fatty acid chains. Samples are shown from 24 hours after re-suspension (top) and 38 hours after re-suspension (bottom). Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.

distributions of highly labeled (12-16 ^{13}C's) chains. Following the second light cycle, this trend was still observed, but the extent of highly labeled chains increased. The changes in isotopomer distributions from the reverse samples was less revealing. At both 24 and 38

hours, reverse samples demonstrated the majority of lipid chains were unlabeled. However, this result was not contradictory, as the NMR results showed the incorporation of ^{13}C labels in the forward experiment started to occur mostly at the end of the first light cycle, and then remained relatively unchanged through the dark cycle as with most other metabolites. There was increased separation between 24 to 38 hours, suggesting that during the second light cycle PUFAs were being de novo synthesized, correlating with the increased fractional abundances of highly labeled PUFA chains at 38 hours when compared to 24.

Omega-3 NMR signals suggested almost no incorporation of bicarbonate supplemented at nitrogen depletion, but rather large incorporation of recycled lipids. The isotopomer distribution for omega-3 (C18:3) fatty acids, shown in Figure B.4, aligned with these findings. Note the high abundance of unlabeled chains in the forward experiment (~58%) at 24 hours and (~48%) at 38 hours. High abundances of unlabeled compounds from forward results demonstrated recycling of lipids accumulated during growth. This is reciprocated by the reverse results. At 24 hours (~35%) and 38 hours (~30%) entirely labeled omega-3 chains were observed, which also aligned with NMR data that showed little to no ^{13}C incorporation at 38 hours and even less at 24. These results also hint at the distinct separation of carbon recycling vs. *de novo* synthesis, as most C18:3 chains appear to be either fully labeled or fully unlabeled, with minimal contributions from partially labeled isotopomers. While other fatty acid chains appeared to have more labeling distribution, it appears that omega-3 fatty acids are distinctly

synthesized from either the bicarbonate amendment or through recycling of cellular carbon, and that both of these may happen independent of each other.

Conclusions

Growth of *C. vulgaris* is achievable through the use of both gaseous CO_2 and solid $NaHCO_3$ salt amendments as primary carbon substrates. While previous research indicated that an enhanced growth scenario exists with a combination of both (Lohman, Gardner et al. 2015), the experiments described here focus on possible fundamental differences of culture-behavior in the presence of these two inorganic carbon sources. The data presented suggest important differences and potential challenges associated with utilizing CO_2 versus HCO_3^- for growth of algal biomass.

Through the use of a powerful NMR technique for high throughput monitoring of algal cultures, inorganic carbon was observed to be fixed from $NaHCO_3$ supplementation and the resultant incorporation into metabolites was studied. Bicarbonate amendment at nitrogen depletion caused the arresting of the cell cycle in *C. vulgaris* and induced lipid accumulation, which was the focus of the ^{13}C labeling experiments here. NMR tracking of metabolites revealed the initial incorporation of bicarbonate into cellular carbohydrate pools, indicated by rapid *de novo* synthesis of sucrose, which reached a biological steady-state after 8 hours. Continued presence of ^{13}C labels in sucrose remained high for the duration of the experiment, indicating that sucrose is likely providing the precursors for the *de novo* synthesis of saturated fatty acid chains and other carbon storage compounds during the remaining hours of the first light cycle. Saturated fatty acid synthesis primarily

utilized carbon from the bicarbonate amendment, while unsaturated fatty acid synthesis appeared to involve more recycling of biomass. Furthermore, the extent of *de novo* synthesis for unsaturated fatty acids appeared to decrease relative to the contributions from recycled carbon as the lever of unsaturation increased. Mass spectrometry was used to resolve the isotopomer distributions, which substantiated the findings from the NMR measurements.

C. vulgaris is quickly becoming a prominent organism for biofuel production due to its high growth rate and ability to accumulate relatively high amounts of lipid in short periods of time under nitrogen stressed conditions. Pairing this to the strategic addition of bicarbonate salts is quickly becoming an attractive method to further enhance its attractiveness as a candidate for biofuel production. The incorporation of bicarbonate into lipid synthesis shown here is proof that the bicarbonate amendment serves to do more than simply add exogenous salt stress such as with the use of sodium chloride. Furthermore, the presence of bicarbonate showed that metabolites in cells were stabilized rather than consumed over dark periods. This could provide benefit for culture health which could be at risk during scaled-up cultivation due to light-dark circadian rhythms associated with natural sunlight.

Competing Interests

A patent entitled "Bicarbonate Trigger for Inducing Lipid Accumulation in Algal Systems" (Pat. No 9,096,875) was co-authored by contributing authors Robert D. Gardner and Brent M. Peyton.

Acknowledgements

A portion of this research was supported by the U.S. Department of Energy (DOE) Office of Energy Efficiency and Renewable Energy (EERE) Biomass Program under Contract No. DE-EE0005993. Support for TCP was also provided by Church and Dwight Co., Inc. NMR Spectroscopy support was provided by the Center for NMR Spectroscopy at Washington State University (WSU) in collaboration with Dr. Gregory L. Helms. Instrumental support was provided through the Environmental and Biofilm Mass Spectrometry Facility at the College of Engineering (COE), and the Center for Biofilm Engineering (CBE), at Montana State University (MSU). A special thanks all members of the MSU Algal Biofuels Group for their introspective discourse on algal biofuel related topics.

References

Bigelow, N. W., W. R. Hardin, J. P. Barker, S. A. Ryken, A. C. MacRae and R. A. Cattolico (2011). "A comprehensive GC–MS sub-microscale assay for fatty acids and its applications." Journal of the American Oil Chemists' Society **88**(9): 1329-1338.

Brennan, L. and P. Owende (2010). "Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products." Renewable and Sustainable Energy Reviews **14**(2): 557-577.

Chisti, Y. (2008). "Biodiesel from microalgae beats bioethanol." Trends in Biotechnology **26**(3): 126-131.

Cooksey, K. E. (2015). "Regulation of the initial events in microalgal triacylglycerol (TAG) synthesis: hypotheses." Journal of Applied Phycology **27**(4): 1385-1387.

Davey, P. T., W. C. Hiscox, B. F. Lucker, J. V. O'Fallon, S. Chen and G. L. Helms (2012). "Rapid triacylglyceride detection and quantification in live micro-algal cultures via liquid state 1H NMR." Algal Research **1**(2): 166-175.

Davis, R., A. Aden and P. T. Pienkos (2011). "Techno-economic analysis of autotrophic microalgae for fuel production." Applied Energy **88**(10): 3524-3531.

Gardner, R. D., K. E. Cooksey, F. Mus, R. Macur, K. Moll, E. Eustance, R. P. Carlson, R. Gerlach, M. W. Fields and B. M. Peyton (2012). "Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte Scenedesmus sp and the diatom Phaeodactylum tricornutum." Journal of Applied Phycology **24**(5): 1311-1320.

Gardner, R. D., E. Lohman, R. Gerlach, K. E. Cooksey and B. M. Peyton (2013). "Comparison of CO(2) and bicarbonate as inorganic carbon sources for triacylglycerol and starch accumulation in Chlamydomonas reinhardtii." Biotechnol Bioeng **110**(1): 87-96.

Gardner, R. D., E. J. Lohman, K. E. Cooksey, R. Gerlach and B. M. Peyton (2013). "Cellular Cycling, Carbon Utilization, and Photosynthetic Oxygen Production during Bicarbonate-Induced Triacylglycerol Accumulation in a Scenedesmus sp." Energies **6**(11): 6060-6076.

Griffiths, M. J. and S. T. L. Harrison (2009). "Lipid productivity as a key characteristic for choosing algal species for biodiesel production." Journal of Applied Phycology **21**(5): 493-507.

Guarnieri, M. T., A. Nag, S. L. Smolinski, A. Darzins, M. Seibert and P. T. Pienkos (2011). "Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga." PLoS One **6**(10): e25851.

Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert and A. Darzins (2008). "Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances." The Plant Journal **54**(4): 621-639.

Jennings, M. E. and D. E. Matthews (2005). "Determination of Complex Isotopomer Patterns in Isotopically Labeled Compounds by Mass Spectrometry." Analytical Chemistry **77**(19): 6435-6444.

Lardon, L., A. Hélias, B. Sialve, J.-P. Steyer and O. Bernard (2009). "Life-cycle assessment of biodiesel production from microalgae." Environmental science & technology **43**(17): 6475.

Lohman, E. J., R. D. Gardner, L. Halverson, R. E. Macur, B. M. Peyton and R. Gerlach (2013). "An efficient and scalable extraction and quantification method for algal derived biofuel." J Microbiol Methods **94**(3): 235-244.

Lohman, E. J., R. D. Gardner, T. Pedersen, B. M. Peyton, K. E. Cooksey and R. Gerlach (2015). "Optimized inorganic carbon regime for enhanced growth and lipid accumulation in Chlorella vulgaris." Biotechnol Biofuels **8**: 82.

Mus, F., J.-P. Toussaint, K. E. Cooksey, M. W. Fields, R. Gerlach, B. M. Peyton and R. P. Carlson (2013). "Physiological and molecular analysis of carbon source supplementation and pH stress-induced lipid accumulation in the marine diatom Phaeodactylum tricornutum." Applied microbiology and biotechnology **97**(8): 3625-3642.

