BIOFUEL PRODUCTION BY TWO GREEN MICROALGAE UTILIZING WASTEWATER AND RECYCLED NUTRIENTS FOR GROWTH

by

Luke Daniel Halverson

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

of

Master of Science

in

Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 2014

©COPYRIGHT

by

Luke Daniel Halverson

2014

All Rights Reserved

DEDICATION

I dedicate this thesis to my parents, Dan and Genette, who have always put their children first and have given me the support, encouragement and love needed for me to be where I am today.

ACKNOWLEDGMENTS

Special thanks to Dr. Egan Lohman and Dr. Rob Gardner who introduced me to algal research and continually provided me with guidance and support.

Special thanks to my advisor Dr. Robin Gerlach who provided me with the opportunity to complete this master's degree and has been an invaluable resource during my time as a graduate student.

Thanks my other committee members, Dr. Brent Peyton and Dr. Matthew Fields, for giving me many insights and ideas throughout my graduate research.

Thanks to my undergraduate assistants, especially Todd Pederson and Ashley Berninghaus, for their dedication to lab work and willingness to help.

Thanks to the MSU Algal Research Group for providing me with feedback and support towards my research.

Thanks to the Center for Biofilm Engineering for instrument support.

TABLE OF CONTENTS

1. INTRODUCTION 1

Background 1
Wastewater as a Low-Quality Source of Nitrogen and Phosphorus 1
Carbon Requirements Associated with Algal Growth 3
Integration of Recycle Streams 4
Project Rationale 6
Aims 7

2. METHODS 9

Strains and Culturing Conditions 9
Wastewater Collection 10
Culture Analysis 10
Analysis of Media Components 11
Chlorophyll Measurements 11
Harvesting 11
Lipid Extraction 12
Transesterification for FAME Analysis 13
Lipid Analysis Using GC-FID 13
FAME Analysis using GC-MS 14

3. INITIAL GROWTH STUDIES USING STRAINS WC-1 AND SLA-04 IN MUNICIPAL WASTEWATERS 16

Introduction 16
WC-1 17
SLA-04 23
Generational Variance 27
Conclusions 27

4. GROWTH, NUTRIENT UTILIZATION AND BIOFUEL POTENTIAL OF WC-1 AND SLA-04 GROWN IN WASTEWATER UNDER SEVERAL INORGANIC CARBON REGIMES 29

Introduction 29
Growth of WC-1 and SLA-04 in Primary Effluent 29
Nitrogen and Phosphorus Uptake by WC-1 and SLA-04 34
Lipid Productivity and Biodiesel Potential of WC-1 and SLA-04 36

TABLE OF CONTENTS-CONTINUED

Conclusions 41

5. GROWTH OF WC-1 AND SLA-04 IN RECYCLED HARVEST WATER SUPPLEMENTED WITH ANAEROBIC DIGESTATE 42

Introduction 42
Methods 42
WC-1 Growth in Recycled Harvest Water 44
2nd and 3rd Generation Lipid Content of WC-1 52
SLA-04 Growth in Recycled Harvest Water 54
Conclusions 57

6. CONCLUSIONS 58

Project Rationale 58
Attainment of Project Aims 59
(i) Identification of Robust Microalgae 59
(ii) Growth on Varying Inorganic Carbon Sources 60
(iii) Nutrient and Water Recycling 61
Future Work 62

REFERENCES 63

APPENDICES 71

APPENDIX A: Co-Author Publication Abstracts 72
APPENDIX B: Tabulated Data 76
APPENDIX C: Organism Selection 102
APPENDIX D: Coal Bed Methane Water Study 107
APPENDIX E: Bold's Basal Medium 117

LIST OF TABLES

Table Page

1. Components and concentrations of the microelement solution added to 2nd generation cultures of WC-1 grown in anaerobic digestate diluted 1:25 (AD4). 19

2. Names and descriptions of each condition used for growth of WC-1 and SLA-04. The "Gas Sparge" column is for lighted hours only. Ambient air was sparged during the dark hours for each condition. 30

3. Maximum specific growth rate, doubling time, and final cell dry weights for WC-1 and SLA-04 32

4. Ammonium, nitrate, and phosphate uptake by WC-1 and SLA-04. Average concentrations in mg/L (±s.d.) are given for primary clarifier effluent prior to inoculation and when the cultures were harvested 8 days later. 35

5. The average number of cell doublings in each generation of WC-1 growth in recycled harvest water. Cell doublings=$\log(C_f/C_i)/\log(2)$ where C_f is the cell concentration at harvest and C_i is the initial cell concentration. 1st generation values are the averages of triplicate treatments ± standard deviation, and 2nd and 3rd generation values are the averages of duplicate treatments ± range. 47

6. Average number of cell doublings (± standard deviation, n=3 (1st generation), ± range, n=2 (2nd and 3rd generation)) for each condition in 1st, 2nd and 3rd generation growth of SLA-04. 55

B.1. Average Cell Concentrations for 1st Generation WC-1 Growth 77

B.2. Standard Deviation of Cell Concentrations for 1st Generation WC-1 Growth 77

B.3. Average pH for 1st Generation WC-1 Growth 77

B.4. Standard Deviation of pH for 1st Generation WC-1 Growth 78

B.5. Average Cell Concentrations for 2nd Generation WC-1 Growth 78

B.6. Standard Deviation of Cell Concentrations for 2nd Generation WC-1 Growth 78

LIST OF TABLES - CONTINUED

Table Page

B.7. Cell Density of 2nd Generation AD4 Cultures Given a Microelement and Iron Solution 79

B.8. Cell Density of 3rd Generation WC-1 Cultures Grown in 1:25 Diluted Anaerobic Digestate with Iron Added at Inoculation or at Seven Days into Growth 79

B.9. Average Cell Concentrations for 1st Generation SLA-04 Growth 80

B.10. Standard Deviation of Cell Concentrations for 1st Generation SLA-04 Growth 80

B.11. Average pH for 1st Generation SLA-04 Growth 80

B.12. Standard Deviation of pH for 1st Generation SLA-04 Growth 80

B.13. Average Cell Concentrations for 2nd Generation SLA-04 Growth 81

B.14. Standard Deviation of Cell Concentrations for 2nd Generation SLA-04 Growth 81

B.15. Cell Concentration of 2nd Generation SLA-04 Cultures Given an Iron Addition at Day 16 82

B.16. Cell Concentration of 2nd Generation SLA-04 Cultures Given a Nitrogen Addition at Day 16 82

B.17. Average Cell Concentrations for Three Generations of WC-1 Growth in Primary Clarifier Effluent 83

B.18. Standard Deviation of Cell Concentrations for Three Generations of WC-1 Growth in Primary Clarifier Effluent 83

B.19. Average Cell Concentrations for Two Generations of SLA-04 Growth in Primary Clarifier Effluent 84

B.20. Standard Deviation of Cell Concentrations for Two Generations of SLA-04 Growth in Primary Clarifier Effluent 84

B.21. Average Cell Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime 85

LIST OF TABLES - CONTINUED

Table Page

B.22. Standard Deviation of Cell Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime 85

B.23. Average pH for WC-1 Grown in PC under Each Inorganic Carbon Regime 85

B.24. Standard Deviation of pH for WC-1 Grown in PC under Each Inorganic Carbon Regime 86

B.25. Average Chlorophyll a + b Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime 86

B.26. Standard Deviation of Chlorophyll a + b Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime 86

B.27. Average Cell Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime 86

B.28. Standard Deviation of Cell Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime 87

B.29. Average pH for SLA-04 Grown in PC under Each Inorganic Carbon Regime 87

B.30. Standard Deviation of pH for SLA-04 Grown in PC under Each Inorganic Carbon Regime 87

B.31. Average Chlorophyll a+b Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime 88

B.32. Standard Deviation of Chlorophyll a+b Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime 88

B.33. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for WC-1 88

B.34. Standard Deviation of FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for WC-1 89

B.35. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for WC-1 89

LIST OF TABLES - CONTINUED

Table Page

B.36. Standard Deviation FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for WC-1 89

B.37. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for SLA-04 90

B.38. Standard Deviation FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for SLA-04....90

B.39. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for SLA-04....90

B.40. Standard Deviation of FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for SLA-04 91

B.41. Average Cell Concentrations for 1st Generation Growth of WC-1 in Recycled Harvest Water 91

B.42. Standard Deviation of Cell Concentrations for 1st Generation Growth of WC-1 in Recycled Harvest Water....91

B.43. Average pH for 1st Generation Growth of WC-1 in Recycled Harvest Water 92

B.44. Standard Deviation of pH for 1st Generation Growth of WC-1 in Recycled Harvest Water 92

B.45. Average Cell Concentrations for 2nd Generation Growth of WC-1 in Recycled Harvest Water 92

B.46. Range of Cell Concentrations for 2nd Generation Growth of WC-1 in Recycled Harvest Water 92

B.47. Average pH for 2nd Generation Growth of WC-1 in Recycled Harvest Water 92

B.48. Range of pH for 2nd Generation Growth of WC-1 in Recycled Harvest Water 93

B.49. Average Cell Concentrations for 3rd Generation Growth of WC-1 in Recycled Harvest Water 93

LIST OF TABLES - CONTINUED

Table Page

B.50. Range of Cell Concentrations for 3rd Generation Growth of WC-1 in Recycled Harvest Water 93

B.51. Average pH for 3rd Generation Growth of WC-1 in Recycled Harvest Water 93

B.52. Range of pH for 3rd Generation Growth of WC-1 in Recycled Harvest Water 94

B.53. Average Ammonium Nitrogen Concentration in 3rd Generation WC-1 Recycled Harvest Water Experiment 94

B.54. Range of Ammonium Nitrogen Concentration in 3rd Generation WC-1 Recycled Harvest Water Experiment 94

B.55. Average Growth Rates of WC-1 in 2nd and 3rd Generation Recycled Harvest Water Experiment 95

B.56. Range of Growth Rates of WC-1 in 2nd and 3rd Generation Recycled Harvest Water Experiment 95

B.57. Average Chlorophyll a+b Concentrations for WC-1 Grown in 3rd Generation Recycled Harvest Water 95

B.58. Range of Chlorophyll a+b Concentrations for WC-1 Grown in 3rd Generation Recycled Harvest Water 95

B.59. Average medium pH and Nitrogen Concentration in RW3+AD+PC+Fe Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1 96

B.60. Range of medium pH and Nitrogen Concentration in RW3+AD+PC+Fe Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1 96

B.61. Average medium pH and Nitrogen Concentration in RW3+AD+Tap Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1 96

LIST OF TABLES - CONTINUED

Table Page

B.62. Range of medium pH and Nitrogen Concentration in RW3+AD+Tap Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1 97

B.63. Average Cell Dry Weight of WC-1 Cultures during 2nd and 3rd Generation Recycled Harvest Water Experiment 97

B.64. Range of Cell Dry Weight of WC-1 Cultures during 2nd and 3rd Generation Recycled Harvest Water Experiment 97

B.65. Average Lipid content (% w/w) of WC-1 Cultures during 2nd Generation Recycled Harvest Water Experiment 97

B.66. Range of Lipid content (% w/w) of WC-1 Cultures during 2nd Generation Recycled Harvest Water Experiment 98

B.67. Average Lipid content (% w/w) of WC-1 Cultures during 3rd Generation Recycled Harvest Water Experiment 98

B.68. Range of Lipid content (% w/w) of WC-1 Cultures during 3rd Generation Recycled Harvest Water Experiment 98

B.69. Average Cell Concentrations for 1st Generation Growth of SLA-04 in Recycled Harvest Water 98

B.70. Standard Deviation of Cell Concentrations for 1st Generation Growth of SLA-04in Recycled Harvest Water 99

B.71. Average pH for 1st Generation Growth of SLA-04 in Recycled Harvest Water 99

B.72. Standard Deviation of pH for 1st Generation Growth of SLA-04 in Recycled Harvest Water 99

B.73. Average Cell Concentrations for 2nd Generation Growth of SLA-04 in Recycled Harvest Water 99

B.74. Range of Cell Concentrations for 2nd Generation Growth of SLA-04in Recycled Harvest Water 100

LIST OF TABLES - CONTINUED

Table Page

B.72. Average pH for 2nd Generation Growth of SLA-04 in Recycled Harvest Water 100

B.73. Range of pH for 2nd Generation Growth of SLA-04 in Recycled Harvest Water 100

B.74. Average Cell Concentrations for 3rd Generation Growth of SLA-04 in Recycled Harvest Water 100

B.75. Range of Cell Concentrations for 3rd Generation Growth of SLA-04in Recycled Harvest Water 101

B.76. Average pH for 3rd Generation Growth of SLA-04 in Recycled Harvest Water 101

B.77. Range of pH for 3rd Generation Growth of SLA-04 in Recycled Harvest Water 101

D.1. Chemical constituency data for CBM water and Bolds basal medium. Specific ion concentrations are presented for both, and the percent difference is calculated. Positive percent differences show produced water has a higher concentration of an ion in the standard Bolds medium, and a negative value means the ion needs to be supplemented. For the most part, the produced water was deficient of most compounds. 110

D.2. Each transfer of the growth matrix is detailed below. The medium began with pure coal bed methane water, and the components of Bolds medium were added one-by-one, pulling out 100mL between additions. The medium was titrated to a pH close to 8.7 for each transfer starting with the second one. 112

LIST OF FIGURES

Figure Page

1. Picture of WC-1 shaker flask growth in Bold's basal medium with the pH adjusted to 8.7 (B8.7), undiluted anaerobic digestate (AD1), 1:5 anaerobic digestate dilution (AD2), 1:10 anaerobic digestate dilution (AD3), 1:25 anaerobic digestate dilution (AD4), 1:50 anaerobic digestate dilution (AD5) and primary clarifier effluent (PC). 17

2. Average cell density (microscopic direct counts) (top) and pH (bottom) with standard deviations for triplicate reactors of first generation growth of WC-1 in Bold's basal medium (B8.7), the three dilutions of anaerobic digestate that supported growth (AD3, AD4 and AD5) and primary clarifier effluent (PC). 18

3. Average cell concentration with standard deviation for triplicate reactors of second generation WC-1 growth in Bold's 8.7 (B8.7), each anaerobic digestate dilution (AD3, AD4 and AD5) and primary clarifier effluent (PC). 20

4. Cell density of 2nd generation WC-1 cultures grown in 1:25 AD supplemented with a microelement solution, an iron solution and both, microelement and iron solutions together. The dotted line at day 13 denotes when both solutions were added to the culture and the dashed line at day 15 denotes when the solutions were added separately to different treatments. One mL of the microelement solution described in Table 1 was added per liter of culture and the iron solution was added to achieve a concentration of 1 mg Fe^{2+}/L in the culture. The values shown for each condition in Figure 4 are for one flask (n=1). 21

5. Cell density of 3rd generation WC-1 grown in 1:25 AD supplemented with iron at different times throughout growth. The dashed line denotes when iron was added to a culture at day 7. 22

6. Average cell density (top) and pH (bottom) with standard deviation of triplicate 1st generation SLA-04 cultures grown in Bold's 8.7 (B8.7), 1:25 dilution of anaerobic digestate (AD4) and primary clarifier effluent (PC). 24

LIST OF FIGURES - CONTINUED

Figure Page

7. Average cell density (top) and pH (bottom) with standard deviation of triplicate 2nd generation SLA-04 cultures grown in Bold's 8.7 (B8.7), 1:25 dilution of anaerobic digestate (AD4) and primary clarifier effluent (PC). 25

8. Second generation growth of SLA-04 in Bold's 8.7 (B8.7), 1:25 diluted anaerobic digestate (AD), and primary clarifier effluent (PC). AD and PC cultures were supplemented with iron (top) and ammonium chloride (bottom) at day 16. 26

9. Generational comparison of cell density for WC-1 (top) and SLA-04 (bottom) grown in untreated primary clarifier effluent without any external additions. 28

