EVALUATION OF A GREEN ALGA ISOLATE FOR GROWTH AND LIPID ACCUMULATION IN COAL BED METHANE WATER FROM THE POWDER RIVER BASIN

by

Logan Henry Hodgskiss

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

of

Master of Science

in

Environmental Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2015

©COPYRIGHT

by

Logan Henry Hodgskiss

2015

All Rights Reserved

DEDICATION

I would like to dedicate this thesis to my parents, Lyle and BethAnn Hodgskiss, my siblings, Taylor, Connor, and Sarah, and my grandma, Maryetta Hodgskiss for always being loving and supporting of my career and my interests.

ACKNOWLEDGEMENTS

First, I would like to thank my research advisor, Matthew W. Fields, for the opportunity to be a part of his lab and learn what research is all about as well as making my first undergraduate microbiology class so intriguing. I would also like to thank Al Cunningham for fulfilling the role of my academic advisor and guiding me through my Master's program. I am also grateful to the remainder of my committee, Robin Gerlach and Brent Peyton, for the feedback and support of my thesis work. The continual support of the Fields Lab, the MSU Algae Group, the MSU CBM Group, and the students, staff, and faculty of the Center for Biofilm Engineering have also been an integral part of the success of my Master's thesis. I would especially like to thank Warren Jones, one of my undergraduate professors, for taking my interest in graduate school seriously and connecting me with Matthew Fields and the rest of the Center for Biofilm Engineering before his unfortunate passing the summer after my undergraduate graduation. His involvement truly made the difference in my career path.

The support of my family and friends has also meant the world to me and I will be eternally grateful for the many special people that have become a part of my life. I would particularly like to thank my mother, for encouraging me in my decision to take a general microbiology class instead of physics II because, "you never know what might come of it."

TABLE OF CONTENTS

1. INTRODUCTION 1

Coal Bed Methane Production Water 1
Microalgae 3
Advantages of Using Microalgae for Biodiesel Production 4
Cellular Cycle 5
Nutrient Uptake 7
Batch Reactor 7
CSTR-like System 8
Potential for Growth in CBM Production Water 10
Nutrients 13
Nitrogen and Phosphorus 13
Carbon Dioxide 15
Salt 17
Light 18
Gene Expression in Microalgae 18
Overview 20
Harvesting Microalgae 20
Summary 22

2. MATERIALS AND METHODS 24

Organism 24
Media Composition 24
Growth Conditions 26
pH 27
Cell Counts 27
Dry Weights 28
Chlorophyll Measurements 28
Anion Measurements 29
Lipid Accumulation 29
Preliminary Experiments 30

3. GROWTH OF A NATIVE ALGAL SPECIES IN COAL BED METHANE WATER FOR BIOFUEL AND BIOMASS ACCUMULATION 34

Contribution of Authors and Co-Authors 34
Manuscript Information Page 35
Abstract 36
Introduction 36

TABLE OF CONTENTS – CONTINUED

Materials and Methods 40
Culturing Conditions 40
Sterile CBM Water 40
Dry Weight 41
Chlorophyll Measurements 41
Lipid Accumulation 42
Anion Concentrations 42
GC-MS Lipid Analysis 43
Results and Discussion 43
Water Chemistry 43
CBM Water without Additions 45
CBM Water with Nitrate 46
CBM Water with Nitrate and Phosphate 46
CBM Water with Nitrate, Phosphate, Magnesium, and Sulfate 46
CBM Water with Nitrate, Phosphate, Magnesium, Sulfate, and Micronutrients 47
Bold's Basal Medium 47
Growth in Sterile CBM Water and BBM 48
Lipid Analysis 55
Summary 56

4. CONCLUSIONS AND FUTURE WORK 58

REFERENCES CITED 63

APPENDICES 74

APPENDIX A: Supporting Material for Chapter Two Materials and Methods 75
APPENDIX B: Supporting Material for CBMW Isolate 89

LIST OF TABLES

Table		Page
1.1	Nutrient concentrations at inflow (IR) and outflow (Q) for algae grown in rice fields with herbicide. Data taken from Aschonitis et al. (2013)	9
2.1	Constituents of Bold's Basal Medium	25
2.2	Constituents of microelement solution for Bold's Basal Medium	25
2.3	Constituents of S1 solution for Bold's Basal Medium	25
2.4	Constituents of S2 solution for Bold's Basal Medium	25
2.5	Constituents of S3 solution for Bold's Basal Medium	26
2.6	Constituents of B12 vitamin solution for Bold's Basal Medium	26
2.7	Constituents of coal bed methane water from the FG-09 well Located in the Powder River Basin	27
3.1	Nutrients observed in CBM water and BBM	44
3.2	Nutrients observed in CBM water only	44
3.3	Nutrients observed in BBM only	45

LIST OF FIGURES

Figure Page

1.1 Percent reduction of contaminants in dairy wastewater by *C. polypyrenoideum*. Cultures were grown in 500 mL of 3:1 (75% wastewater:25% distilled water) wastewater mix in 1 L flasks (Kothari et al., 2013) 8

1.2 Growth of an algal consortium in distilled water (DIW), coal seam gas water with a TDS of 1.7 g/L (CSG1.7), coal seam gas water with a TDS of 11.6 g/L (CSG11.6), sea water diluted to an EC value corresponding to CSG1.7 (SEA1.7), and sea water diluted to an EC value corresponding to CSG11.6 (SEA11.6). Experiments were performed in 1 L fed-batch reactors with added nutrients. Error bars represent one standard deviation between replicate reactors (Buchanan et al., 2013) 11

1.3 Depletion of nitrogen (○) and phosphorus (◊) along with growth of *P. tricornutum* (▲). Arrows represent time points where cells were collected for RNA sequencing (Valenzuela et al., 2012) 14

1.4 **A**.) Lipid production (■) of *P. tricornutum* is observed to increase when cellular growth (▲) becomes stationary. **B**.) Lipid accumulation (■) of *P. tricornutum* increases after nitrogen (○) and phosphorus (◊) depletion (Valenzuela et al., 2012) 16

3.1 Lipid production of CBMW when grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates 49

LIST OF FIGURES – CONTINUED

Figure Page

3.2 pH of CBMW when grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates 50

3.3 Dry weight of CBMW when grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates 51

3.4 (**A)** Lipid accumulation (■) and nitrate depletion (□) in CBM water with 0.5 mM NO_3^- and (**B)** lipid accumulation (■), nitrate depletion (□), and phosphate depletion (Δ) in CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-}. Phosphate concentration is multiplied by 10 for scaling purposes. Error bars represent min and max of technical duplicates 52

LIST OF FIGURES – CONTINUED

Figure Page

3.5 Total chlorophyll (*a, b,* and *c*) of CBMW grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Grey area shows time frame when nitrate was depleted. Error bars represent one standard deviation of biological and technical duplicates 54

3.6 Lipid composition of CBMW grown in CBM water with 0.5 mM NO_3^- (white), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (light grey) and CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (dark grey). Lipid content is % w/w of fatty acid to biomass. Error bars represent one standard deviation among technical and biological duplicates 57

A.1 Nile Red fluorescence was taken at varying dilutions of CBMW to determine a linear range of cell count values 76

A.2 A time assay for the Nile Red fluorescence method was performed on CBMW. Nile Red fluorescence was taken at marked time points after samples were stained with Nile Red to determine the optimum staining time. Cultures were kept in the dark to avoid light interference 76

A.3 pH values of CBMW grown in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates 77

A.4 Cell count of CBMW grown in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates 78

A.5 Chlorophyll levels of CBMW in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates 78

LIST OF FIGURES – CONTINUED

Figure Page

A.6 Lipid accumulation of CBMW grown in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates 79

A.7 pH of CBMW grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates 80

A.8 Cell counts of CBMW grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates 80

A.9 Chlorophyll concentrations of CBMW when grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates 81

A.10 Lipid accumulation of CBMW when grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates 81

A.11 pH values of CBMW grown in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates 83

A.12 Cell counts for CBMW grown in in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates 83

A.13 Chlorophyll concentrations for CBMW when grown in in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates 84

LIST OF FIGURES – CONTINUED

Figure Page

A.14 Lipid accumulation of CBMW when grown in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates....84

A.15 Nitrate concentrations of cultures grown with CBMW in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates....85

A.16 Phosphate concentrations of cultures grown with CBMW in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates....85

A.17 Nutrient depletion and Nile Red fluorescence for CBMW grown in Bold's Basal Medium (Filtered and Unfiltered Experiment)....86

A.18 CBMW when transferred from Bold's Basal Medium to non-sterile CBM water. Clumping occurs approximately 10-15 minutes after inoculation with slight shaking....87

A.19 (**A)** Comparison of CBMW when inoculated into filter sterilized CBM water and (**B)** non-sterile CBM water....87

A.20 Water chemistry of CBM water over approximately one year. Water from the same container was used for all experiments. CBM water was stored in a 5°C, dark, walk-in refrigerator. Red dotted line indicates the time when the clumping behavioral change occurred. The second time point (10/04/2013) had to be diluted (5x) due to volume concerns. The dilution pushed many of the concentrations below the accurate range of the IC machine, possibly accounting for the increase observed in some of the concentrations. Chloride concentrations are on the secondary axis (on the right side of the graph). All other concentrations are on the primary axis (left side of the graph)....88

LIST OF FIGURES – CONTINUED

Figure Page

B.1 Coal bed methane production pond where CBMW was isolated from. The isolate was taken on October 18, 2010 from the CBM water inflow (written communication with Elliott Barnhart). Image taken from Google Earth 90

B.2 CBMW under a brightfield microscope while in 2 mM nitrate Bold's Basal Medium and cultured at 25°C. Cells were stained with a cell mask orange (CMO) stain. CMO appears green while autofluorescence of chlorophyll appears red. Photo taken in the Microscope Facility at the Center for Biofilm Engineering, Montana State University and provided courtesy of Luisa Corredor.....91

B.3 Nile Red fluorescence (yellow) of lipid droplets and autofluorescence of chlorophyll (red) in CBMW cultured in 0.5 mM nitrate Bold's Basal Medium at 25°C. Photo taken in the Microscope Facility at the Center for Biofilm Engineering, Montana State University and provided courtesy of Luisa Corredor.....91

ABSTRACT

Coal bed methane (CBM) production ponds are being constructed more frequently in areas such as the Powder River Basin in Montana and Wyoming where methane production has been active in the past decade. These ponds are currently not being utilized and are holding billions of gallons of water. The extracted water in these ponds is presently being discharged to local stream drainages or infiltrating into the surrounding soil. The environmental impacts of this increase in water can have negative effects on the surrounding areas. The purpose of this thesis is to explore the possibility of using CBM production ponds in the Powder River Basin, in Montana and Wyoming, for the growth of microalgae and the production of biodiesel from their accumulated lipids. Microalgae have been known to grow in other bodies of undesirable water and research has been ongoing on how to effectively use microalgae as a resource by stimulating lipid accumulation through the use of various environmental stressors. Coal bed methane ponds already provide a source of non-potable water for microalgae cultivation. Exploring the possibility of making these ponds a growth medium for microalgae is the first step in determining whether they can be turned into a productive energy resource. A native green alga, CBMW, has been isolated from a CBM production pond in northeastern Wyoming. CBMW has been cultured and grown under laboratory conditions in sterile CBM water and Bold's Basal Medium (BBM). Chlorophyll levels, biomass growth, pH, lipid accumulation, and water chemistry were tracked while CBMW was grown in sterile CBM water to understand how the alga responds to varying environmental conditions. When grown under the right environmental conditions isolate CBMW increased biomass and accumulated lipids. These results suggest that attempting to grow CBMW on a larger scale in CBM production water could be an effective method to produce biodiesel while utilizing a potentially problematic water source.

CHAPTER ONE

INTRODUCTION

Coal bed methane (CBM) is a source of energy that is becoming increasingly popular in the United States (www.eia.gov, 2014). Methane is produced deep within the ground in coal seams due to biogenic or thermogenic activity and can be extracted from coal seams and brought to the surface. The most common method of extraction is to pump water out of the ground causing the underground aquifers to depressurize (McBeth et al. 2003). Once an aquifer is depressurized, methane can desorb from the coal and be collected (Wheaton & Olson, 2001). While de-watering allows the collection of natural gas, the massive amount of produced water is a waste stream that is an environmental hazard. A possible use for CBM water is growing microalgae to produce biomass and lipids for biodiesel production. The purpose of the research presented in this thesis is to explore the potential for growing a native algal species, strain CBMW, in CBM production water ponds in order to turn these ponds into a productive energy resource.

Coal Bed Methane Production Water

The collection of methane for use as an energy source results in large amounts of water being pumped out of underground aquifers. This causes methane to desorb from coal seams making it available for collection (Wheaton & Olson, 2001). In 2014 the Powder River Basin in Wyoming alone produced, 256 million Bbls (8.07 billion gallons) of water due to the extraction of methane (WGOCC, 2014). CBM water is high in

sodium, bicarbonate, and total dissolved solids (TDS) (Healy et al. 2011; Jackson & Reddy, 2007; McBeth et al., 2003) making it difficult to handle once it reaches the surface. Most of the available options are expensive or environmentally unfriendly and are beginning to be more strictly regulated.

Coal bed methane sites are often located in rural areas that would make irrigation a convenient method of disposing of the water. Unfortunately, the sodium adsorption ratio (SAR) is too high in many cases to make this possible. SAR is a ratio of sodium concentration in water relative to the concentrations of calcium and magnesium. Water with an SAR value higher than 10 is not recommended for irrigation purposes (Healy et al., 2011; Richards et al., 1954). Water with SAR levels above 10 can cause sodium ions to accumulate to high levels in the soil, negatively affecting soil properties such as infiltration rate (Ganjegunte et al., 2008).

In some instances, discharging small amounts of water to nearby streams or rivers is acceptable. Wyoming, for example, allows small portions of CBM water to be discharged into streams and drainages ("Guidance and Tech for CBM Permitting under WYPDES"). However, the allowable amounts are not nearly enough to take care of the large volume of water that is being produced. Another possibility is reinjection of the produced water back into the ground (Ross & Zoback, 2008). While this sounds feasible, the geology of the formation has to be impermeable enough to insure that the water will not contaminate nearby aquifers or alter coal play hydrology. This can result in the water needing to be transported over great distances to an acceptable site, which becomes costly and uneconomical. A third option would be to apply reverse osmosis, or a filtering

technique, to remove high concentrations of solutes in the water (Welch, 2009). Once again, this becomes costly because of the large amount of water that would need to be treated and the high energy cost of the process.

Due to the cost, inconvenience, and environmental restrictions of other options, a large majority of the water is stored in unlined ponds where the water evaporates to the atmosphere or infiltrates the soil. The unlined ponds allow the CBM water to percolate into the soil profile creating the potential for many undesired effects on local groundwater. Some of these effects include changes in pH, TDS, and raising the groundwater table (Healy et al., 2011). It is hard to predict what changes will occur to the local water quality because of the diversity of geology and soil chemistry of any given site with a CBM pond. In any case, CBM water stored in ponds raises many concerns about the effects it will have on the surrounding environment (Davis et al., 2009; Reddy et al., 2014; Zou et al., 2006). However, the possibility of utilizing these ponds to grow microalgae could be an environmentally friendly alternative.

Microalgae

Microalgae are single celled organisms with a promising potential for biodiesel production. They can be grown in large bodies of water and produce lipids when cultivated under the right conditions. CBM ponds could be an ideal place to grow microalgae as they are a preexisting water source. The next section will take a closer look at some of the general characteristics of microalgae, particularly in the areas of lipid

accumulation and nutrient uptake, as well as the possibility of growing microalgae in CBM water.

Advantages of Using Microalgae for Biodiesel Production

Microalgae are very efficient producers of bio-oil compared to more traditional crops such as cotton and soybeans. A comparison and analysis performed by Schenk et al. showed that algae have the potential to produce up to 98,500 L/ha/year (10,500 gal/ac/year) of biodiesel compared to 325 L/ha/year (34.7 gal/ac/year) for cotton and 446 L/ha/year (47.7 gal/ac/year) for soybeans. This correlates to anywhere from 15-300 times more biodiesel that can be produced (Schenk et al., 2008). These calculations show that significantly less area would be required for microalgae to produce an equivalent amount of biodiesel when compared to cotton, soybean, and other traditional sources. Microalgae can also be grown in ponds on non-arable land (Singh et al., 2011). This becomes advantageous because the production of algal biodiesel would not compete with land that might be needed to grow food. From an agricultural standpoint, algae also have a very quick life cycle. Algae could potentially be harvested for lipids every 1-10 days (Schenk et al., 2008) with the remaining biomass being used as fertilizer (Mulbry et al., 2005) or organic matter for an anaerobic digester system (Golueke et al., 1957). As algae grow, they also fix carbon dioxide from the atmosphere making it a beneficial process to help balance carbon dioxide emissions.

Cellular Cycle

Microalgae have not always been looked at as a beneficial resource. Until recently, mass algae growth in water was considered harmful because of the end effects of the water quality and the toxins that some diatom and cyanobacteria species produce (Hernandez-Becerril et al., 2007). Algae thrive in eutrophic zones of water where there are plenty of nutrients available (Heisler et al., 2008). The algae will continue to grow until the nutrients run out or the growth becomes so thick that light becomes a limiting resource. At this point, algae will start to decay. Once algal growth declines, dead cells are utilized by heterotrophs in the water as a carbon source. Heterotrophs also consume the dissolved oxygen in the water since it is the preferred terminal electron acceptor of many bacteria in aquatic systems. The resulting anoxic water is deadly to plants and animals that rely on dissolved oxygen to survive. Ultimately, it is not the algae's metabolism that is directly causing the lack of oxygen, but merely the presence of algae as a carbon source (Schenk et al., 2008).

In some species of microalgae, lipid accumulation naturally occurs when the algae begin to experience nutrient deplete conditions (Gardner et al., 2011; Lohman et al., 2014; Valenzuela et al., 2012; X. P. Zhou et al., 2013). In nature, this is a good survival mechanism for algae. The algae begin converting carbon dioxide to neutral lipids to use later. When light becomes limiting due to culture density or other factors, the algae can start to utilize the accumulated lipids to prolong survival (Manoharan et al., 1999).

Fixation of atmospheric carbon dioxide is a part of algal metabolism and their main carbon source. The energy required to fix carbon dioxide comes from the process

of photosynthesis (White et al., 2012). Algae contain light harvesting complex proteins that absorb energy from photons. These proteins are known as chlorophyll and carotenoids. Chlorophyll is what gives green algae their characteristic green color. Other algae, such as red and brown algae, are primarily colored from carotenoids. Light energy captured by these pigments is used in a structure called photosystem II (PSII) for the following reaction (White et al., 2012):

$$H_2O \rightarrow 2H^+ + 2e^- + \frac{1}{2}O_2 \qquad \text{Eq. 1.1}$$

The hydrogen ions and electrons produced are crucial to the cellular processes of algae. Electrons are sent down the photoelectron transport chain of the cell and the hydrogen ions are used to produce a proton gradient. This gradient is then used to produce ATP. Electrons are transferred onto NADP+ to form NADPH via photosystem I (PSI) at the end of the photoelectron transport chain (White et al., 2012). NADPH is an important cofactor in biomass production and lipid biosynthesis (White et al., 2012). ATP and NADPH are also essential for the Calvin Cycle, the pathway used for carbon fixation (White et al., 2012).

A key enzyme in the Calvin Cycle is Rubisco. This enzyme combines carbon dioxide and ribulose-1,5-bisphosphate (RuBP) (White et al., 2012). After the fixation of carbon, the carbon can be allocated by the cell for a variety of processes and eventually be used to make acetyl-CoA, one of the building blocks of lipids (White et al., 2012).

Nutrient Uptake

A beneficial attribute of many microalgae species is their ability to improve water quality by utilizing unwanted nutrients found in the water. This has been observed in a variety of systems including batch cultures (Kothari et al., 2013) as well as arrangements that more closely resemble a continuously stirred tank reactor (CSTR) (Aschonitis et al., 2013).

Batch Reactor

One study observed the effect of nutrient utilization by algae in concentrated dairy wastewater using *Chlamydomonas polypyrenoideum*. Dairy wastewater is very high in TDS, nitrate, and phosphate. In this particular example, these values were 781 mg/L, 78.31 mg/L, and 5.58 mg/L respectively (Kothari et al., 2013). The water sample also contained ammonia and nitrite. Due to the high levels of contaminants, the wastewater was diluted at different levels with distilled water to see which combination was the most effective for growth and to ensure that the high concentrations of the dairy wastewater would not be toxic to the algae. The optimal result was a ratio of 3:1 (dairy wastewater: distilled water). 500 mL of the 3:1 mix were placed in 1 L flasks, inoculated with algae, and allowed to grow for 15 days (Kothari et al., 2013). The results can be seen below in Figure 1.1.

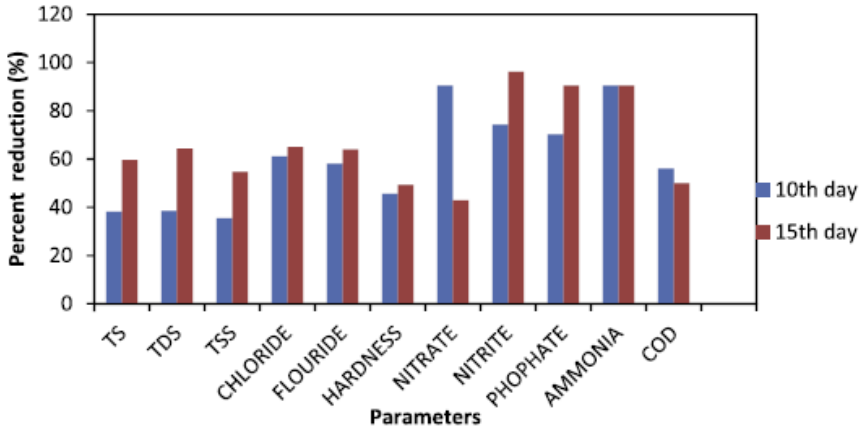


Figure 1.1 Percent reduction of contaminants in dairy wastewater by *C. polypyrenoideum*. Cultures were grown in 500 mL of 3:1 (75% wastewater:25% distilled water) wastewater mix in 1 L flasks (Kothari et al., 2013).