Ördög, V., W. Stirk, P. Bálint, J. Staden and C. Lovász (2012). "Changes in lipid, protein and pigment concentrations in nitrogen-stressed Chlorella minutissima cultures." Journal of Applied Phycology **24**(4): 907-914.

Schenk, P. M., S. R. Thomas-Hall, E. Stephens, U. C. Marx, J. H. Mussgnug, C. Posten, O. Kruse and B. Hankamer (2008). "Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production." BioEnergy Research **1**(1): 20-43.

Valenzuela, J., A. Mazurie, R. P. Carlson, R. Gerlach, K. E. Cooksey, B. M. Peyton and M. W. Fields (2012). "Potential role of multiple carbon fixation pathways during lipid accumulation in Phaeodactylum tricornutum." Biotechnology for biofuels **5**(1): 1.

Wensel, P., G. Helms, B. Hiscox, W. C. Davis, H. Kirchhoff, M. Bule, L. Yu and S. Chen (2014). "Isolation, characterization, and validation of oleaginous, multi-trophic, and haloalkaline-tolerant microalgae for two-stage cultivation." Algal Research **4**: 2-11.

Williams, P. J. l. B. and L. M. L. Laurens (2010). "Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics." Energy & Environmental Science **3**(5): 554.

CHAPTER FOUR

CONCLUSIONS

Project Outcomes

Fundamental knowledge of the use of bicarbonate salts in algal cultivation strategies is continuously developing. Research on new strains and use of novel methods has elaborated on the possibilities for use of bicarbonate, but this field will continue to expand as more of the scientific community becomes aware of and begins to accept the conclusions from previous authors' work. This project advanced the current understanding bicarbonate induced lipid accumulation, and exposed future questions for successive work. The aims of the project here were to:

(1) Investigate the use of low concentrations of bicarbonate for potential application to maximize biomass production prior to lipid accumulation an industrially relevant strain *Nannochloropsis gaditana.* Additionally, to pair this application strategy with bicarbonate amendments at near nitrogen depletion to investigate changes in lipid accumulation under nitrogen deplete conditions.

(2) Implementation of NMR based fluxomics for metabolic pathway elucidation under bicarbonate supplementation in the green alga *Chlorella vulgaris*. Use high-resolution magic angle spinning (HR-MAS) ^{1}H-NMR to allow for real time tracking of metabolic information in live algal cells and elucidate fundamental lipidomic shifts after bicarbonate amendment at near nitrogen depletion under a diel cycle.

Nannochloropsis gaditana
Bicarbonate Studies

N. gaditana now numbers among the species specifically investigated for bicarbonate application strategies here at Montana State University. Low concentrations of bicarbonate were not observed to significantly improve growth over pH controlled conditions alone, regardless of aeration strategy. Nitrogen depletion was an effective trigger for lipid accumulation when cultures had previously been grown under nitrogen replete conditions with the use of elevated inorganic carbon supplementation (*i.e.* 5% CO_2 supplied continuously or on demand to control pH). However, chlorophyll content was oppositely effected in these conditions during nitrogen deplete conditions, showing degradation immediately following nitrogen depletion in all cultures. The pairing of high concentration bicarbonate amendment at near nitrogen depletion with pH controlled strategies was observed to produce the highest lipid content at harvest of the conditions tested. However, *N. gaditana* may not be the best potential candidate for biofuel production, as it takes relatively long (>10 days) to be grown to cultures densities of ~$1 g \cdot L^{-1}$ under even the best conditions studied here.

The results obtained from this study have been prepared for submission to a scientific journal (J Appl Phycol) and have contributed to tasks outlined in the grant funded by Church and Dwight Co., Inc. Furthermore, the study outlined various strategies for the application of bicarbonate and can be used as reference for future studies to be conducted regarding the use of bicarbonate. While the experiments here lacked engineering analysis (*i.e.* mass balances, mass transfer rates, experimental modeling, etc.), task aims were fulfilled and results are slated for publication.

Chlorella vulgaris
$NaH^{13}CO_3$ Labeling Studies.

The ^{13}C labeling studies described in chapter 3 should be considered a 'draft' compilation of results, aiming towards the highly relevant concurrent investigations of bicarbonate induced lipid accumulation in a model green alga, *Chlorella vulgaris.* These results are preliminarily established here, for future publishing in parallel to results from transcriptomic investigations of the duplicated experiment. Growth analysis of *C. vulgaris* was performed when different inorganic carbon substrates were utilized. CO_2 demonstrated better growth in the experiments performed here, but $NaHCO_3$ supplementation was also sufficient to grow biomass during nitrogen replete conditions. However, growth using only $NaHCO_3$ was limited by rapid pH increases during light hours which had to be manually controlled.

NMR investigations revealed similar findings to a previous study (Gardner et al. – Unpublished). Bicarbonate supplemented in concert with nitrogen depletion was quickly utilized, with carbon incorporation into sucrose metabolism observed in the first hour after re-suspension. Generation of sucrose continually increased until a biological steady state was reached after 8 hours, and then remained relatively unchanged. This bicarbonate incorporated carbon pool was utilized for the *de novo* synthesis of saturated fatty acids during the latter half of the first light cycle and throughout the second. Unsaturated fatty acid chains appeared to incorporate more carbon from recycled biomass. The results showed increased contributions from carbon recycling as unsaturation increased in fatty acid chains. Mass spectrometry experiments were used to elaborate on the contributions from recycling and provide support for the NMR

conclusions. However, without results from the reverse NMR experiments, it has been difficult to fully summarize the experimental set.

Use of the ^{1}H HR-MAS NMR proved to be a useful way to monitor metabolic production of neutral lipids under bicarbonate induced lipid accumulation. Fixation of inorganic carbon was observed to progress into a biological steady-state carbon pool, which was maintained during the subsequent production of fatty acid chains. Metabolites appeared to be maintained in cultures during dark cycles which possibly is the result of some bicarbonate stabilization effect. These result should provide invaluable information for the soon to be completed transcriptomic investigation which was run analogously. The combination of these inquiries should provide ample evidence for the bicarbonate use under stress conditions, specifically aimed at elucidating the effects of bicarbonate under nitrogen deplete conditions and ultimately, the fate of bicarbonate.

Concluding Remarks

Bicarbonate salts have emerged as a viable alternative to CO_2 for the cultivation of microalgae for biomass production, and offer the ability to enhancing lipid content when used in cultivation under stress conditions. Much of the previous work existing on the use of bicarbonate has been focused on the resulting changes to neutral lipid bodies which are relevant for the focus of biofuels. However, due to the low economic feasibility of using algal biomass as a feedstock for biofuels, other products should be investigated for potential enhancements from bicarbonate.

Funding Acknowledgements

This technology has earned funding, both in previous years and for this work, from Church and Dwight Co., Inc. to expand on the prospects of using sodium bicarbonate ($NaHCO_3$) and other bicarbonate salts in microalgae culturing systems. Additional funding support for these projects, and many others, was provided by the US Department of Energy, DOE ASAP DE-EE0005993.

CHAPTER FIVE

FUTURE WORK

Integration of Bicarbonate Induced Lipid Production and Continuous Growth

While batch growth is useful for monitoring controlled effects in the culture of microalgae, the overall productivity of cultures may suffer from these conditions when compared to continuous culture. Moving forward, it is suggested that a semi-continuous growth scheme is tested in combination with bicarbonate-induced lipid accumulation. This would involve a multi-step cultivation process, whereby during an early phase of growth (Phase 1), culture would be grown to high culture densities (~$1 g \cdot L^{-1}$) under optimized batch conditions as outlined by Lohman, Gardner et al. (2015) until near nitrogen depletion. At this point, culture would be switched to semi-continuous operation (phase 2), during which, a portion of high density culture would be transferred daily from a primary reactor to secondary reactors and amended with high-concentration bicarbonate. This operation mode would be continued for sufficient time to enable the harvest of lipid rich biomass coming out of secondary reactors, while harvested biomass from these reactors would be replaced at near-nitrogen starved conditions from the primary reactor. The primary reactor would then be replenished with new medium and seeded with remaining culture. A rudimentary diagram of this is presented below in Figure 5.1.