10. Growth, pH, and chlorophyll a+b concentrations for WC-1 (left) and SLA-04 (right). Dashed line indicates the time of 50 mM HCO_3^- addition for treatments labeled "+ HCO3". (a) and (b) show the cell densities achieved by WC-1 and SLA-04, respectively. (c) and (d) show the medium pH for WC-1 and SLA-04, respectively. In pH controlled conditions, the pH was maintained at 8.1-8.5 until the bicarbonate addition. At which time, the cultures amended with 50 mM HCO_3^- had the pH controlled to 10.1-10.5, and cultures without added bicarbonate were controlled at 8.1-8.5. (e) and (f) show the average chlorophyll a + b concentrations for WC-1 and SLA-04, respectively. 33

11. Lipid and FAME content, in %w/w (top) and concentration (bottom), of WC-1. 39

12. Lipid and FAME content, in %w/w (top) and concentration (bottom), of SLA-04. 40

13. Schematic of recycled harvest water studies. 44

14. Average cell density and pH values of triplicate treatments during 1st generation (top row) growth (±standard deviation) and duplicate treatments during 2nd (middle row) and 3rd (bottom row) generation growth (± range) for WC-1. 46

LIST OF FIGURES - CONTINUED

Figure Page

15. Nitrogen (measured as ammonia) utilization by WC-1 over time during the 3rd generation experiment. Initial nitrogen concentrations were roughly twice as high as the intended initial nitrogen concentration of 41 mg N/L likely due to an error in measuring the nitrogen content of the collected anaerobic digestate. 47

16. Average growth rate of WC-1 for 2nd and 3rd generation growth experiments. Growth rates were calculated between day 2 and day 4 for each treatment during the respective generation of growth. 48

17. Chlorophyll a and b concentrations of WC-1 for the 3rd generation growth experiment. The chlorophyll content of anaerobic digestate cultures with added iron reached a much higher concentration than AD cultures without added iron. 49

18. Biomass yields (g biomass/g nitrogen) of AD-amended WC-1 cultures at the time of harvest for 2nd and 3rd generation growth. 3rd generation yields experienced a significant decrease likely due to ammonia volatilization. 50

19. Cell dry weight values of WC-1 at the time of harvest for 2nd and 3rd generation growth. 3rd generation cell dry weights generally decreased for anaerobic digestate cultures with the exception of RW+AD+PC+Fe. 52

20. pH (primary vertical axis) and nitrogen concentration (secondary vertical axis) over time for RW3+AD+PC+Fe (top) and RW3+AD+Tap (bottom). The pH of the iron-amended condition (top) increased above the pKa of ammonium/ammonia (pKa=9.25), and nitrogen was depleted by day 6. The pH of the condition without iron (bottom) remained below 9.25 for the majority of the experiment, and nitrogen was present at the time of harvest (day 10). 51

LIST OF FIGURES - CONTINUED

Figure Page

21. Extractable lipid content (% w/w) of WC-1 at the time of harvest of the 2nd generation growth experiment (t=8 days). Graph shows free fatty acids (FFA), monoacylglycerides (MAG), diacylglycerides (DAG), triacylglycerides (TAG) and total extractable lipid (TOTAL) contents of each condition. The lipid profile of WC-1 grown in freshly collected primary clarifier effluent is also shown. Recycled harvest water conditions were harvested just after nitrogen depletion, and the "Fresh PC Effluent" cultures were harvested roughly 5 days after nitrogen depletion. 53

22. Extractable lipid content (% w/w) of WC-1 at the harvest of the 3rd generation growth experiment (t=10 days). Graph shows free fatty acids (FFA), monoacylglycerides (MAG), diacylglycerides (DAG), triacylglycerides (TAG) and total extractable lipid (TOTAL) contents of each condition. 54

23. Average cell density and pH values of triplicate treatments during 1st generation growth (±standard deviation) and duplicate treatments during 2nd and 3rd generation growth (± range) for SLA-04. 56

C.1. Transmitted light micrograph (left) and Nile Red fluorescence image (right) of Scenedesmus sp. WC-1. 103

C.2. Average growth curve for WC-1 grown in triplicate in Bold's 8.7 basal medium on 5% CO_2. 104

C.3. Transmitted light micrograph (left) and Nile Red fluorescence image (right) of *Chlorella* sp. SLA-04. 104

C.4. Average growth curve for SLA-04 grown in triplicate in Bold's 8.7 basal medium on 5% CO2. 105

C.3. Transmitted light micrograph (left) and Nile Red fluorescence image (right) of *Navicula* sp. RGd-1. 106

D.1. The growth chart showing how each of the nine cultures grew over a two week span. 113

D.2. The Nile Red Fluorescence values taken from Day 2 to Day 15. 114

ABSTRACT

Microalgae have received considerable attention in recent years as a viable feedstock for biofuel production. In order for algal biofuel to displace a significant amount of traditional fossil-based fuel, non-potable water must be used to avoid additional stress on dwindling freshwater supplies. Additionally, adequate nutrient sources must be available, and productive, robust strains need to be utilized. The work performed towards this thesis aims to determine the ability of two green microalgae and one diatom to grow and produce biofuel and biofuel precursors in untreated wastewater. *Scenedesmus* sp. strain WC-1 (WC-1), *Chlorella* sp. strain SLA-04 (SLA-04) and *Navicula* sp. strain RGd-1 (RGd-1) were initially screened for growth in untreated primary clarifier effluent and diluted anaerobic digestate. WC-1 and SLA-04 were able to grow in each condition, but RGd-1 was unable to sustain growth. After WC-1 and SLA-04 displayed successful growth, experiments were performed attempting to increase the lipid content in WC-1 and SLA-04 by varying the form and concentration of dissolved inorganic carbon present in the cultures. The addition of supplementary inorganic carbon did not increase cellular triacylglyceride (TAG) content as expected, but WC-1 and SLA-04 were able to achieve considerable fatty acid methyl ester (FAME) content. The final experiments conducted toward this thesis involved the use of recycled harvest water amended with anaerobic digestate for nutrients. WC-1 grew without inhibition during the first two generations of growth in recycled harvest water, but high ammonium concentrations due to an error during the addition of anaerobic digestate in the third generation caused reduced growth rates. SLA-04 was able to grow without inhibition during each of the three generations of growth in recycled harvest water. The results of this work may strengthen the outlook of microalgae's potential as a biofuel feedstock. Identifying robust algae that can utilize low-cost nutrients while requiring minimal supplies of freshwater is a large step towards the commercialization of algal biofuels.

CHAPTER 1

INTRODUCTION

Background

As traditional fossil fuels become scarcer and supplies become more unstable, an alternative to crude oil-based fuels must be found. Large increases of greenhouse gases in the atmosphere and energy shortage concerns have led to research in biofuel as a replacement energy source (Dismukes et al. 2008; Greenwell et al. 2010; Groom et al. 2008; Hill et al. 2006; Sheehan et al. 1998). Corn, soybean, and sugar cane have been the predominant feedstocks for the production of bioethanol and biodiesel. Although biofuel is able to be produced from these crops, they compete for arable land used for the production of food (DOE 2010). Microalgae are considered a viable feedstock for biofuel due to their high biomass productivity, high lipid yield, and ability to grow on non-arable land (Chisti 2007; Hu et al. 2008). Microalgae have been studied for their biofuel potential since the 1940's, but many hurdles need to be overcome to ensure commercial viability. Technical constraints include water availability, nutrient limitation, carbon sources and culture resistance to inhibitory predators (Davis et al. 2011; DOE 2010; Pate et al. 2011; Singh and Olsen 2011).

Wastewater as a Low-Quality Source of Nitrogen and Phosphorus

Freshwater availability is becoming more and more of a concern as populations increase worldwide. Current studies estimate that 1.5 billion people currently do not

have access to freshwater, and nearly half of the world's population will live in water stressed regions by 2030 (Amarasinghe et al. 2007; Bhatnagar et al. 2010). For large scale microalgae cultivation to be possible, alternatives to freshwater must be used to support algal growth. Various sources of wastewater, saline water, and produced water have been used to cultivate microalgae while trying to avoid putting additional strains on freshwater supply.

If biofuel from microalgae is going to be used to replace a significant amount of traditional fossil-based fuel, the nitrogen and phosphorus demand would significantly exceed current global production rates (Batan et al. 2010). Therefore, alternative nutrient sources must be utilized. It is known that certain strains of microalgae can grow on multiple forms of nitrogen such as nitrate, urea, and ammonium. Ammonium is considered to be the preferred form of nitrogen because no redox or hydrolysis reaction is needed during assimilation, unlike nitrate or urea, respectively (Bekheet and Syrett 1977; Cai et al. 2013; Leftley and Syrett 1973). Instead, ammonium is utilized directly through the GS/GOGAT cycle to be incorporated into amino acids (Fernández et al. 2004). It has been shown that microalgae generally prefer ammonium over nitrate when both are present in culture medium (Maestrini et al. 1986). Ammonium rich streams such as primary settled wastewater and anaerobic digestate have the potential to be used as alternative nitrogen feedstocks compared to conventional, energy-intensive ones (i.e. Haber-Bosch).

Phosphorus is also an essential nutrient necessary for algal growth. Phosphorus, generally in the form of $H_2PO_4^-$ or HPO_4^{2-}, is transported across the plasma membrane to

be incorporated into organic compounds, including energy metabolites (e.g. ATP), nucleic acids, lipids, and proteins (Cai et al. 2013; Martinez et al. 1999). Although orthophosphate is generally the preferential form of phosphorus utilized by microalgae, some species have been found to also use phosphorus-containing esters for growth (Kuenzler 1965). World mineral supplies of phosphorus are expected to be depleted in 50 to 100 years (Cordell et al. 2009), so the use of low-quality or recycled phosphorus is critical for large-scale algae production.

Carbon Requirements Associated with Algal Growth

Besides nitrogen and phosphorus, inorganic carbon is also a critical nutrient that must be supplemented economically. Algal cultivation models have traditionally focused on industrial carbon dioxide as the source of carbon for growth (Aresta et al. 2005; Collet et al. 2011). The concept of using industrial CO_2 may be an inefficient process that both produces a large greenhouse gas footprint due to the release of unabsorbed CO_2 and requires the installation of massive piping infrastructure (Moroney and Somanchi 1999; Pate et al. 2011). Bicarbonate has been suggested as a possible alternative to CO_2 sparging (Ip et al. 1982; Lohman et al. 2014). Bicarbonate addition to an algal culture would supply dissolved inorganic carbon needed for growth, and a higher concentration of bicarbonate addition near the end of the growth cycle has been shown to trigger an increase in neutral lipid content (Gardner et al. 2012). Studies have shown that the addition of bicarbonate just prior to medium nitrogen depletion can result in a large increase in triacylglyceride (TAG), a constituent of nonpolar lipid responsible for a large part of an alga's biodiesel potential. Furthermore, adding bicarbonate increases alkalinity

and medium pH. The use of alkaliphilic algae under these conditions is proposed to minimize contamination from invasive microorganisms that may greatly reduce the productivity of an algal culture (Richmond et al. 1982; Vijverberg et al. 1996).

Integration of Recycle Streams

After initially growing algae using wastewater, nutrients and water must be recycled in order to support subsequent algal growth. Acquiring the massive amount of nutrients to continually supply large-scale algae cultivation facilities has been an ongoing issue facing the commercialization of algal biofuels (Clarens et al. 2011; Liu et al. 2013). Nutrient recycling has been considered a necessity in implementing economically feasible microalgae cultivation facilities (Biller et al. 2012). Current attempts to recycle nutrients in pilot-scale facilities using hydrothermal liquefaction (HTL) have been reported at 12.5 ± 3% (Liu et al. 2013), and this inefficient reuse of nutrients could create a large cost towards production. The raffinate stream leaving an HTL unit has an unfavorable nitrogen to carbon ratio (N:C ratio of 1:2) for efficient anaerobic digestion (ideal N:C ratio of 1:25) (Frank et al. 2012; Fricke et al. 2007; Yu et al. 2011), so alternative methods of biomass processing may need to be examined. Anaerobic digestion has been the main focus for nutrient recycling (Sialve et al. 2009). The liquid portion of activated sludge, the anaerobic digestate, contains high levels of ammonium and phosphate that could be used as nutrients for algal growth (Wang et al. 2010).

In addition to nutrients, the recycling of water has also been a major concern in commercializing large-scale algal facilities. A recent report issued by the DOE (2010)

criticized the massive volumes of freshwater that would be needed for algal biofuel production. One possible way to reduce water demand is to reuse the water remaining after the algae have been removed, known as harvest water. Several studies have suggested that harvest water should be recycled for both water reuse and nutrient recovery (Chowdhury et al. 2012; Guieysse et al. 2013; Lam and Lee 2012; Resurreccion et al. 2012; Zhu and Ketola 2012). The water footprint associated with algal biofuels has been estimated to be approximately 4000 kg-water/kg-biodiesel when no harvest water is recycled (Yang et al. 2011). With a harvest water recycle, the water footprint can be reduced to 600 kg-water/kg-biodiesel, corresponding to an 84% decrease in freshwater usage (Yang et al. 2011). Furthermore, using wastewater while recycling 100% of available harvest water can reduce freshwater usage up to 90% and nitrogen use by 94% (Yang et al. 2011).

Although recycling harvest water can be beneficial, the reuse of water introduces several problems toward algae production. Recycling harvest water multiple times can cause algal cultures to become susceptible to contamination by fungi and bacteria (Lam and Lee 2012; Zhu and Ketola 2012). Growth can also be inhibited while using recycled harvest water due to the build-up of metabolites released during microalgae growth (Zhu et al. 2013). Yu et al. (2012) showed that microalgae continuously release extracellular organic matter, such as carbohydrates, amino acids, proteins, lipids and organic acids, into the medium. Soluble algal products (SAP) present in harvest water have been shown to inhibit growth in both *Scenedesmus* and *Chlorella* species (Lívanský et al. 1996; Pratt and Fong 1940).

Project Rationale

Increasing demand for alternatives to fossil fuels has led to extensive research in algae-derived biofuels. Microalgae have shown promise as a biofuel feedstock, but adequate resources such as water, nitrogen, phosphorus and carbon must be available without compromising supplies used for traditional crops. Wastewater containing nitrogen and phosphorus has been shown to sustain microalgal growth, but robust strains that can sustain productive growth must be identified. In order to outcompete other microorganisms found in wastewater, alkaliphilic strains may be able to be used in conditions unfavorable for competing organisms. Furthermore, various forms of carbon may be able to be used to provide the dissolved inorganic carbon needed to create alkaline conditions and provide carbon for algal growth. Gaseous carbon dioxide has traditionally been the inorganic carbon source of focus. Issues with collecting, concentrating and pumping carbon dioxide have been shown to be a major cost associated with algal growth (Lackner et al. 2012; Liu et al. 2013), so other forms of carbon, such as bicarbonate, have been proposed as alternative forms of carbon to supply algal cultures during growth and lipid accumulation stages (Gardner et al. 2012, 2013; Radmer and Ollinger 1980). Additionally, water and nutrient resources must be recycled to further optimize the process and avoid a potentially insurmountable demand of these already precious resources.

Additional research must be completed to fully realize microalgae's potential as a sustainable biofuel feedstock. Identifying robust, productive strains that can generate large yields of biofuel while utilizing low-quality nutrients is a vital step towards making

algae-derived biofuel a realistic possibility. Furthermore, the recycling of water and nutrients must be implemented to create a system that can produce algal biofuel while minimizing the consumption of resources used for traditional crops.