From the figure, it can be observed that there was significant reduction in the contaminant concentrations. In particular, nitrate and phosphate, were removed by approximately 90% and 74% respectively (Kothari et al., 2013). These are very promising results for the potential of algal growth in contaminated waters. The algae were also found to contain a lipid content of 42% by weight (Kothari et al., 2013). Growing microalgae in a lab scale batch system is beneficial in determining how the species of interest will respond to various nutrient stimuli. This information is helpful when moving towards pilot, commercial, or industrial scale systems.

CSTR-like System

A second study looked at a system that more closely resembled a continuous stirred-tank reactor (CSTR) as there was a constant flow of water into and out of the system. In this case, algae were being grown in rice fields concurrently with the rice crop. Three different green algae species were observed to make up more than 95% of

the algal biomass (Aschonitis et al., 2013). Water from flood irrigation continuously flows into and out of the rice field bringing nutrients along with it including nitrogen, phosphate, calcium, magnesium, sodium, potassium, and bicarbonate (Aschonitis et al., 2013). The results of the influent and effluent concentrations can be seen below in Table 1.1.

Table 1.1 Nutrient concentrations at inflow (IR) and outflow (Q) for algae grown in rice fields with herbicide. Data taken from Aschonitis et al. (2013).

Nutrient (kg ha^{-1})	IR	Q
N	38.82	7.16
P	2.76	0.07
C	-	-
Ca	997.53	296.27
K	31.16	13.18
Na	270.47	87.66
Mg	329.42	133.06

Once again, significant reductions in nutrient levels occurred. These results were despite of the fact that the water also contained herbicides. The herbicides did not appear to have a negative effect on the algae likely due to the algae's short lifespan (Aschonitis et al., 2013). Growth in the rice fields during flooding was approximately 8.2 kg/ha/day (Aschonitis et al., 2013). The observation that microalgae did so well when combined with multiple species and a crop might open doors for future research on the applications of microalgae. This study also demonstrated that microalgae can be successfully grown in outdoor systems where keeping the biomass axenic is not possible. This is an

important realization when considering growing microalgae in CBM water as the water itself will contain a variety of microflora (Barnhart et al., 2013).

Potential for Growth in CBM Production Water

Based on the previous discussions of nutrient utilization by microalgae in contaminated and low quality water, there is potential for microalgae to grow well in CBM production water. However, the previously discussed studies focused mainly on water sources with high levels of nitrogen and phosphorous. CBM water, on the other hand, has very high levels of sodium and TDS (Healy et al., 2011; Jackson & Reddy, 2007) and low levels of nitrogen and phosphorous. Nevertheless, a study in Australia verified that it is possible to grow microalgae in very brackish water (Buchanan et al., 2013).

One of the goals of the Australian study was to see if algae would grow in coal seam gas (CSG) water with different TDS concentrations. This particular study did not focus on a specific algal species. Algae were obtained from three different sources: seawater with added nutrients, lake water (fresh water combined with some secondary treated effluent), and a laboratory reactor with secondary treated effluent. Algae from all three sources were mixed together to make the inoculum. Two samples of CSG water were used. One was from a holding pond and had a TDS of 1.7 g/L. The other was concentrated CSG water from a reverse osmosis process and had a TDS of 11.6 g/L. Seawater diluted to the same TDS concentrations with deionized water and one sample of pure deionized water was also used. All five samples were in separate, 1 L fed-batch reactors, for the duration of the experiment (Buchanan et al., 2013).

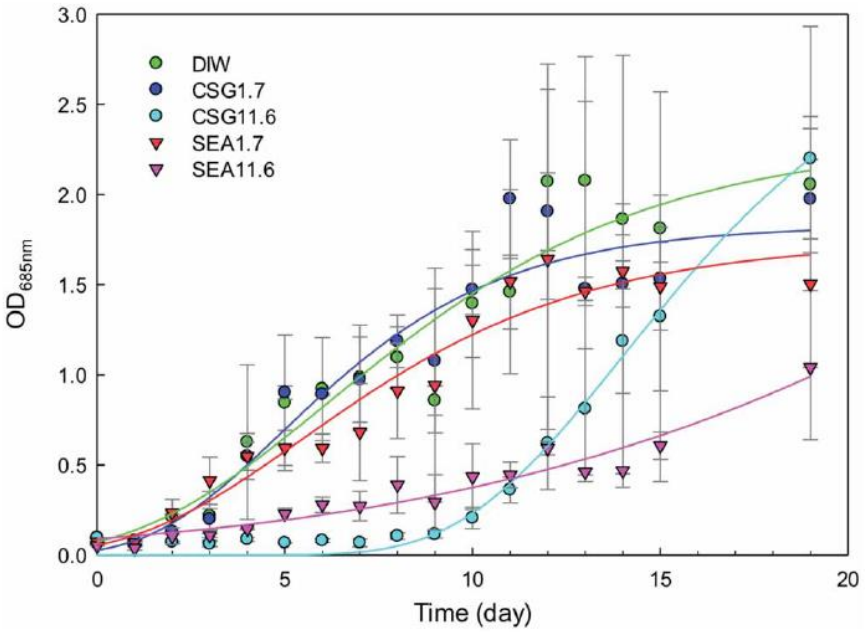


Figure 1.2 Growth of an algal consortium in distilled water (DIW), coal seam gas water with a TDS of 1.7 g/L (CSG1.7), coal seam gas water with a TDS of 11.6 g/L (CSG11.6), sea water diluted to an EC value corresponding to CSG1.7 (SEA1.7), and sea water diluted to an EC value corresponding to CSG11.6 (SEA11.6). Experiments were performed in 1 L fed-batch reactors with added nutrients. Error bars represent one standard deviation between replicate reactors (Buchanan et al., 2013).

As can be seen in Figure 1.2 above, both samples of CSG water supported the growth of algae. However, it was noted in the paper that the CSG water had to be supplemented with nutrients found in f/2 medium, such as nitrogen (Buchanan et al., 2013).

Along with testing the growth of algae in the water samples, the study also evaluated the diversity of algal species in the water using terminal-restriction fragment length polymorphism (T-RFLP). It was concluded that CSG water supported the same type of algal communities that were observed in fresh- and marine water. However, on day 15, the CSG water sample with 11.6 g/L of TDS had an algal community that was

different from the other samples. The conclusion was that the conditions in the CSG water sample with a TDS of 11.6 g/L (i.e., high inorganic carbon, high TDS, etc.) could have selected for a unique community (Buchanan et al., 2013). A unique algal community selected for a given set of parameters could be advantageous (Schenk et al., 2008). Parameters such as high salinity or pH could support the growth of certain algal communities while preventing the growth of other microorganisms. Unwanted contamination by other microorganisms can cause algal populations to crash and disappear from a pond (Schenk et al., 2008). Since CBM ponds are open to the environment, contamination could be a point of concern if these ponds are used to grow microalgae.

A separate study evaluated the potential for growing a selected marine microalgae species, *Dunalliella tertiolecta*, in coal seam gas water in Australia (Aravinthan & Harrington, 2014). This study demonstrated that growth and lipid production were possible after amending the water to a salinity of 10 g NaCl/L, adding additional bicarbonate, and providing nutrients found in a defined medium. This salinity is significantly higher than that of CBM water found in the Powder River Basin. Working with a native algal species isolated from CBM water should allow CBM water to be used as a growth medium without having to significantly modify it through various amendments. Another obstacle to consider, especially in Montana, is the low temperatures during the winter months. Based on modeling programs, it has been speculated that the optimum growth temperature for algae in other studies is approximately 28°C and the minimum water temperature for algal growth is around 5°C

(Aschonitis et al., 2013). This would not make it possible to grow algae in CBM ponds during the winter in Montana. The effects of various temperatures on the growth of CBMW would need to be evaluated to identify an optimal growing season for this species in Montana and Wyoming. Other limitations that have significant effects on algal growth and lipid production are nutrient concentrations and availability.

Nutrients

The nutrients that microalgae have to grow on play a role in how fast they grow, how much lipids they produce, and even what kinds of lipids are accumulated (Chia et al., 2013; Gonzalez-Garcinuno et al., 2014). As mentioned earlier, microalgae begin to produce and accumulate lipids when they are under environmental stress. Understanding how microalgae react to different nutrient stressors provides the opportunity to utilize microalgae the most efficiently. Every species is different, but there are some nutrients that have relatively common impacts throughout most studied algal species.

Nitrogen and Phosphorus

Nitrogen and phosphorus are two nutrients that are required for algal cells to replicate and thrive. Algal cells can obtain nitrogen from various forms such as nitrate, nitrite, ammonium, and urea (Eustance et al., 2013; Kothari et al., 2013). Nitrate is a common source of nitrogen, and it has been suggested that algal cells have a higher affinity for nitrate as opposed to nitrite and urea (Kothari et al., 2013). Nitrogen is vital for cell growth due to its role in the molecular structure of RNA, DNA, and chlorophyll molecules. Without nitrogen, the cell will not be able to produce these molecules, which

are essential to divide and produce more cells. Phosphorus, like nitrogen, is also vital for the cell and plays key roles in the molecular structure of ATP, phospholipid molecules, and nucleic acids.

Algal cells become environmentally stressed under nitrogen and/or phosphorus deplete conditions. Since the cells cannot replicate due to nitrogen and/or phosphorus depletion, some cells begin to accumulate lipids as a carbon and/or reduced electron storage molecule (Valenzuela et al., 2012). The diatom *Phaeodactylum tricornutum* is an example of a microalgae species that behaves in this manner. The slowing and leveling out of the growth rate of *P. tricornutum* in response to nitrate and phosphate depletion can be seen below in Figure 1.3.

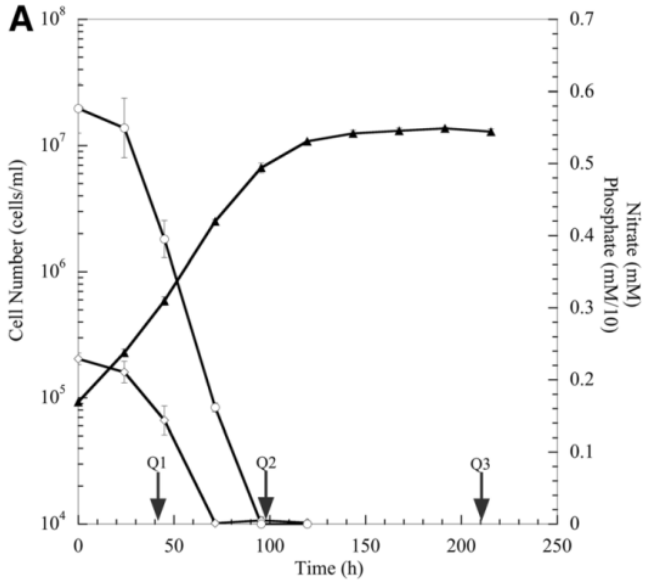


Figure 1.3 Depletion of nitrogen (○) and phosphorus (◊) along with growth of *P. tricornutum* (▲). Arrows represent time points where cells were collected for RNA sequencing (Valenzuela et al., 2012).

Once cells entered the stationary phase, they began redirecting carbon in the cell to make lipids as can be observed by the Nile Red fluorescence measurements. When nitrogen and phosphorus are present, carbon is used to make algal biomass leading to replication. At low levels of nitrogen and/or phosphorus, carbon is allocated towards fatty acids since this process does not require a significant source of nitrogen and phosphorus and still provides a source of $NADP^+$ that can be used as a terminal electron acceptor in the process of photosynthesis. The correlation between lipid production and nitrogen and phosphorus depletion can be seen below in Figure 1.4. Nitrogen depletion is one of the most well-known methods to induce lipid accumulation and was found to be used many times throughout the literature (Fields et al., 2014; Gardner et al., 2011; Huang et al., 2013; Jiang et al., 2012; Valenzuela et al., 2012; Yeesang & Cheirsilp, 2011).

Carbon Dioxide

Increasing the concentration of available carbon dioxide supplied to microalgae cultures has been evaluated as a way to change the availability of carbon to algal cells (Lohman et al., 2014). Lipid accumulation and growth were evaluated on their responses to the increase in carbon dioxide. In a recent study, algal growth responded very well to the increase in the partial pressure of carbon dioxide (Gardner et al., 2012). This makes intuitive sense because there would be more carbon available to be used for cellular growth via the Calvin Cycle. However, lipid accumulation had the opposite effect and decreased with the increase in carbon dioxide. This was thought to be due to the change in the pH of the system. It was also observed that lipid production can be stimulated by high pH levels but will decrease at low pH levels (Gardner et al., 2012). This could be

due to enzymes becoming denatured, because of the low pH, that are important in the production of lipids. However, it should be noted that an increase in pH of the medium does not necessarily correlate to an increase of pH within the cell (Goyal & Gimmler, 1989). The increase in carbon dioxide caused the pH of the water to decrease due to the effects of the carbonate buffering system.

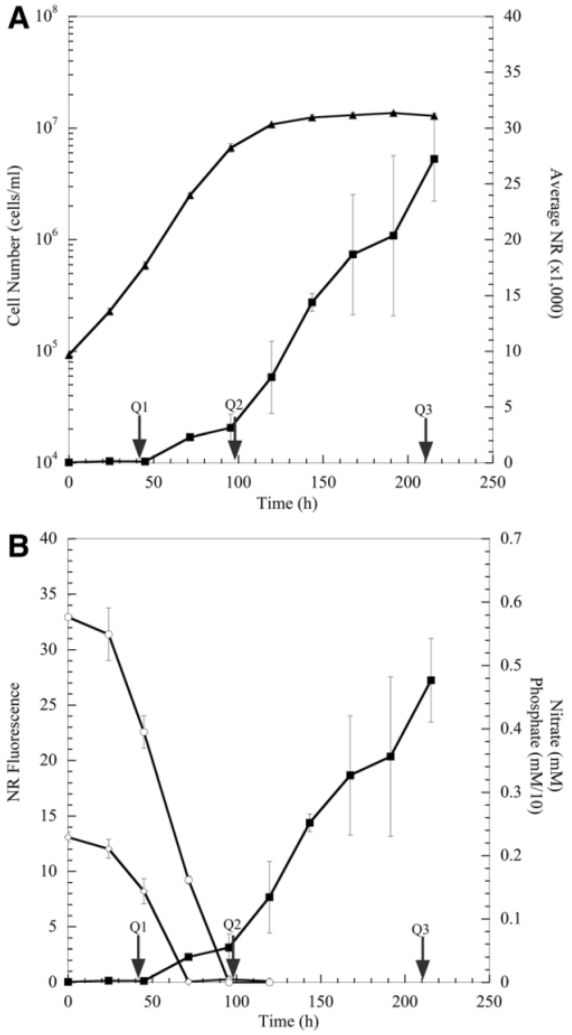


Figure 1.4 **A**.) Lipid production (■) of *P. tricornutum* is observed to increase when cellular growth (▲) becomes stationary. **B**.) Lipid accumulation (■) of *P. tricornutum* increases after nitrogen (○) and phosphorus (◊) depletion (Valenzuela et al., 2012).

Knowing that increased carbon dioxide increases growth and decreases lipid production, it was possible to show that keeping high levels of carbon dioxide up until nitrogen depletion, and then switching back to lower levels of carbon dioxide, enabled the cells to grow quickly and accumulate more lipids. This maximized cell growth and lipid accumulation (Gardner et al., 2012). However, a situation like this would not be possible in a CBM pond setting because there would not be an economic way to increase the carbon dioxide partial pressure since the ponds are open to the atmosphere and located in remote rural areas.

Salt

Stimulating lipid production with the addition of salt was shown in a recent study. Adding NaCl to an algal culture created enough stress on the algae to induce lipid accumulation (X. P. Zhou et al., 2013). The timing of the addition of NaCl proved to be important. NaCl added at the early growth stages of the algae inhibited growth. With growth inhibited, not as much lipid was accumulated. However, if NaCl was added at the late stage of exponential growth, lipid production was stimulated with minor changes to the growth of the algae (X. P. Zhou et al., 2013). The NaCl likely inhibited growth in the late exponential stage as well, but had a minor effect since the algae were already about to enter the stationary stage of growth. It is conceivable that treating a portion of CBM water by reverse osmosis could provide a high salt medium that could induce lipid accumulation. But as previously mentioned, reverse osmosis is an expensive process and not readily available in the rural areas of the Powder River Basin.

Light

Although light is not a nutrient, it is very important to the successful growth and lipid accumulation of algae via phototrophy. In a lab setting, light can be supplied to algae 24 hours a day. This constant supply of light provides a consistent photo energy supply allowing the algae to continuously grow and accumulate lipids once nitrogen is depleted. However, in a realistic setting, such as a CBM pond, there will be a light:dark cycle that correlates with the rising and setting of the sun. This has led experiments to be conducted in a similar setting to more accurately reflect the light availability that microalgae would experience in outdoor conditions.

In these experiments, the lipid accumulation of the algae seemed to decline, especially during the dark cycle. Upon further investigation, it was determined that this is likely because the algae began to utilize the accumulated lipids as an energy source when there was no light (Gardner et al., 2012). This should be considered when looking at the feasibility of growing algae in CBM ponds, particularly in Montana where light:dark cycles vary dramatically based on the time of year.

Gene Expression in Microalgae

A review of the literature revealed interesting discussions on the gene expression of various proteins in the different stages of growth for certain species of microalgae. In one study the genes expressed in the exponential compared to the stationary phase were examined for the algal species *P. tricornutum.* During exponential growth genes are up-expressed for nitrate and phosphate transporters (Valenzuela et al., 2012). This is

intuitive because the algae cells would need to be bringing in large amounts of these nutrients to continue growing. During the stationary phase, genes are up-expressed for a putative nucleotidase and alkaline phosphatases, which are used in the recycling of nitrogen and phosphorus within the cell (Valenzuela et al., 2012). Other transcriptomic studies have shown that genes coding for nitrogen assimilation and the pentose phosphate pathway are up-expressed during nitrogen deplete conditions (Park et al., 2015). Simultaneously, genes that encode for proteins essential to lipid accumulation are also up-expressed while genes for fatty acid degradation are down-expressed (Park et al., 2015; Rismani-Yazdi et al., 2012). These findings support the observed response of lipid accumulation by microalgae when under nutrient stress. In some instances, the availability of carbon can become a point of concern for algal cultures containing a high number of cells (Valenzuela et al., 2012). Processes involving carbon cycling, known as CO_2-concentration mechanisms (CCMs), can be utilized by the cell in response to this situation and vary depending upon growth stage of the population (Valenzuela et al., 2012). Methods to recycle three carbon and four carbon intermediates were thought to be utilized during the stationary phase of growth of *P. tricornutum* (when cell numbers are high) in response to limited carbon availability (Valenzuela et al., 2012). The combined work of these studies, as well as others, point to a massive shift in gene expression, the transcriptome, and the proteome of algal cells to allow for a lipid accumulation event.

Overview

As can be seen from the above discussions, there are a variety of ways to manipulate the growth and lipid production of various microalgae species. It would be fair to say that one of the fundamental methods for stimulating lipid accumulation is a depletion of nitrogen. However, the depletion of phosphorus has also been observed to play a role (K. H. Liang et al., 2013; Valenzuela et al., 2012). Lipid accumulation can be enhanced by other factors such as carbon dioxide addition (Gardner et al., 2012; Lohman et al., 2014), salt addition just before nitrogen depletion (X. P. Zhou et al., 2013), and/or pH control (Gardner et al., 2011). Significant limiting factors for lipid accumulation include the level of carbon dioxide available and the characteristics of the light:dark cycle that are used. Various combinations of all the above nutrients and growth conditions can be used to maximize lipid accumulation and biomass production from algae. It is important to remember that every species is different and could behave uniquely to environmental stress and stimuli. For these reasons, isolating a native algal species from a CBM production pond is advantageous as the species is already adapted to growing in the intended environment.

Harvesting Microalgae

One of the biggest issues with the use of biological biomass as a renewable chemical feedstock is harvesting. There are a variety of ways to harvest microalgae. The end goal of growing microalgae can have a direct effect on the chosen harvest process.

Flocculation is one of the more common harvesting methods. There are two types of flocculation: chemical and biological. In chemical flocculation, chemicals such as alum salts, (Cui et al., 2014) ferric salts, and polymeric coagulants (de Godos et al., 2011; Eldridge et al., 2012) are used to induce flocculation. It has been pointed out that a disadvantage of some chemical flocculation techniques is the resulting salt and/or coagulant content in the algal biomass, which can create issues if the biomass will be reused for any other biological processes (Molina Grima et al., 2003) such as fertilizer or as a feedstock for anaerobic digestion. If lipid extraction is the only end goal, this method might be feasible. In biological flocculation, natural methods such as pH stress (J. X. Liu et al., 2014) and co-culturing with bacteria (Lee et al., 2013) can be used to induce flocculation. Filtration is another option to concentrate algal biomass (Frappart et al., 2011). Unfortunately, this method becomes very expensive with large amounts of water making it very uneconomical. Centrifugation can also be used to harvest microalgae (Dassey & Theegala, 2013), but comes with the same economic disadvantage. One of the more creative ideas in the literature involved feeding the algae to tilapia fish. The fish are not efficient digesters of the algae and a majority of the biomass is passed out of the fish as excrement making it easier to collect (Schenk et al., 2008). A unique method for harvesting is the use rotating algal bioreactors (RABRs) that have been designed at Utah State University (Christenson & Sims, 2012) that is in essence a biofilm. Biofilms offer the advantage of inherently being a method for biomass accumulation; however, methods of collection would still need to be developed (Fields et al., 2014). The development of new harvesting techniques and/or the combination of

existing techniques is an active area of research that could have a significant impact on the economy of larger-scale cultivation operations. A unique solution might have to be designed for CBM ponds that are located in rural areas and do not have easy access to some of the technologies mentioned above.