The primary aim of this work will be to determine if semi-continuous growth can be maintained under nutrient replete conditions, and if lipid accumulation can be

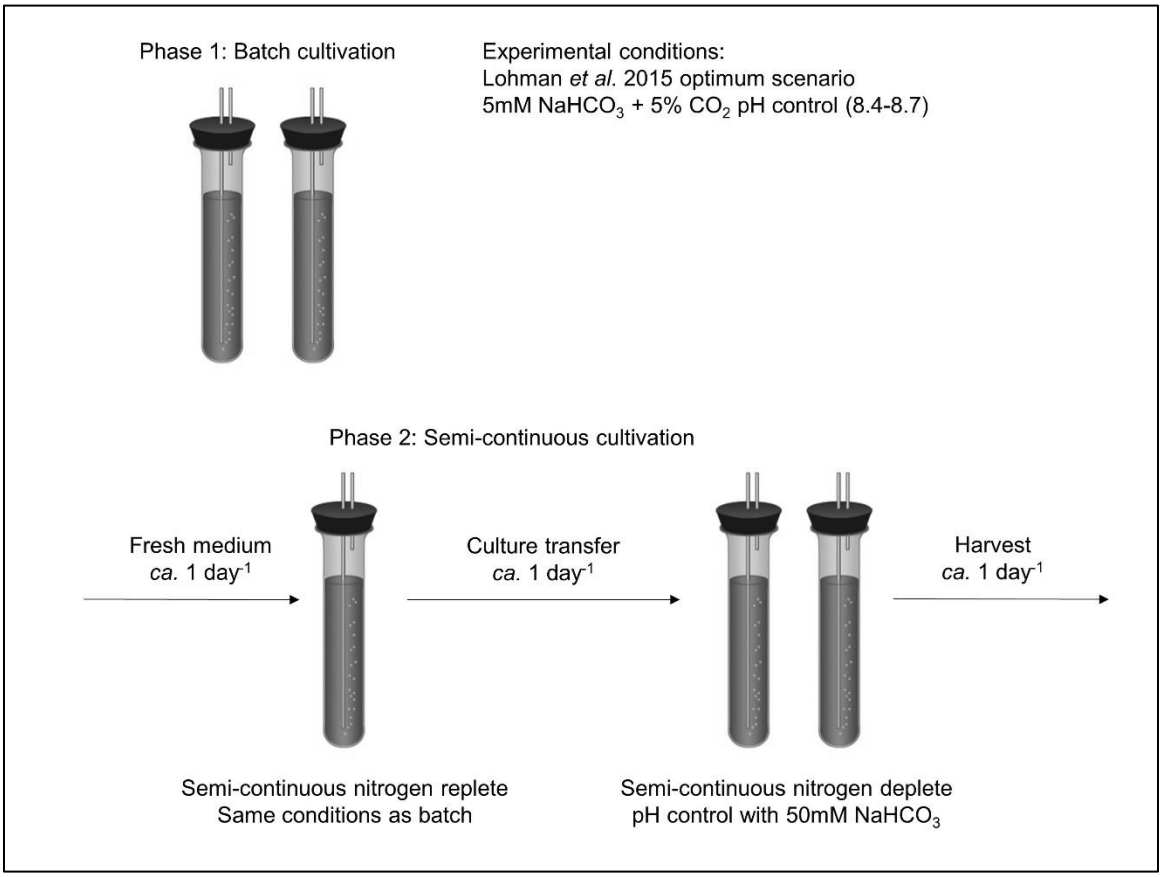

Figure 5.1. Proposed process for integration of semi-continuous operation and bicarbonate-induced lipid accumulation.

observed in secondary reactors after transfer and bicarbonate amendment. Rudimentary calculations are carried out below to provide a base case for what could potentially be achievable.

First, the maximum possible dilution rate for this culture is considered. If the system was to be run under true continuous conditions, dilution rate would be equivalent to specific growth rate, as is with a chemostat. Using the value obtain from Lohman, Gardner et al. (2015), these researchers found specific growth rate in their optimized scenario to be 1.7 day^{-1}.

$$D = \frac{F}{V} = \mu \qquad \text{(Eq. 5.1)}$$

$$D = \mu = 1.7day^{-1} = \frac{F}{1.25L}$$

$$F = 2.125\ L \cdot day^{-1}$$

Assuming a photobioreactor volume of 1.25L, the maximum feasible flowrate to avoid washout would be 2.125 $L \cdot day^{-1}$. If future researchers desire to utilize a full continuous operation mode, it is not the intent here to claim that it would not be possible; however, they must consider multiple difficulties associated with chemostat operation. First, the mass balance assumption of a chemostat is that the concentration of substrate inside of the reactor is equivalent to its effluent. With consideration to bicarbonate-induced lipid accumulation, timing of nitrogen depletion for bicarbonate addition could prove difficult if culture is maintained for full residence time under near-nitrogen starved conditions. Second, to maintain growth of photosynthetic culture under continuous growth, 24-hour light would need to be implemented. This could lead to damaging free-radical stress on cultures and pre-mature lipid accumulation or culture crash. Therefore, a semi-continuous operation method has been proposed. Under these conditions, cultures would follow batch kinetics during growth between successive culture transfers. Again, using a theoretical example and assuming that at the transition of phase 1 to phase 2, half of the high density culture ($1g \cdot L^{-1}$) would be transferred to a secondary reactor for lipid accumulation to initiate semi-continuous growth conditions, an example is provided. The transferred culture would be replaced with new medium and the resulting biomass concentration is calculated.

$$C_1V_1 = C_2V_2 \qquad \text{(Eq. 5.2)}$$

$$C_2 = \frac{C_1V_1}{V_2} = \frac{(1g \cdot L^{-1})(0.625L)}{(1.25L)}$$

$$C_2 = 0.5g \cdot L^{-1}$$

The resulting concentration of biomass in the primary reactor would be $0.5g{\cdot}L^{-1}$, which can then be used to estimate the amount of time it would take to return to $1g{\cdot}L^{-1}$. Since this is a simple doubling of culture density, it would be expected that this time would be equivalent to the doubling time, but the calculation is demonstrated below nonetheless using the growth expression for batch kinetics.

$$X = X_o e^{\mu t} \qquad \text{(Eq. 5.3)}$$

$$t = \frac{\ln(\frac{X}{X_o})}{\mu} \qquad \text{(Eq. 5.4)}$$

$$t = \frac{\ln(\frac{X}{X_o})}{\mu} = \frac{\ln(2)}{\mu} = t_d$$

$$t_d = \frac{\ln(2)}{\mu} = \frac{0.693}{1.7day^{-1}} = 0.4\ day$$

As expected, this would take approximately 0.4 days (t_d), or roughly 9.8 hours. Under 14:10 L/D conditions, this would likely nitrogen starve cultures before the next transfer to a secondary reactor; however, these calculations are not meant to provide a precise answer for the amount of culture to transfer or how often transfers should be carried out. The intention here is to provide a basis for the approach to carrying out this task. Considerations will have to made into the long term viability of maintaining nitrogen

replete cultures under semi-continuous operation. The concentration of nitrogen remaining in reactors will need to be accounted for when determining how much nitrogen will need to be replenished by fresh medium additions. This same effect will have to be examined for other essential nutrients, which may be consumed by high density cultures, such as phosphorus, iron, potassium, and other macro- or micronutrients. A well written review on nutrient supply in algal cultivation is provided by Markou, Vandamme et al. (2014).

It is recommended that sampling of reactors be done immediately before and after daily culture transfers, including but not limited to, cell concentrations, pH, chlorophyll, nitrogen, phosphorus, dissolved inorganic carbon (DIC), and biomass for cell dry weight (CDW) and lipid characterization. It is also recommended that transfer of culture be done near the end of light cycles to maximize biomass concentrations. Overall, it is envisioned that these data could provide a basis for either continuous growth of microalgae, or prove useful at scaled up conditions where batch systems will likely become obsolete.

REFERENCES CITED

Ahmad, A. L., N. H. M. Yasin, C. J. C. Derek and J. K. Lim (2011). "Microalgae as a sustainable energy source for biodiesel production: A review." Renewable and Sustainable Energy Reviews **15**(1): 584-593.

Allen, E. J. and E. W. Nelson (1910). "On the Artificial Culture of Marine Plankton Organisms." Journal of the Marine Biological Association of the United Kingdom (New Series) **8**(05): 421-474.

Amin, S. (2009). "Review on biofuel oil and gas production processes from microalgae." Energy Conversion and Management **50**(7): 1834-1840.

Barreiro, D. L., W. Prins, F. Ronsse and W. Brilman (2013). "Hydrothermal liquefaction (HTL) of microalgae for biofuel production: state of the art review and future prospects." Biomass and Bioenergy **53**: 113-127.

Bhateria, R. and R. Dhaka (2015). "Algae as biofuel." Biofuels **5**(6): 607-631.

Bigelow, N. W., W. R. Hardin, J. P. Barker, S. A. Ryken, A. C. MacRae and R. A. Cattolico (2011). "A comprehensive GC–MS sub-microscale assay for fatty acids and its applications." Journal of the American Oil Chemists' Society **88**(9): 1329-1338.

Biller, P. and A. Ross (2011). "Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content." Bioresource technology **102**(1): 215-225.

Brennan, L. and P. Owende (2010). "Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products." Renewable and Sustainable Energy Reviews **14**(2): 557-577.

Camacho-Rodríguez, J., M. C. Cerón-García, J. M. Fernández-Sevilla and E. Molina-Grima (2015). "The influence of culture conditions on biomass and high value product generation by Nannochloropsis gaditana in aquaculture." Algal Research **11**: 63-73.

Chen, Y., L. Zhang, C. Xu and S. Vaidyanathan (2016). "Dissolved inorganic carbon speciation in aquatic environments and its application to monitor algal carbon uptake." Science of The Total Environment **541**: 1282-1295.

Chi, Z., J. V. O'Fallon and S. Chen (2011). "Bicarbonate produced from carbon capture for algae culture." Trends in Biotechnology **29**(11): 537-541.