Aims

The purpose of this research is to evaluate the growth characteristics, nutrient utilization, lipid production and biofuel potential of two alkaliphilic green microalgae *Scenedesmus* sp. WC-1 and *Chlorella* sp. SLA-04 cultured in untreated municipal wastewater. A diatom, *Navicula* sp. RGd-1, was also screened for growth, but the organism was unable to successfully grow in the untreated wastewater. WC-1 and SLA-04 were also grown in recycled harvest water amended with anaerobic digestate to evaluate the feasibility of reusing water after growth while utilizing low-quality nutrients. Each strain was initially grown in primary clarifier effluent and in various dilutions of anaerobic digestate to determine whether growth was possible in untreated wastewater (Chapter 3). After successful growth was shown, WC-1 and SLA-04 were cultivated in primary effluent under four inorganic carbon regimes to evaluate the effect that the inorganic carbon species and concentration present in the growth medium had on specific cultures (Chapter 4). The four inorganic carbon regimes used were: (1) a continuous sparge with 5% CO_2 in air, (2) 5% CO_2 until nitrogen depletion when 50 mM HCO_3^- were supplemented and ambient air was sparged, (3) pH control using 5% CO_2, and (4) pH control using 5% CO_2 with 50 mM HCO_3^- addition near nitrogen depletion. Each condition received an ambient air sparge during the night hours. When pH control was used, the pH was controlled between 8.1 and 8.5 to maintain nearly all of the dissolved

inorganic carbon as bicarbonate prior to the 50 mM HCO_3^- addition. After the 50 mM bicarbonate addition, the pH was maintained between 10.1 and 10.5 in condition (4). The 50 mM bicarbonate addition near nitrogen depletion was performed in an attempt to increase lipid accumulation based on previous studies (Gardner et al. 2012). WC-1 and SLA-04 were also grown in sterile Bold's basal medium supplied with continuous 5% CO_2, with and without a 50 mM HCO_3^- addition at nitrogen depletion, to compare growth characteristics of wastewater cultures to those of controlled conditions. Chapter 4 has the potential to be converted into a peer-reviewed manuscript after further investigation into using bicarbonate to trigger lipid accumulation in wastewater cultures. The final experiments performed reused harvest water amended with anaerobic digestate as a nutrient source (Chapter 5). The harvest water was recycled multiple times to evaluate whether nutrient limitations would be observed and negatively affect growth and lipid production. Chapter 5 could also become a peer-reviewed manuscript after additional work has been performed to determine the least amount of make-up water needed to sustain optimal algal growth.

The overall goals of this thesis were the following:

(i) Identify robust microalgae that are capable of sustaining growth in untreated municipal wastewater.

(ii) Vary the species and concentration of the inorganic carbon supplied to algal cultures to optimize growth and lipid production.

(iii) Integrate recycle streams to decrease the water and nutrient demand required by prospective large-scale algal growth facilities.

CHAPTER 2

METHODS

Strains and Culturing Conditions

Scenedesmus sp. WC-1 (WC-1) was isolated from an alkaline (pH 8.7) stream in Yellowstone National Park, USA and confirmed unialgal as previously described (Gardner et al. 2010). *Chlorella* sp. SLA-04 (SLA-04) was isolated from Soap Lake in Washington State in the USA. Both strains were cultured using Bold's basal medium with the pH adjusted to 8.7 using KOH prior to autoclaving. Cultures were tested for contamination by inoculation into medium agar plates supplemented with 0.05% glucose and yeast extract followed by dark incubation for 20 days. Batch experiments for each organism were conducted in triplicate using 70 mm x 500 mm glass tubes containing 1.25 L medium submersed in a water bath to control temperature. Temperature was maintained at 24°C ± 1°C. Light (400 μmol photons m^{-2} s^{-1}) was maintained on a 14:10 light/dark cycle using a light bank containing T5 fluorescent bulbs. Aeration (400 mL min^{-1}) was supplied by humidified compressed air (supplemented with 5% CO_2 (v/v) for continuous CO_2 and pH controlled conditions). Gas flow was controlled using individual rotameters for each bioreactor. ACS grade sodium bicarbonate was used in all experiments involving a bicarbonate addition (Sigma-Aldrich, St. Louis, MO).

Wastewater Collection

Municipal wastewater was collected from the effluent of the primary clarifier at the Bozeman Water Reclamation Facility (BWRF) located in Bozeman, MT, USA. The BWRF treats roughly 6-8 MGD of municipal wastewater. Wastewater samples were collected in 20 L plastic containers and stored at 4°C until the time of experiment. No pretreatment was performed on the wastewater prior to experiments.

Culture Analysis

An optical hemacytometer and transmitted light microscope were used to determine cell concentrations with a minimum of 400 cells counted per sample for statistical reliability. Dry weight yields were determined at the end of the experiment by collecting 50 mL of culture into a pre-weighed 50 mL Falcon tube (Fisher Scientific, Palatine, IL) followed by centrifugation at 4800 x g and 4°C for 10 minutes (Thermo Scientific, Sorvall Legend XTR, Waltham, MA). The concentrated biomass was rinsed with deionized H_2O, 18 MΩ, to remove media salts and excess bicarbonate, before centrifuging again. Remaining algae pellets were frozen and lyophilized (Labconco lyophilizer, Kansas City, MO) for 48 hours. CDW's were collected by subtracting the weight of the biomass-free Falcon tube from the weight of the Falcon tube containing the freeze-dried biomass.

Analysis of Media Components

Medium pH was measured using a standard bench top pH meter. Ammonium was measured using a modified 2-phenylphenol method previously described by Rhine and Mulvaney (1998). Nitrate and phosphate were measured using ion chromatography (Dionex, Sunnyvale, CA).

Chlorophyll Measurements

Chlorophylls a, b, and carotenoids were determined using the methanol extraction and optical absorption correlation previously described by Ördög et al. (2011). One mL of culture was centrifuged at 16,000 x g for 5 minutes, after which the supernatant was discarded. One mL of methanol was added to the pellet, and the sample was vortexed and lightly sonciated to disperse the pellet. The suspension was heated at 70°C for 10 minutes in a water bath followed by centrifugation at 16,000 x g for 3 minutes. Absorption was read at 666 nm, 654 nm, and 470 nm. Calculations for chlorophyll were conducted as described Ördög et al. (2011).

Harvesting

Cultures were harvested at the end of each experiment. Two aliquots of 50 mL were dispensed into 50 mL Falcon tubes (Fisher Scientific, Palatine, IL) and centrifuged at 4800 x g and 4°C for 10 minutes (Thermo Scientific, Sorvall Legeng XTR, Waltham, MA). The concentrated biomass was rinsed with deionized H_2O to remove media salts and excess bicarbonate, before centrifuging again. Remaining algae pellets were frozen

and lyophilized (Labconco lyophilizer, Kansas City, MO) for 48 hours and stored at -20°C for subsequent lipid analysis.

Lipid Extraction

Extraction and analysis of extractable lipid was conducted as previously described by Lohman et al. (2013). Approximately 30 mg of dried biomass was combined with 1 mL of chloroform in a 1.5 mL stainless steel microvial with a silicone cap (BioSpec Products, Bartlesville, OK). Three types of beads (0.6 g of 0.1 mm zirconium/silica beads, 0.4 g of 1.0 mm glass beads, and two 2.7 mm glass beads) were added to each vial before capping. A FastPrep bead beater (Bio101/Thermo Savant, Carlsbad, CA) was used to agitate the vials for six 20 s pulses at power level 6.5 followed by a 1 min cooling period between pulses. Total agitation time was 2 min. The mixture of solvent, residual biomass and beads was transferred to a 15 mL Pyrex test tube with a Teflon lined screw cap (Kimble-Chase, Vineland, NJ), and the steel microvial was rinsed twice with 1 mL of chloroform, which was also added to the test tube, bringing the total solvent volume to 3 mL. One mL of 15% NaCl (v/v) was added to enhance phase separation. Samples were then centrifuged at 1200 x g for 2 min to separate residual biomass. One mL of the organic phase was removed from the bottom of the test tube using a glass syringe and transferred to a 2 mL GC vial for GC-FID analysis.

Transesterification for FAME Analysis

Transesterification of fatty acids was conducted using a previously described method (Griffiths et al. 2010) with the modifications described in Lohman et al. (2013). FAME composition was analyzed using GC-MS. In brief, approximately 30 mg of dried biomass was transferred to a 15 mL Pyrex test tube with a Teflon lined screw cap (Kimble-Chase, Vineland, NJ). One mL toluene and 2 mL sodium methoxide (Fisher Scientific, Pittsburgh, PA) were added to each test tube as well as 10 μL of a 10 mg/mL standard mixture of C11:0 and C17:0 TAG to monitor transesterification efficacy. Samples were heated in an oven for 30 min at 90°C while being vortexed every 10 min. Samples were cooled to room temperature before 2 mL of 14% boron tri-fluoride in methanol (Sigma-Aldrich, St. Louis, MO) were added, and each sample was heated again for 30 min. Samples were then allowed to cool to room temperature before 10 μL of a 10 mg/mL standard mixture of C23:0 FAME was added to monitor the partitioning of FAME into the organic phase. Additionally, 0.8 mL of hexane and 0.8 mL of a saturated NaCl-deionized water solution was added. Samples were heated for 10 min at 90°C to facilitate FAME partitioning into the organic phase, vortexed for 10 s, and centrifuged at 1200 x g for 2 min to enhance phase separation. One mL of the organic phase was removed from the top layer using a glass syringe and transferred to a 2 mL GC vial for GC-MS analysis.

Lipid Analysis Using GC-FID

Gas chromatography-flame ionization detection (GC-FID) was used to analyze extractable lipid content according to a previously published protocol (Lohman et al. 2013). Briefly, a 1 μL splitless injection was performed using an autosampler into a GC-FID (Agilent 6890N, Santa Clara, CA) equipped with a 15 m (fused silica) RTX biodiesel column (Restek, Bellefonte, PA). The initial column temperature was held at 100°C for 1 min, before being increased to 370°C at a rate of 10°C/min. The injector temperature was held constant at 320°C. Helium was used as the carrier gas and column flow was held at 1.3 mL/min for 22 min, increased to 1.5 mL/min, held for 2 min, increased to 1.7 mL/min and held for 12 min. All flow rate increases were set to 0.2 mL/min^2. Calibration curves were constructed for each of the following standards: C12:0, C14:0, C16:0, C18:0, and C20:0 FFAs; C12:0, C14:0, C16:0, and C18:0 MAGs; C12:0, C14:0, C16:0, and C18:0 DAGs; along with C11:0, C12:0, C14:0, C16:0, C17:0, C18:0, and C20:0 TAGs (Sigma-Aldrich, St. Louis, MO) for quantification ($r^2 > 0.99$). This GC method quantifies the amounts of free fatty acids (FFA), monoacylglycerides (MAG), diacylglycerides (DAG) and TAG in a single analysis as detailed in Lohman et al. (2013).

FAME Analysis Using GC-MS

Gas chromatography-mass spectrometry (GC-MS) analysis was performed as previously described by Lohman et al. (2013) to analyze total cellular fatty acids (FAME). Briefly, 1 μL split (2:1) injections were performed using an autosampler into a GC–MS (Agilent 6890N GC and Agilent 5973 Networked, MS) equipped with a 60 m x

0.25 mm Agilent DB-23 column (0.25 μm phase thickness). The injector temperature was 280°C and the detector temperature was 150°C. The initial column temperature was 50°C (held for 1 min) and was increased to 175°C at a rate of 25 °C/min, immediately followed by a ramp at 4°C/min to a final temperature of 230°C which was held for 10 min before run termination. Helium was used as the carrier gas and column flow was held at 0.5 mL/min. Quantities of FAMEs were determined by quantifying each response peak with the nearest eluting calibration standard based on retention time, using MSD ChemStation software (Ver. D.02.00.275). A 28-component fatty acid methyl ester standard prepared in methylene chloride (NLEA FAME mixture; Restek, Bellefonte, PA) was used for GC-MS retention time identification and response curve generation ($r^2 > 0.99$).

CHAPTER 3

INITIAL GROWTH STUDIES USING STRAINS WC-1 AND SLA-04 IN MUNICIPAL WASTEWATER

Introduction

The first experiments performed during this thesis work served as an initial screening to evaluate whether strains *Scenedesmus* sp. strain WC-1 (WC-1) and *Chlorella* sp. strain SLA-04 (SLA-04) could be grown in untreated wastewater. An isolated diatom, *Navicula* sp. strain RGd-1 (RGd-1), was also initially screened for growth in wastewater (Appendix C). Despite several attempts to amend wastewater with essential nutrients, RGd-1 was unable to sustain growth in wastewater, and no further research was conducted on the organism. The wastewater streams of interest were primary clarifier effluent (PC) and the liquid phase of anaerobic digestate (AD) collected at the Bozeman Water Reclamation Facility (BWRF). Each strain was initially grown in PC with no additions and in AD diluted with deionized water as following: undiluted (AD1), 1:5 (AD2), 1:10 (AD3), 1:25 (AD4) and 1:50 (AD5). Growth studies were performed in triplicate using 250 mL shaker flasks fitted with cloth caps to facilitate gas transfer (Figure 1). At least two generations of growth were carried out to ensure that growth in wastewater was not due to nutrients carried over during inoculation.

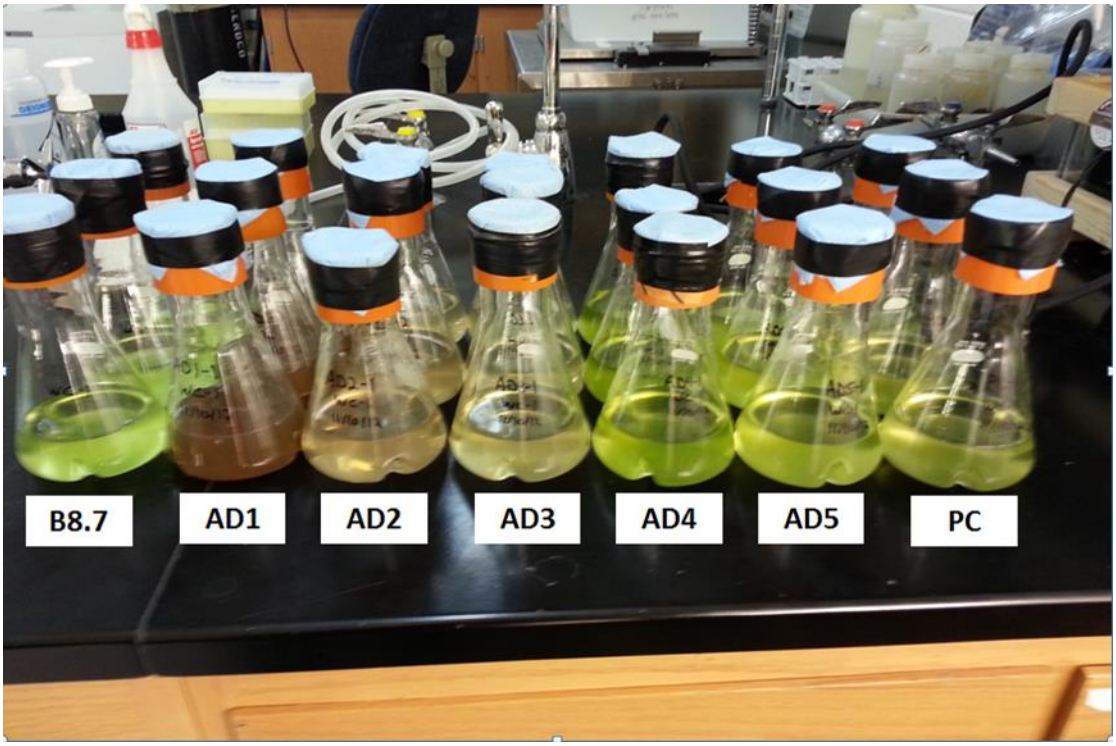
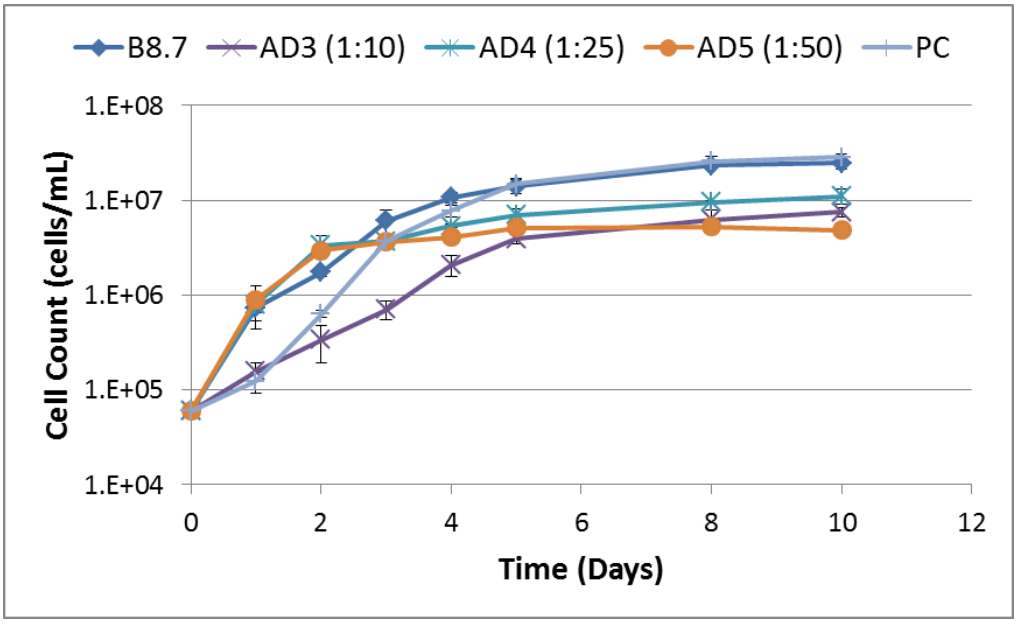


Figure 1. Picture of WC-1 shaker flask growth in Bold's basal medium with the pH adjusted to 8.7 (B8.7), undiluted anaerobic digestate (AD1), 1:5 anaerobic digestate dilution (AD2), 1:10 anaerobic digestate dilution (AD3), 1:25 anaerobic digestate dilution (AD4), 1:50 anaerobic digestate dilution (AD5) and primary clarifier effluent (PC).