Summary

Based on this review of the current literature involving microalgae, it would be feasible to grow microalgae in coal bed methane production water ponds. Turning these ponds into a productive resource would be beneficial and economical based on the vast amount of water that is contained. Research has shown that certain species of algae are able to grow in the high salinity conditions found in CBM water with some supplemental nutrients added (Aravinthan & Harrington, 2014; Buchanan et al., 2013). It is even possible that the high salinity could select for a specific species and help prevent contamination from unwanted organisms (Buchanan et al., 2013). Microalgae are naturally occurring and accumulate lipids as a response to environmental stress. This trait of microalgae can be exploited and used to provide a source for biodiesel production. Microalgae can be stimulated to accumulate lipids in many different ways including nitrogen and phosphorus depletion (Valenzuela et al., 2012), addition of carbon dioxide (Lohman et al., 2014), increasing the pH (Gardner et al., 2011), and the addition of salts (X. P. Zhou et al., 2013). Of these methods, nitrogen depletion is the most studied and likely the most critical. Combining depletion of nitrogen with other methods could maximize algal growth and lipid accumulation. Different species of algae can react

uniquely to various methods and efforts should be made to characterize what will work best with the species of microalgae that will be used.

CHAPTER TWO

MATERIALS AND METHODS

Organism

CBMW is a green alga that was isolated from a coal bed methane (CBM) production pond in northeastern Wyoming. The isolate was taken from the CBM water inflow at a pond with coordinates of 44° 52.613'N 106° 54.700'W. At the time of sampling, the inflow temperature was 14°C while the pond measured 15°C (written communication with Elliott Barnhart). The use of a native algal species has the benefit of already being adapted to the intended growth environment. Most studies look for a species that will do well in contaminated or low quality water and then proceed to determine if lipid production is high. In this case, the environment has naturally selected a strain of microalgae.

Medium Composition

CBMW was maintained in liquid and agar plate cultures of Bold's Basal Medium (BBM). BBM is a generic medium designed to support the growth of a wide variety of green algae species. The composition of the medium and added microelements can be seen below. Liquid medium and plates were autoclaved and allowed to gas exchange for 24 hours before being used to cultivate CBMW.

Table 2.1 Constituents of Bold's Basal Medium.

Bold's (1 liter)	
Reagent	
KH_2PO_4	175 mg
$CaCl_2*2H_2O$	25 mg
$MgSO_4*7H_2O$	75 mg
$NaNO_3$	250 mg
K_2HPO_4	75 mg
NaCl	25 mg
H_3BO_3	11.42 mg
Microelements (ME)	1 mL
S1	1 mL
S2	1 mL
S3	1 mL
B12	0.5 mL

Table 2.2 Constituents of microelement solution for Bold's Basal Medium

ME (1 liter)	g
$ZnSO_4*7H_2O$	8.82
$MnCl_2*4H_2O$	1.44
$MoO_3Na2*2H_2O$	0.71
$CuSO_4*5H_2O$	1.57
$Co(NO_3)_2*6H_2O$	0.49

Table 2.3 Constituents of S1 solution for Bold's Basal Medium.

S1 (1 liter)	g
Na_2EDTA	50
KOH	3.1

Table 2.4 Constituents of S2 solution for Bold's Basal Medium.

S2 (1 liter)	g
$FeSO_4*7H_2O$	4.98
H_2SO_4 (con)	1 (mL)

Table 2.5 Constituents of S3 solution for Bold's Basal Medium.

S3 (1 liter)	g
Inositol	5
Thymine	3
Thiamine*HCl (B1)	0.5
Nicotinic acid (niacin)	0.1
Ca pantothenate	0.1
p-Aminobenzoic acid	0.01
Biotin (vitamin H)	0.001
Folic Acid	0.002

Table 2.6 Constituents of B12 vitamin solution for Bold's Basal Medium.

B12 (1 liter)	g
Cyanocobalamin (B12)	0.004

Experiments were performed using BBM, sterile CBM water with and without added nutrients, and non-sterile CBM water with and without added nutrients. The major ion constituents of CBM water can be seen below in Table 2.7. CBM water was obtained directly from the FG-09 extraction well located at 45° 26' 5.8914" N 106° 23' 31.416" W in the Powder River Basin.

Growth Conditions

CBMW was maintained in a 20°C incubator with a light:dark cycle of 14 hours of light followed by 10 hours of dark. Light intensity was measured and found to be 7871.9 lux. A 14:10 light:dark cycle was selected over a 12:12 light:dark cycle to better simulate the longer days and shorter nights of the Powder River Basin (PRB) during the summer months. Liquid flasks were placed on a Labnet Orbit 1900 Heavy Duty Shaker at 125 rpm to encourage mass transfer of carbon dioxide into the medium. 500 mL Erlenmeyer

flasks containing 250 mL of BBM or CBM were used to maintain cultures and perform experiments.

Table 2.7 Constituents of coal bed methane water from the FG-09 well located in the Powder River Basin.

Molecule	CBM Concentration (mg/L)
Ca	3.9
Mg	1.5
Na	590
K	4.8
Sr	0.3
Si	3.6
B	0.33
Mn	0.02
Fe	0.02
Ba	0.34
F	3.8
NO_2	0
Br	0.33
NO_3	0
Cl	63.6
SO_4	9

pH

pH was measured with a standard benchtop Oakton pH 11 Series meter. The pH meter was calibrated using pH standards of 4.0, 7.0, and 10.0 before each use.

Cell Counts

Cell counts were originally used to track the growth of CBMW in preliminary experiments. Cell counts were taken using a standard hemocytometer. Once CBMW

began to flocculate and clump in culture, cell counts were no longer an accurate measure of growth or biomass and were no longer used.

Dry Weight

Dry weight measurements were taken using 0.2 μm filters. Filters were 25 mm in diameter and were made of either nitrocellulose or acetate cellulose. 4-10 mL of culture were filtered using a vacuum pump. The exact volume used was recorded for each sample. Each sample was rinsed with approximately 5 mL of deionized water to remove excess salts. Once filtered, samples were dried for 48 hours in a 90°C oven. The difference in weight before filtering and after drying was used as a measure of culture biomass concentrations.

Chlorophyll Measurements

1 mL samples of culture were placed in 1.7 mL microcentrifuge tubes and centrifuged for 15 minutes at 16,162xg. After centrifuging, 950 μL of the supernatant was pipeted out being careful not to disturb the cell pellet at the bottom of the tube. Vortexing and/or sonicating were used to resuspend the cell pellet in 1 mL of 100% methanol. The microcentrifuge tubes were then stored in a 4°C refrigerator inside a cardboard box for at least 24 hours. The box was used to prevent light from interacting with the extracted chlorophyll as suggested by Ritchie (2008). After approximately 24 hours, the samples were spun down in a centrifuge for 5 minutes at 16,162xg to remove the biomass from the supernatant. 1 mL of supernatant was then carefully pipeted out

and analyzed on a Shimadzu UV-1700 UV-VIS spectrophotometer. A blank of 100% methanol was analyzed before each group of readings was taken. The spectrophotometer read absorbance values at wavelengths of 665.2 nm, 652 nm, and 632 nm. The wavelength measurements were used to calculate the concentration of chlorophyll in µg/L according to the following equations (Ritchie, 2008):

$$Chla = [-2.0780(Abs\,632) - 6.5079(Abs\,652) + 16.2127(Abs\,665)] \times dilution\ factor \quad \text{Eq. 2.1}$$

$$Chlb = [-2.9450(Abs\,632) + 32.1228(Abs\,652) - 13.8255(Abs\,665)] \times dilution\ factor \quad \text{Eq. 2.2}$$

$$Chlc = [34.0115(Abs\,632) - 12.7873(Abs\,652) - 1.4489(Abs\,665)] \times dilution\ factor \quad \text{Eq.2.3}$$

$$\sum Chl = Chla + Chlb + Chlc \quad \text{Eq.2.4}$$

Anion Measurements

Fluoride, chloride, nitrite, bromide, nitrate, phosphate, and sulfate were tracked during the course of selected experiments using a DIONEX ICS-1100 and Chromeleon Chromatography Management System. If samples contained concentrations higher than 100 mg/L, they were diluted using nano-pure water. An ASRS 4mm suppressor was used with an anion exchange column and an eluent feed of 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate.

Lipid Accumulation

Accumulation of lipids was tracked using the Nile Red method developed by Cooksey et al. (1987). 4 µL of Nile Red solution (0.25 mg Nile red/ml of acetone) were mixed in 1 mL of sample and then placed in the dark for 5 minutes. Nile Red

fluorescence was measured using a Synergy H1 hybrid reader. Gen5 microplate reader software was used to evaluate the fluorescence at an excitation of 480 nm and emission of 580 nm.

Preliminary Experiments

Preliminary experiments were performed to help narrow down nutrients and conditions of specific interest when growing CBMW in CBM production water. Each preliminary experiment was done with one biological replicate. Two technical replicates were taken for each measurement of pH, cell counts, chlorophyll, Nile Red, and water chemistry.

CBMW was grown with various nutrients added that were lacking when compared to the nutrient composition of BBM. These added nutrients included nitrate, phosphate, micronutrients, vitamin B12, calcium, and boron. Nitrate, phosphate, micronutrients, and vitamin B12 were determined to be the additions of primary interest. CBM water already contains small amounts of calcium and boron, and the addition of more of these two nutrients was not observed to make a marked difference in the growth of CBMW. After comparing water chemistries, the only nutrients that were lacking in CBM water that had not been tested were magnesium and sulfate. A growth condition including these nutrients was included in the final experiments.

The amounts of added nitrogen and phosphorus were adjusted in various experiments. In some experiments, the same concentrations as found in BBM (2.94 mM N and 1.7 mM P) were used. With one exception, lipids were not observed to

accumulate, presumably because of the large amount of nitrogen and phosphorus coupled with the short length of the experiments. In a subsequent experiment, 0.3 mM N and 0.85 mM P were tested. Without the added micronutrients, the large amount of phosphorus compared to nitrate seemed to negatively affect the growth of CBMW. It was determined that a nitrogen concentration of 0.5 mM and a phosphorus concentration of 0.05 mM would be used for the final experiments. These amounts were previously observed to successfully stimulate lipid accumulation in the marine diatom *P. tricornutum* (Valenzuela et al., 2012). To accurately compare results to BBM, the amounts of nitrogen and phosphorus were reduced to these levels in BBM in the final experiments as well.

Originally, each preliminary experiment was performed in non-sterile CBM water to better simulate the natural environment. BBM was used as a standard growth medium to compare to the growth of CBMW in CBM water. In all experiments, cell counts in CBM water were far below that in BBM. This was due in part to a lack of nutrients found in CBM water, but was also suspected to be affected by the flocculating behavior of CBMW in CBM water. These small flocs were too large to be evenly distributed by the volume of sample used for cell counts on a hemocytometer. This caused there to be a very large number of cells in some instances and very few in others. The small flocs were presumably a by-product of growing CBMW in non-sterile water as bacteria can have an aggregating effect on algal growth (Cho et al., 2015; Lee et al., 2013; D. D. Zhou et al., 2015). To test this hypothesis, CBMW was grown in both sterile CBM water and non-sterile CBM water, both having nutrients added that would be found in BBM. Cell

counts in sterile CBM water were comparable to cell counts in BBM with the same concentrations of added nutrients. Cell counts in non-sterile water were significantly lower. Due to these results, a dry cell weight method was selected to track increases in biomass for the final experiments to give a more accurate comparison of non-sterile CBM water to BBM.

Before the final experiments could be carried out, a radical change in the behavior of CBMW in non-sterile CBM water was observed. Rather than clumping on a micrometer scale, large millimeter sized clumps were formed. Previously, a one milliliter aliquot from a culture flask was enough to be a representative sample for the entire flask. With the formation of the new, much larger clumps, obtaining a representative sample was no longer possible. The vast majority of the microalgae in the flasks were growing within these clumps, leaving the rest of the flask very clear with hardly any turbidity and no sign of the green hue common to microalgal growth. This presented a significant obstacle when trying to determine growth parameters such as chlorophyll content, biomass growth, and Nile Red fluorescence readings. The CBM water used for all of the previously mentioned experiments, and the final experiments, came from the same container of CBM water stored in a 5°C walk-in, dark refrigerator. Water chemistry of this container was tracked for the past year to determine if a significant change in water chemistry concentrations had occurred. Based on the results, this was not the case. While clumping behavior is problematic when trying to evaluate the growth of organisms in batch culture, it is also very intriguing for other aspects of microalgal biodiesel production, such as harvesting. The implications of this are discussed further in Chapter

4. The only method that would prevent large clumping of CBMW was to use filter sterilized CBM water. Although there were still small flocs present, they were much smaller and were evenly distributed allowing for representative samples to be taken from the flask cultures. Because of this, all final experiments were performed in sterile CBM water.

Data and observations from these preliminary experiments were the driving force behind the growth conditions and methods used for the final experiments. Details and results of the final experiments are presented in the following chapter.

CHAPTER THREE

GROWTH OF A NATIVE ALGAL SPECIES IN COAL BED METHANE WATER FOR BIOFUEL AND BIOMASS ACCUMULATION

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Logan H. Hodgskiss

Contributions: Experimental design, data analysis, and manuscript preparation.

Co-Author: Matthew W. Fields

Contributions: Primary investigator, experimental design, and manuscript editing.

Manuscript Information Page

Logan H. Hodgskiss and Matthew W. Fields
Journal: Environmental Science and Technology
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

Abstract

Coal bed methane (CBM) production in the Powder River Basin has resulted in massive amounts of water being extracted from underground coal seams. This water is presently stored in unlined ponds or discharged to local stream drainages resulting in undesirable environmental effects to the local area. Utilizing the CBM water for agricultural purposes can be problematic due to the high total dissolved solids and sodium content of the water. An alternative use of CBM water is as a growth medium for microalgae for biomass and lipid accumulation. A green alga isolate, CBMW, has been isolated from a CBM production pond in northeastern Wyoming and has been shown to grow and accumulate lipids when a source of nitrogen is added to sterile CBM water. A native algal strain provides the advantage of a species that is already acclimated to the environmental conditions of the Powder River Basin. A lipid content of 26.9% was observed with CBMW being grown in filter sterilized CBM water with added nitrate. The composition of lipids accumulated is similar to that of other biological sources of biodiesel.

Introduction

Natural gas is a primary source of energy in the United States, second only to petroleum in overall consumption in the past several years (www.eia.gov). Coal bed methane (CBM) has become a significant component of the natural gas industry and represented approximately 4.75% of the natural gas production of the U.S. in 2013 (www.eia.gov). Natural gas found in coal seams under the earth's surface is predominantly made up of methane, but can also include ethane, carbon dioxide, and nitrogen gas (Scott, 1994). Methane in the subsurface is typically adsorbed to coal due to the high hydrostatic pressure found in these seams (Wheaton & Olson, 2001). Extracting methane involves depressurizing the coal seam by withdrawing large amounts of water to the surface to allow the methane to desorb from the coal (McBeth et al., 2003). A resource assessment performed by the United States Geological Survey in 2002 indicated

that one of the most promising sources of CBM is the Powder River Basin located in northeast Wyoming and southeast Montana (USGS, 2002). According to the Wyoming Oil and Gas Conservation Commission (WOGCC, in 2014 the Powder River Basin produced 246,601,576 Mcf (1000 cubic feet) of gas and an associated 256,305,005 Bbls (8,073,617,107 gallons) of water (WOGCC, 2014).

Water produced by CBM extraction is typically used for agricultural purposes, discharged into stream drainages, or stored in unlined impoundments. This can be problematic due to the high concentrations of Na^{+} and total dissolved solids (TDS), and low concentrations of Mg^{2+} and Ca^{2+} found in the majority of the produced water (Healy et al., 2011; Jackson & Reddy, 2007; McBeth et al., 2003). Sodium, calcium, and magnesium ions can be used to approximate the quality of water used for irrigation and agricultural purposes via the sodium adsorption ratio (SAR):

$$SAR = \frac{Na^{+}}{\sqrt{\frac{1}{2}(Ca^{2+}+Mg^{2+})}} \qquad \text{Eq. 3.1}$$

In this equation, concentrations are in meq/L for sodium, calcium, and magnesium. The SAR can give an indication of the leaching effect the water will have on the soil with a low SAR being acceptable and SAR values above 10 being potentially harmful (Buckland et al., 2002; Richards et al., 1954; Vance et al., 2008). Produced water in the Powder River Basin contain SAR values ranging from 7 to 43 depending on the location of the well (Healy et al., 2011; Jackson & Reddy, 2007; Reddy et al., 2014; Vance et al., 2008). Irrigation with CBM water for multiple seasons has been observed to have negative effects on the soil, particularly if it is fine textured (Vance et al., 2008). These negative effects can include an increase in SAR values of the soil itself and a decreased

rate of infiltration (Vance et al., 2008). The effects on plant growth have also been evaluated with less conclusive results (Vance et al., 2008). Studies have also been performed to evaluate the consequences of discharging CBM water into local streams or using the water as a livestock and wildlife drinking source. These studies have indicated that CBM water can be suitable for livestock and aquatic life, but cautioned that continued use or accumulation of trace elements, such as arsenic, in ponds due to evaporation could become a pressing issue (Davis et al., 2009; Reddy et al., 2014).

Although direct discharge of CBM water does not appear to be harmful, the leaching of CBM water from unlined ponds can result in unintended consequences. This additional CBM water can raise the water table significantly causing it to interact with soil that has been previously untouched by groundwater (Healy et al., 2011). The primary concern in this case is the resulting selenium concentrations picked up by the water from the untouched soils. In one case study, levels as high as 300 μg/L were recorded (Healy et al., 2011). This is significantly higher than the recommended concentration of 5 μg/L established by the United States Environmental Protection Agency (EPA) ("External Peer Review Draft Aquatic Life Ambient Water Quality Criterion For Selenium – Freshwater 2014," 2014). Apart from groundwater issues, the presence of CBM water has also been directly correlated with an increase in mosquitoes that are a carrier for the West Nile virus (Zou et al., 2006) resulting in public health concerns for the area.

A new alternative use for CBM water could be the growth of microalgae for biodiesel and biomass production. Microalgae have been shown to accumulate lipids

when under environmental stress (Gardner et al., 2011; Valenzuela et al., 2012; X. P. Zhou et al., 2013). The lipids accumulated can be converted into biodiesel through a variety of processes and help offset the demand for traditional oil and gas. Microalgae have a distinct advantage over traditional biodiesel crops, such as corn and soybeans, because of their high lipid content to biomass ratio and their ability to be grown in large quantities without competing for arable land that can be used for agriculture (Schenk et al., 2008; Singh et al., 2011). Growing microalgae in other low water quality sources for the purposes of remediation and/or biofuel production can be found throughout the literature. These water sources include dairy wastewater (Kothari et al., 2013), municipal wastewater (Gressler et al., 2014; Mata et al., 2013), and rice fields treated with herbicides (Aschonitis et al., 2013). A primary concern when cultivating microalgae is selecting a species that is a good fit for the intended environment. A recent study successfully grew a microalgae species in coal seam gas water in Australia (Aravinthan & Harrington, 2014) in which a marine algae species was selected to accommodate the high salt content of the water. To this date, the use of CBM water from the Powder River Basin for this purpose has not been evaluated.

We have isolated a native green alga species from a CBM production pond in northeastern Wyoming and evaluated its potential for growth and its ability to accumulate fatty acids that can be converted into biodiesel. A native species provides the benefit of an organism that is already adapted to the semi-arid climate of the Powder River Basin. We have identified the key nutrients required for this species to thrive in CBM water and

established its ability to accumulate lipids when certain environmental stimuli are present.

Materials and Methods

Culturing Conditions

CBMW was isolated from a CBM production pond in northeastern Wyoming at coordinates of 44° 52.613'N 106° 54.700'W. Cultures were maintained on Bold's Basal Medium (BBM) agar plates and in liquid flasks in a 20°C incubator with a 14h:10h light:dark cycle. Light was supplied at an intensity of 7871.9 lux. Flasks were placed on a shaker rotating at 125 rpm to encourage mass transfer of carbon dioxide. Experiments were inoculated from a stock culture of CBMW grown in liquid BBM. Each inoculum was centrifuged at 1750xg for five minutes and washed twice with sterile CBM water or BBM depending on the intended use. CBM water was supplied from the FG-09 well located at 45° 26' 5.8914" N 106° 23' 31.416" W in the Powder River Basin. This water was used for all experiments. CBM water was filter sterilized using a bottle top 0.2 μm, polyethersulfone (PES), Nalgene Rapid-Flow vacuum filter from Thermo Scientific.

Sterile CBM Water

Axenic experiments were performed with biological duplicates in 500 mL Erlenmeyer flasks containing 250 mL of sterile CBM water or Bold's Basal Medium (BBM; 0.5 mM nitrate, 0.05 mM phosphate). Five different nutrient conditions were evaluated using CBM water: CBM water without additions; CBM water with 0.5 mM nitrate; CBM water with 0.5 mM nitrate and 0.05 mM phosphate; CBM water with 0.5

mM nitrate, 0.05 mM phosphate, and 0.3 mM magnesium sulfate; and CBM water with 0.5 mM nitrate, 0.05 mM phosphate, 0.3 mM magnesium sulfate, and micronutrients used in BBM. All flasks were inoculated with 15 mL of inoculum from the same stock solution of CBMW. Values for pH were measured using a standard bench top Oakton pH 11 Series meter.

Dry Weight

25 mm diameter nitrocellulose or acetate cellulose 0.2 μm filters were used to periodically determine the dry weight biomass of the cultures. Culture samples were vacuum filtered and rinsed with water to remove excess salts before being allowed to dry in an oven set at approximately 90°C for 48 hours. Filters were weighed before and after use to determine dry weight biomass.