Chi, Z., Y. Xie, F. Elloy, Y. Zheng, Y. Hu and S. Chen (2013). "Bicarbonate-based Integrated Carbon Capture and Algae Production System with alkalihalophilic cyanobacterium." Bioresource Technology **133**: 513-521.

Chisti, Y. (2007). "Biodiesel from microalgae." Biotechnology advances **25**(3): 294-306.

Chisti, Y. (2008). "Biodiesel from microalgae beats bioethanol." Trends in Biotechnology **26**(3): 126-131.

Cooksey, K. E. (2015). "Regulation of the initial events in microalgal triacylglycerol (TAG) synthesis: hypotheses." Journal of Applied Phycology **27**(4): 1385-1387.

Davey, P. T., W. C. Hiscox, B. F. Lucker, J. V. O'Fallon, S. Chen and G. L. Helms (2012). "Rapid triacylglyceride detection and quantification in live micro-algal cultures via liquid state 1H NMR." Algal Research **1**(2): 166-175.

Davis, R., A. Aden and P. T. Pienkos (2011). "Techno-economic analysis of autotrophic microalgae for fuel production." Applied Energy **88**(10): 3524-3531.

Dong, H.-P., E. Williams, D.-Z. Wang, Z.-X. Xie, R.-C. Hsia, A. Jenck, R. Halden, J. Li, F. Chen and A. R. Place (2013). "Responses of Nannochloropsis oceanica IMET1 to Long-Term Nitrogen Starvation and Recovery." Plant physiology **162**(2): 1110.

Fields, M. W., A. Hise, E. J. Lohman, T. Bell, R. D. Gardner, L. Corredor, K. Moll, B. M. Peyton, G. W. Characklis and R. Gerlach (2014). "Sources and resources: importance of nutrients, resource allocation, and ecology in microalgal cultivation for lipid accumulation." Applied Microbiology and Biotechnology **98**(11): 4805-4816.

Gardner, R., P. Peters, B. Peyton and K. E. Cooksey (2010). "Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the chlorophyta." Journal of Applied Phycology **23**(6): 1005-1016.

Gardner, R. D., K. E. Cooksey, F. Mus, R. Macur, K. Moll, E. Eustance, R. P. Carlson, R. Gerlach, M. W. Fields and B. M. Peyton (2012). "Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte Scenedesmus sp and the diatom Phaeodactylum tricornutum." Journal of Applied Phycology **24**(5): 1311 1320.

Gardner, R. D., E. Lohman, R. Gerlach, K. E. Cooksey and B. M. Peyton (2013). "Comparison of CO(2) and bicarbonate as inorganic carbon sources for triacylglycerol and starch accumulation in Chlamydomonas reinhardtii." Biotechnol Bioeng **110**(1): 87-96.

Gardner, R. D., E. J. Lohman, K. E. Cooksey, R. Gerlach and B. M. Peyton (2013). "Cellular Cycling, Carbon Utilization, and Photosynthetic Oxygen Production during Bicarbonate-Induced Triacylglycerol Accumulation in a Scenedesmus sp." Energies **6**(11): 6060-6076.

Giordano, M., J. Beardall and J. A. Raven (2005). "CO2 CONCENTRATING MECHANISMS IN ALGAE: Mechanisms, Environmental Modulation, and Evolution." Annual Review of Plant Biology **56**(1): 99-131.

Griffiths, M. J. and S. T. L. Harrison (2009). "Lipid productivity as a key characteristic for choosing algal species for biodiesel production." Journal of Applied Phycology **21**(5): 493-507.

Guarnieri, M. T., A. Nag, S. L. Smolinski, A. Darzins, M. Seibert and P. T. Pienkos (2011). "Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga." PLoS One **6**(10): e25851.

Guihéneuf, F. and D. B. Stengel (2013). "LC-PUFA-Enriched Oil Production by Microalgae: Accumulation of Lipid and Triacylglycerols Containing n-3 LC-PUFA Is Triggered by Nitrogen Limitation and Inorganic Carbon Availability in the Marine Haptophyte Pavlova lutheri." Marine Drugs **11**(11): 4246-4266.

Hallenbeck, P. C., M. Grogger, M. Mraz and D. Veverka (2015). "The use of Design of Experiments and Response Surface Methodology to optimize biomass and lipid production by the oleaginous marine green alga, Nannochloropsis gaditana in response to light intensity, inoculum size and CO2." Bioresource Technology **184**: 161-168.

Hill, J., E. Nelson, D. Tilman, S. Polasky and D. Tiffany (2006). "Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels." Proceedings of the National Academy of Sciences **103**(30): 11206-11210.

Ho, S.-H., C.-Y. Chen, D.-J. Lee and J.-S. Chang (2011). "Perspectives on microalgal CO2-emission mitigation systems — A review." Biotechnology Advances **29**(2): 189-198.

Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert and A. Darzins (2008). "Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances." The Plant Journal **54**(4): 621-639.

IAE (2008). From 1st- to 2nd- Generation Biofuel Technologies, International Energy Association.

Jennings, M. E. and D. E. Matthews (2005). "Determination of Complex Isotopomer Patterns in Isotopically Labeled Compounds by Mass Spectrometry." Analytical Chemistry **77**(19): 6435-6444.

Keymer, P. C., P. A. Lant and S. Pratt (2014). "Modelling microalgal activity as a function of inorganic carbon concentration: accounting for the impact of pH on the bicarbonate system." Journal of Applied Phycology **26**(3): 1343-1350.

Kumar, A., S. Ergas, X. Yuan, A. Sahu, Q. Zhang, J. Dewulf, F. X. Malcata and H. van Langenhove (2010). "Enhanced CO2 fixation and biofuel production via microalgae: recent developments and future directions." Trends in Biotechnology **28**(7): 371-380.

Lam, M. K., K. T. Lee and A. R. Mohamed (2012). "Current status and challenges on microalgae-based carbon capture." International Journal of Greenhouse Gas Control **10**: 456-469.

Lardon, L., A. Hélias, B. Sialve, J.-P. Steyer and O. Bernard (2009). "Life-cycle assessment of biodiesel production from microalgae." Environmental science & technology **43**(17): 6475.

Laurens, L. M. L., K. Cooksey, J. Sears, M. Edwards, T. Lundquist, C. Behnke, N. Schultz, S. Howell, G. Clapper, R. Gardner, E. Slaby, J. Olivares, R. McCormick and R. A. Cattolico (2015). "Industrial Algal Measurements Version 7.0." 33.

Li, H., Z. Liu, Y. Zhang, B. Li, H. Lu, N. Duan, M. Liu, Z. Zhu and B. Si (2014). "Conversion efficiency and oil quality of low-lipid high-protein and high-lipid low-protein microalgae via hydrothermal liquefaction." Bioresource technology **154**: 322-329.

Lohman, E. J., R. D. Gardner, L. Halverson, R. E. Macur, B. M. Peyton and R. Gerlach (2013). "An efficient and scalable extraction and quantification method for algal derived biofuel." J Microbiol Methods **94**(3): 235-244.

Lohman, E. J., R. D. Gardner, L. D. Halverson, B. M. Peyton and R. Gerlach (2014). "Carbon partitioning in lipids synthesized by Chlamydomonas reinhardtii when cultured under three unique inorganic carbon regimes." Algal Research-Biomass Biofuels and Bioproducts **5**: 171-180.

Lohman, E. J., R. D. Gardner, T. Pedersen, B. M. Peyton, K. E. Cooksey and R. Gerlach (2015). "Optimized inorganic carbon regime for enhanced growth and lipid accumulation in Chlorella vulgaris." Biotechnol Biofuels **8**: 82.

Ma, Y., Z. Wang, C. Yu, Y. Yin and G. Zhou (2014). "Evaluation of the potential of 9 Nannochloropsis strains for biodiesel production." Bioresour Technol **167**: 503 509.

Markou, G., D. Vandamme and K. Muylaert (2014). "Microalgal and cyanobacterial cultivation: the supply of nutrients." Water Res **65**: 186-202.

Mata, T. M., A. A. Martins and N. S. Caetano (2010). "Microalgae for biodiesel production and other applications: A review." Renewable and Sustainable Energy Reviews **14**(1): 217-232.

Moll, K. M., R. D. Gardner, E. O. Eustance, R. Gerlach and B. M. Peyton (2014). "Combining multiple nutrient stresses and bicarbonate addition to promote lipid accumulation in the diatom RGd-1." Algal Research-Biomass Biofuels and Bioproducts **5**: 7-15.

Moroney, J. V. and R. A. Ynalvez (2007). "Proposed carbon dioxide concentrating mechanism in Chlamydomonas reinhardtii." Eukaryot Cell **6**(8): 1251-1259.

Mus, F., J.-P. Toussaint, K. E. Cooksey, M. W. Fields, R. Gerlach, B. M. Peyton and R. P. Carlson (2013). "Physiological and molecular analysis of carbon source supplementation and pH stress-induced lipid accumulation in the marine diatom Phaeodactylum tricornutum." Applied microbiology and biotechnology **97**(8): 3625-3642.

Nichols, H. W. and H. C. Bold (1965). "Trichosarcina polymorpha Gen. et Sp. Nov." Journal of Phycology **1**(1): 34-38.