WC-1

Growth curves and medium pH for the first growth screen of WC-1 in wastewater are shown in Figure 2. It should be noted that the undiluted AD (AD1) and the 1:5 dilution of AD (AD2) did not support growth likely due to high turbidity or high ammonium concentrations. Growth was possible in all other dilutions of AD and primary clarifier effluent to varying degrees. PC cultures grew very similarly to the Bold's 8.7 control medium shown by growth and pH curves tracking closely. Growth in diluted AD achieved significantly lower cell densities compared to PC and Bold's 8.7.



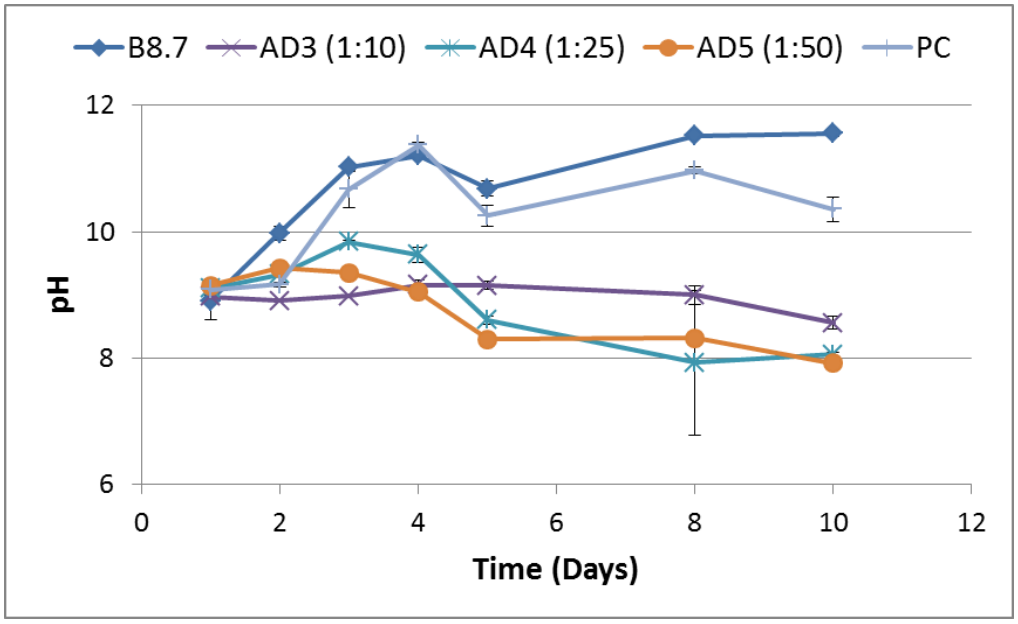


Figure 2. Average cell density (microscopic direct counts) (top) and pH (bottom) with standard deviations for triplicate reactors of first generation growth of WC-1 in Bold's basal medium (B8.7), the three dilutions of anaerobic digestate that supported growth (AD3, AD4 and AD5) and primary clarifier effluent (PC).

A similar trend in growth was also seen in the second generation of growth in wastewater and diluted anaerobic digestate (Figure 3). PC and B8.7 cultures grew to similar cell concentrations, and cultures grown in diluted AD reached a markedly lower cell concentration. After observing two generations of inhibited growth in diluted AD cultures, the possibility of a nutrient deficiency in those cultures was explored. Three separate additions were made to AD cultures to test for one or more deficient nutrients. The addition of an iron solution to reach 1 mg Fe^{2+}/L, the addition of one mL of a microelement solution (composition shown in Table 1) and the addition of both the iron solution and the microelement solution were made to AD4 cultures after stationary growth was observed. The addition of both solutions occurred at day 13 of growth, and the individual additions occurred at day 15.

Table 1. Components and concentrations of the microelement solution added to 2nd generation cultures of WC-1 grown in anaerobic digestate diluted 1:25 (AD4).

Component	**$ZnSO_4*7H_2O$**	**$MnCl_2*4H_2O$**	**MoO_3**	**$CuSO_4*5H_2O$**	**$Co(NO_3)_2*6H_2O$**
Concentration (g/L)	**8.82**	**1.44**	**0.71**	**1.57**	**0.49**

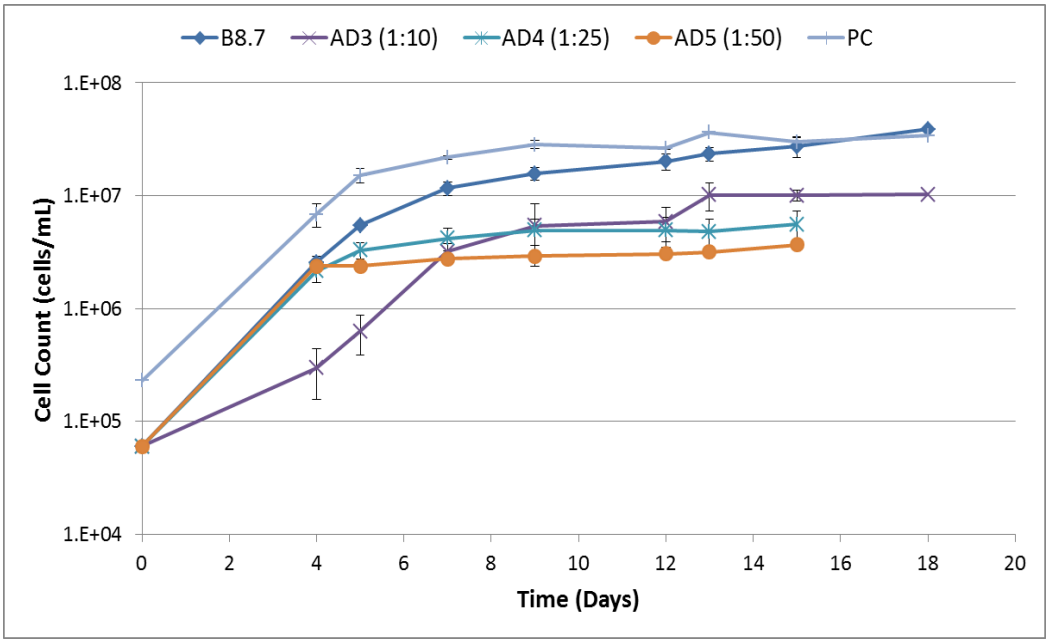


Figure 3. Average cell concentration with standard deviation for triplicate reactors of second generation WC-1 growth in Bold's 8.7 (B8.7), each anaerobic digestate dilution (AD3, AD4 and AD5) and primary clarifier effluent (PC).

The addition of the microelement solution and the iron solution together resulted in a large increase in cell density in the 1:25 AD dilution cultures (Figure 4). The culture increased from approximately 4.3 x 10^6 cells/mL at the time of the addition to roughly 1.5 x 10^7 cells/mL at day 18. After seeing an increase in cell numbers upon the addition of both solutions, the solutions were added individually to see if one of the additions had more of an effect than the other. Figure 4 also shows the growth curves of the culture given the iron solution and the culture given the microelement solution. After the addition of iron, the cell density increased from 7.0 x 10^6 to 2.0 x 10^7cells/mL, but the culture given the microelement solution did not experience the same increase in cell density. These results suggest that cultures grown on AD are limited in growth due to an iron deficiency. Studies have shown that iron is important for chlorophyll synthesis,

nitrate assimilation and redox reactions involved in the electron transport chain (Greene et al. 1992; Terry and Abadía 1986). Iron deficiency commonly leads to reduced pigment concentrations which results in a decrease in the maximum chlorophyll specific rate of photosynthesis (Belkhodja et al. 1994; Greene et al. 1991; Terry and Abadía 1986), and this decrease may affect the efficiency of excitation energy transfer in photosystem II. Another consequence of iron stress is the possibility of a significant decrease in photosynthetic components on the acceptor side of the plastoquinone pool such as cytochrome b_6f, cytochrome oxidase, photosystem I and ferredoxin (Behrenfeld et al. 2006; Ivanov et al. 2000; Roche et al. 1996; Sandmann 1985; Vassiliev et al. 1995).

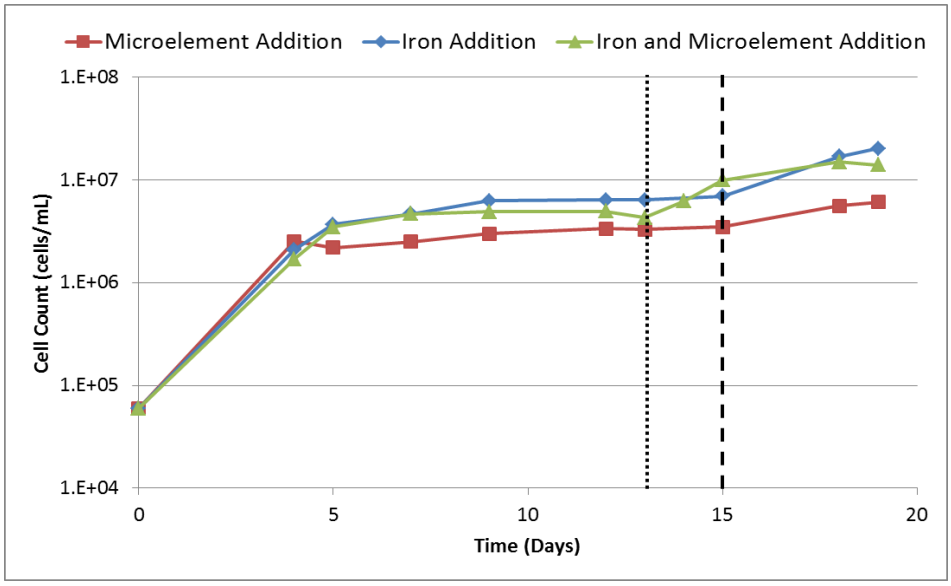


Figure 4. Cell density of 2nd generation WC-1 cultures grown in 1:25 AD supplemented with a microelement solution, an iron solution and both, microelement and iron solutions together. The dotted line at day 13 denotes when both solutions were added to the culture and the dashed line at day 15 denotes when the solutions were added separately to different treatments. One mL of the microelement solution described in Table 1 was added per liter of culture and the iron solution was added to achieve a concentration of 1 mg Fe^{2+}/L in the culture. The values shown for each condition in Figure 4 are for one flask (n=1).

In the 3rd generation growth study, the effect of the timing of the iron addition was evaluated. The iron addition was administered either at inoculation or after 7 days of growth when a lag in growth was observed. Figure 5 shows the growth curves for each of the iron-supplemented conditions as well as the growth curve of the culture without added iron. The culture that had iron added at inoculation grew steadily until it reached a maximum cell density at day 9 followed by a stationary phase. The two cultures without any externally added iron grew slowly until day 7 when iron was supplemented in one of the two cultures. Upon the addition of iron, the culture increased in cell density until day 10 or 12 when stationary growth began. Although the cell density increased when iron was added at day 7, the culture was unable to reach the same cell concentration as the culture with external iron added at inoculation. This result suggests that optimized growth is achieved when iron is available throughout the entire growth phase.

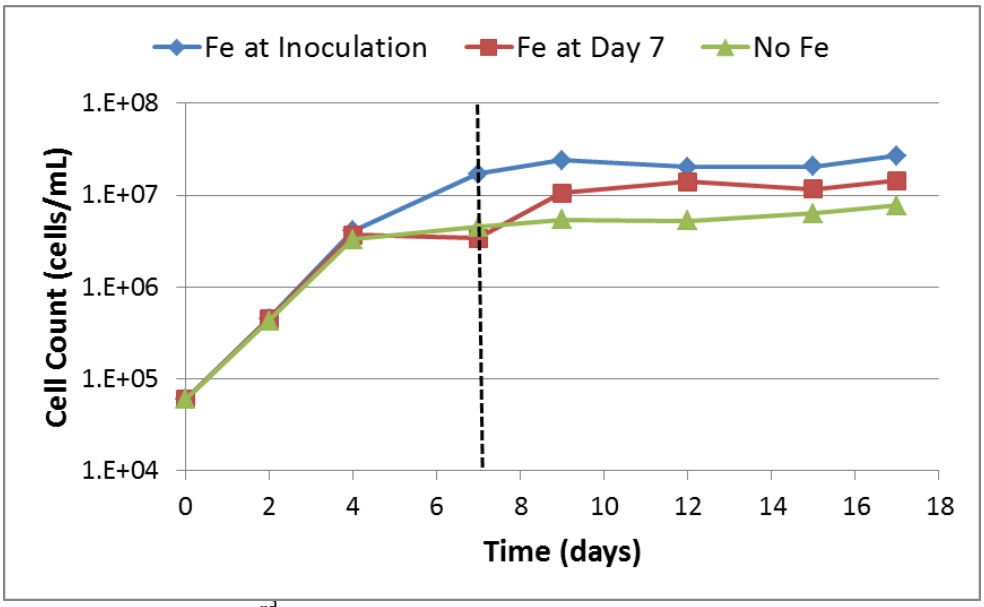


Figure 5. Cell density of 3rd generation WC-1 grown in 1:25 AD supplemented with iron at different times throughout growth. The dashed line denotes when iron was added to a culture at day 7.

SLA-04

Two generations of growth were performed for SLA-04. In each generation, sterile Bold's 8.7, primary clarifier effluent and anaerobic digestate diluted 1:25 with added iron (1 mg Fe^{2+}/L medium concentration) were used as growth media. The 1:25 AD dilution supplemented with iron condition was used because this condition showed optimal growth during the WC-1 experiments. Figure 6 shows the cell density and pH of the three conditions used in the first generation of growth. The growth of SLA-04 was very similar for each condition based on growth curves corresponding closely to one another. The pH trends of Bold's basal medium (B8.7) and primary clarifier effluent (PC) conditions behave similarly, but a significant decrease in pH can be seen in the diluted anaerobic digestate condition (AD4). The large drop in pH can be attributed to growth solely on ammonium (NH_4^+) as a nitrogen source. When ammonium is utilized for algal growth, protons are transported out of the cell to maintain cell neutrality, and the increase in protons in the medium causes a decrease in pH (Fuggi et al. 1981). The acidification of the growth medium decreases the dissolved inorganic carbon (DIC) of the medium and these conditions have been shown to decrease algal growth (Eustance et al. 2013). Although the pH of the AD4 condition decreased to roughly 6.3, the cell density reached nearly the same level as the B8.7 and PC growth conditions.