Chlorophyll Measurements

1 mL culture samples were centrifuged at 16,162xg for 15 minutes in micro centrifuge tubes. 950 μL of supernatant was removed using a pipet and frozen at -80°C for later water chemistry analysis. The cell pellet was submerged in 1 mL of 100% methanol and disrupted by sonication and vortexing before being placed in a cardboard box (to prevent any light influence) and put in a 4°C fridge for a least 24 hours. After 24 hours, the samples were spun down at 16,162xg for 5 minutes. The supernatant was extracted and analyzed in disposable plastic cuvettes using a Shimadzu UV-1700 UV-VIS spectrophotometer at wavelengths of 665.2 nm, 652 nm, and 632 nm. Chlorophyll concentrations in μg/mL were estimated using the following equations (Ritchie, 2008):

$$Chla = [-2.0780(Abs\,632) - 6.5079(Abs\,652) + 16.2127(Abs\,665)] \times dilution\ factor \quad \text{Eq. 3.2}$$

$$Chlb = [-2.9450(Abs\,632) + 32.1228(Abs\,652) - 13.8255(Abs\,665)] \times dilution\ factor \quad \text{Eq. 3.3}$$

$$Chlc = [34.0115(Abs\,632) - 12.7873(Abs\,652) - 1.4489(Abs\,665)] \times dilution\ factor \quad \text{Eq. 3.4}$$

$$\sum Chl = Chla + Chlb + Chlc \quad \text{Eq. 3.5}$$

Lipid Accumulation

Lipid accumulation was tracked using a Nile Red staining method (Cooksey et al., 1987). 1 mL of culture sample was stained with 4 μL of a Nile Red stock solution having a concentration of 0.25 mg Nile Red/ml of acetone. Samples were stored in the dark for 5 minutes. The set time of 5 minutes was determined by performing a time assay with CBMW to determine the optimum time to measure Nile Red fluorescence as described by Chen et al (Chen et al., 2009). After 5 minutes, 200 μL duplicates were pipeted into a 96 well plate and fluorescence was read using a Synergy H1 hybrid reader. Gen5 microplate reader software was used to evaluate the fluorescence at an excitation of 480 nm and emission of 580 nm. Samples were diluted 2, 4, or 10 times as necessary to keep Nile Red measurements within a linear range (Chen et al., 2009).

Anion Concentrations

Nitrate and phosphate concentrations of culture samples were determined using a DIONEX ICS-1100 and Chromeleon Chromatography Management System with an ASRS 4 mm suppressor. A 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate eluent mix was used.

GC-MS Lipid Analysis

Cultures were harvested once a Nile Red peak was observed. Flasks were transferred to 50 mL sterile Falcon tubes and centrifuged at 4,816xg for 10 minutes. The supernatant was then poured off and the biomass was flash frozen at -80°C until the sample was lyophilized.

After lyophilization, biomass was weighed out in glass tubes, and submerged in 1 mL of toluene and 2 mL of sodium methoxide. The samples were vortexed and placed in a 90 °C oven for 30 minutes (while in the oven, samples were taken out every 10 minutes to be vortexed). After 30 minutes, 2 mL of 14% boron trifluoride in methanol were added to each sample and vortexed. Another 30 minute incubation in at 90°C oven followed (samples were vortexed every 10 minutes during this incubation). When finished, 0.8 mL of hexane and 0.8 mL of 15% NaCl were added to each sample. Samples were incubated for 10 minutes and then spun in a centrifuge at 2,500xg for 2 minutes. The top layer in the tube (containing the lipids) was extracted and analyzed on an Agilent Technologies 6890N Network GC system to determine lipid concentration and composition. This method was performed as described in Lohman et al. (2013).

Results and Discussion

Water Chemistry

Water from the FG-09 well was analyzed for its chemical content by the United States Geological Survey (USGS) and can be seen in Tables 3.1-3.3 compared with nutrients found in BBM. While CBM water and BBM share several nutrients, the

concentrations are varied (Table 3.1). CBM has a significant amount of manganese and iron when compared to standardized BBM. Typically, iron availability can be a point of concern for algal growth and supplementation of iron will increase biomass productivity (Z. Y. Liu et al., 2008). Lack of iron does not appear to be a growth limiting factor when using CBM water as a growth medium. However, magnesium and sulfate, two key nutrients in algal physiology, were relatively low.

Table 3.1 Nutrients observed in CBM water and BBM.

Medium	Nutrient (mM)				
	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	B
CBM	0.10	0.06	25.65	0.12	0.03
BBM	0.17	0.30	1.10	0.06	0.18
Supplemented CBM	0.17	0.36	26.75	0.19	0.18
	Mn^{2+}	Fe	Cl^{-}	SO_4^{2-}	
CBM	3.6E-04	3.58E-04	1.79	0.09	
BBM	7.28E-06	1.79E-05	0.94	0.38	
Supplemented CBM	3.72E-04	3.76E-04	2.13	0.41	

Tables 3.2 and 3.3 highlight the absolute differences between CBM and BBM, particularly the inability to detect nitrate and phosphate in CBM water.

Table 3.2 Nutrients observed in CBM water only.

Medium	Nutrient (mM)				
	Ba^{2+}	Sr^{2+}	Si	F^{-}	Br-
CBM	2.48E-03	3.42E-03	0.13	0.2	4.13E-03
BBM	0	0	0	0	0
Supplemented CBM	2.48E-03	3.42E-03	0.13	0.2	4.13E-03

Table 3.3 Nutrients observed in BBM only.

Medium	Nutrient (mM)				
	NO_3^-	PO_4^{3-}	Zn^{2+}	Mo	Cu
CBM	0	0	0	0	0
BBM	0.50	0.05	3.07E-05	3.15E-06	6.29E-06
Supplemented CBM	0.50	0.50	3.07E-05	3.15E-06	6.29E-06
	Co				
CBM	0				
BBM	1.68E-06				
Supplemented CBM	1.68E-06				

From this water chemistry, an SAR value of 64.29 was calculated. This value is much higher than most of the water that has been analyzed in the Powder River Basin (Healy et al., 2011; Reddy et al., 2014; Vance et al., 2008) and would likely be harmful to soil health if used for irrigation purposes (Richards et al., 1954).

The amendments made to CBM water for this study were based on these comparisons and previously determined nutritional requirements for microalgae.

CBM Water without Additions CBM water is the natural environment of CBMW. For this reason, one of the growth conditions consisted of medium containing nothing but sterile CBM water. A primary difference between this growth condition and naturally occurring growth is the lack of other microorganisms interacting with CBMW. Some microalgae are not capable of synthesizing vitamins such as B12 and rely on the production of these vitamins from bacteria in the phycosphere (Croft et al., 2005). This growth condition may hinder the success of CBMW if it cannot generate these, or other, vitamins.

CBM Water with Nitrate Aa shown in Table 3.3, one of the primary nutrients missing from CBM water is nitrogen. Many previous experiments have demonstrated the importance of a nitrogen source for the successful cultivation of microalgae (Huang et al., 2013; Jiang et al., 2012; Kunikane et al., 1984; Yeesang & Cheirsilp, 2011). Some species are capable of utilizing a variety of nitrogen sources such as nitrate, ammonium, and urea (Eustance et al., 2013). The depletion of nitrate has also repeatedly been shown to initiate the accumulation of lipids within many microalgae species (Gardner et al., 2011; Valenzuela et al., 2013; Valenzuela et al., 2012).

CBM Water with Nitrate and Phosphate Along with nitrogen, Table 3.3 shows phosphate is also missing from CBM water. Phosphate is imperative in the energy processes that occur within a cell. Phosphate is the main constituent of the high energy transfer molecule ATP, and is also a building block of lipid bilayers via phospholipids and is essential to the phosphate backbone of nucleic acids. The depletion of phosphate in combination with nitrate has also been shown to be a trigger for lipid accumulation within some microalgae (K. H. Liang et al., 2013; Valenzuela et al., 2012).

CBM Water with Nitrate, Phosphate, Magnesium, and Sulfate CBM water contains small amounts of magnesium and sulfate but not in significant concentrations when compared to that found in BBM (Table 3.1). This led to a fourth growth condition where these nutrients were supplemented along with nitrate and phosphate. Sulfate plays an important role in the function of photosystem II in microalgae (Wykoff et al., 1998) while magnesium is the central atom of chlorophyll, the energy harvesting molecule of

the cell. Supplementation of magnesium to algal cultures has been observed to have a positive impact on lipid accumulation (Gorain et al., 2013), likely due to the direct link of magnesium and cellular energy levels via chlorophyll.

CBM Water with Nitrate, Phosphate, Magnesium, Sulfate, and Micronutrients

The final amendment to CBM water was the addition of the micronutrients found in BBM along with the previously mentioned macronutrients. Many of these micronutrients are important cofactors for enzymes that play an important role in microalgae metabolism. Examples include the role of zinc in carbonic anhydrase, the role of copper in plastocyanin, and the role of molybdenum in nitrate reductase. These affect the processes of carbon capture (Morel et al., 1994), energy transfer (Katoh & Takamiya, 1961), and nitrogen metabolism (Evans & Hall, 1955) respectively. A lack of these micronutrients in CBM water could be non-ideal when attempting to grow microalgae on a large scale.

Bold's Basal Medium CBMW was also grown in BBM as a control. The amount of nitrate and phosphate that the medium recipe calls for was reduced from 2.94 mM to 0.5 mM and 1.7 mM to 0.05 mM respectively. These concentrations will be the same as those found in the CBM water cultures with these added nutrients. This amount of nitrate and phosphate has been used to trigger growth and lipid accumulation in microalgae grown for the purpose of biodiesel production (Valenzuela et al., 2012).

Growth in Sterile CBM Water and BBM

The results of lipid accumulation, pH, and dry weight measurements for CBMW grown in CBM water with added nutrients and BBM can be seen in Figures 3.1-3.3 respectively. The growth condition that achieved the highest dry weight was the amendment of CBM water with nitrate (Figure 3.3). Cell division and growth is dependent on a nitrogen source to build purines, pyrimidines, and amino acids for the replication of DNA and translation of mRNA. This is reflected in the poor growth observed in CBM water lacking nitrate. All cultures with added nitrate achieved a relatively similar amount of biomass. Even though phosphate was not added to the amendment with only CBM water and nitrate, it is possible that the microalgae in the inoculum had stores of phosphate that could be utilized once it was transferred to a medium lacking this nutrient (Rhee, 1973).

Lipid accumulation was observed in conditions with added nitrate, added nitrate and phosphate, and added nitrate, phosphate, magnesium and sulfate (Figure 3.1). Of these conditions, each that had phosphate reached a lipid accumulation peak more quickly than the condition without added phosphate. Phosphate is required for the formation of $NADP^+$ and NADPH and therefore lipid biosynthesis. $NADP^+$ is also the terminal electron acceptor in photosynthesis. Under environmental conditions where phosphate is more plentiful, less scavenging of phosphate might be required to create $NADP^+$ and other phosphorus containing molecules such as nucleic acids and ATP.

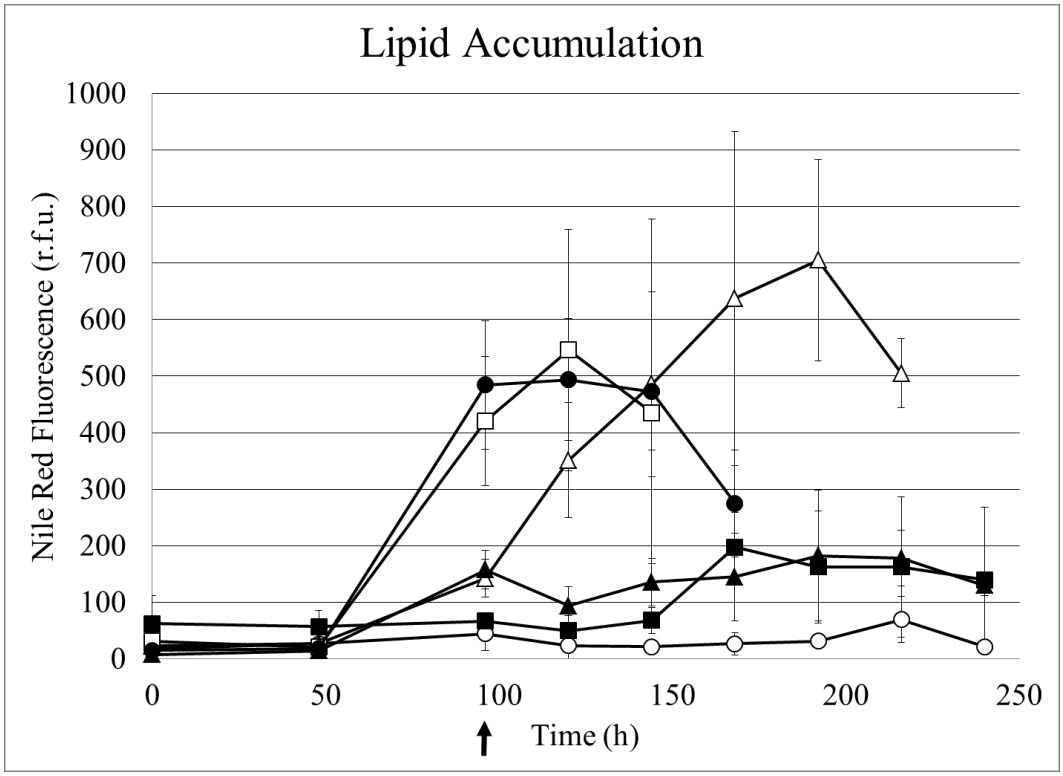


Figure 3.1 Lipid production of CBMW when grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates.

Situations where phosphate is depleted might initiate a need for the cell to begin replacing phospholipids with sulfolipids in lipid membranes to retrieve the phosphate needed for the creation of DNA, RNA, NADPH, and ATP. This remodeling of lipid membranes and other cellular structures has been recently studied in a diatom species once nitrate and phosphate have been depleted (Abida et al., 2015). Comparable levels of lipid were measured in each of the three conditions that accumulated lipids (CBM with nitrate; CBM with nitrate and phosphate; and CBM with nitrate, phosphate, and magnesium sulfate) in subsequent experiments using the Nile Red method.

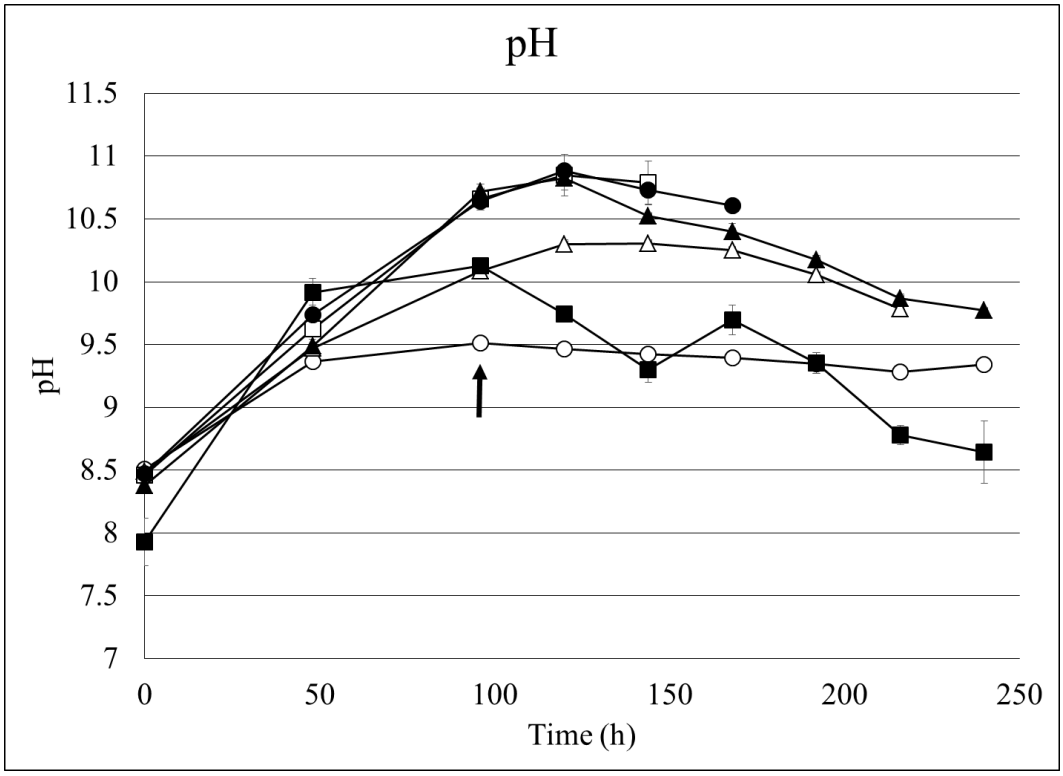


Figure 3.2 pH of CBMW when grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates.

In each condition that accumulated lipids, a strong correlation was observed between nitrate and phosphate (if present) depletion and the beginning of lipid accumulation (Figure 3.4). This correlation has been previously well documented (Valenzuela et al., 2012). Theoretically, a lack of nitrate causes an inability to produce nucleotides and proteins therefore halting cell division and growth. However, when light is present the cell is still producing energy and is in need of $NADP^+$ as the terminal electron acceptor for the process of photosynthesis. The pool of available $NADP^+$ is typically restored by the oxidation of NADPH to $NADP^+$ when biomass is being

produced. In a nitrogen deplete situation where the production of biomass has halted, the cell can regenerate the pool of $NADP^+$ through the biosynthesis of lipids. This is supported by transcriptomic studies that have shown a depletion of nitrogen causes an overexpression of lipid biosynthesis and pentose phosphate pathway genes along with a down expression of fatty acid degradation genes (Park et al., 2015; Rismani-Yazdi et al., 2012; Valenzuela et al., 2012).

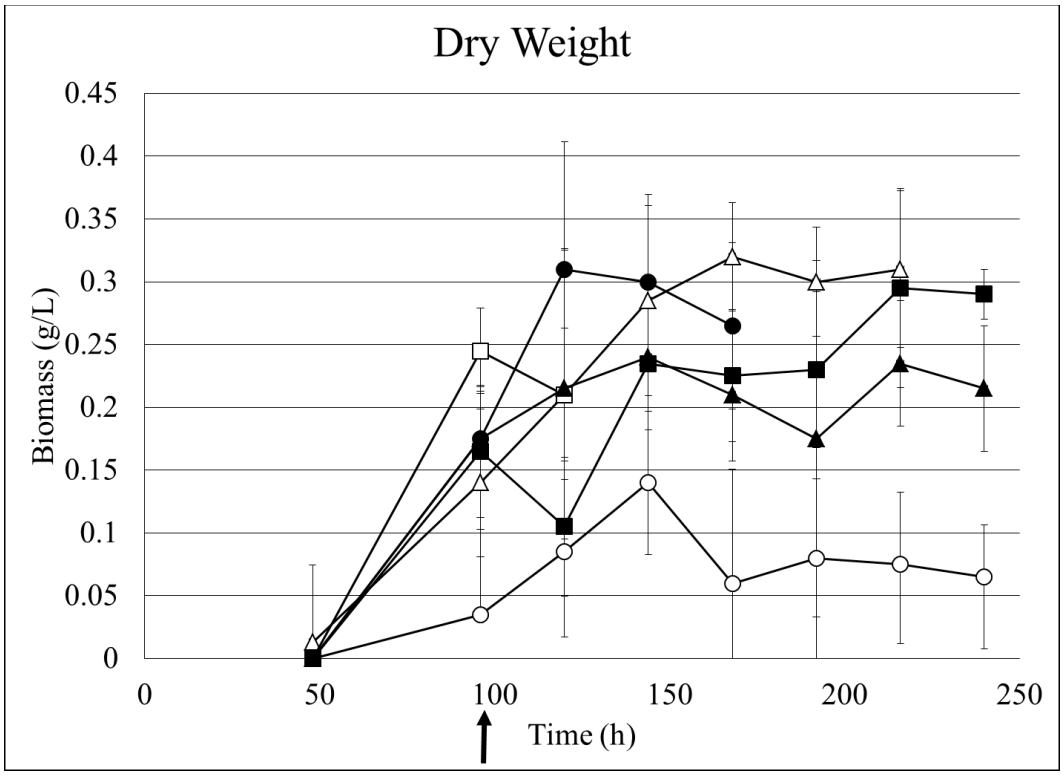


Figure 3.3 Dry weight of CBMW when grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates.

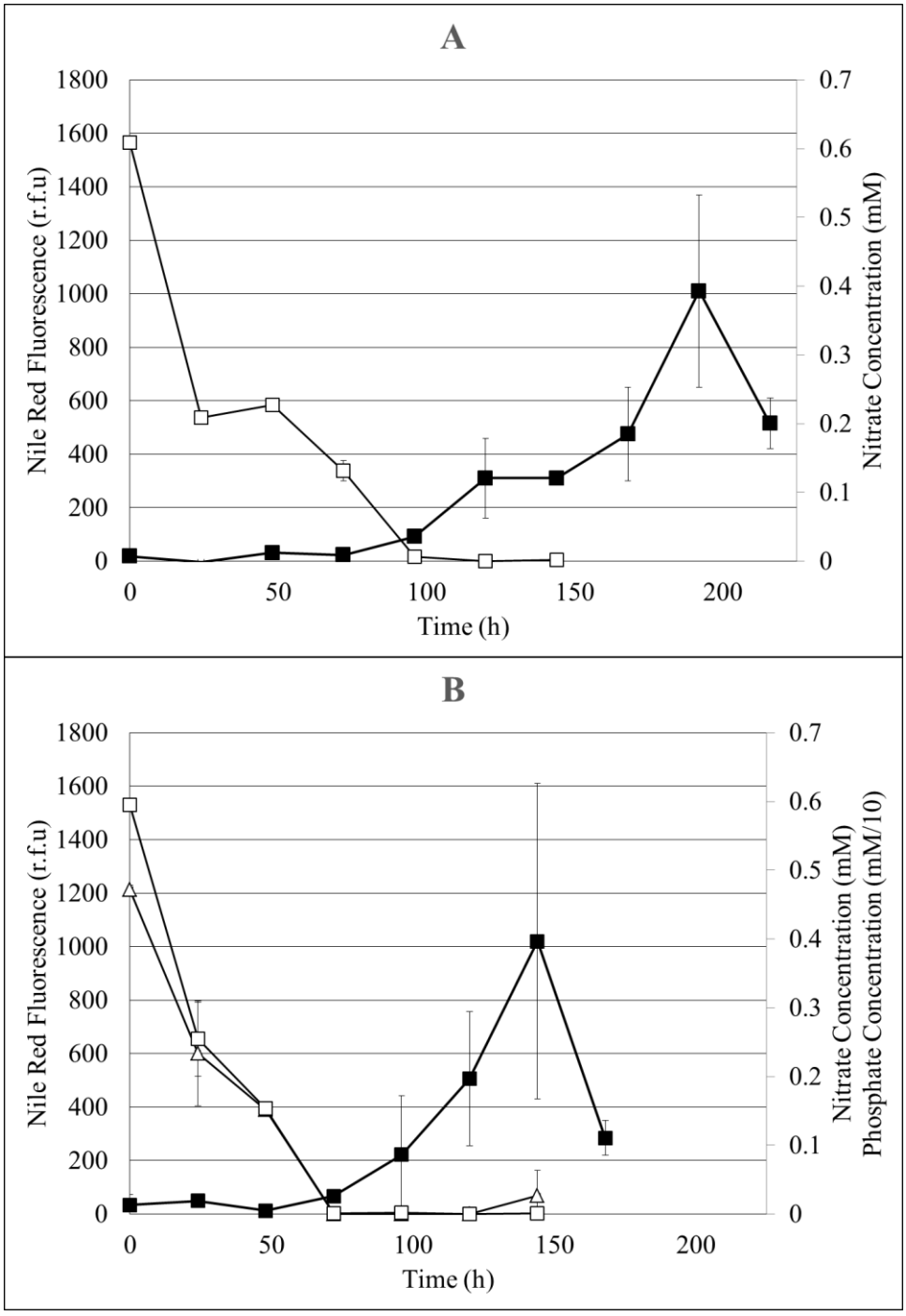


Figure 3.4 **(A)** Lipid accumulation (■) and nitrate depletion (□) in CBM water with 0.5 mM NO_3^- and **(B)** lipid accumulation (■), nitrate depletion (□), and phosphate depletion (Δ) in CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-}. Phosphate concentration is multiplied by 10 for scaling purposes. Error bars represent minimum and maximum of technical duplicates.