Nunez, M. and A. Quigg (2016). "Changes in growth and composition of the marine microalgae Phaeodactylum tricornutum and Nannochloropsis salina in response to changing sodium bicarbonate concentrations." Journal of Applied Phycology **28**(4): 2123-2138.

Ördög, V., W. Stirk, P. Bálint, J. Staden and C. Lovász (2012). "Changes in lipid, protein and pigment concentrations in nitrogen-stressed Chlorella minutissima cultures." Journal of Applied Phycology **24**(4): 907-914.

Pancha, I., K. Chokshi, T. Ghosh, C. Paliwal, R. Maurya and S. Mishra (2015). "Bicarbonate supplementation enhanced biofuel production potential as well as nutritional stress mitigation in the microalgae Scenedesmus sp. CCNM 1077." Bioresour Technol **193**: 315-323.

Provasoli, L., J. J. A. McLaughlin and M. R. Droop (1957). "The development of artificial media for marine algae." Archiv für Mikrobiologie **25**(4): 392-428.

Radakovits, R., R. E. Jinkerson, S. I. Fuerstenberg, H. Tae, R. E. Settlage, J. L. Boore and M. C. Posewitz (2012). "Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropis gaditana." Nat Commun **3**: 686.

Raven, J. A., J. Beardall and M. Giordano (2014). "Energy costs of carbon dioxide concentrating mechanisms in aquatic organisms." Photosynthesis Research **121**(2):111-124.

Ren, M. and K. Ogden (2014). "Cultivation of Nannochloropsis gaditana on mixtures of nitrogen sources." Environmental Progress & Sustainable Energy **33**(2): 551-555.

Rocha, J. M., J. E. Garcia and M. H. Henriques (2003). "Growth aspects of the marine microalga Nannochloropsis gaditana." Biomol Eng **20**(4-6): 237-242.

Sawayama, S., S. Inoue, Y. Dote and S.-Y. Yokoyama (1995). "CO2 fixation and oil production through microalga." Energy Conversion and Management **36**(6–9): 729-731.

Schenk, P. M., S. R. Thomas-Hall, E. Stephens, U. C. Marx, J. H. Mussgnug, C. Posten, O. Kruse and B. Hankamer (2008). "Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production." BioEnergy Research **1**(1): 20-43.

Sheehan, J., T. Dunahay, J. Benemann and P. Roessler (1998). "A look back at the US Department of Energy's aquatic species program: biodiesel from algae." National Renewable Energy Laboratory **328**.

Simionato, D., M. A. Block, N. La Rocca, J. Jouhet, E. Maréchal, G. Finazzi and T. Morosinotto (2013). "The Response of Nannochloropsis gaditana to Nitrogen Starvation Includes De Novo Biosynthesis of Triacylglycerols, a Decrease of Chloroplast Galactolipids, and Reorganization of the Photosynthetic Apparatus." Eukaryotic Cell **12**(5): 665-676.

Sydney, E. B., W. Sturm, J. C. de Carvalho, V. Thomaz-Soccol, C. Larroche, A. Pandey and C. R. Soccol (2010). "Potential carbon dioxide fixation by industrially important microalgae." Bioresource Technology **101**(15): 5892-5896.

Terry, K. L. and L. P. Raymond (1985). "System design for the autotrophic production of microalgae." Enzyme and Microbial Technology **7**(10): 474-487.

USDOE (2013). Replacing the Whole Barrel-To Reduce U.S. Dependence on Oil.

USEPA (2016). Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2014.

Uusitalo, J. (1996). "Algal carbon uptake and the difference between alkalinity and high pH (" alkalinization"), exemplified with a pH-drift experiment." Scientia Marina **60**: 129-134.

Valenzuela, J., A. Mazurie, R. P. Carlson, R. Gerlach, K. E. Cooksey, B. M. Peyton and M. W. Fields (2012). "Potential role of multiple carbon fixation pathways during lipid accumulation in Phaeodactylum tricornutum." Biotechnology for biofuels **5**(1): 1.

Wensel, P., G. Helms, B. Hiscox, W. C. Davis, H. Kirchhoff, M. Bule, L. Yu and S. Chen (2014). "Isolation, characterization, and validation of oleaginous, multi-trophic, and haloalkaline-tolerant microalgae for two-stage cultivation." Algal Research **4**: 2-11.

White, D., A. Pagarette, P. Rooks and S. Ali (2013). "The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgae cultures." Journal of Applied Phycology **25**(1): 153-165.

Williams, P. J. l. B. and L. M. L. Laurens (2010). "Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics." Energy & Environmental Science **3**(5): 554.

Yang, Y. and K. Gao (2003). "Effects of CO2 concentrations on the freshwater microalgae, Chlamydomonas reinhardtii, Chlorella pyrenoidosa and Scenedesmus obliquus (Chlorophyta)." Journal of Applied Phycology **15**(5): 379-389.

APPENDICES

APPENDIX A

TABULATED DATA FROM *N. GADITANA* STUDIES

Table A.1. Air-1 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	8.42E+05	1.69E+05	7.36	0.04	40.4	5.4	0.00	0.00
1.0	1.11E+06	1.77E+05	7.27	0.05	44.2	7.2	0.02	0.06
2.0	2.09E+06	8.43E+05	7.31	0.09	42.8	6.3	0.04	0.03
3.0	2.74E+06	4.23E+05	7.52	0.08	52.4	1.2	0.05	0.03
4.0	5.42E+06	5.34E+05	7.83	0.09	47.6	18.1	0.12	0.00
5.0	9.35E+06	2.34E+06	8.14	0.16	36.8	5.6	0.19	0.07
6.0	1.56E+07	1.75E+06	8.60	0.17	41.2	5.1	0.58	0.21
7.0	2.36E+07	7.18E+06	8.87	0.16	31.6	8.1	0.94	0.36
8.0	3.55E+07	3.52E+06	9.04	0.19	21.7	15.4	1.43	0.71
9.0	4.52E+07	1.24E+07	9.26	0.21	15.6	15.3	2.07	0.91
10.0	5.57E+07	3.43E+07	9.38	0.19	12.0	5.2	2.93	1.35
11.0	6.04E+07	2.15E+07	9.48	0.08	11.2	5.5	3.18	0.31
12.0	7.13E+07	3.13E+07	9.53	0.10	10.0	3.8	3.38	1.01
13.0	8.23E+07	2.71E+07	9.64	0.14	7.9	2.3	4.43	2.41
14.0	8.21E+07	2.84E+07	9.58	0.10	6.4	6.7	5.16	2.50
15.0	8.40E+07	2.27E+07	9.68	0.10	6.7	10.0	5.72	2.21
16.0	9.50E+07	2.49E+07	9.61	0.06	3.5	5.6	7.11	1.71
17.0	1.07E+08	5.59E+07	9.65	0.10	2.5	4.4	5.64	1.28
18.0	9.36E+07	1.79E+07	9.84	0.06	n/d	n/d	6.88	2.53
19.0	1.04E+08	3.97E+06	9.82	0.07	n/d	n/d	6.89	1.94
20.0	1.23E+08	3.70E+07	9.73	0.12	n/d	n/d	7.25	1.80
21.0	1.71E+08	3.58E+07	9.78	0.09	n/d	n/d	7.41	1.50

Table A.2. Air-2 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	8.38E+05	2.47E+05	7.36	0.04	40.4	5.4	0.00	0.00
1.0	1.11E+06	1.77E+05	7.27	0.05	44.2	7.2	0.02	0.06
2.0	2.09E+06	8.43E+05	7.31	0.09	42.8	6.3	0.04	0.03
3.0	2.74E+06	4.23E+05	7.52	0.08	52.4	1.2	0.05	0.03
4.0	5.42E+06	5.34E+05	7.83	0.09	47.6	18.1	0.12	0.00
5.0	9.35E+06	2.34E+06	8.14	0.16	36.8	5.6	0.19	0.07
6.0	1.56E+07	1.75E+06	8.60	0.17	41.2	5.1	0.58	0.21
7.0	2.36E+07	7.18E+06	8.87	0.16	31.6	8.1	0.94	0.36
8.0	3.55E+07	3.52E+06	9.04	0.19	21.7	15.4	1.43	0.71
9.0	4.52E+07	1.24E+07	9.26	0.21	15.6	15.3	2.07	0.91
10.0	5.57E+07	3.43E+07	9.38	0.19	12.0	5.2	2.93	1.35
11.0	6.04E+07	2.15E+07	9.48	0.08	11.2	5.5	3.18	0.31
12.0	7.13E+07	3.13E+07	9.53	0.10	10.0	3.8	3.38	1.01
13.0	8.23E+07	2.71E+07	9.64	0.14	7.9	2.3	4.43	2.41
14.0	8.21E+07	2.84E+07	9.58	0.10	6.4	6.7	5.16	2.50
15.0	8.40E+07	2.27E+07	9.68	0.10	6.7	10.0	5.72	2.21
16.0	9.50E+07	2.49E+07	9.61	0.06	3.5	5.6	7.11	1.71
17.0	1.07E+08	5.59E+07	9.65	0.10	2.5	4.4	5.64	1.28
18.0	1.07E+08	3.01E+07	8.52	0.01	n/d	n/d	6.70	0.99
19.0	1.30E+08	4.56E+07	9.89	0.04	n/d	n/d	7.61	1.50
20.0	1.35E+08	6.12E+07	10.32	0.02	n/d	n/d	7.05	0.37
21.0	1.19E+08	2.12E+07	10.30	0.06	n/d	n/d	4.77	0.35