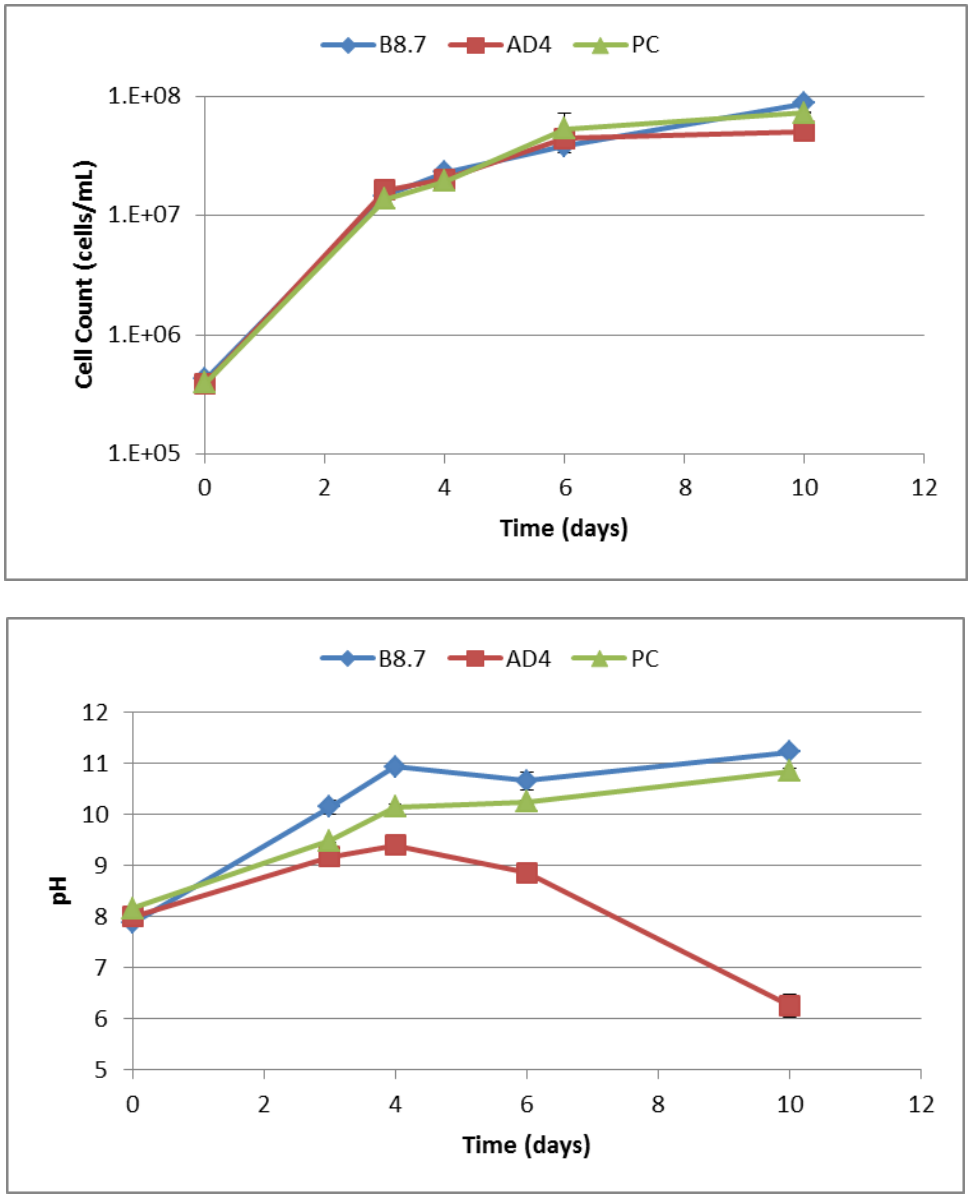


Figure 6. Average cell density (top) and pH (bottom) with standard deviation of triplicate 1st generation SLA-04 cultures grown in Bold's 8.7 (B8.7), 1:25 dilution of anaerobic digestate (AD4) and primary clarifier effluent (PC).

The same three conditions were used to grow SLA-04 in the second generation as were used in the first generation of growth. All three conditions grew similarly until roughly 12 days into the experiment when the wastewater conditions (AD4 and PC)

reached a stationary cell concentration of approximately 6.7 x 10^7 cells/mL compared to 1.4 x 10^8 cells/mL reached in the B8.7 condition (Figure 7). The wastewater conditions were checked for a possible deficiency of iron and nitrogen. The iron addition used the same iron solution as previously described, and nitrogen was given in the form of NH_4Cl to reach 25 mg N/L. When iron was added to each of the wastewater conditions, no subsequent increase in growth was observed (Figure 8). The addition of nitrogen to the PC condition (PC+NH4) resulted in the cell concentration to increase and reach a similar level as in the B8.7 condition, but the addition of nitrogen did not affect the cell concentration in the AD4 condition (Figure 8). This result suggests nitrogen, either as a lab grade solution or as a recycled wastewater stream such as anaerobic digestate, may need to be added to primary clarifier effluent cultures to reach optimal cell density.

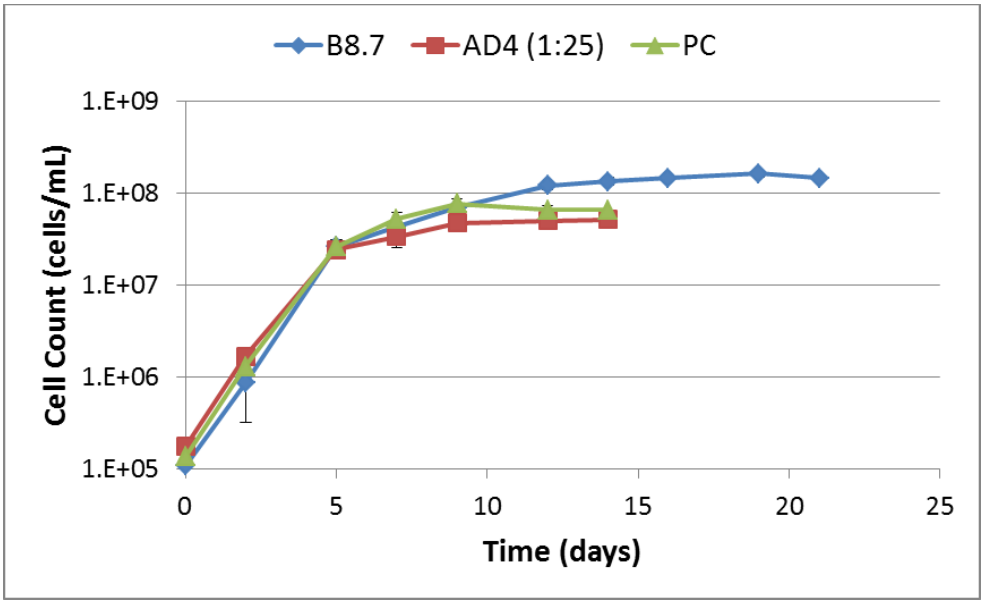


Figure 7. Average cell density (top) and pH (bottom) with standard deviation of triplicate 2nd generation SLA-04 cultures grown in Bold's 8.7 (B8.7), 1:25 dilution of anaerobic digestate (AD4) and primary clarifier effluent (PC).

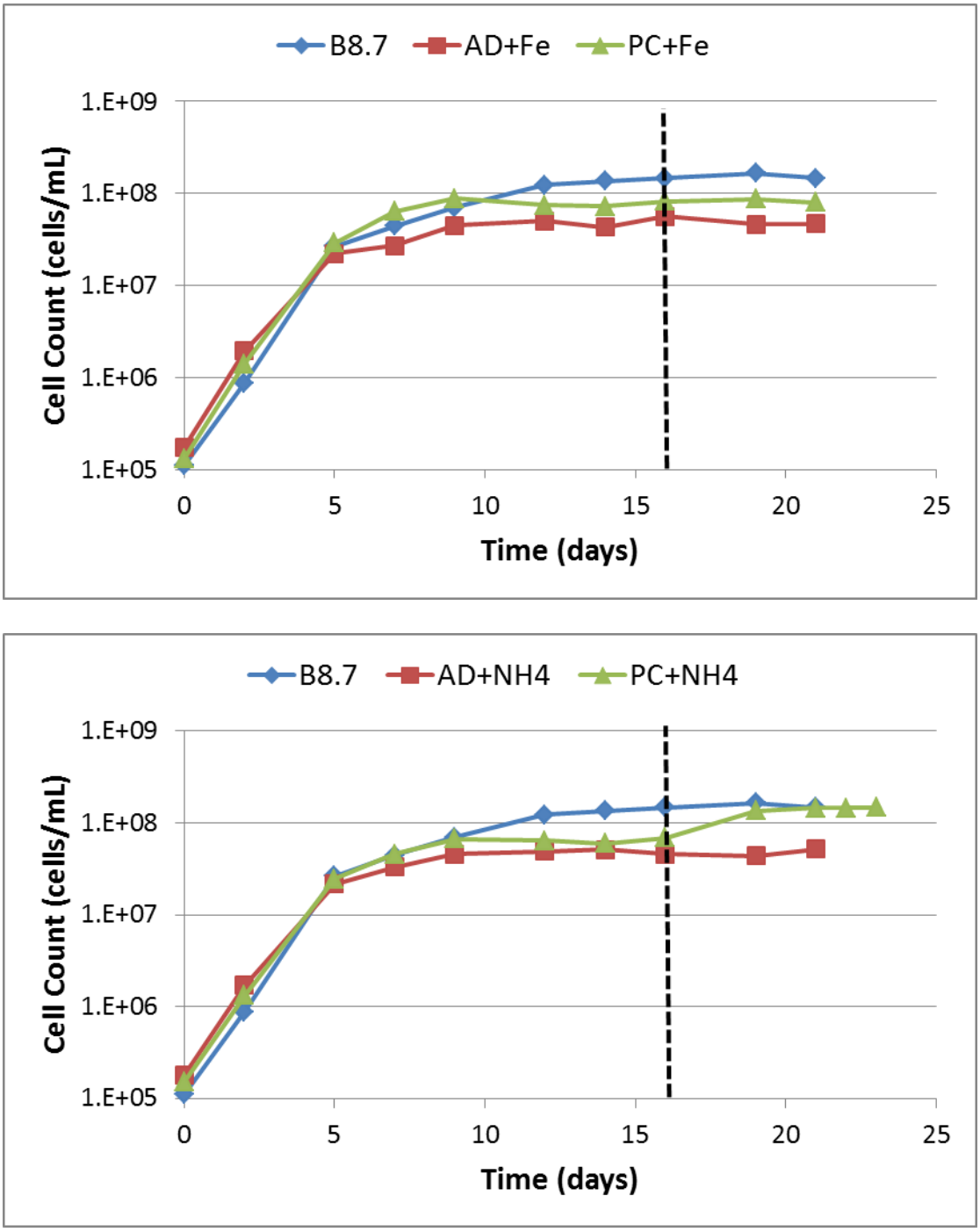


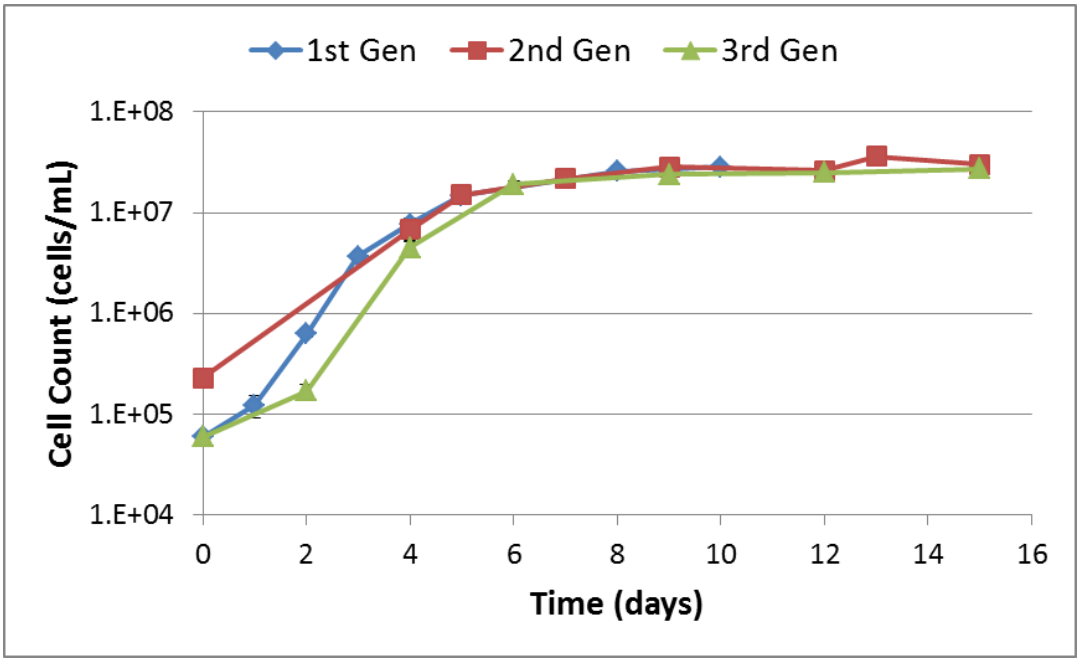
Figure 8. Second generation growth of SLA-04 in Bold's 8.7 (B8.7), 1:25 diluted anaerobic digestate (AD), and primary clarifier effluent (PC). AD and PC cultures were supplemented with iron (top) and ammonium chloride (bottom) at day 16.

Generational Variance

After the initial studies using WC-1 and SLA-04 in both PC and diluted AD were performed, the remaining experiments performed towards this thesis were focused on using PC as the primary growth medium and AD as a recycled nutrient stream for nutrient supplementation. The multiple generations of growth performed in the initial studies can be compared to judge whether WC-1 and SLA-04 will behave consistently in wastewater. The three generations of WC-1 growth in primary clarifier effluent had very similar growth characteristics (Figure 9). WC-1 reached stationary growth at roughly 6 days after inoculation for each generation at a cell concentration of approximately 2.4 x 10^7 cells/mL. SLA-04 also behaved very similarly for both generations of growth in PC (Figure 9). SLA-04 cultures reached stationary phase by day 8 or 9 at a cell concentration of roughly 7.7 x 10^7 cells/mL.

Conclusions

The initial growth studies performed in this chapter demonstrated that strains WC-1 and SLA-04 were able to be grown in untreated municipal wastewater. Both WC-1 and SLA-04 grew consistently in PC over two or three generations of growth, and each strain was able to grow in diluted anaerobic digestate with an external iron addition. Subsequent studies involving WC-1 and SLA-04 were then performed attempting to increase lipid accumulation via the timely addition of inorganic carbon (Chapter 4) and recycle harvest water amended with anaerobic digestate (Chapter 5).



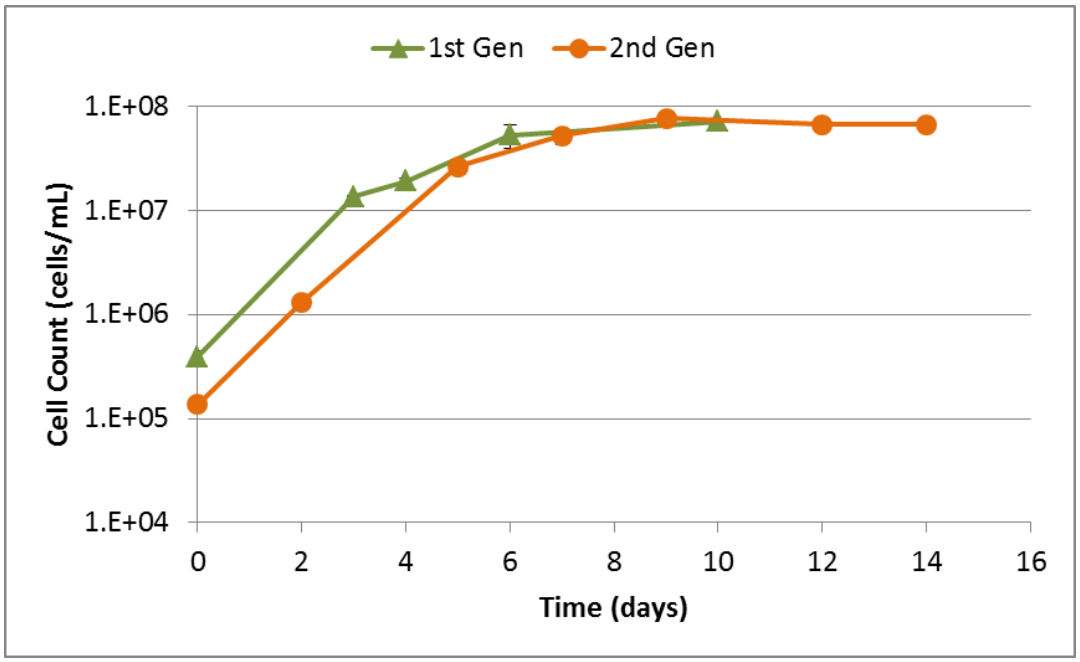


Figure 9. Generational comparison of cell density for WC-1 (top) and SLA-04 (bottom) grown in untreated primary clarifier effluent without any external additions.