A higher pH was observed for each condition that accumulated a significant amount of lipids (Figure 3.2). pH stress has been observed to trigger lipid accumulation in other microalgae (R. Gardner et al., 2011; Skrupski et al., 2013). It can be difficult to determine whether the lipid accumulation was a result of high pH, nitrate depletion, or a combination of the two conditions. In this study, nitrate depletion was assumed to be the cause of lipid accumulation since this trigger was observed before the spike in Nile Red fluorescence, whereas the highest pH values were observed after the nitrate depletion.

A lack of lipid accumulation in CBM water without added nutrients can likely be attributed to the poor growth from an absence of nitrate and phosphate. In the condition with nitrate, phosphate, magnesium, sulfate, and micronutrients, and the condition with Bold's medium, where lipid accumulation was not observed, nitrate and phosphate were confirmed to be depleted at the same time as the other growth conditions that did accumulate lipids. The reason for the lack of lipid accumulation is difficult to determine. A possible explanation could be the presence of the many micronutrients in these conditions. A recent study evaluating the proteome of *Chlamydomonas reinhardtii* under conditions lacking zinc, copper, manganese, and iron found that some enzymes involved in lipolysis within vesicles were negatively affected under deplete conditions (Hsieh et al., 2013). Perhaps the presence of these micronutrients allowed enzymes that degrade lipids to stay functional and therefore prevent a lipid accumulation event.

For all cultures except CBM with only nitrate, chlorophyll was observed to decrease when nitrate was depleted (Figure 3.5), and this response to nitrate depletion has also been previously documented (Rodhe, 1946). A possible explanation for the decline

in chlorophyll could be the damaging effects of reactive oxygen species formed from oxidative stress during photosynthesis. A recent study showed an increase in antioxidant enzymes after nitrogen depletion indicating that the microalgae were experiencing oxidative stress (Zhang et al., 2013). Concurrently, microalgae have been observed to degrade proteins and RNA as well as down regulate protein synthesis to adapt to a lack of nitrogen in their environment (Park et al., 2015). The combination of these two responses could lead to an undesirable situation for the cell where energy harvesting machinery is damaged due to normal photosynthetic reactions, but cannot be replaced due to a lack of nitrogen containing amino acids within the cell.

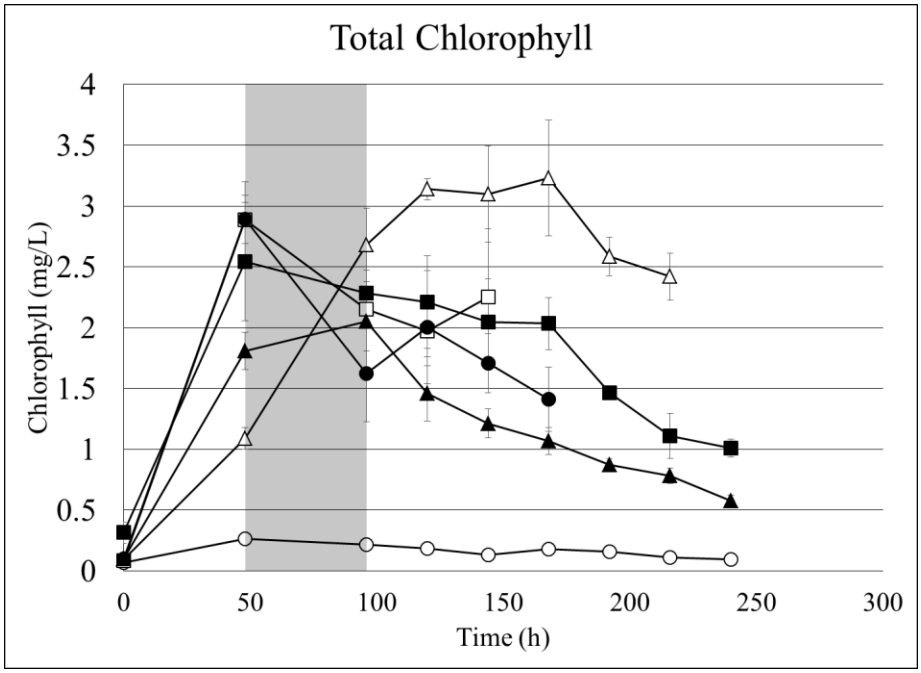


Figure 3.5 Total chlorophyll (*a, b,* and *c*) of CBMW grown in CBM water (○), CBM water with 0.5 mM NO_3^- (Δ), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (□), CBM water with 0.5 mM NO_3^- 0.05, mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (●), CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, 0.3 mM $MgSO_4$, and micronutrients (▲), and BBM with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (■). Grey area shows time frame when nitrate was depleted. Error bars represent one standard deviation of biological and technical duplicates.

Lipid Analysis

Gas chromatography-mass spectrometry (GC-MS) analysis on the extracted lipids of transesterified samples revealed a lipid content of 26.9%, 20.4%, and 24.8% weight of fatty acid/weight of biomass (w/w) for the conditions with added nitrate, added nitrate and phosphate, and added nitrate, phosphate, and magnesium sulfate respectively. These values correspond well to other values found in the literature (Jiang et al., 2012; M. H. Liang & Jiang, 2013; Song et al., 2013). All three lipid contents match very closely to values observed in *Dunalliella tertiolecta* (20% and 24% lipid content) when grown in coal seam gas water from Australia (Aravinthan & Harrington, 2014). A primary difference between the two studies was the need to add a significant amount of salt to the coal seam gas water for *D. tertiolecta* to optimally grow (Aravinthan & Harrington, 2014) as it is a marine green algae species. This highlights an advantage to using CBMW for growth in CBM water. Although primary nutrients such as nitrogen and phosphorus are needed, other amendments to the water are unnecessary to encourage CBMW to grow since CBM water is its natural habitat.

The decrease in lipid content observed in the nitrate and phosphate condition could be a result of a non-optimal phosphate concentration. Varying the phosphorus concentration has been observed to have an effect on the amount of lipids produced by microalgae (K. H. Liang et al., 2013). The rebound in lipid content observed in the condition with nitrate, phosphate, and magnesium sulfate, could point to an interesting interaction between sulfate and phosphate. Sulfate containing compounds have been shown to have inhibitory effects on enzymes that play a direct role in phosphate activity

(Tran et al., 2005; Wu, 1971). The increase in sulfate concentration in the CBM water could be hindering the ability of CBMW to effectively utilize the available phosphate leading to a more pronounced lipid accumulation event.

The lipid profile for the three evaluated conditions can be seen in Figure 3.6. The predominant fatty acids in each case were palmitic acid (C16:0), oleic acid (C18:1 (ω-9)), linoleic acid (C18:2 (ω-9,12)), and α-linolenic acid (18:3 (ω-9,12,15)). These fatty acids match those predominantly found in other plant-based sources of biodiesel such as canola and palm oil (Hoekman et al., 2012), implying that they could be effectively utilized as a biodiesel source. When compared to other algal species, CBMW is lacking in palmitic acid accumulation and contains a higher amount of C: 18 unsaturated fatty acids, especially α-linolenic acid (K. H. Liang et al., 2013; Song et al., 2013). It is not possible to determine from the current data if this difference in lipid profile composition is a product of species specific metabolism or of CBM water as a growth medium.

Summary

In summary, a green algae species, CBMW, was isolated from a coal bed methane production pond and, when nitrate was added, was shown to reach biomass dry weight levels comparable to that found in Bold's Basal Medium. CBMW accumulated lipids when under stress of nitrate limitation unless micronutrients were present in the same concentrations as those in BBM. The highest lipid content achieved was 26.9% w/w when only nitrate was added to CBM water. The addition of other nutrients resulted in lower lipid content values. The benefits of isolating a native algal species can be observed through the limited amendments that need to be made to CBM water to initiate

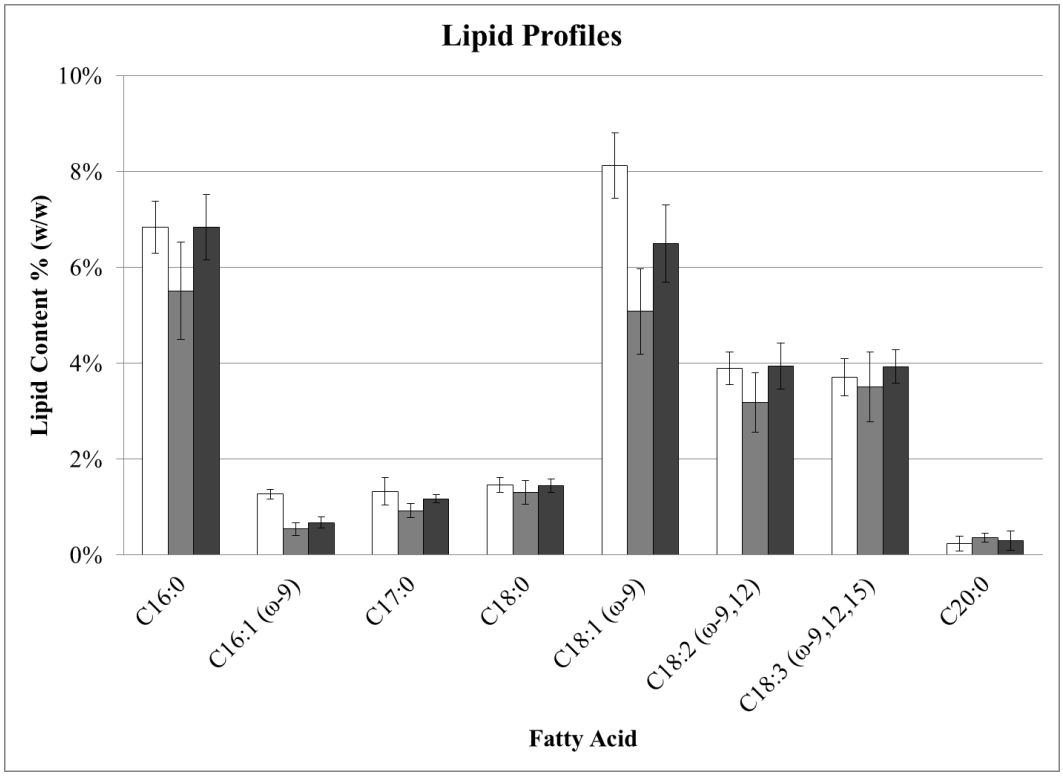


Figure 3.6 Lipid composition of CBMW grown in CBM water with 0.5 mM NO_3^- (white), CBM water with 0.5 mM NO_3^- and 0.05 mM PO_4^{3-} (light grey) and CBM water with 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, and 0.3 mM $MgSO_4$ (dark grey). Lipid content is % w/w of fatty acid to biomass. Error bars represent one standard deviation among technical and biological duplicates.

growth and lipid accumulation. However, more research needs to be done to characterize this species. Unpublished results have indicated that CBMW can grow on ammonium as well as nitrate. Assessing its ability to assimilate other nitrogen sources could prove beneficial when determining how to best supplement CBM water in the Powder River Basin. Concurrently, moving from lab scale to pilot scale experiments with outdoor conditions and non-sterile CBM water could give a more practical assessment of the robustness of this algal species.

CHAPTER FOUR

CONCLUSIONS AND FUTURE WORK

The rising consumption of coal bed methane as a source of energy has spurred the development of extraction wells in many parts of the world rich in shallow underground coal seams. The Powder River Basin in southeastern Montana and northeastern Wyoming has been included in this development which has resulted in billions of gallons of groundwater being extracted and stored in unlined ponds (WOGCC, 2014). The displacement of this groundwater has caused a great deal of concern over how to manage such a vast volume, particularly if it is unsuitable for irrigation or other environmental and agricultural purposes. This thesis has shown that this water can be used as a medium to grow a native microalgae species, CBMW, for the purposes of lipid and biomass accumulation.

In order to stimulate growth, a nitrogen source must be added to the water. As shown in previous studies, the depletion of the nitrogen source can act as a trigger for the accumulation of lipids (Breuer et al., 2012; Mata et al., 2013; Valenzuela et al., 2012). Lipid accumulation was not observed in cultures lacking a nitrogen source or that had micronutrients added to the culture. Although a phosphate source was not needed to stimulate growth or lipid accumulation, it should be noted that it is possible that the inoculum from the stock culture contained phosphate stores in the form of polyphosphates (Rhee, 1973). While these results are promising, there are still several aspects of growing microalgae in CBM water that need to be evaluated.

Practically speaking, the growth of CBMW in CBM water will not be axenic as it was in the final experiments. CBM water contains many microorganisms that could readily interact with CBMW (Barnhart et al., 2013). Although many of the current studies on biomass and lipid accumulation focus on axenic cultures, there is a growing interest in the effects of bacteria on the growth of microalgae for these purposes. One such study has demonstrated that selecting for certain species of bacteria can increase algal cell growth, lipid content, and even play a role in what kinds of lipids are produced (Cho et al., 2015). Studying the effects of growing CBMW in its native ecological environment could also prove to be beneficial since it has already adapted to surviving with the naturally existing bacteria. As mentioned in Chapter 2, we have performed some preliminary experiments with CBMW in non-sterile water and have made some intriguing observations, particularly in the aggregation of axenic CBMW when introduced to non-sterile CBM water.

Harvesting microalgae has become one of the biggest obstacles in the development of microalgal biodiesel production (Rios et al., 2013). While methods such as filtration (Frappart et al., 2011) and centrifugation (Dassey & Theegala, 2013) are effective, they are costly and inefficient. A better and more economical way to remove the microalgae from the water must be used in order to make biodiesel production from microalgae economically feasible. An interesting characteristic of CBMW is its tendency to flocculate in CBM water when transferred from BBM. The flocculation of CBMW is rapid and can easily be observed without the use of a microscope. To the best of our knowledge, a flocculation on this scale, without the use of chemical coagulants, has not

been observed in the literature. Growing microalgae in co-cultures of bacteria have been shown to cause microalgae to aggregate (Cho et al., 2015; D. D. Zhou et al., 2015), but not to this extent. Based on our work, the primary cause of this flocculation is due directly and/or indirectly to microbial populations present in the CBM water. When CBM water was filter sterilized, only small flocs were observed. The large clumps observed in non-sterile CBM water will last for weeks and will reassemble if disturbed by vigorous shaking. Harnessing this flocculation capability could have a big impact on our ability to efficiently remove microalgae from any source of water (Vandamme et al., 2013) and ease the transition of scaling up the production of CBMW.

Along with non-axenic growth and optimizing harvesting techniques, developing pilot scale experiments for the growth of CBMW is also necessary to move forward in utilizing CBM water for microalgal growth. An ideal pilot scale experiment would involve growing CBMW in large outdoor containers while tracking growth and lipid accumulation. Growing photosynthetic organisms in natural sunlight could possibly promote higher photosynthetic efficiency and thus have a positive effect on the health of the culture. Preliminary tests with CBMW grown under natural sunlight during the fall season produced a much stronger chlorophyll response than cultures grown in an incubator. However, this did not hold true during winter months, likely due to the significantly shorter periods of natural sunlight in Montana. It would also be beneficial to observe how well CBMW can adapt to varying environmental changes that would be inherent with growth outside of controlled laboratory conditions. Theoretically, CBMW would have a high tolerance for the outdoor conditions of Montana, Wyoming, and the

Powder River Basin as this is its natural habitat. However, more work needs to be done on evaluating the optimal growth temperature of CBMW as the temperature can fluctuate drastically during a 24 day as well as throughout the year.

Lastly, evaluating the capability of CBMW to assimilate various nitrogen sources would be beneficial to determine the best way to encourage growth in CBM water of the Powder River Basin. Acquiring nitrate for the sole purpose of stimulating CBMW growth could be costly and make the concept of utilizing CBM water ponds uneconomical. If this algal species can utilize ammonia and/or urea as a nitrogen source, it could be possible to combine CBM water with other low quality water sources that are readily available in the Powder River Basin. These other water sources would primarily take the form of livestock waste lagoons. Runoff from cattle feedlots and other confined livestock areas is required to be collected and stored in lined or unlined ponds to prevent contamination of other water sources. These ponds are typically very high in ammonium and phosphate and are allowed to evaporate with the sludge periodically being mechanically removed. It should be noted that the occurrence and total volume of livestock waste ponds is nowhere near that of CBM ponds. However, the combination of CBM water with a dose of highly concentrated livestock waste could provide the ideal combination of water that CBMW is found in with a nitrogen and phosphorus source that is needed to optimize growth and lipid accumulation. The growth of other species of microalgae on livestock wastewater has previously been documented (Kothari et al., 2013; Prajapati et al., 2014).

It should be pointed out that the use of these water sources for microalgal growth would still result in water being a by-product once the microalgae is harvested. This water could potentially be reused for microalgae growth and allowed to continually evaporate into the atmosphere. The reuse and evaporation of this water would create a concentrating effect of the salts over time in the CBM water. This will have to be taken into consideration for the design of a system that utilizes CBM water and microalgae. Another item to consider is the addition of nitrogen and/or phosphorus to CBM water. As many of these ponds are unlined, the addition of these nutrients could allow for nitrogen and phosphorus to leach into the subsurface. To prevent this, it might be necessary to construct an adjacent lined pond or other water holding structure next to the source of CBM water where nitrogen and phosphorus could be safely added and microalgae allowed to grow without causing unintended harm to the environment.

While the utilization of CBM water for microalgal growth and lipid accumulation is a relatively new area of research, there is a broad and increasing knowledge base of how to best stimulate microalgae responses for these purposes. Applying what is already known and investigating what methods are best suited for growth in CBM water could lead to an efficient utilization of massive amounts of unused water in the Powder River Basin and other areas of the world. The usage of this water has the potential to alleviate environmental stress caused by the large influx of water to the surrounding areas and help offset the growing demand for alternative energy sources.

REFERENCES CITED

Abida, H., Dolch, L. J., Mei, C., Villanova, V., Conte, M., Block, M. A., . . . Marechal, E. (2015). Membrane Glycerolipid Remodeling Triggered by Nitrogen and Phosphorus Starvation in Phaeodactylum tricornutum. *Plant Physiology, 167*(1), 118-136. doi: 10.1104/pp.114.252395

Aravinthan, V., & Harrington, D. (2014). Coal seam gas water as a medium to grow Dunalliella tertiolecta microalgae for lipid extraction. *Desalination and Water Treatment, 52*(4-6), 947-958. doi: 10.1080/19443994.2013.827295

Aschonitis, V. G., Lekakis, E. H., Petridou, N. C., Koukouli, S. G., & Pavlatou-Ve, A. (2013). Nutrients fixation by algae and limiting factors of algal growth in flooded rice fields under semi-arid Mediterranean conditions: case study in Thessaloniki plain in Greece. *Nutrient Cycling in Agroecosystems, 96*(1), 1-13. doi: 10.1007/s10705-013-9573-x

Barnhart, E. P., De Leon, K. B., Ramsay, B. D., Cunningham, A. B., & Fields, M. W. (2013). Investigation of coal-associated bacterial and archaeal populations from a diffusive microbial sampler (DMS). *International Journal of Coal Geology, 115*, 64-70. doi: 10.1016/j.coal.2013.03.006

Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., & Wijffels, R. H. (2012). The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology, 124*, 217-226. doi: 10.1016/j.biortech.2012.08.003

Buchanan, J. J., Slater, F. R., Bai, X., & Pratt, S. (2013). Algal growth and community structure in a mixed-culture system using coal seam gas water as the water source. *Environmental Technology, 34*(6), 695-701. doi: 10.1080/09593330.2012.715676

Buckland, G. D., Bennett, D. R., Mikalson, D. E., de Jong, E., & Chang, C. (2002). Soil salinization and sodication from alternate irrigations with saline-sodic water and simulated rain. *Canadian Journal of Soil Science, 82*(3), 297-309.