Table A.3. Air-3 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	1.05E+06	9.59E+04	8.40	0.05	47.7	2.1	0.36	0.06
1.0	1.85E+06	1.86E+05	8.83	0.04	44.0	2.5	0.07	0.03
1.9	3.75E+06	1.05E+06	8.84	0.06	48.1	3.3	0.14	0.01
3.0	1.10E+07	3.99E+06	9.50	0.14	44.9	1.9	0.26	0.09
4.0	2.16E+07	7.52E+06	10.00	0.08	36.4	4.3	0.67	0.28
5.0	3.62E+07	1.16E+07	10.43	0.10	27.3	3.3	1.25	0.20
6.0	4.83E+07	1.32E+07	10.52	0.05	21.7	2.3	1.29	0.55
7.0	6.31E+07	1.37E+07	10.49	0.05	11.4	1.5	0.97	0.17
8.0	8.32E+07	1.65E+07	10.53	0.04	12.8	3.3	1.07	0.19
9.0	9.38E+07	1.67E+07	10.50	0.16	9.0	3.8	1.24	0.19
10.0	8.61E+07	1.35E+07	10.39	0.16	10.5	4.5	1.16	0.19
11.0	9.24E+07	2.16E+07	10.41	0.17	8.3	9.5	1.23	0.20
12.0	8.97E+07	2.38E+07	10.34	0.29	4.8	5.0	1.28	0.20
13.0	9.52E+07	1.55E+07	10.28	0.30	2.7	4.2	1.27	0.34
14.0	9.27E+07	2.01E+07	9.42	0.02	n/d	n/d	1.97	0.51
15.0	9.55E+07	1.05E+07	9.64	0.22	n/d	n/d	1.66	0.62
16.0	8.19E+07	6.53E+07	9.64	0.57	n/d	n/d	1.08	1.35

Table A.4. CO_2-1 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	8.02E+05	2.38E+05	6.53	0.02	39.8	10.3	0.01	0.03
1.0	9.43E+05	1.00E+05	6.03	0.03	48.9	21.5	0.00	0.00
2.0	1.42E+06	2.70E+05	6.06	0.08	37.6	12.5	0.00	0.00
3.0	1.60E+06	1.44E+05	6.19	0.09	48.1	4.9	0.00	0.00
4.0	1.28E+06	5.04E+05	6.33	0.13	45.8	12.2	0.00	0.00
5.0	2.27E+06	9.85E+05	6.24	0.13	39.8	13.4	0.00	0.00
7.0	5.17E+06	3.56E+06	6.32	0.17	35.3	6.2	0.17	0.18
8.0	8.94E+06	9.61E+06	6.39	0.20	12.7	8.2	0.27	0.69
9.0	1.61E+07	9.20E+06	6.53	0.38	13.1	13.8	0.35	0.66
10.0	2.68E+07	2.16E+07	6.91	0.27	11.4	5.8	0.75	1.25
11.0	4.51E+07	3.93E+07	6.62	0.39	7.4	6.8	1.30	1.76
12.0	6.57E+07	4.79E+07	6.70	0.27	6.1	10.7	1.92	2.11
13.0	1.14E+08	1.76E+07	7.02	0.16	1.8	4.4	2.80	2.03
14.0	1.01E+08	6.71E+07	7.10	0.08	n/d	n/d	3.44	1.59
15.0	1.22E+08	2.64E+07	9.70	0.08	n/d	n/d	3.34	2.11
16.0	1.36E+08	1.60E+07	9.41	0.18	n/d	n/d	2.88	1.94
17.0	1.22E+08	4.66E+07	9.47	0.11	n/d	n/d	2.97	1.78
18.0	1.15E+08	6.01E+07	9.51	0.09	n/d	n/d	2.93	2.09

Table A.5. CO_2-2 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	1.71E+06	1.09E+06	7.98	0.02	48.4	4.5	0.38	0.27
1.0	1.96E+06	2.35E+05	7.74	0.04	47.2	3.3	0.08	0.00
1.9	3.63E+06	5.45E+05	7.70	0.22	49.5	4.2	0.17	0.19
3.0	1.05E+07	3.00E+06	7.87	0.25	46.4	0.2	0.19	0.03
4.0	2.35E+07	5.88E+06	8.14	0.66	41.7	6.4	0.51	0.09
5.0	3.78E+07	5.98E+06	7.73	0.32	31.4	3.1	1.36	0.20
6.0	8.06E+07	1.90E+07	8.12	0.40	7.3	4.2	3.79	0.77
6.6	1.36E+08	3.66E+07	8.27	0.38	n/d	n/d	4.78	0.49
7.0	1.27E+08	8.97E+06	8.25	0.28	n/d	n/d	4.47	0.32
7.6	1.54E+08	3.03E+07	8.03	0.20	n/d	n/d	3.71	0.37
8.0	1.69E+08	4.15E+07	7.85	0.05	n/d	n/d	2.80	0.45
8.6	1.73E+08	4.75E+07	7.90	0.05	n/d	n/d	2.84	0.76
9.0	1.81E+08	2.68E+07	8.10	0.07	n/d	n/d	2.35	0.34
9.6	1.86E+08	1.84E+07	8.57	0.11	n/d	n/d	2.22	0.43

Table A.6. CO_2/HCO_3-1 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	8.68E+05	1.18E+05	7.35	0.03	41.6	6.8	0.04	0.07
1.0	1.03E+06	1.96E+05	7.33	0.02	42.5	10.9	0.04	0.08
2.0	2.25E+06	2.91E+05	7.30	0.11	45.6	13.5	0.03	0.01
3.0	2.88E+06	2.65E+05	7.51	0.08	48.6	6.0	0.11	0.18
4.0	6.12E+06	1.39E+06	7.87	0.09	48.7	13.7	0.13	0.07
5.0	1.12E+07	1.63E+06	7.82	0.09	40.0	4.5	0.33	0.04
6.0	2.25E+07	4.67E+06	7.87	0.16	38.5	2.2	0.80	0.33
7.0	4.38E+07	5.88E+06	8.13	0.13	18.6	2.8	1.82	0.42
8.0	8.47E+07	2.23E+07	8.04	0.12	8.3	3.6	3.80	1.58
9.0	1.00E+08	7.00E+06	8.25	0.13	0.2	0.4	7.36	1.28
10.0	1.39E+08	5.48E+07	8.18	0.15	0.6	0.0	6.74	2.22
11.0	1.45E+08	5.16E+07	8.33	0.07	n/d	n/d	6.22	1.14
12.0	1.40E+08	5.40E+07	8.16	0.18	n/d	n/d	4.69	1.83
13.0	1.59E+08	6.88E+07	8.31	0.11	n/d	n/d	4.04	2.15

Table A.7. CO_2/HCO_3-2 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	8.27E+05	6.90E+04	8.04	0.01	34.1	12.8	0.05	0.05
1.0	1.24E+06	1.38E+05	8.05	0.13	43.8	14.2	0.02	0.03
2.0	2.21E+06	6.87E+05	8.02	0.07	37.3	2.5	0.03	0.00
3.0	3.57E+06	5.23E+05	8.06	0.01	47.4	5.1	0.07	0.03
4.0	6.41E+06	1.77E+06	8.00	0.03	40.6	6.0	0.15	0.05
5.0	1.45E+07	3.16E+06	7.97	0.11	37.8	6.2	0.40	0.22
6.0	2.57E+07	1.58E+07	8.15	0.15	36.8	6.2	1.02	0.90
7.0	4.77E+07	1.83E+07	8.45	0.45	18.2	5.7	2.08	2.02
8.0	8.69E+07	6.87E+07	8.35	0.34	10.2	14.0	3.99	4.45
9.0	9.70E+07	6.08E+07	8.40	0.38	10.2	21.5	5.22	4.39
10.0	1.47E+08	7.13E+07	8.36	0.18	0.5	0.2	6.12	2.32
11.0	1.43E+08	8.22E+07	8.42	0.12	n/d	n/d	4.88	2.92
12.0	1.46E+08	1.35E+08	8.44	0.19	n/d	n/d	3.00	2.37
13.0	1.35E+08	8.35E+07	8.48	0.24	n/d	n/d	2.45	3.45