CHAPTER 4

GROWTH, NUTRIENT UTILIZATION AND BIOFUEL POTENTIAL OF WC-1 AND SLA-04 GROWN IN WASTEWATER UNDER SEVERAL INORGANIC CARBON REGIMES

Introduction

The experiments presented in this chapter aimed to evaluate growth, nutrient utilization, lipid accumulation, and biodiesel potential by *Scenedesmus* sp. strain WC-1 (WC-1) and *Chlorella* sp. strain SLA-04 (SLA-04) when cultured in wastewater supplied with various forms and concentrations of inorganic carbon. WC-1 and SLA-04 were also cultured in sterile Bold's 8.7 medium to compare growth characteristics of wastewater cultures to those of controlled conditions. The conditions used for each strain are outlined in Table 2.

Growth of WC-1 and SLA-04 in Primary Effluent

Maximum specific growth rates of WC-1 were very similar between growth conditions (Table 3). Bold's 8.7 (5% CO2) cultures achieved the highest cell density and cell dry weight (Figure 10a, Table 3). The 50 mM addition of bicarbonate to Bold's 8.7 (5% CO2)+HCO3 resulted in a cessation of cell replication which is consistent with previously reported findings (Gardner et al. 2012). As a result of arrested growth, the cell dry weight of Bold's 8.7 (5% CO2)+HCO3 reached only 53% of the cell dry weight of Bold's 8.7 (5% CO2).

Table 2. Names and descriptions of each condition used for growth of WC-1 and SLA-04. The "Gas Sparge" column is for lighted hours only. Ambient air was sparged during the dark hours for each condition.

Name	Growth Medium	Gas Sparge	Bicarbonate Addition
Bold's 8.7 (5% CO2)	Sterile Bold's 8.7 basal medium	5% CO_2 throughout experiment	None
Bold's 8.7 (5% CO2) + HCO3	Sterile Bold's 8.7 basal medium	5% CO_2 until nitrogen depletion when ambient air was supplied	50 mM at nitrogen depletion
PC (5% CO2)	Primary Clarifier Effluent	5% CO_2 throughout experiment	None
PC (5% CO2) + HCO3	Primary Clarifier Effluent	5% CO_2 until nitrogen depletion when ambient air was supplied	50 mM at nitrogen depletion
PC (pH Control)	Primary Clarifier Effluent	5% CO_2 to keep pH between 8.1 and 8.5 before bicarbonate addition	None
PC (pH Control) + HCO3	Primary Clarifier Effluent	5% CO_2 to keep pH between 8.1 and 8.5 before bicarbonate addition 5% CO_2 to keep pH between 10.1 and 10.5 before bicarbonate addition	50 mM at nitrogen depletion

Cultures of WC-1 grown in primary clarifier effluent behaved very similarly under each inorganic carbon regime (Figure 10a). PC (5% CO2) reached a final cell dry weight 45% greater than that of PC (5% CO2)+HCO3 (Table 3). Conversely, PC (pH Control)+HCO3 cultures reached a cell dry weight 72% greater than PC (pH Control) cultures. Interestingly, the 50 mM bicarbonate addition to wastewater cultures did not stop cellular replication as it did in Bold's 8.7 cultures. All wastewater cultures continued to grow until one day after the addition of 50 mM HCO_3^- (Figure 10a).

SLA-04 cultures reached similar cell concentrations and cell dry weights whether grown in Bold's 8.7 or municipal wastewater (Figure 10b, Table 2). Similar to WC-1, SLA-04 grown in Bold's 8.7 without the addition of 50 mM HCO_3^- achieved the highest cell dry weight, but the difference was less significant than with WC-1. The bicarbonate addition near nitrogen depletion resulted in a 9.5-23.8% decrease in cell dry weight in SLA-04 for each condition (Table 3). SLA-04 had relatively high growth rates when cultured in primary effluent (roughly 1.5 day^{-1}) compared to similar studies; growth rates of *Chlorella* species cultured in wastewater have been reported previously as 0.17-1.37 day^{-1} (Ji et al. 2013; Lau et al. 1995; Li et al. 2011; Wang et al. 2010).

Chlorophyll concentrations were measured over time for WC-1 and SLA-04 cultures to estimate relative photosynthetic activity (Figure 10e,f). WC-1 grown in Bold's 8.7 had significantly more chlorophyll per volume of culture compared to wastewater cultures. Chlorophyll concentrations of SLA-04 were also greater in cultures grown in Bold's 8.7 compared to those grown in wastewater, which suggests algae cultured in wastewater have a decreased ability for electron transport and carbon fixation. The cause of decreased chlorophyll content is likely low iron concentrations in the primary clarifier effluent. Iron plays a major role in chlorophyll biosynthesis, inorganic nitrogen assimilation and redox reactions in the electron transport chain (Terry and Abadía 1986). Iron limitation commonly leads to a reduction in pigment concentration and limits the efficiency of excitation energy transfer in photosystem II (Greene et al. 1991, 1992).

Table 3. Maximum specific growth rate, doubling time, and final cell dry weights for WC-1 and SLA-04

Organism	Condition	μ_{max} (day^{-1})	T_d (days)	CDW (g/L)
WC-1	Bold's 8.7 (5% CO2)	1.69 ± 0.01	0.41 ± 0.00	2.23 ± 0.00
	Bold's 8.7 (5% CO2) + HCO3	1.65 ± 0.15	0.42 ± 0.04	1.18 ± 0.31
	PC (5% CO2)	1.50 ± 0.05	0.46 ± 0.02	1.52 ± 0.16
	PC (5% CO2) + HCO3	1.49 ± 0.27	0.47 ± 0.08	1.05 ± 0.33
	PC (pH Control)	1.56 ± 0.07	0.44 ± 0.02	0.75 ± 0.01
	PC (pH Control) + HCO3	1.62 ± 0.07	0.43 ± 0.02	1.29 ± 0.35
SLA-04	Bold's 8.7 (5% CO2)	2.07 ± 0.43	0.34 ± 0.07	1.68 ± 0.01
	Bold's 8.7 (5% CO2) + HCO3	1.65 ± 0.32	0.42 ± 0.08	1.28 ± 0.09
	PC (5% CO2)	2.22 ± 0.22	0.31 ± 0.03	1.46 ± 0.05
	PC (5% CO2) + HCO3	2.28 ± 0.02	0.30 ± 0.00	1.32 ± 0.03
	PC (pH Control)	1.55 ± 0.07	0.45 ± 0.02	1.36 ± 0.04
	PC (pH Control) + HCO3	1.77 ± 0.04	0.39 ± 0.01	1.19 ± 0.05

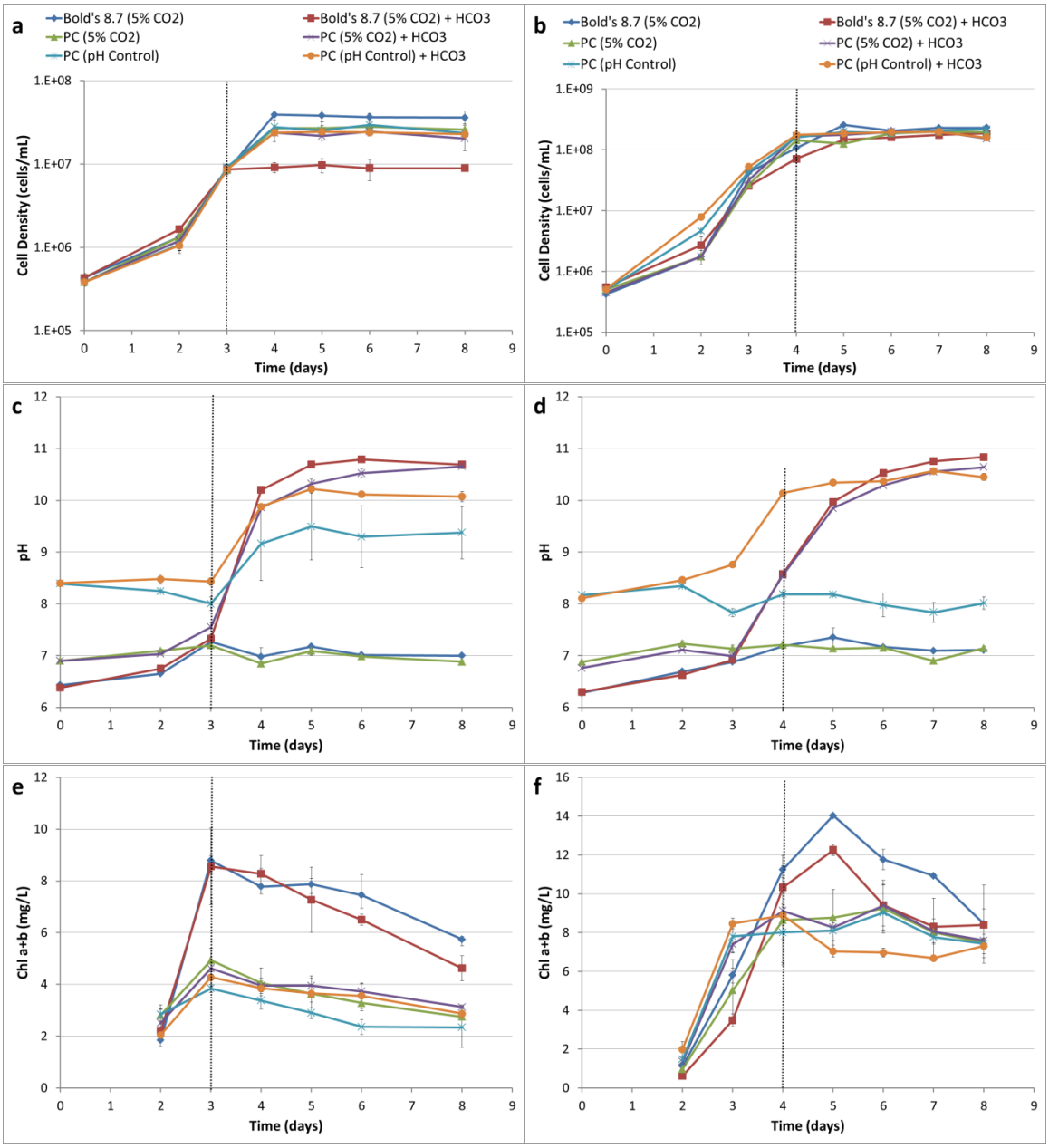


Figure 10. Growth, pH, and chlorophyll a+b concentrations for WC-1 (left) and SLA-04 (right). Dashed line indicates the time of 50 mM HCO_3^- addition for treatments labeled "+ HCO3". (a) and (b) show the cell densities achieved by WC-1 and SLA-04, respectively. (c) and (d) show the medium pH for WC-1 and SLA-04, respectively. In pH controlled conditions, the pH was maintained at 8.1-8.5 until the bicarbonate addition. At which time, the cultures amended with 50 mM HCO_3^- had the pH controlled to 10.1-10.5, and cultures without added bicarbonate were controlled at 8.1-8.5. (e) and (f) show the average chlorophyll a + b concentrations for WC-1 and SLA-04, respectively.

Nitrogen and Phosphorus Uptake by WC-1 and SLA-04

Ammonium, nitrate, and phosphate concentrations were evaluated at inoculation and at harvest (Table 4). WC-1 utilized both the ammonium and nitrate initially present in the primary clarifier effluent, and the levels of each were essentially depleted by the time of harvest (96.5-99% nitrogen removal). WC-1 also reduced phosphate levels from roughly 2.3 to 0-0.6 mg PO_4^{3-}/L (73.9 to >99% phosphate reduction). Xin, Hong-Ying, and Jia (2010) reported similar wastewater nutrient removal. The *Scenedesmus* sp. used in that study removed 98.5% of the nitrogen and 98% of the phosphorus initially present. SLA-04 also consumed essentially all of the ammonium and nitrate initially present in the wastewater. Initial phosphate levels in the SLA-04 experiment were roughly 5x the initial phosphate levels in the WC-1 experiment. The phosphate levels may have been high during the SLA-04 experiments due to the time of collection. The primary clarifier effluent used in the WC-1 experiment was collected in early summer when mountain runoff can dilute wastewater entering the treatment facility. Water used in the SLA-04 experiment was collected later in the summer when it is likely the amount of water (melt water and/or groundwater) entering the sewer system is much lower than during periods of mountain runoff.

At harvest, 2.3-4.4 mg PO_4^{3-}/L remained in the SLA-04 cultures (61.3-79.7% phosphate reduction). Lau, Tam, and Wong (1995) reported similar nutrient removals of 98% NH_4^+ reduction (33 mg/L initial NH_4^+ concentration reduced to 0.5 mg/L at harvest) and 93% phosphate reduction (3.9 mg PO_4^{3-}/L reduced to 0.3 mg PO_4^{3-}/L) by *Chlorella* vulgaris grown in wastewater. Another study by Wang et al. (2010) showed a 74.7%

ammonium reduction (33.4 mg NH_3-N/L reduced to 8.5 mg NH_3-N/L) and a 90.6% phosphate reduction (5.66 mg P/L reduced to 0.5 mg P/L) using an isolated *Chlorella* species grown in primary effluent. Variations in nutrient removal are likely due to differences in N:P ratios. Optimal N:P ratios for freshwater algae have been estimated between 6.8 and 10 (Darley 1982; Martin et al. 1985; Reynolds 1984). With an imbalanced N:P ratio, nitrogen or phosphorus is likely to become limiting leading to an inefficient removal of the replete nutrient.

Table 4. Ammonium, nitrate, and phosphate uptake by WC-1 and SLA-04. Average concentrations in mg/L (±s.d.) are given for primary clarifier effluent prior to inoculation and when the cultures were harvested 8 days later.

	Before Inoculation	**At harvest (8 days after inoculation)**			
WC-1	Primary Clarifier Effluent	PC (5% CO2)	PC (5% CO2) + HCO3	PC (pH Control)	PC (pH Control) + HCO3
Ammonium (mg/L)	5.91 ± 0.98	BQL	BQL	BQL	BQL
Nitrate (mg/L)	0.55 ± 0.12	BQL	BQL	BQL	BQL
Phosphate (mg/L)	2.29 ± 0.11	BQL	BQL	BQL	0.59 ± 0.01
SLA-04					
Ammonium (mg/L)	6.74 ± 0.28	BQL	BQL	BQL	BQL
Nitrate (mg/L)	1.42 ± 0.39	BQL	BQL	BQL	BQL
Phosphate (mg/L)	11.24 ± 2.23	4.35 ± 1.28	2.28 ± 0.92	2.56 ± 0.71	2.37 ± 0.89

BQL denotes that measured values were below the limit of quantification.
Quantification limits: Ammonium=0.5 mg/L; Nitrate=0.5 mg/L; Phosphate=0.5 mg/L

Lipid Productivity and Biodiesel Potential of WC-1 and SLA-04

The addition of $NaHCO_3$ to WC-1 cultures near nitrogen depletion resulted in an increase in TAG content (% w/w) for cultures grown in wastewater but had no effect on the TAG content of cells grown in Bold's 8.7 (Figure 11). FAME analyses of WC-1 cultures reflect the results of the extractable lipid analyses. Total FAME increased on a percent w/w basis in wastewater cultures amended with 50 mM HCO_3^-, but Bold's 8.7 cultures show no change in FAME content with $NaHCO_3$ addition. The bicarbonate addition to the Bold's 8.7 cultures may have occurred after nitrate depletion in the medium which may explain why no increase in TAG content was observed. Gardner et al. (2012) showed that HCO_3^- addition must occur just prior to nitrate depletion to result in a significant TAG increase. The highest lipid content observed in WC-1 grown in wastewater was roughly 37 % (w/w) for PC (5% CO2)+HCO3. Xin, Hong-Ying, and Jia (2010) reported similar lipid content, 33%, in an isolated *Scenedesmus* species grown in secondary effluent. Ji et al. (2013) achieved a lipid content of 27% in *Scenedesmus obliquus* grown in municipal wastewater.