Chen, W., Zhang, C. W., Song, L. R., Sommerfeld, M., & Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods, 77*(1), 41-47. doi: 10.1016/j.mimet.2009.01.001

Chia, M. A., Lombardi, A. T., Melao, M. D. G., & Parrish, C. C. (2013). Effects of cadmium and nitrogen on lipid composition of Chlorella vulgaris (Trebouxiophyceae, Chlorophyta). *European Journal of Phycology, 48*(1), 1-11. doi: 10.1080/09670262.2012.750687

Cho, D. H., Ramanan, R., Heo, J., Lee, J., Kim, B. H., Oh, H. M., & Kim, H. S. (2015). Enhancing microalgal biomass productivity by engineering a microalgal-bacterial community. *Bioresource Technology, 175*, 578-585. doi: 10.1016/j.biortech.2014.10.159

Christenson, L. B., & Sims, R. C. (2012). Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products. *Biotechnology and Bioengineering, 109*(7), 1674-1684. doi: 10.1002/bit.24451

Cooksey, K. E., Guckert, J. B., Williams, S. A., & Callis, P. R. (1987). FLUOROMETRIC-DETERMINATION OF THE NEUTRAL LIPID-CONTENT OF MICROALGAL CELLS USING NILE RED. *Journal of Microbiological Methods, 6*(6), 333-345. doi: 10.1016/0167-7012(87)90019-4

Croft, M. T., Lawrence, A. D., Raux-Deery, E., Warren, M. J., & Smith, A. G. (2005). Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature, 438*(7064), 90-93. doi: http://www.nature.com/nature/journal/v438/n7064/suppinfo/nature04056_S1.html

Cui, Y., Yuan, W., & Cheng, J. (2014). Understanding pH and Ionic Strength Effects on Aluminum Sulfate-Induced Microalgae Flocculation. *Applied Biochemistry and Biotechnology, 173*(7), 1692-1702. doi: 10.1007/s12010-014-0957-4

Dassey, A. J., & Theegala, C. S. (2013). Harvesting economics and strategies using centrifugation for cost effective separation of microalgae cells for biodiesel applications. *Bioresource Technology, 128*, 241-245. doi: 10.1016/j.biortech.2012.10.061

Davis, W. N., Bramblett, R. G., Zale, A. V., & Endicott, C. L. (2009). A Review of the Potential Effects of Coal Bed Natural Gas Development Activities on Fish Assemblages of the Powder River Geologic Basin. *Reviews in Fisheries Science, 17*(3), 402-422. doi: 10.1080/10641260902737067

de Godos, I., Guzman, H. O., Soto, R., Garcia-Encina, P. A., Becares, E., Munoz, R., & Vargas, V. A. (2011). Coagulation/flocculation-based removal of algal-bacterial biomass from piggery wastewater treatment. *Bioresource Technology, 102*(2), 923-927. doi: 10.1016/j.biortech.2010.09.036

Eldridge, R. J., Hill, D. R. A., & Gladman, B. R. (2012). A comparative study of the coagulation behaviour of marine microalgae. *Journal of Applied Phycology, 24*(6), 1667-1679. doi: 10.1007/s10811-012-9830-4

Eustance, E., Gardner, R. D., Moll, K. M., Menicucci, J., Gerlach, R., & Peyton, B. M. (2013). Growth, nitrogen utilization and biodiesel potential for two chlorophytes grown on ammonium, nitrate or urea. *Journal of Applied Phycology, 25*(6), 1663-1677. doi: 10.1007/s10811-013-0008-5

Evans, H. J., & Hall, N. S. (1955). ASSOCIATION OF MOLYBDENUM WITH NITRATE REDUCTASE FROM SOYBEAN LEAVES. *Science, 122*(3176), 922-923. doi: 10.1126/science.122.3176.922

External Peer Review Draft Aquatic Life Ambient Water Quality Criterion For Selenium – Freshwater 2014. (2014). *United States Environmental Protection Agency*. Retrieved from: http://water.epa.gov/scitech/swguidance/standards/criteria/aqlife/selenium/upload/External-Peer-Review-Draft-Aquatic-Life-Ambient-Water-Quality-Criterion-For-Selenium-Freshwater-2014.pdf

Fields, M. W., Hise, A., Lohman, E. J., Bell, T., Gardner, R. D., Corredor, L., . . . Gerlach, R. (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. doi: 10.1007/s00253-014-5694-7

Frappart, M., Masse, A., Jaffrin, M. Y., Pruvost, J., & Jaouen, P. (2011). Influence of hydrodynamics in tangential and dynamic ultrafiltration systems for microalgae separation. *Desalination, 265*(1-3), 279-283. doi: 10.1016/j.desal.2010.07.061

Ganjegunte, G. K., King, L. A., & Vance, G. F. (2008). Cumulative soil chemistry changes from land application of saline-sodic waters. *Journal of Environmental Quality, 37*(5), S128-S138. doi: 10.2134/jeq2007.0424

Gardner, R., Peters, P., Peyton, B., & Cooksey, K. E. (2011). Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the chlorophyta. *Journal of Applied Phycology, 23*(6), 1005-1016. doi: 10.1007/s10811-010-9633-4

Gardner, R. D., Cooksey, K. E., Mus, F., Macur, R., Moll, K., Eustance, E., . . . Peyton, B. M. (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. doi: 10.1007/s10811-011-9782-0

Golueke, C. G., Oswald, W. J., & Gotaas, H. B. (1957). ANAEROBIC DIGESTION OF ALGAE. *Applied Microbiology, 5*(1), 47-55.

Gonzalez-Garcinuno, A., Tabernero, A., Sanchez-Alvarez, J. M., del Valle, E. M. M., & Galan, M. A. (2014). Effect of nitrogen source on growth and lipid accumulation in Scenedesmus abundans and Chlorella ellipsoidea. *Bioresource Technology, 173*, 334-341. doi: 10.1016/j.biortech.2014.09.038

Gorain, P. C., Bagchi, S. K., & Mallick, N. (2013). Effects of calcium, magnesium and sodium chloride in enhancing lipid accumulation in two green microalgae. *Environmental Technology, 34*(13-14), 1887-1894. doi: 10.1080/09593330.2013.812668

Goyal, A., & Gimmler, H. (1989). OSMOREGULATION IN DUNALIELLA-TERTIOLECTA - EFFECTS OF SALT STRESS, AND THE EXTERNAL PH ON THE INTERNAL PH. *Archives of Microbiology, 152*(2), 138-142. doi: 10.1007/bf00456091

Gressler, P. D., Bjerk, T. R., Schneider, R. D. S., Souza, M. P., Lobo, E. A., Zappe, A. L., . . . Moraes, M. S. A. (2014). Cultivation of Desmodesmus subspicatus in a tubular photobioreactor for bioremediation and microalgae oil production. *Environmental Technology, 35*(2), 209-219. doi: 10.1080/09593330.2013.822523

, Guidance and Tech for CBM Permitting under WYPDES. Guidance and Tech for CBM Permitting under WYPDES. *Wyoming Department of Environmental Quality*. Retrieved from http://deq.wyoming.gov/wqd/coalbed-methane/resources/guidance-tech/

Healy, R. W., Bartos, T. T., Rice, C. A., McKinley, M. P., & Smith, B. D. (2011). Groundwater chemistry near an impoundment for produced water, Powder River Basin, Wyoming, USA. *Journal of Hydrology, 403*(1-2), 37-48. doi: 10.1016/j.jhydrol.2011.03.042

Heisler, J., Glibert, P. M., Burkholder, J. M., Anderson, D. M., Cochlan, W., Dennison, W. C., . . . Suddleson, M. (2008). Eutrophication and harmful algal blooms: A scientific consensus. *Harmful Algae, 8*(1), 3-13. doi: 10.1016/j.hal.2008.08.006

Hernandez-Becerril, D. U., Alonso-Rodriguez, R., Alvarez-Gongora, C., Baron-Campis, S. A., Ceballos-Corona, G., Herrera-Silveira, J., . . . Rodriguez-Salvadoro, R. (2007). Toxic and harmful marine phytoplankton and microalgae (HABs) in Mexican Coasts. *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering, 42*(10), 1349-1363. doi: 10.1080/10934520701480219

Hoekman, S. K., Broch, A., Robbins, C., Ceniceros, E., & Natarajan, M. (2012). Review of biodiesel composition, properties, and specifications. *Renewable & Sustainable Energy Reviews, 16*(1), 143-169. doi: 10.1016/j.rser.2011.07.143

Hsieh, S. I., Castruita, M., Malasarn, D., Urzica, E., Erde, J., Page, M. D., . . . Loo, J. A. (2013). The Proteome of Copper, Iron, Zinc, and Manganese Micronutrient Deficiency in Chlamydomonas reinhardtii. *Molecular & Cellular Proteomics, 12*(1), 65-86. doi: 10.1074/mcp.M112.021840

Huang, X. X., Huang, Z. Z., Wen, W., & Yan, J. Q. (2013). Effects of nitrogen supplementation of the culture medium on the growth, total lipid content and fatty acid profiles of three microalgae (Tetraselmis subcordiformis, Nannochloropsis oculata and Pavlova viridis). *Journal of Applied Phycology, 25*(1), 129-137. doi: 10.1007/s10811-012-9846-9

Jackson, R. E., & Reddy, K. J. (2007). Geochemistry of coalbed natural gas (CBNG) produced water in powder river basin, wyoming: Salinity and sodicity. *Water Air and Soil Pollution, 184*(1-4), 49-61. doi: 10.1007/s11270-007-9398-9

Jiang, Y. L., Yoshida, T., & Quigg, A. (2012). Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae. *Plant Physiology and Biochemistry, 54*, 70-77. doi: 10.1016/j.plaphy.2012.02.012

Katoh, S., & Takamiya, A. (1961). NEW LEAF COPPER PROTEIN PLASTOCYANIN, A NATURAL HILL OXIDANT. *Nature, 189*(476), 665-&. doi: 10.1038/189665a0

Kothari, R., Prasad, R., Kumar, V., & Singh, D. P. (2013). Production of biodiesel from microalgae Chlamydomonas polypyrenoideum grown on dairy industry wastewater. *Bioresource technology, 144*, 499-503. doi: 10.1016/j.biortech.2013.06.116

Kunikane, S., Kaneko, M., & Maehara, R. (1984). GROWTH AND NUTRIENT-UPTAKE OF GREEN-ALGA, SCENEDESMUS-DIMORPHUS, UNDER A WIDE-RANGE OF NITROGEN PHOSPHORUS RATIO .1. EXPERIMENTAL-STUDY. *Water Research, 18*(10), 1299-1311. doi: 10.1016/0043-1354(84)90036-8

Lee, J., Cho, D.-H., Ramanan, R., Kim, B.-H., Oh, H.-M., & Kim, H.-S. (2013). Microalgae-associated bacteria play a key role in the flocculation of Chlorella vulgaris. *Bioresource Technology, 131*, 195-201. doi: 10.1016/j.biortech.2012.11.130

Liang, K. H., Zhang, Q. H., Gu, M., & Cong, W. (2013). Effect of phosphorus on lipid accumulation in freshwater microalga Chlorella sp. *Journal of Applied Phycology, 25*(1), 311-318. doi: 10.1007/s10811-012-9865-6

Liang, M. H., & Jiang, J. G. (2013). Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. *Progress in Lipid Research, 52*(4), 395-408. doi: 10.1016/j.plipres.2013.05.002

Liu, J. X., Tao, Y. J., Wu, J. H., Zhu, Y., Gao, B. Y., Tang, Y., . . . Zhang, Y. M. (2014). Effective flocculation of target microalgae with self-flocculating microalgae induced by pH decrease. *Bioresource Technology, 167*, 367-375. doi: 10.1016/j.biortech.2014.06.036

Liu, Z. Y., Wang, G. C., & Zhou, B. C. (2008). Effect of iron on growth and lipid accumulation in Chlorella vulgaris. *Bioresource Technology, 99*(11), 4717-4722. doi: 10.1016/j.biortech.2007.09.073

Lohman, E. J., Gardner, R. D., Halverson, L., Macur, R. E., Peyton, B. M., & Gerlach, R. (2013). An efficient and scalable extraction and quantification method for algal derived biofuel. *Journal of Microbiological Methods, 94*(3), 235-244. doi: 10.1016/j.mimet.2013.06.007

Lohman, E. J., Gardner, R. D., Halverson, L. D., Peyton, B. M., & Gerlach, R. (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. doi: 10.1016/j.algal.2014.08.001

Manoharan, K., Lee, T. K., Cha, J. M., Kim, J. H., Lee, W. S., Chang, M., . . . Cho, J. H. (1999). Acclimation of Prorocentrum minimum (Dinophyceae) to prolonged darkness by use of an alternative carbon source from triacylglycerides and galactolipids. *Journal of Phycology, 35*(2), 287-292. doi: 10.1046/j.1529-8817.1999.3520287.x

Mata, T. M., Almeida, R., & Caetano, N. S. (2013). Effect of the Culture Nutrients on the Biomass and Lipid Productivities of Microalgae Dunaliella tertiolecta. *Icheap-11: 11th International Conference on Chemical and Process Engineering, Pts 1-4, 32*, 973-978. doi: 10.3303/cet1332163

McBeth, I. H., Reddy, K. J., & Skinner, Q. D. (2003). Coalbed methane product water chemistry in three Wyoming watersheds. *Journal of the American Water Resources Association, 39*(3), 575-585. doi: 10.1111/j.1752-1688.2003.tb03676.x

Molina Grima, E., Belarbi, E. H., Acién Fernández, F. G., Robles Medina, A., & Chisti, Y. (2003). Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances, 20*(7–8), 491-515. doi: http://dx.doi.org/10.1016/S0734-9750(02)00050-2

Morel, F. M. M., Reinfelder, J. R., Roberts, S. B., Chamberlain, C. P., Lee, J. G., & Yee, D. (1994). ZINC AND CARBON CO-LIMITATION OF MARINE-PHYTOPLANKTON. *Nature, 369*(6483), 740-742. doi: 10.1038/369740a0

Mulbry, W., Westhead, E. K., Pizarro, C., & Sikora, L. (2005). Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer. *Bioresource Technology, 96*(4), 451-458. doi: 10.1016/j.biortech.2004.05.026

Park, J. J., Wang, H. X., Gargouri, M., Deshpande, R. R., Skepper, J. N., Holguin, F. O., . . . Gang, D. R. (2015). The response of Chlamydomonas reinhardtii to nitrogen deprivation: a systems biology analysis. *Plant Journal, 81*(4), 611-624. doi: 10.1111/tpj.12747

Prajapati, S. K., Choudhary, P., Malik, A., & Vijay, V. K. (2014). Algae mediated treatment and bioenergy generation process for handling liquid and solid waste from dairy cattle farm. *Bioresource Technology, 167*, 260-268. doi: 10.1016/j.biortech.2014.06.038

Reddy, K. J., Whitman, A. J., & Kniss, A. R. (2014). Potential beneficial uses of coalbed natural gas (CBNG) water. *Environmental Science-Processes & Impacts, 16*(1), 148-158. doi: 10.1039/c3em00255a

Rhee, G. Y. (1973). CONTINUOUS CULTURE STUDY OF PHOSPHATE UPTAKE, GROWTH-RATE AND POLYPHOSPHATE IN SCENEDESMUS SP. *Journal of Phycology, 9*(4), 495-506. doi: 10.1111/j.0022-3646.1973.00495.x

Richards, L. A., Allison, L. E., Bernstein, L., Bower, C. A., Brown, J. W., Fireman, M., . . . Wilcox, L. v. (1954). *Diagnosis and Improvement of Saline and Alkali Soils*. Washington D.C.: United States Department of Agriculture.

Rios, S. D., Torres, C. M., Torras, C., Salvado, J., Mateo-Sanz, J. M., & Jimenez, L. (2013). Microalgae-based biodiesel: Economic analysis of downstream process realistic scenarios. *Bioresource Technology, 136*, 617-625. doi: 10.1016/j.biortech.2013.03.046

Rismani-Yazdi, H., Haznedaroglu, B. Z., Hsin, C., & Peccia, J. (2012). Transcriptomic analysis of the oleaginous microalga Neochloris oleoabundans reveals metabolic insights into triacylglyceride accumulation. *Biotechnology for Biofuels, 5*, 16. doi: 10.1186/1754-6834-5-74

Ritchie, R. J. (2008). Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. *Photosynthetica, 46*(1), 115-126. doi: 10.1007/s11099-008-0019-7

Rodhe, W. (1946). Environmental requirements of fresh-water plankton algae. *Symbolae Bot Upsalienses, 10*((1)), 1-149.

Ross, H. E., & Zoback, M. D. (2008). Sub-hydrostatic pore pressure in coalbed and sand aquifers of the Powder River Basin, Wyoming and Montana, and implications for disposal of coalbed-methane-produced water through injection. *Rocky Mountain Geology, 43*(2), 155-169.

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., . . . Hankamer, B. (2008). Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *Bioenergy Research, 1*(1), 20-43. doi: 10.1007/s12155-008-9008-8

Scott, A. R. (1994). COMPOSITION OF COALBED GASES. *In Situ, 18*(2), 185-208.

Singh, A., Nigam, P. S., & Murphy, J. D. (2011). Renewable fuels from algae: An answer to debatable land based fuels. *Bioresource Technology, 102*(1), 10-16. doi: 10.1016/j.biortech.2010.06.032

Skrupski, B., Wilson, K. E., Goff, K. L., & Zou, J. T. (2013). Effect of pH on neutral lipid and biomass accumulation in microalgal strains native to the Canadian prairies and the Athabasca oil sands. *Journal of Applied Phycology, 25*(4), 937-949. doi: 10.1007/s10811-012-9930-1

Song, M. M., Pei, H. Y., Hu, W. R., & Ma, G. X. (2013). Evaluation of the potential of 10 microalgal strains for biodiesel production. *Bioresource Technology, 141*, 245-251. doi: 10.1016/j.biortech.2013.02.024

Tran, K. L., Aronov, P. A., Tanaka, H., Newman, J. W., Hammock, B. D., & Morisseau, C. (2005). Lipid sulfates and sulfonates are allosteric competitive inhibitors of the N-terminal phosphatase activity of the mammalian soluble epoxide hydrolase. *Biochemistry, 44*(36), 12179-12187. doi: 10.1021/bi050842g

USGS (Producer). (2002). National Oil and Gas Assessment. *Energy Resources Program*. Retrieved from http://energy.usgs.gov/OilGas/AssessmentsData/NationalOilGasAssessment.aspx#.VPUqCvnF8mu

Valenzuela, J., Carlson, R. P., Gerlach, R., Cooksey, K., Peyton, B. M., Bothner, B., & Fields, M. W. (2013). Nutrient resupplementation arrests bio-oil accumulation in Phaeodactylum tricornutum. *Applied Microbiology and Biotechnology, 97*(15), 7049-7059. doi: 10.1007/s00253-013-5010-y

Valenzuela, J., Mazurie, A., Carlson, R. P., Gerlach, R., Cooksey, K. E., Peyton, B. M., & Fields, M. W. (2012). Potential role of multiple carbon fixation pathways during lipid accumulation in Phaeodactylum tricornutum. *Biotechnology for Biofuels, 5*. doi: 10.1186/1754-6834-5-40

Vance, G. F., King, L. A., & Ganjegunte, G. K. (2008). Soil and plant responses from land application of saline-sodic waters: Implications of management. *Journal of Environmental Quality, 37*(5), S139-S148. doi: 10.2134/jeq2007.0442

Vandamme, D., Foubert, I., & Muylaert, K. (2013). Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends in Biotechnology, 31*(4), 233-239. doi: 10.1016/j.tibtech.2012.12.005

Welch, J. (2009). Reverse osmosis treatment of CBM produced water continues to evolve. *Oil & Gas Journal, 107*(37), 45-50.

WGOCC (Producer). (2014). *Wyoming Oil and Gas Conservation Commission*. Retrieved from http://wogcc.state.wy.us/

Wheaton, J. R., & Olson, J. R. (2001). *Overview of coal-bed methane development and a discussion of Montana impacts.* Paper presented at the Proceedings of National Meetings of American Society of Mining and Reclamation, Albuquerque, NM.

White, D., Drummond, J., & Fuqua, C. (2012). *The Phsiology and Biochemistry of Prokaryotes Fourth Edition*. New York, NY: Oxford University Press.

Wu, R. (1971). INHIBITION OF POLYNUCLEOTIDE KINASE BY AGAR, DEXTRAN SULFATE AND OTHER POLYSACCHARIDE SULFATES. *Biochemical and Biophysical Research Communications, 43*(4), 927-&. doi: 10.1016/0006-291x(71)90706-6

Wykoff, D. D., Davies, J. P., Melis, A., & Grossman, A. R. (1998). The regulation of photosynthetic electron transport during nutrient deprivation in Chlamydomonas reinhardtii. *Plant Physiology, 117*(1), 129-139. doi: 10.1104/pp.117.1.129

Yeesang, C., & Cheirsilp, B. (2011). Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. *Bioresource Technology, 102*(3), 3034-3040. doi: 10.1016/j.biortech.2010.10.013

Zhang, Y. M., Chen, H., He, C. L., & Wang, Q. (2013). Nitrogen Starvation Induced Oxidative Stress in an Oil-Producing Green Alga Chlorella sorokiniana C3. *Plos One, 8*(7), 12. doi: 10.1371/journal.pone.0069225

Zhou, D. D., Li, Y. B., Yang, Y., Wang, Y., Zhang, C. F., & Wang, D. (2015). Granulation, control of bacterial contamination, and enhanced lipid accumulation by driving nutrient starvation in coupled wastewater treatment and Chlorella regularis cultivation. *Applied Microbiology and Biotechnology, 99*(3), 1531-1541. doi: 10.1007/s00253-014-6288-0

Zhou, X. P., Xia, L., Ge, H. M., Zhang, D. L., & Hu, C. X. (2013). Feasibility of biodiesel production by microalgae Chlorella sp (FACHB-1748) under outdoor conditions. *Bioresource Technology, 138*, 131-135. doi: 10.1016/j.biortech.2013.03.169

Zou, L., Miller, S. N., & Schmidtmann, E. T. (2006). Mosquito larval habitat mapping using remote sensing and GIS: Implications of coalbed methane development and West Nile virus. *Journal of Medical Entomology, 43*(5), 1034-1041. doi: 10.1603/0022-2585(2006)43[1034:mlhmur]2.0.co;2

APPENDICES

APPENDIX A

SUPPORTING MATERIAL FOR CHAPTER TWO MATERIALS AND METHODS

Nile Red Assay Time and Concentration Assays for CBMW

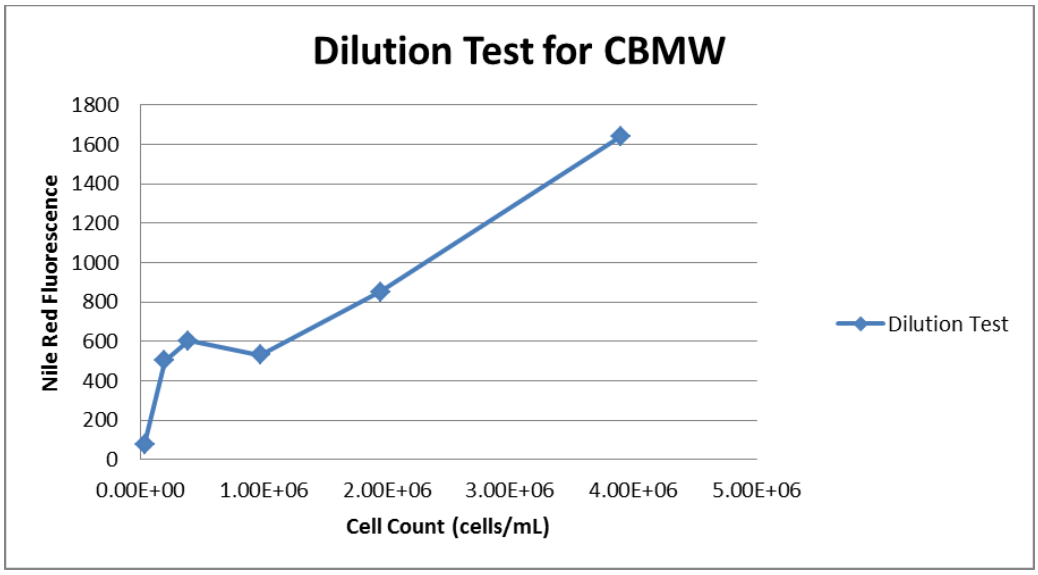


Figure A.1 Nile Red fluorescence was taken at varying dilutions of CBMW to determine a linear range of cell count values.