Table A.8. CO_2/HCO_3-3 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	1.74E+06	6.98E+05	8.32	0.05	48.1	1.2	0.35	0.05
1.0	2.01E+06	2.65E+05	8.23	0.01	46.8	0.7	0.07	0.03
1.9	4.04E+06	9.85E+05	8.25	0.03	49.5	2.5	0.14	0.11
3.0	1.04E+07	2.34E+06	8.38	0.03	46.6	2.4	0.19	0.09
4.0	2.52E+07	5.94E+06	7.68	0.35	41.0	2.2	0.56	0.22
5.0	4.37E+07	6.90E+06	8.12	0.08	30.8	5.3	1.47	0.53
6.0	8.15E+07	2.27E+07	8.20	0.14	9.0	7.6	3.85	0.84
6.6	1.17E+08	3.56E+07	8.29	0.05	n/d	n/d	4.35	0.85
7.0	1.07E+08	2.22E+07	8.45	0.03	n/d	n/d	4.21	0.65
7.6	1.23E+08	4.75E+07	8.29	0.08	n/d	n/d	3.82	0.49
8.0	1.40E+08	3.81E+07	8.43	0.04	n/d	n/d	3.42	0.41
8.6	1.40E+08	1.74E+07	8.30	0.08	n/d	n/d	2.90	0.47
9.0	1.40E+08	2.62E+07	8.31	0.07	n/d	n/d	2.71	0.33
9.6	1.64E+08	1.97E+07	8.35	0.04	n/d	n/d	2.41	0.41

Table A.9. CO_2/HCO_3-4 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	1.58E+06	7.83E+05	7.66	0.16	49.9	0.3	0.36	0.16
1.0	2.00E+06	5.87E+05	7.59	0.19	46.6	0.6	0.08	0.00
1.9	2.92E+06	2.17E+05	7.12	0.09	49.3	4.1	0.09	0.03
3.0	5.71E+06	1.06E+06	6.86	0.35	47.5	2.9	0.11	0.05
4.0	1.20E+07	2.28E+06	7.43	0.15	43.6	1.0	0.19	0.03
5.0	1.92E+07	8.20E+05	7.39	0.07	37.9	1.6	0.57	0.04
6.0	5.01E+07	8.00E+06	7.38	0.18	20.6	7.0	1.82	0.45
7.0	8.46E+07	3.59E+07	7.48	0.08	n/d	n/d	3.11	0.91
8.0	1.20E+08	1.18E+07	10.39	0.03	n/d	n/d	2.68	0.00
9.0	1.21E+08	6.56E+06	10.30	0.08	n/d	n/d	2.55	0.33
10.0	1.10E+08	2.59E+07	10.21	0.08	n/d	n/d	2.22	0.21

Table A.10. CO_2/HCO_3-5 average values ± 95% confidence interval for cell density [cells mL^{-1}], pH, nitrate concentration [mg N (NO_3^-) L^{-1}], and total chlorophyll [mg L^{-1}].

Time [days]	Cell Counts [cells·mL^{-1}]		pH		Nitrate Concentration [mg N (NO_3^-)·L^{-1}]		Total Chlorophyll [mg Chl·L^{-1}]	
	Average	95% CI	Average	95% CI	Average	95% CI	Average	95% CI
0.0	1.63E+06	5.34E+05	6.70	0.14	48.3	0.4	0.41	0.11
1.0	1.72E+06	1.50E+05	6.44	0.36	47.0	2.2	0.07	0.04
1.9	2.67E+06	4.85E+05	6.13	0.02	48.2	2.7	0.08	0.11
3.0	6.15E+06	1.26E+06	5.98	0.24	47.8	1.4	0.08	0.00
4.0	1.12E+07	2.43E+06	6.57	0.07	43.9	1.0	0.19	0.08
5.0	1.97E+07	1.80E+06	6.67	0.09	39.3	4.0	0.53	0.23
6.0	3.82E+07	4.57E+06	6.67	0.10	24.9	5.1	1.39	0.60
7.0	7.17E+07	1.54E+07	7.08	0.07	n/d	n/d	2.69	0.87
7.6	1.18E+08	3.74E+07	8.54	0.08	n/d	n/d	3.21	0.42
8.0	1.09E+08	2.29E+07	9.32	0.10	n/d	n/d	3.37	0.22
8.6	1.05E+08	3.25E+06	9.58	0.04	n/d	n/d	3.09	0.34
9.0	1.11E+08	1.88E+07	10.09	0.07	n/d	n/d	2.83	0.10
9.6	1.20E+08	6.56E+06	10.12	0.29	n/d	n/d	2.33	0.24
10.0	1.09E+08	8.00E+06	10.44	0.21	n/d	n/d	1.94	0.40
10.6	1.06E+08	1.46E+07	10.24	0.11	n/d	n/d	1.68	0.21
0.0	1.63E+06	5.34E+05	6.70	0.14	48.3	0.4	0.41	0.11

APPENDIX B

SUPPLEMENTAL MATERIAL FROM *C. VULGARIS* STUDIES

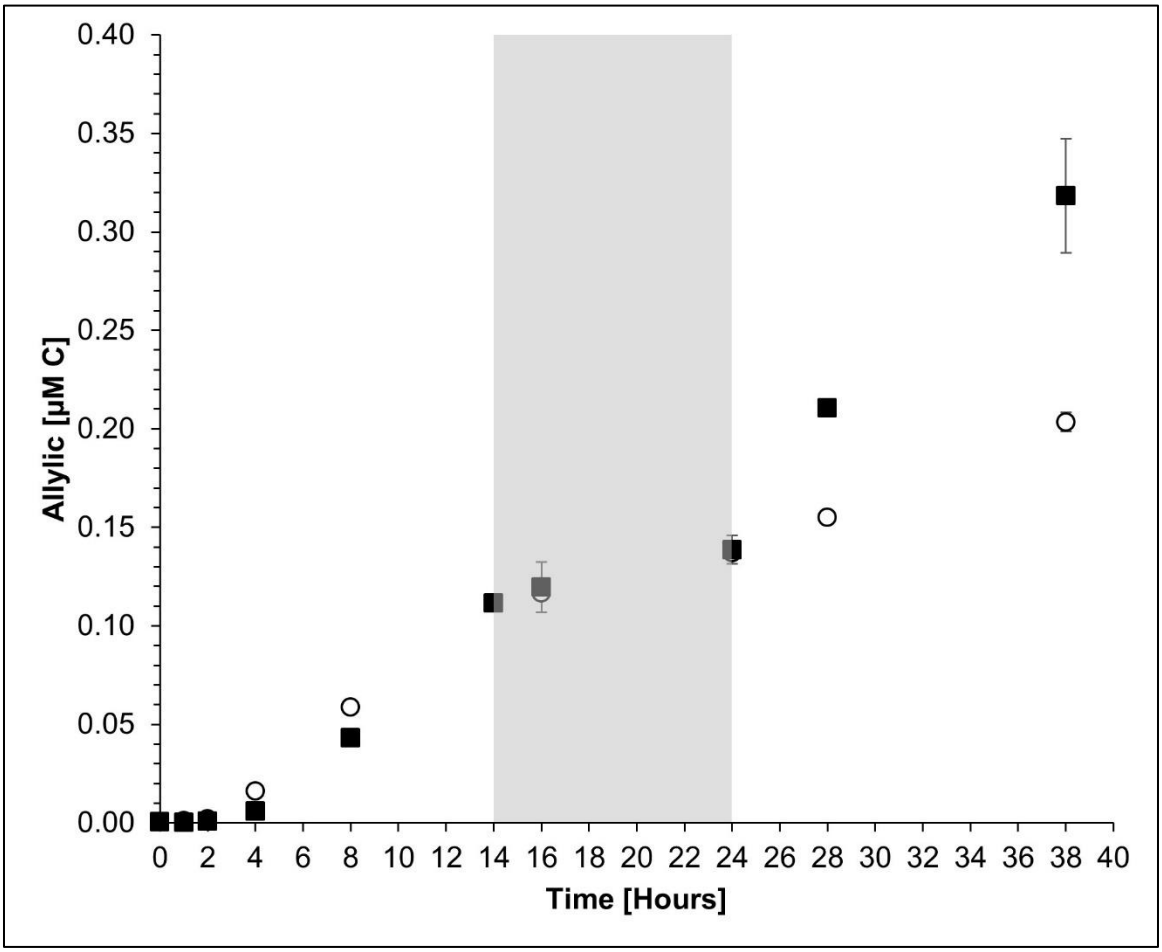

Figure B.1. Monounsaturated fatty acid (MUFA) chain accumulation monitored in *C. vulgaris* using ^{1}H HR-MAS NMR and $H^{13}CO_3^-$ labeling. Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.