When extractable lipid content was evaluated on a concentration basis (in g/L culture medium), it becomes evident that the cultures with Bold's 8.7 and sparged with 5% CO_2 were the most productive with respect to FAME, TAG and FFAs (Figure 11b). Total extractable lipid concentration (in g/L) was lower in both Bold's 8.7 cultures supplied with $NaHCO_3$ and wastewater cultures sparged with 5% CO_2 and amended with 50 mM $NaHCO_3$ compared to cultures not amended with $NaHCO_3$. Gardner et al. (2012) showed that the addition of HCO_3^- to algal cultures can stop cellular replication when the

pH is allowed to increase due to inorganic carbon fixation after HCO_3^- addition. Cessation of cellular replication in the current study resulted in a decrease in cell dry weight productivity which explains the lower extractable lipid concentration in both Bold's 8.7 cultures supplied with $NaHCO_3$ and wastewater cultures under 5% CO_2 supplied with $NaHCO_3$ (dry weights are shown in Table 3). FAME concentration in WC-1 grown in Bold's 8.7 and wastewater under 5% CO_2 was also higher without $NaHCO_3$ addition. Unlike Bold's 8.7 (5% CO2)+HCO3 and PC (5% CO2)+HCO3, cell dry weight increased in PC (pH Control)+HCO3 compared to PC (pH Control) (Table 3). The increased cell dry weight resulted in higher lipid and FAME concentrations in pH controlled cultures amended with 50 mM HCO_3^- than in the pH controlled cultures not given 50 mM HCO_3^-. The increased cell dry weight may be due to the increase of dissolved inorganic carbon present in the growth medium. Furthermore, the speciation of the available inorganic carbon might play a role. In cultures that stop cellular replication, the pH was above 10.5 and carbonate is the dominant inorganic carbon species. With the pH value being maintained between 8.1 and 8.5 through pH control, bicarbonate accounted for nearly all of the inorganic carbon in the growth medium. Therefore, if the pH goes well above 9.3, high concentrations of CO_3^{2-} ions are present and might stop cellular growth in WC-1 during the uptake of bicarbonate.

Unlike WC-1, strain SLA-04 did not show an increase in TAG or FAME content upon the addition of $NaHCO_3$. TAG and FAME contents were actually significantly lower in cultures amended with 50 mM $NaHCO_3$ near nitrogen depletion on both a percent w/w basis and a concentration basis (Figure 12a,b). Cell dry weight decreased in

all cultures supplied with 50mM HCO_3^- (Table 3) which exacerbates the differences in TAG and FAME concentrations. This result may indicate medium nitrate was depleted at the time of $NaHCO_3$ addition (Table 4) or SLA-04 not being able to utilize added inorganic carbon for lipid production under these conditions. In this study, the determination of the time of nitrogen depletion was based on medium ammonium concentration because ammonium constitutes the majority of the nitrogen present in primary clarifier effluent. Previous studies found bicarbonate addition just prior to nitrate depletion was the ideal time to trigger lipid accumulation (Gardner et al. 2012), so the form of nitrogen being assimilated may be a factor in whether timely bicarbonate addition will increase cellular TAG concentration.

When using primary clarifier effluent, multiple forms of nitrogen are present (i.e. ammonium, nitrate and organic nitrogen), so the correct time to provide cultures with 50 mM HCO_3^- may be difficult to discern. Maestrini et al. (1986) reported that algae prefer ammonium over nitrate, and nitrate is not assimilated until ammonium is nearly depleted. Therefore, using ammonium depletion as an indicator for the addition of 50 mM HCO_3^- may not be ideal because cultures are likely still replete in nitrate at that time. Instead, both ammonium and nitrate should be monitored in wastewater cultures, and the 50 mM HCO_3^- addition may be more effective when both forms of nitrogen are nearly depleted.

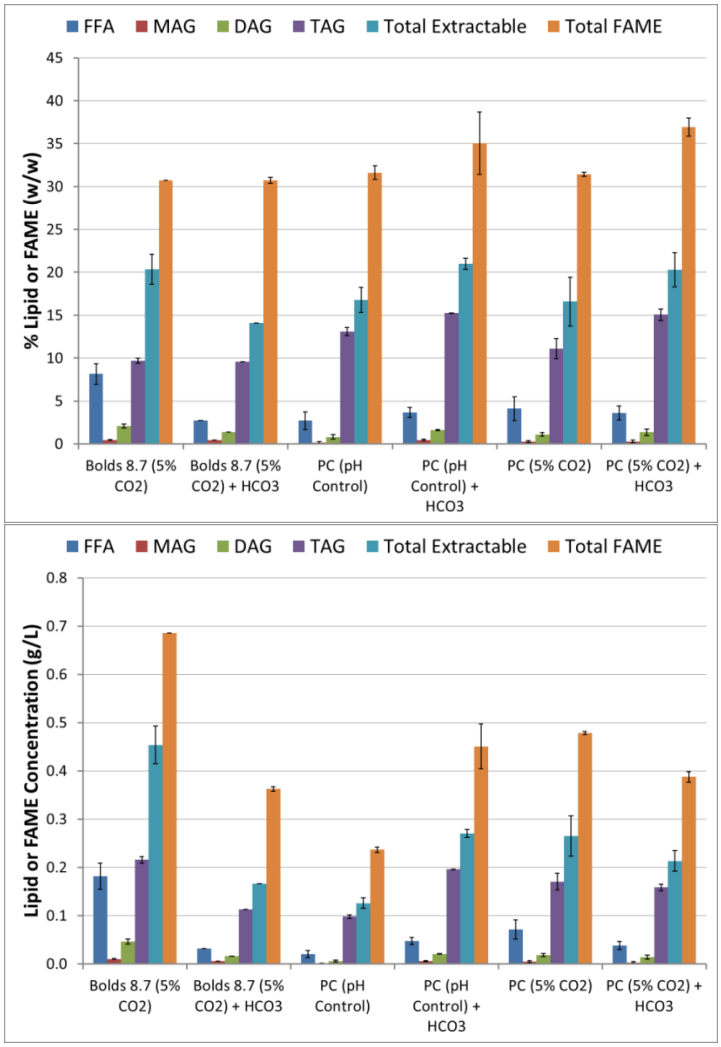


Figure 11. Lipid and FAME content, in %w/w (top) and concentration (bottom), of WC-1.

Although the bicarbonate "trigger" did not have the desired effect, SLA-04 produced high lipid and FAME concentrations in both sterile Bold's 8.7 medium and wastewater cultures. SLA-04 grown in wastewater under a continuous sparge of 5% CO_2 achieved 27% (w/w) and 0.39 g/L of total extractable lipid. FAME content and FAME concentration of SLA-04 under the same conditions reached 50% (w/w) and 0.72 g

FAME/L, respectively. Ji et al. (2013) reported a lipid content and lipid concentration of 30% (w/w) and 0.087 g/L, respectively, for *Chlorella vulgaris* grown in municipal wastewater. The large difference in lipid concentration between the current study and the Ji et al. manuscript is due to a substantial difference in cell dry weight (1.2-1.7 g/L compared to 0.3 g/L). Li et al. (2011) achieved 11% (w/w) and 0.12 g/L FAME in an isolated *Chlorella* species grown in untreated wastewater.

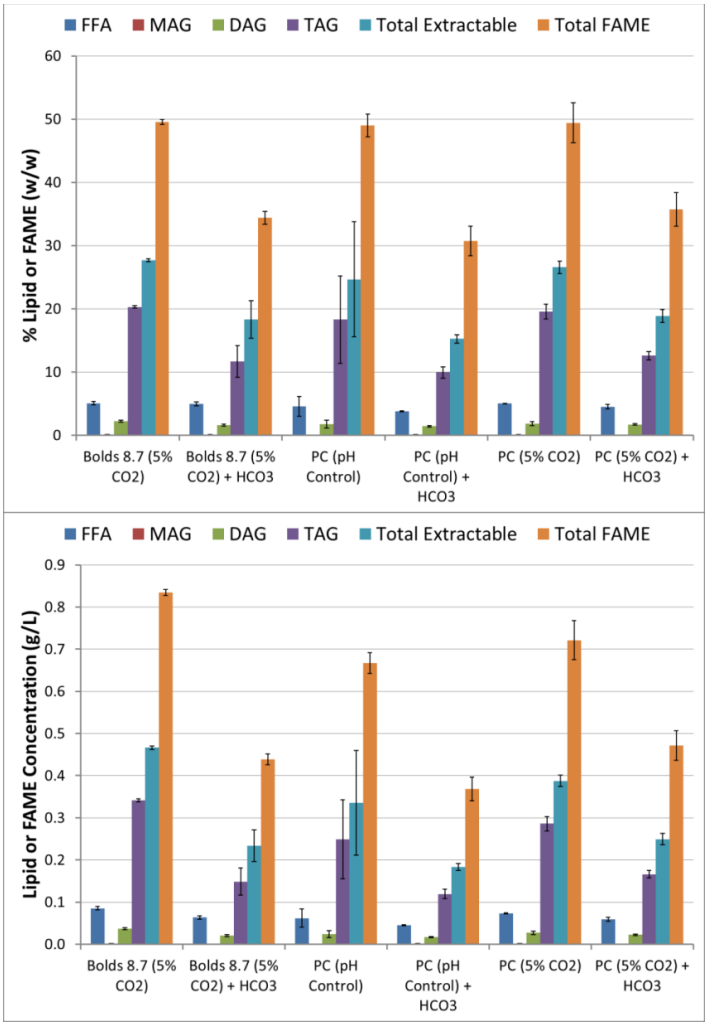


Figure 12. Lipid and FAME content, in %w/w (top) and concentration (bottom), of SLA-04.

Conclusions

This study evaluated the growth, nutrient utilization, lipid production and biodiesel potential of two freshwater microalgae grown in municipal wastewater. Experiments were conducted in tubular bioreactors supplemented with several forms of inorganic carbon to determine what effect the inorganic carbon differences displayed. Strains WC-1 and SLA-04 grew in untreated primary clarifier effluent with growth characteristics similar to those in sterile Bold's basal medium. The two strains utilized the ammonium, nitrate, and phosphate present in the wastewater, and high removal efficiencies were achieved. Although the addition of bicarbonate generally had unexpected results with respect to TAG synthesis, considerable FAME production was achieved by both strains.

CHAPTER 5

GROWTH OF WC-1 AND SLA-04 IN RECYCLED HARVEST WATER SUPPLEMENTED WITH ANAEROBIC DIGESTATE

Introduction

The experiments presented in this chapter evaluated whether strains WC-1 and SLA-04 could grow in recycled harvest water supplemented with anaerobic digestate. Recycling harvest water may be a necessary component of large-scale algae production facilities (Chowdhury et al. 2012; Guieysse et al. 2013; Zhu et al. 2013), and determining if algae can grow in water that has been reused multiple times is a crucial step toward implementing the recycle stream. Furthermore, results shown in Chapter 3 suggested WC-1 and SLA-04 sustained growth through the utilization of anaerobically digested nutrients, so amending recycled harvest water with anaerobic digestate may provide low-cost, bioavailable nutrients for algal growth. The experiments performed in this chapter aimed to build upon the results of Chapters 3 and 4 by continuing to develop methods for algal growth using low-quality water and nutrient sources.

Methods

Strains WC-1 and SLA-04 were grown in 1.5 L tubular, air-lift reactors using primary clarifier effluent from the Bozeman Water Reclamation Facility (BWRF) as growth medium. Cells were cultured until medium nitrogen, measured as ammonium, was depleted and growth reached the stationary phase. The cultures were then

centrifuged to pellet the biomass, and the mostly algae-free supernatant (harvest water) was used for the next generation of growth. Four growth conditions were initially evaluated using recycled harvest water in 250 mL shaker flasks equipped with cloth caps to facilitate gas transfer: (1) recycled harvest water without any additions, (2) recycled harvest water supplemented with $NaNO_3$ to reach 41 mg $N\text{-}NO_3^-$/L (equal to the nitrate concentration of Bold's basal medium), (3) recycled harvest water supplemented with anaerobic digestate to reach 41 mg $N\text{-}NH_4^+$/L, and (4) recycled harvest water supplemented with anaerobic digestate to reach 41 mg $N\text{-}NH_4^+$/L and $FeCl_3$ to reach 1 mg Fe/L (equal to the iron concentration of Bold's basal medium). Iron was added in condition (4) because previous work (Chapter 3) had demonstrated decreased growth and decreased chlorophyll content due to iron deficiency. No growth was observed in condition (1), so no results are shown for growth in harvest water without any additions. Once medium nitrogen was depleted in first generation growth using recycled harvest water, cultures were centrifuged again to pellet the biomass, and the harvest water was removed to be reused. For generations 2 and 3, make-up water was added back to the harvest water to compensate for water lost from sampling, during harvest and through evaporation for all cultures other than those with NO_3^- addition. Make-up water was added either in the form of primary clarifier effluent (PC) or tap water (Tap) to achieve a 1:2 dilution of recycled harvest water. The initial nitrogen concentrations in all cultures were intended to be approximately 41 mg N/L either in the form of NH_4^+ or NO_3^-. A schematic of the experimental design is shown in Figure 13. Cell concentrations, pH, chlorophyll and medium nitrogen concentrations were monitored throughout each

experiment. Cell dry weight (CDW) and lipid profiles were also analyzed at the end of 2nd and 3rd generation growth of WC-1.

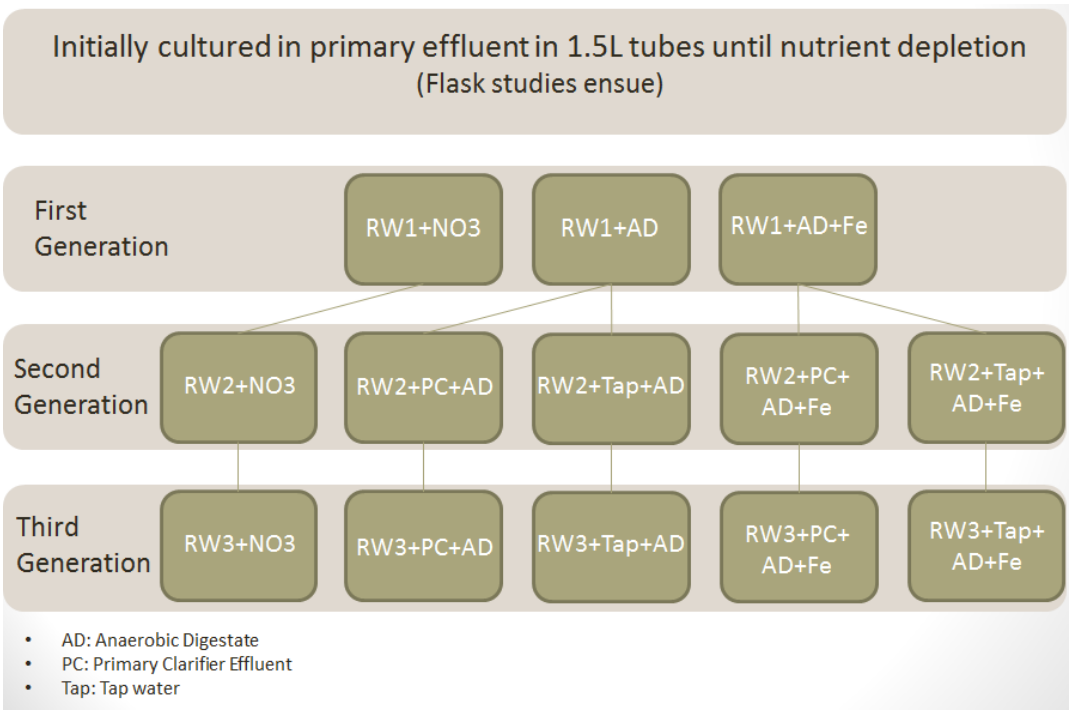


Figure 13. Schematic of recycled harvest water studies.