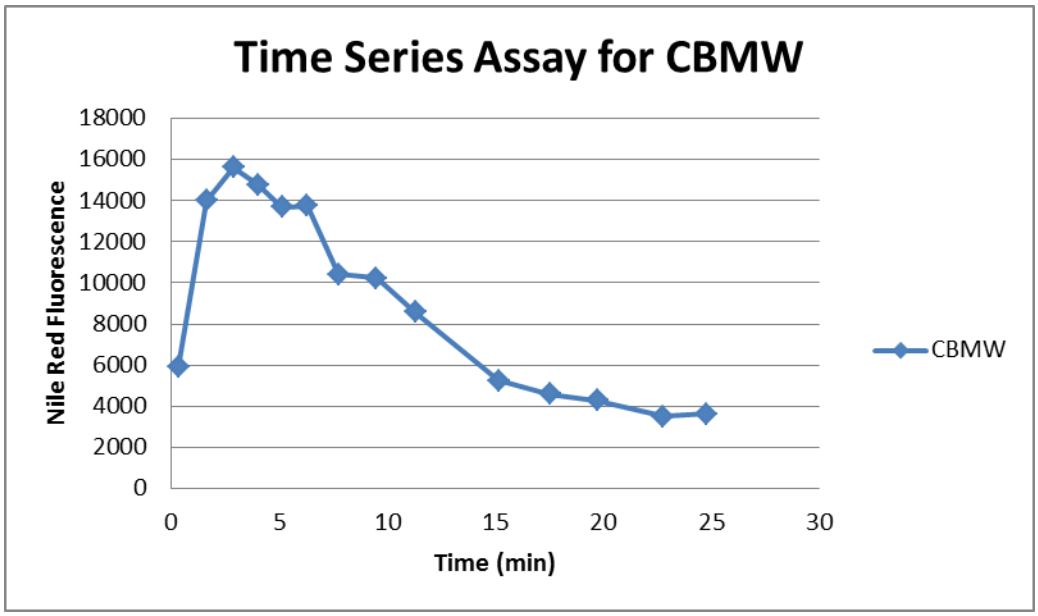


Figure A.2 A time assay for the Nile Red fluorescence method was performed on CBMW. Nile Red fluorescence was taken at marked time points after samples were stained with Nile Red to determine the optimum staining time. Cultures were kept in the dark to avoid light interference.

Preliminary Experiments

Nutrients Experiment with CBMW

This experiment was performed to identify key nutrients missing from CBM water that could stimulate growth of CBMW. Each condition was performed with one biological replicate and two technical replicates for pH, cell counts, chlorophyll concentrations, and Nile Red fluorescence. 5 mL of unwashed stock culture was added to each flask with an approximate cell count concentration of 1.22×10^6 cells/mL. CBM water was not sterilized. In figure legends: "NO3" represents 2.94 mM NO_3^-; "PO4" represents 1.7 mM PO_4^{3-}; "S1", "S2", "S3", "me", and "B12" represent Solution 1, Solution 2, Solution 3, microelement solution, and vitamin B12 solution used for Bold's Basal Medium respectively.

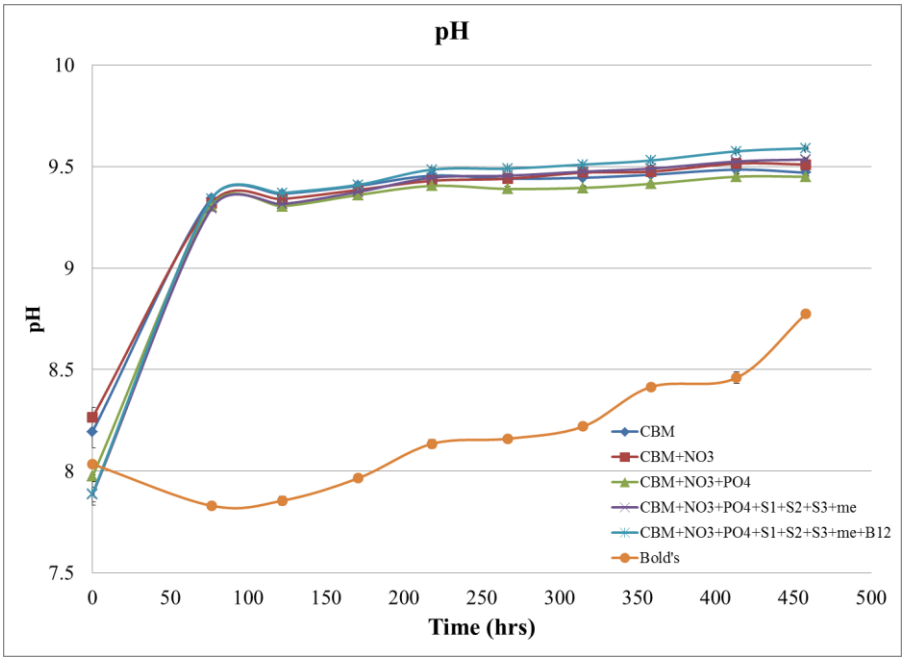


Figure A.3 pH values of CBMW grown in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates.

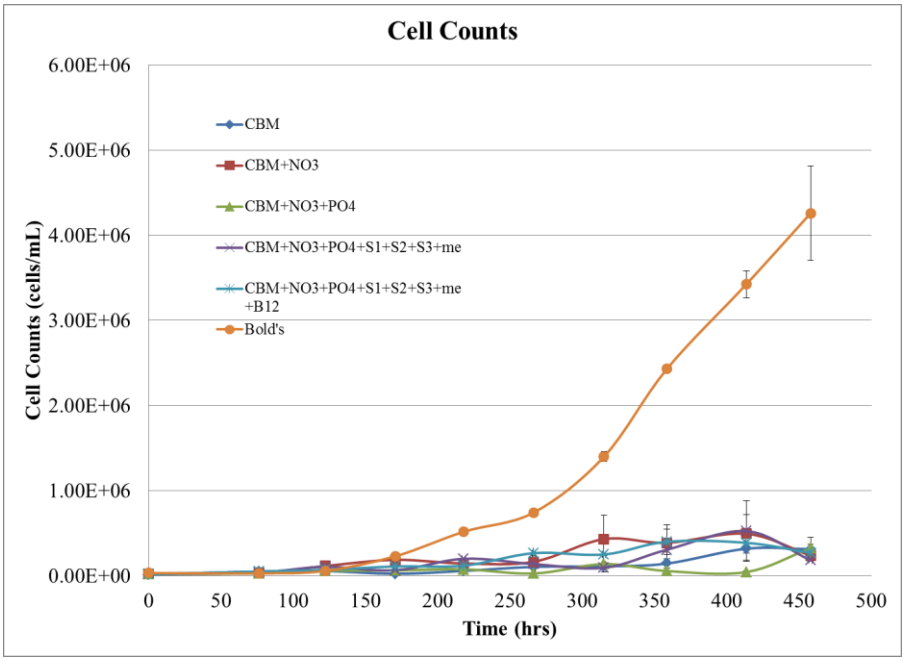


Figure A.4 Cell count of CBMW grown in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates.

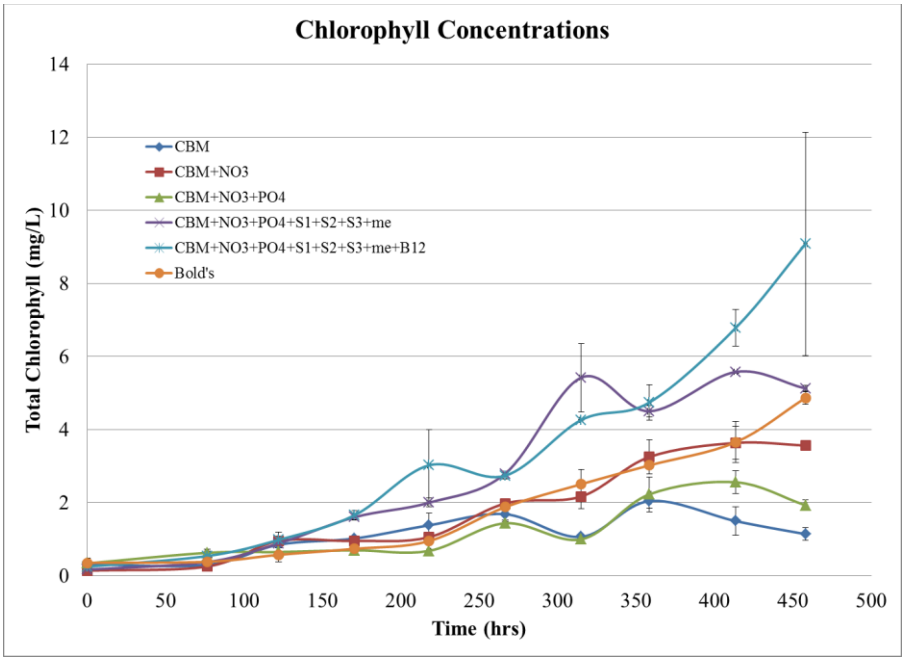


Figure A.5 Chlorophyll levels of CBMW in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates.

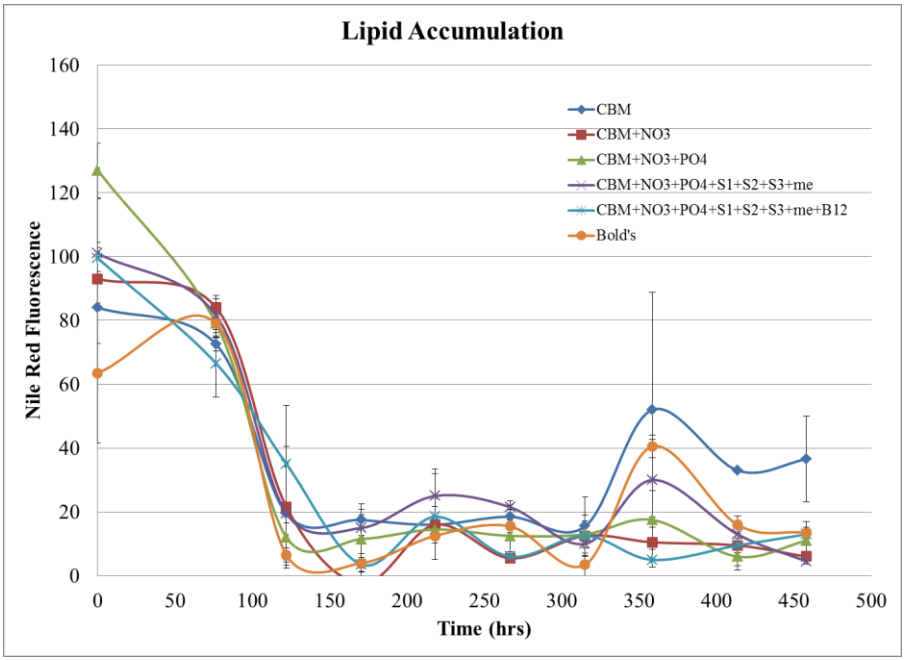


Figure A.6 Lipid accumulation of CBMW grown in non-sterile CBM water with and without added nutrients found in BBM. Error bars represent one standard deviation of technical duplicates.

From these results, it was determined that there could still be nutrients lacking in CBM water that is hindering the growth of CBMW. Calcium and boron were identified as nutrients that could be of interest.

Boron and Calcium Experiment with CBMW

This experiment was performed to identify if calcium and boron lacking in CBM water was hindering the growth of CBMW. Each condition was performed with one biological replicate and two technical replicates for pH, cell counts, chlorophyll, and Nile Red fluorescence. 5 mL of unwashed stock culture was added to each flask with an approximate cell count concentration of 4.4×10^6 cells/mL. CBM water was not sterilized. In figure legends: "all" represents 0.3 mM NO_3^-, 0.86 mM PO_4^{3-}, Solution 1, Solution 2,

Solution 3, microelement solution, and vitamin B12 solution used for Bold's; "B" represents 0.185 mM boron; "Ca" represents 0.17 mM Ca.

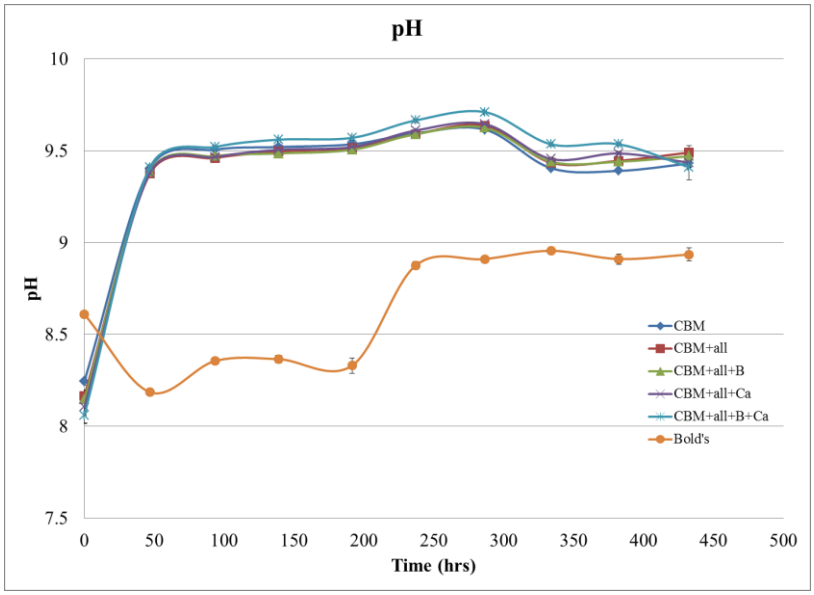


Figure A.7 pH of CBMW grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

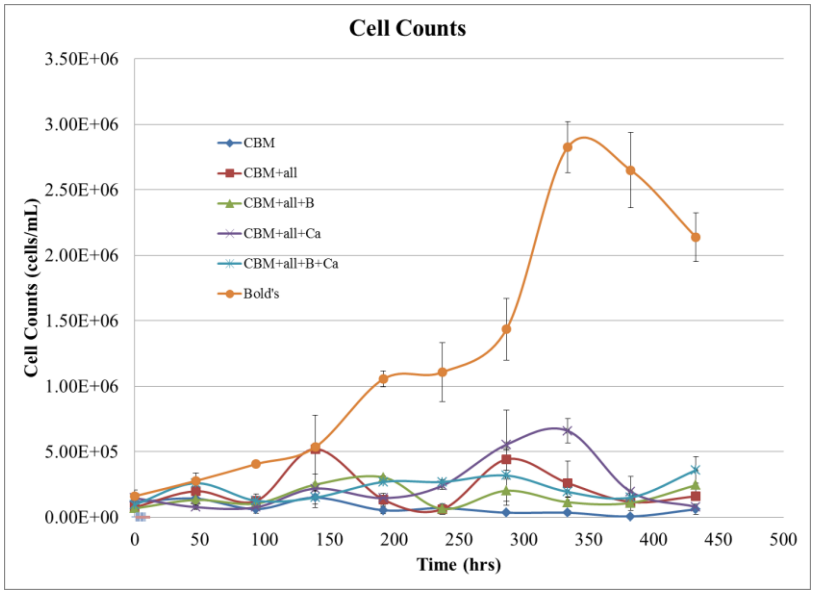


Figure A.8 Cell counts of CBMW grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

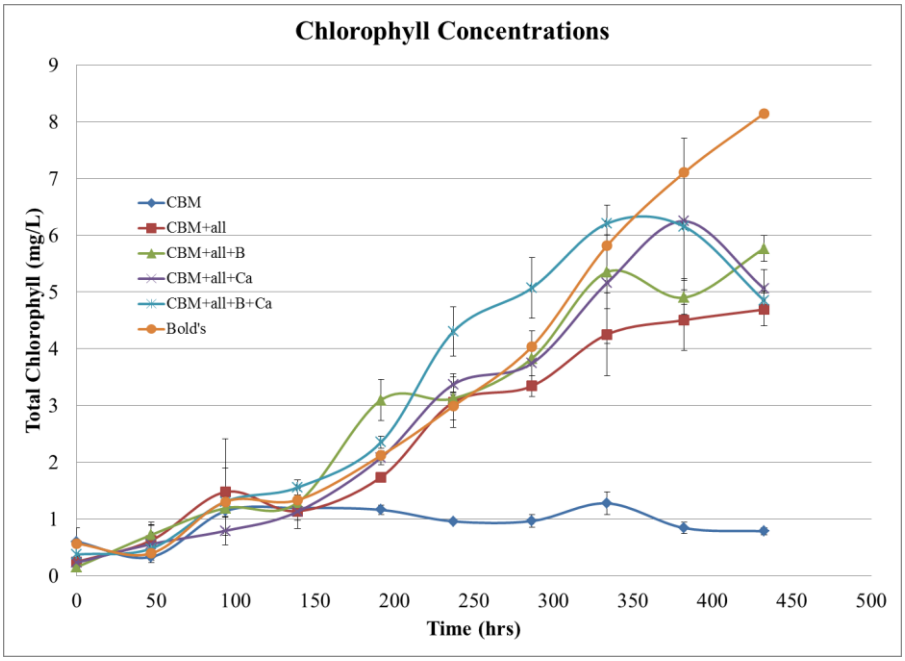


Figure A.9 Chlorophyll concentrations of CBMW when grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

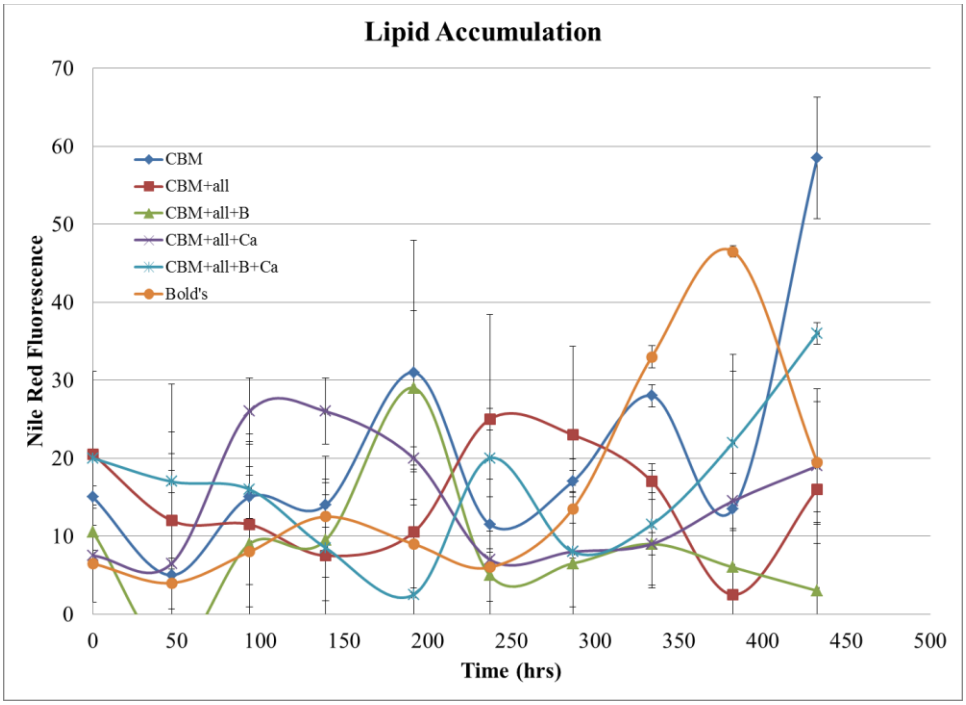


Figure A.10 Lipid accumulation of CBMW when grown in non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

From these results, it was determined that it was possible that comparable cell counts between CBM water and Bold's Basal Medium were not being observed due to the fact that the water was not sterile. Exploring the results of sterile vs. non-sterile CBM water was determined as the next variable of interest.

Filtered and Unfiltered Experiment

This experiment was performed to identify if sterile vs. non-sterile CBM water was hindering the cell count results of CBMW. Each condition was performed with one biological replicate and two technical replicates for pH, cell counts, chlorophyll, Nile Red fluorescence, and anion water chemistry. 5 mL of unwashed stock culture were added to each flask with an approximate cell count concentration of $4.13x10^6$ cells/mL. In figure legends: "all" represents 0.5 mM NO_3^-, 0.05 mM PO_4^{3-}, Solution 1, Solution 2, Solution 3, microelement solution, and vitamin B12 solution used for Bold's; "B" represents 0.185 mM boron; "Ca" represents 0.17 mM Ca; "(filtered)" represents a CBM water condition that was filter sterilized with syringe 0.2 μm filters; "Bold's (reduced)" represents Bold's Basal Medium with nitrate reduced from 2.94 mM to 0.5 mM and phosphate reduced from 1.7 mM to 0.05 mM.