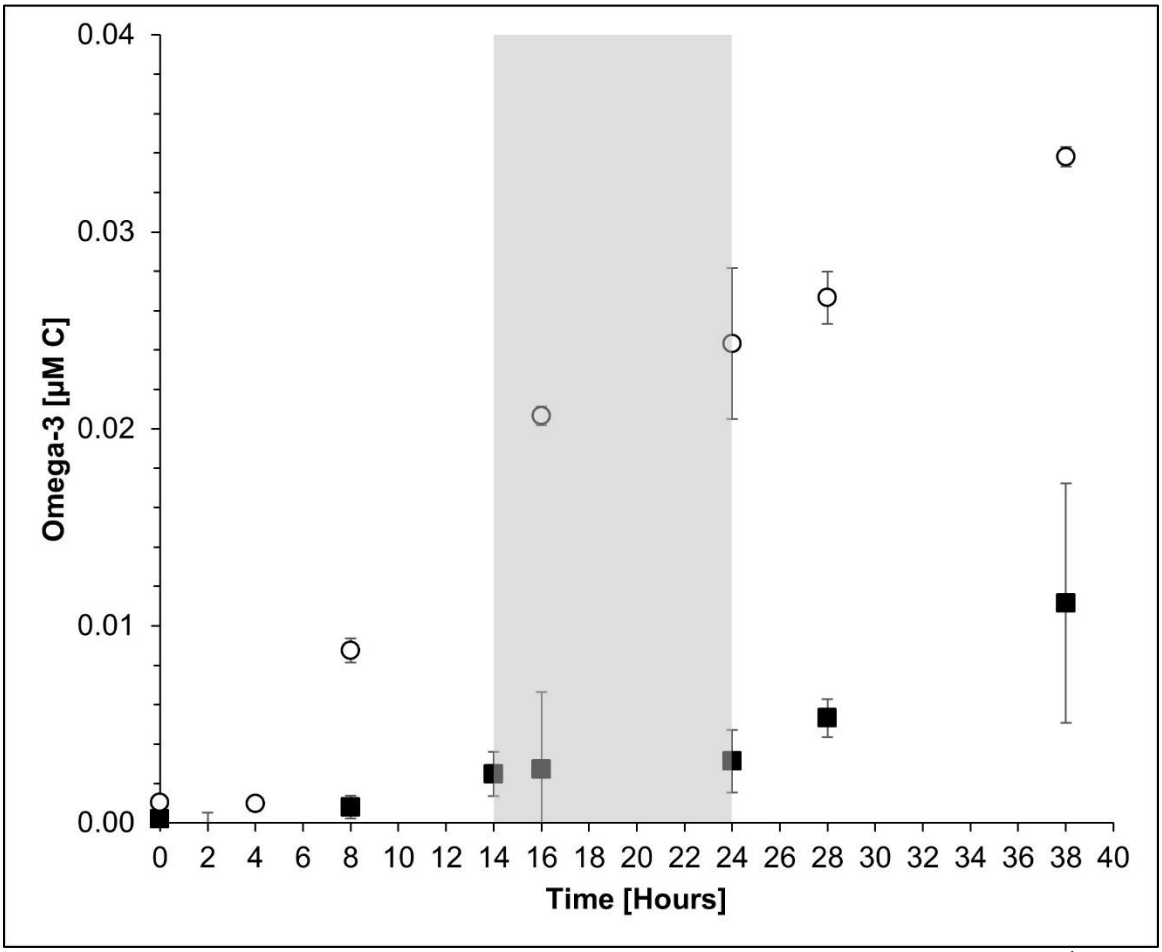

Figure B.2. Omega-3 fatty acid chain accumulation monitored in *C. vulgaris* using ^{1}H HR-MAS NMR and $H^{13}CO_3^-$ labeling. Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.

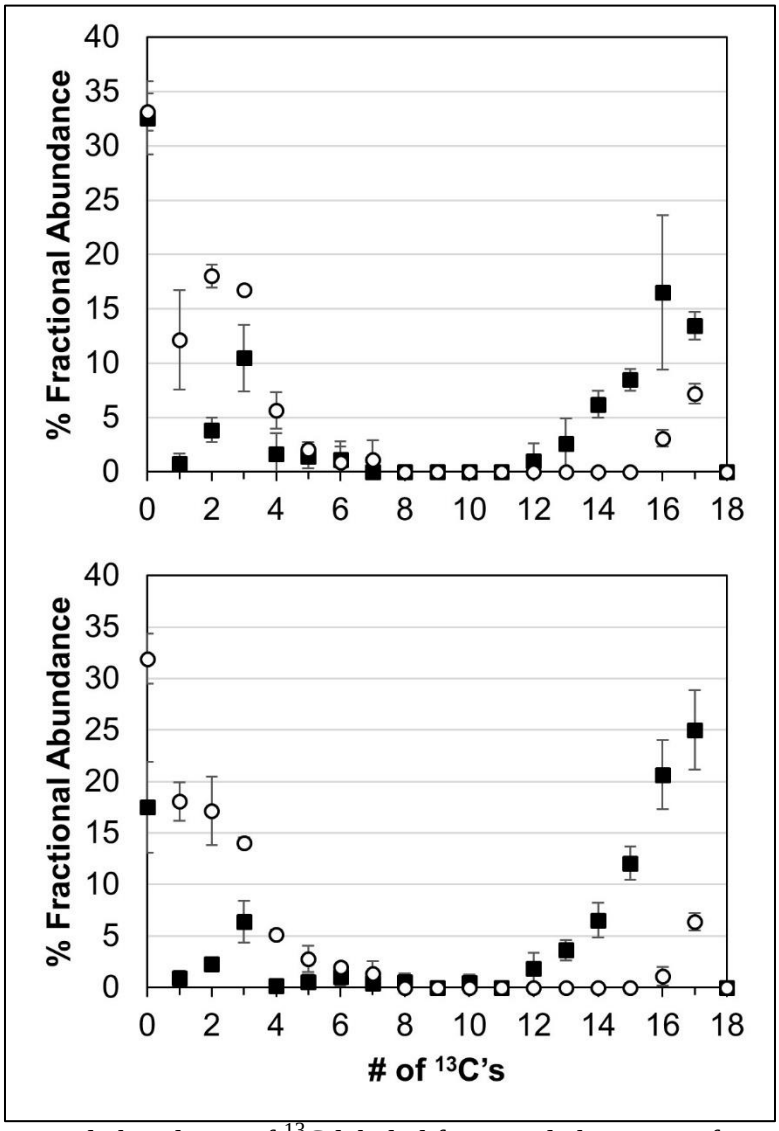

Figure B.3. Fractional abundance of ^{13}C labeled fatty acid chains as a function of number of added ^{13}C labels for unsaturated (C18:2) fatty acid chains. Samples are shown from 24 hours after re-suspension (top) and 38 hours after re-suspension (bottom). Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.

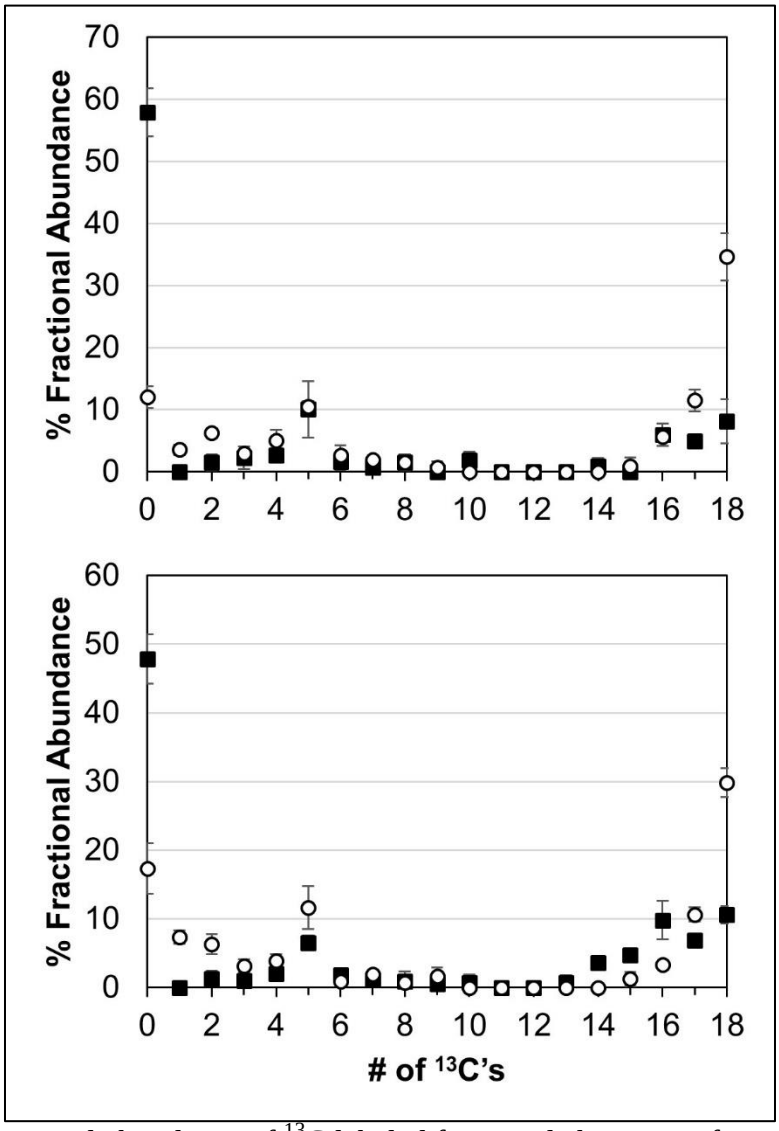

Figure B.4. Fractional abundance of ^{13}C labeled fatty acid chains as a function of number of added ^{13}C labels for omega-3 (C18:3) fatty acid chains. Samples are shown from 24 hours after re-suspension (top) and 38 hours after re-suspension (bottom). Forward experiment (■) expressing *de novo* metabolite synthesis during nitrogen deplete culturing from labeled bicarbonate supplied at re-suspension. Reverse experiment (○) expressing recycling of metabolites accumulated during nitrogen replete culturing from labeled bicarbonate supplied during exponential growth of cultures prior to re-suspension.