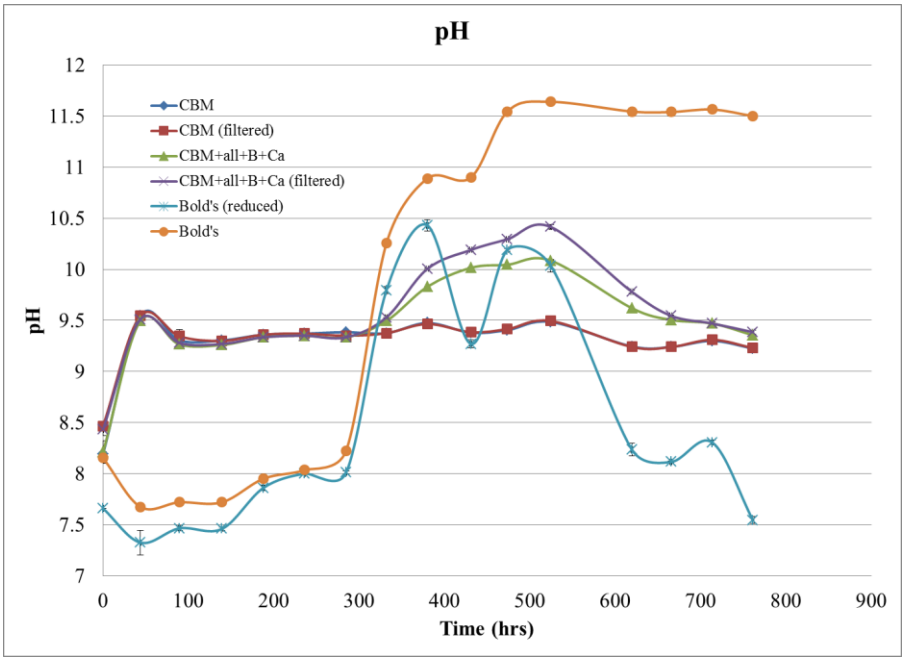


Figure A.11 pH values of CBMW grown in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

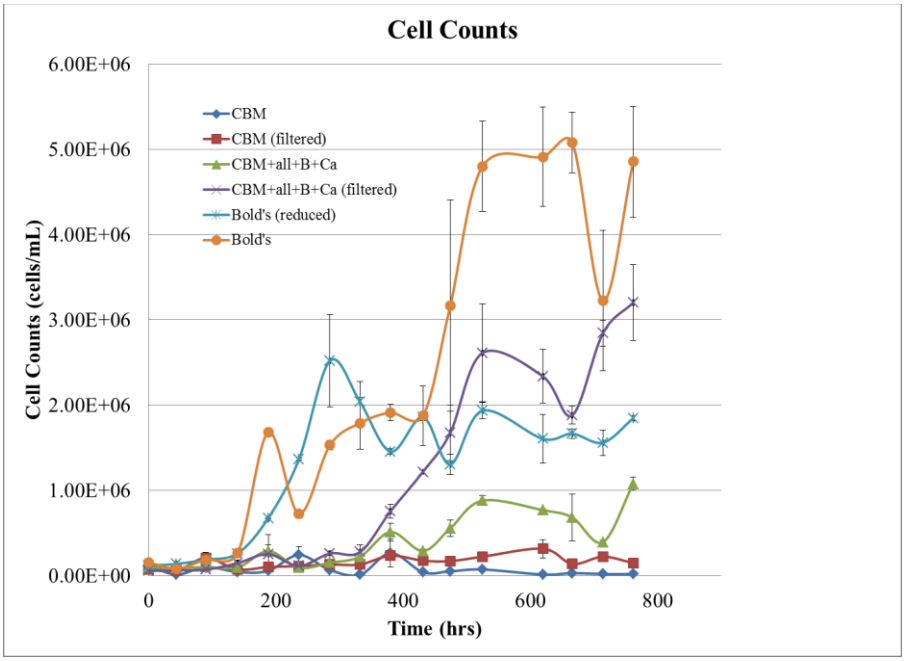


Figure A.12 Cell counts for CBMW grown in in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

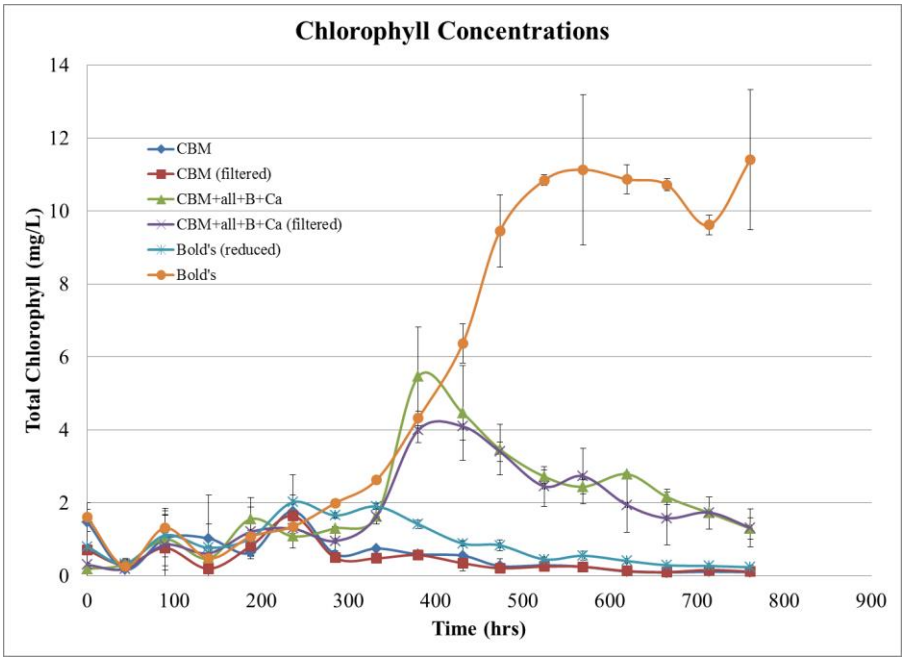


Figure A.13 Chlorophyll concentrations for CBMW when grown in in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

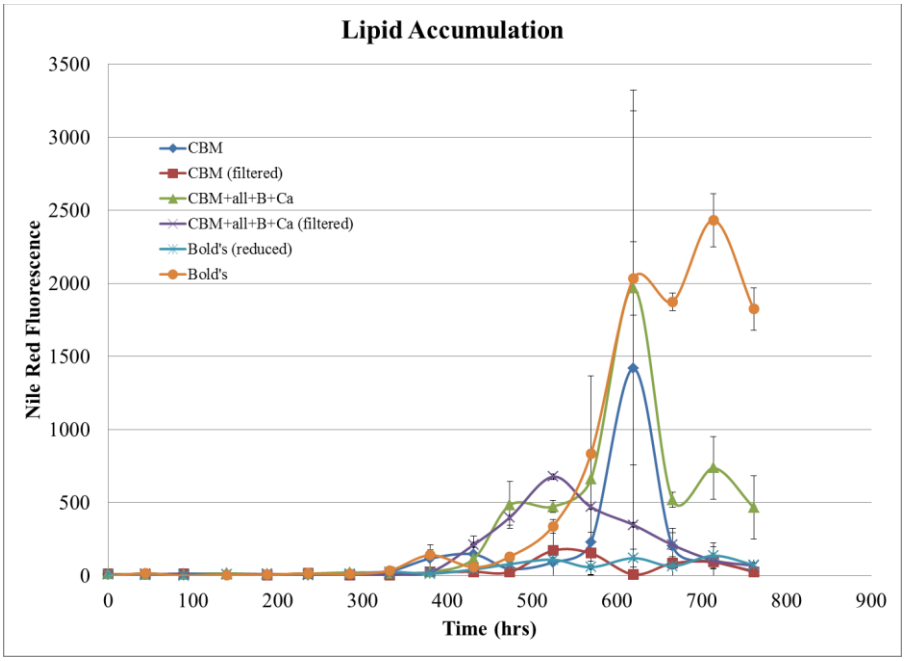


Figure A.14 Lipid accumulation of CBMW when grown in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

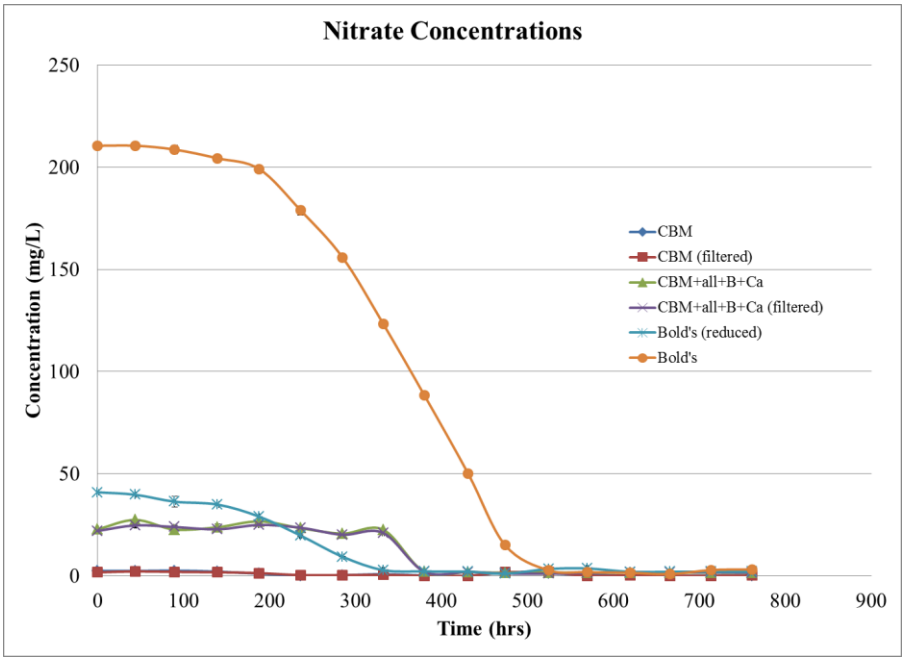


Figure A.15 Nitrate concentrations of cultures grown with CBMW in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

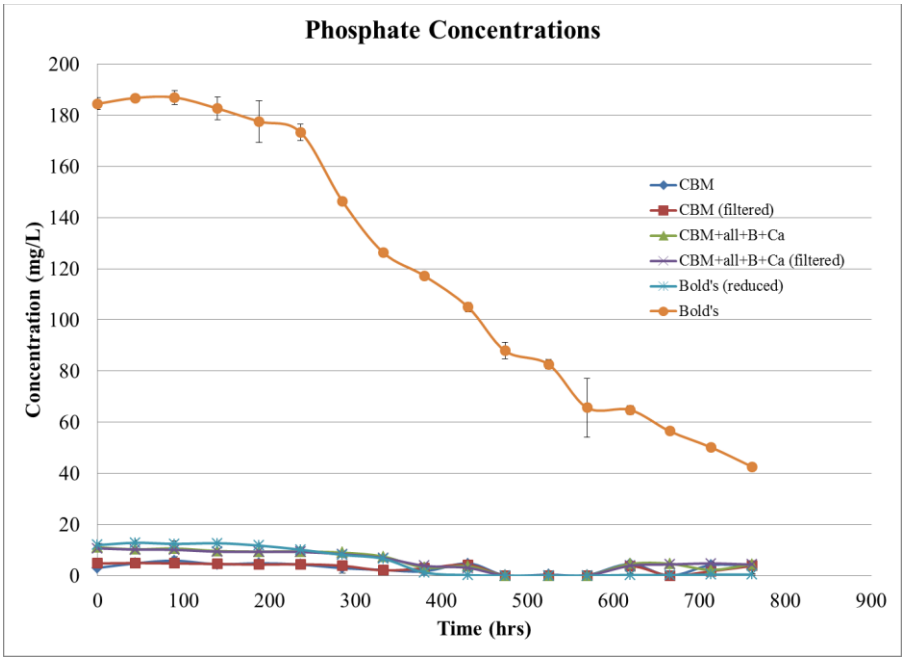


Figure A.16 Phosphate concentrations of cultures grown with CBMW in sterile and non-sterile CBM water with and without added nutrients, calcium, and boron found in BBM. Error bars represent one standard deviation of technical duplicates.

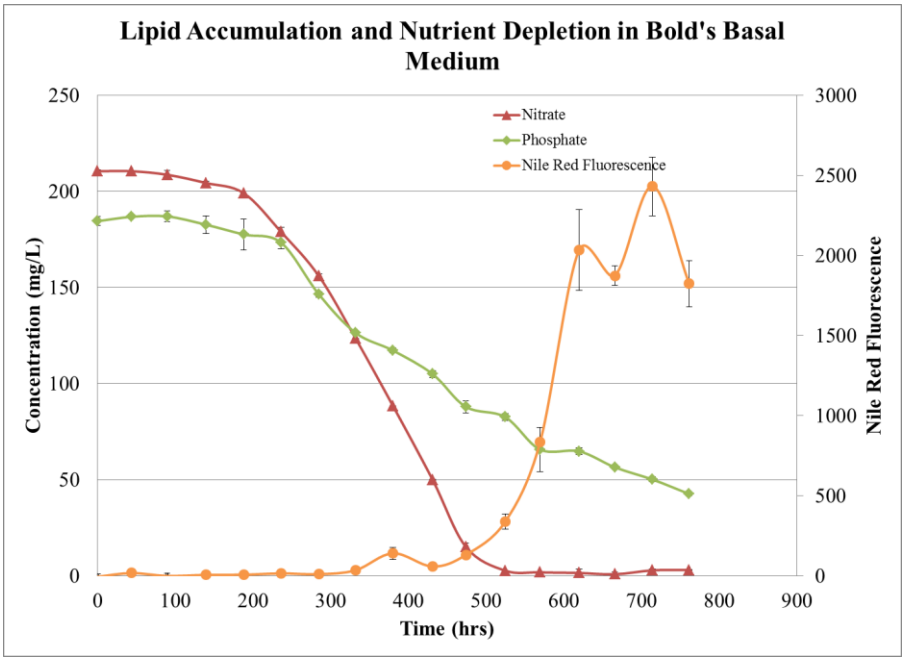


Figure A.17 Nutrient depletion and Nile Red fluorescence for CBMW grown in Bold's Basal Medium (Filtered and Unfiltered Experiment).

The results from this experiment indicate that the difference in observed cell counts was due to the CBM water not being sterile. Nile Red fluorescence was observed in this experiment. The only condition without significant standard deviation was Bold's Basal Medium with 2.94 mM nitrate and 1.7 mM phosphate. This experiment was maintained longer than previous experiments, likely accounting for the lack of lipid accumulation in previous experiments.

Clumping Behavior of CBMW



Figure A.18 CBMW when transferred from Bold's Basal Medium to non-sterile CBM water. Clumping occurs approximately 10-15 minutes after inoculation with slight shaking.

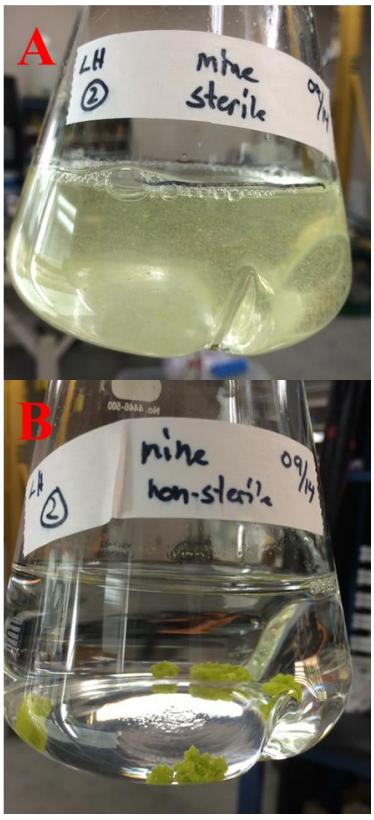


Figure A.19 (**A)** Comparison of CBMW when inoculated into filter sterilized CBM water and (**B**) non-sterile CBM water.

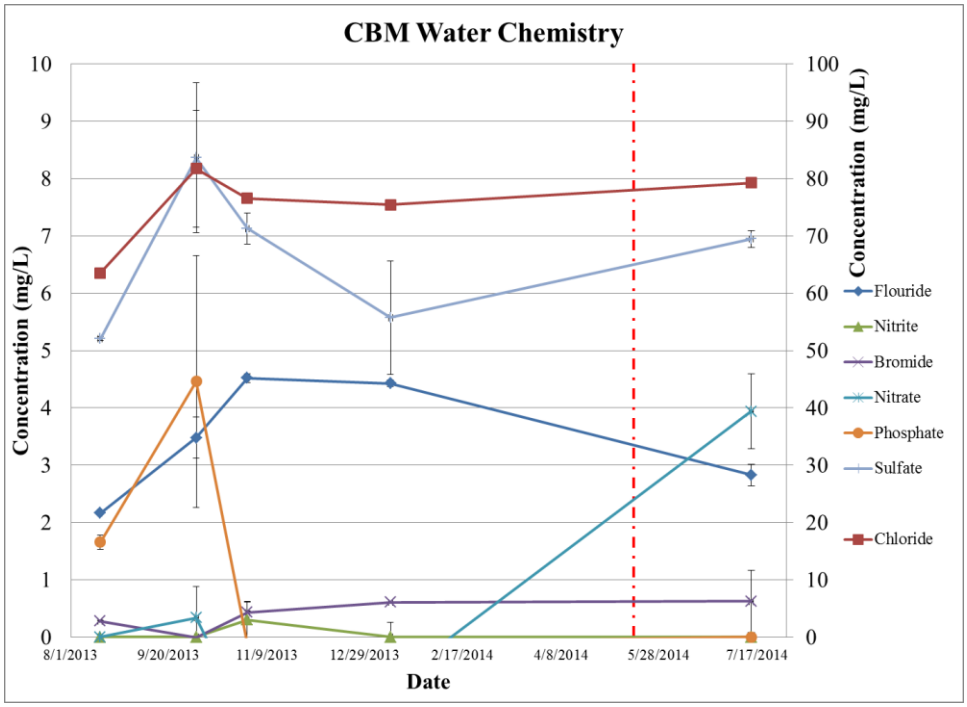


Figure A.20 Water chemistry of CBM water over approximately one year. Water from the same container was used for all experiments. CBM water was stored in a 5°C, dark, walk-in refrigerator. Red dotted line indicates the time when the clumping behavioral change occurred. The second time point (10/04/2013) had to be diluted (5x) due to volume concerns. The dilution pushed many of the concentrations below the accurate range of the IC machine, possibly accounting for the increase observed in some of the concentrations. Chloride concentrations are on the secondary axis (on the right side of the graph). All other concentrations are on the primary axis (left side of the graph).

APPENDIX B

SUPPORTING MATERIAL FOR CBMW ISOLATE



Figure B.1 Coal bed methane production pond where CBMW was isolated from. The isolate was taken on October 18, 2010 from the CBM water inflow (written communication with Elliott Barnhart). Image taken from Google Earth.

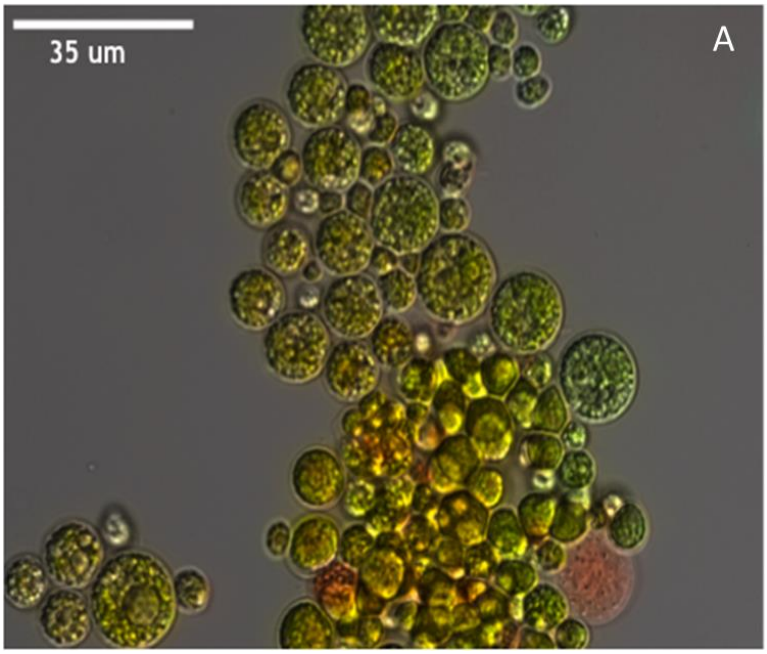


Figure B.2 CBMW under a brightfield microscope while in 2 mM nitrate Bold's Basal Medium and cultured at 25°C. Cells were stained with a cell mask orange (CMO) stain. CMO appears green while autofluorescence of chlorophyll appears red. Photo taken in the Microscope Facility at the Center for Biofilm Engineering, Montana State University and provided courtesy of Luisa Corredor.

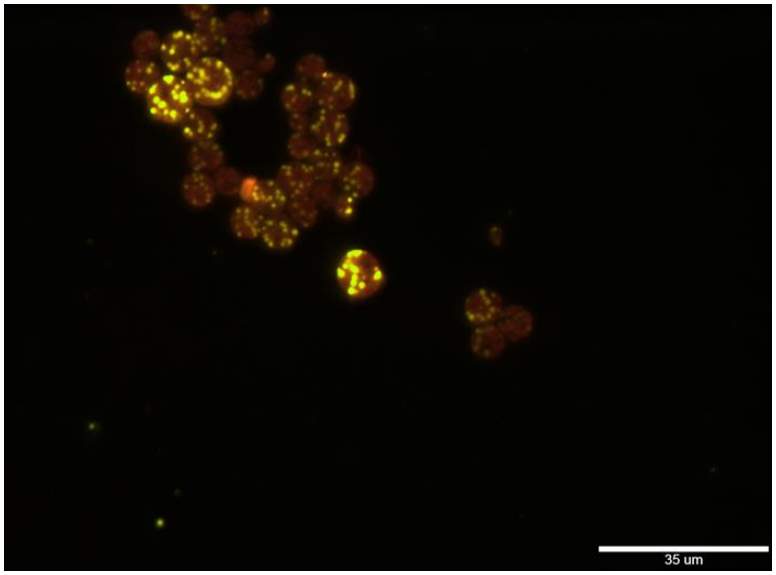


Figure B.3 Nile Red fluorescence (yellow) of lipid droplets and autofluorescence of chlorophyll (red) in CBMW cultured in 0.5 mM nitrate Bold's Basal Medium at 25°C. Photo taken in the Microscope Facility at the Center for Biofilm Engineering, Montana State University and provided courtesy of Luisa Corredor.