WC-1 Growth in Recycled Harvest Water

The average cell concentrations and pH values of each condition during three generations of WC-1 growth in recycled harvest water are shown in Figure 14. The first and second generation experiments were carried out for 8 days and third generation growth was evaluated for 10 days. Recycling harvest water caused no noticeable inhibition between the 1st and 2nd generations of growth based on cell concentration and cell doublings. The average number of cell doublings either increased or remained constant during the 2nd generation compared to the 1st generation (Table 5). Furthermore,

the 2nd generation cultures with added $NaNO_3$ (RW2+NO3) exhibited approximately 1.5 more cell doublings than the 1st generation cultures amended with $NaNO_3$ (RW+NO3).

Irregular trends (e.g. premature stationary phase followed by subsequent growth in RW3+AD+PC cultures) were observed during 3rd generation growth in recycled harvest water amended with anaerobic digestate (Figure 14). As previously stated, cultures were supposed to begin with approximately 41 mg N/L either in the form of NH_4^+ or NO_3^-, but the initial nitrogen concentration in the 3rd generation growth experiment was nearly twice the intended concentration for cultures supplemented with anaerobic digestate (Figure 15). The large initial nitrogen concentration was likely due to an error in measuring the nitrogen content of collected anaerobic digestate. Third generation growth displayed a general decrease in cell doublings and growth rates for cultures amended with anaerobic digestate (Table 5, Figure 16). However, the growth rate actually increased in the cultures grown on nitrate in the 3rd generation. The observation of decreasing growth rates in the cultures amended with anaerobic digestate suggests ammonia inhibition due to the higher ammonium concentrations rather than an effect of recycling harvest water. If the recycled harvest water was the cause of inhibition, one would expect the cultures amended with $NaNO_3$ to also show decreased cell doublings and growth rates in the 3rd generation experiment.

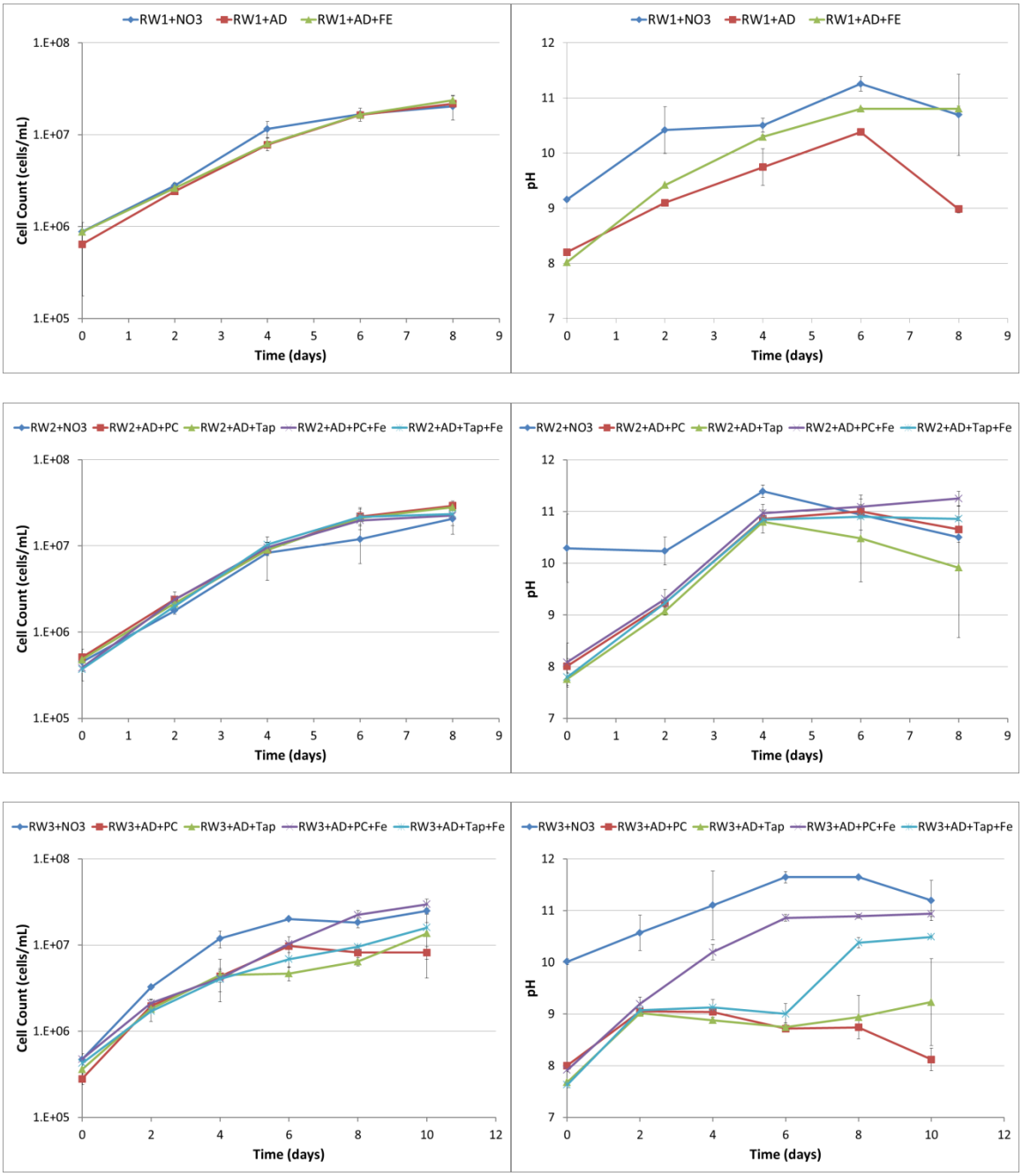


Figure 14. Average cell density and pH values of triplicate treatments during 1st generation (top row) growth (±standard deviation) and duplicate treatments during 2nd (middle row) and 3rd (bottom row) generation growth (± range) for WC-1.

Table 5. The average number of cell doublings in each generation of WC-1 growth in recycled harvest water. Cell doublings=$\log(C_f/C_i)/\log(2)$ where C_f is the cell concentration at harvest and C_i is the initial cell concentration. 1st generation values are the averages of triplicate treatments ± standard deviation, and 2nd and 3rd generation values are the averages of duplicate treatments ± range.

	RW+NO3	RW+AD	RW+AD+Fe		
1st Generation	4.03 ± 0.40	5.61 ± 1.86	4.76 ± 0.17		
	RW+NO3	**RW+PC+AD**	**RW+TAP+AD**	**RW+PC+AD+Fe**	**RW+TAP+AD+Fe**
2nd Generation	5.54 ± 0.35	5.84 ± 0.03	5.87 ± 0.12	5.87 ± 0.01	5.95 ± 0.69
3rd Generation	5.72 ± 0.12	4.87 ± 0.04	5.17 ± 0.56	5.97 ± 0.42	5.23 ± 0.17

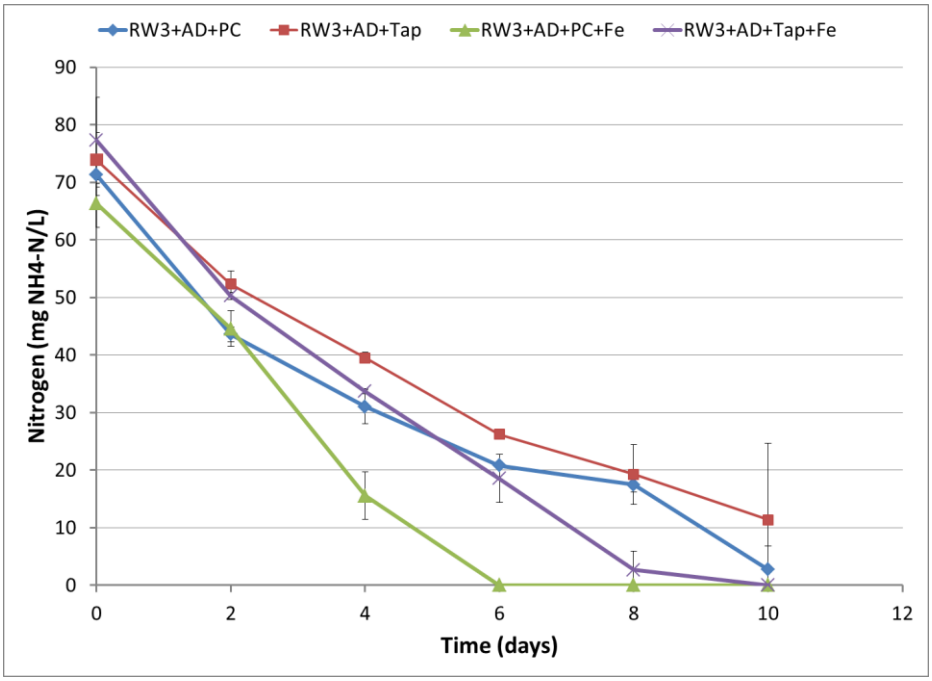


Figure 15. Nitrogen (measured as ammonia) utilization by WC-1 over time during the 3rd generation experiment. Initial nitrogen concentrations were roughly twice as high as the intended initial nitrogen concentration of 41 mg N/L likely due to an error in measuring the nitrogen content of the collected anaerobic digestate.

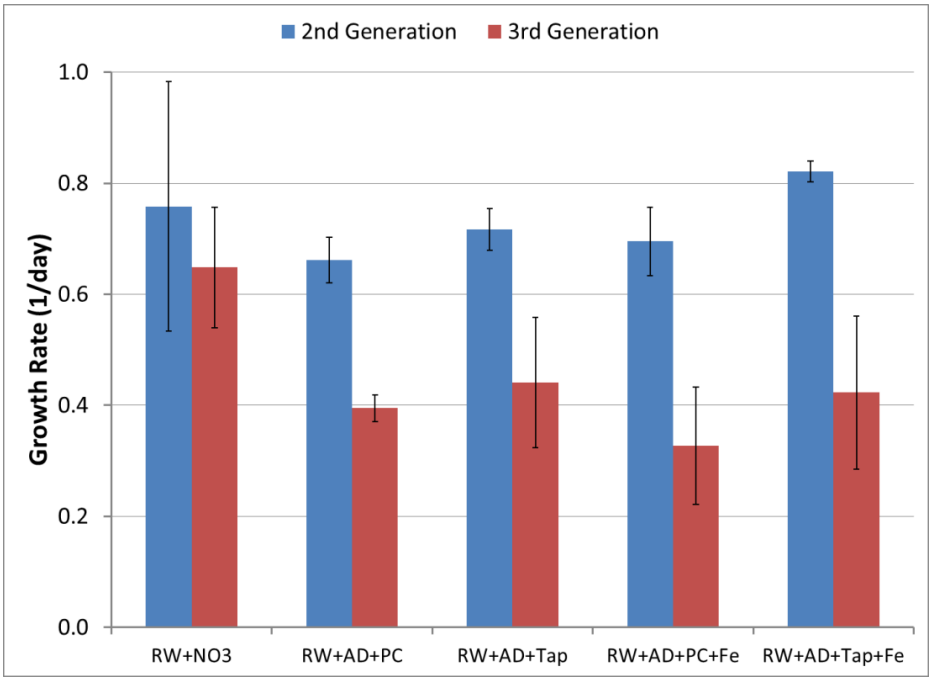


Figure 16. Average growth rate of WC-1 for 2nd and 3rd generation growth experiments. Growth rates were calculated between day 2 and day 4 for each treatment during the respective generation of growth.

The importance of iron addition for the cultures amended with anaerobic digestate can be seen in the 3rd generation of growth. Cultures amended with iron achieved the highest chlorophyll content (Figure 17), approximately as high as cultures grown in recycled harvest water with added nitrate (RW3+NO3). Higher chlorophyll content in cultures with added iron may indicate an iron limitation in the anaerobic digestate relative to the other nutrients. An iron limitation could reduce the ability of these cultures to fix carbon efficiently (Chapter 3). Correspondingly, the supplementation with iron might have increased the ability of cultures to fix carbon as indicated by higher pH values in the growth medium (Figure 14). For instance, the RW3+AD+PC+Fe and the RW3+AD+Tap+Fe cultures both displayed higher pH values compared to the non-Fe-

supplemented treatments (Figure 14). As the pH increased above the pKa of ammonia/ammonium (pKa=9.25), the majority of the nitrogen in those systems would have been in the form of ammonia (NH_3). It is possible that the free NH_3 in the cultures was volatized leading to reduced inhibitory effects.

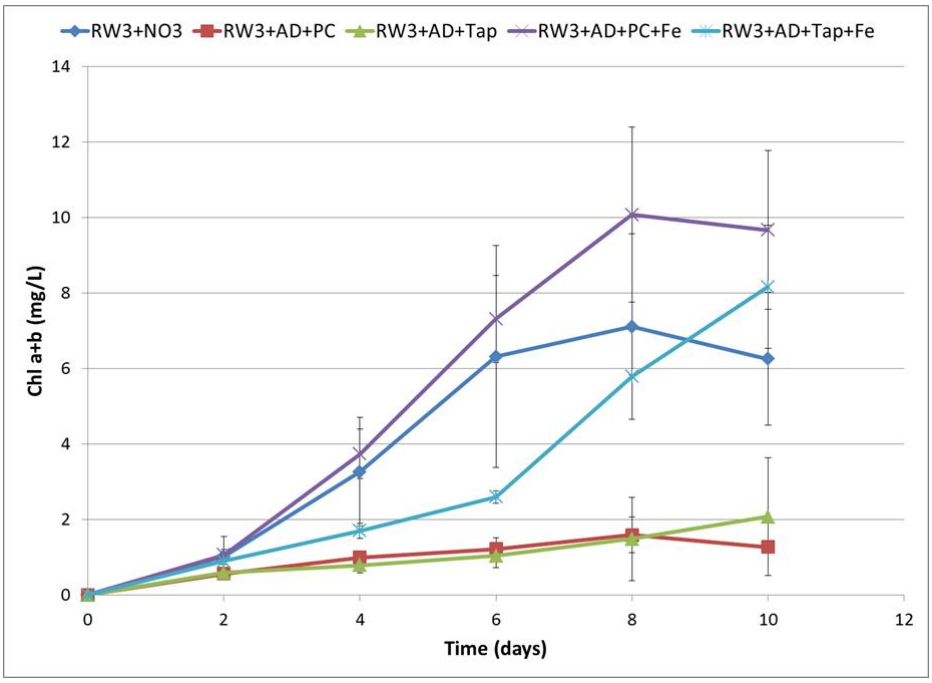


Figure 17. Chlorophyll a and b concentrations of WC-1 for the 3rd generation growth experiment. The chlorophyll content of anaerobic digestate cultures with added iron reached a much higher concentration than AD cultures without added iron.

Lower yields (g-biomass/g-nitrogen) in the third generation WC-1 growth experiment compared to the second generation experiment suggests a large amount of nitrogen was volatilized out of 3rd generation cultures instead of being assimilated into biomass (Figure 18). 3rd generation biomass yields were 55% to 88% less than 2nd generation yields for AD-amended cultures. Furthermore, Figure 19 shows pH and nitrogen concentration over time for a treatment that received an iron addition

(RW3+AD+PC+Fe) and a treatment without an iron addition (RW3+AD+Tap). The pH of the iron-added cultures increased above 9.2 after two days into the experiment, and ammonia was depleted by day 6. In the treatment without added iron, the pH remained below 9.2 throughout the experiment, and ammonia was still present after ten days of growth. Therefore, the addition of iron may have led to higher photosynthetic activity that increased the pH to a level where free NH_3 constituted the majority of nitrogen present which volatilized from the solution. In all cases, the supplementation with iron increased growth and lead to higher cell dry weights at harvest for 3rd generation growth (Figure 20).

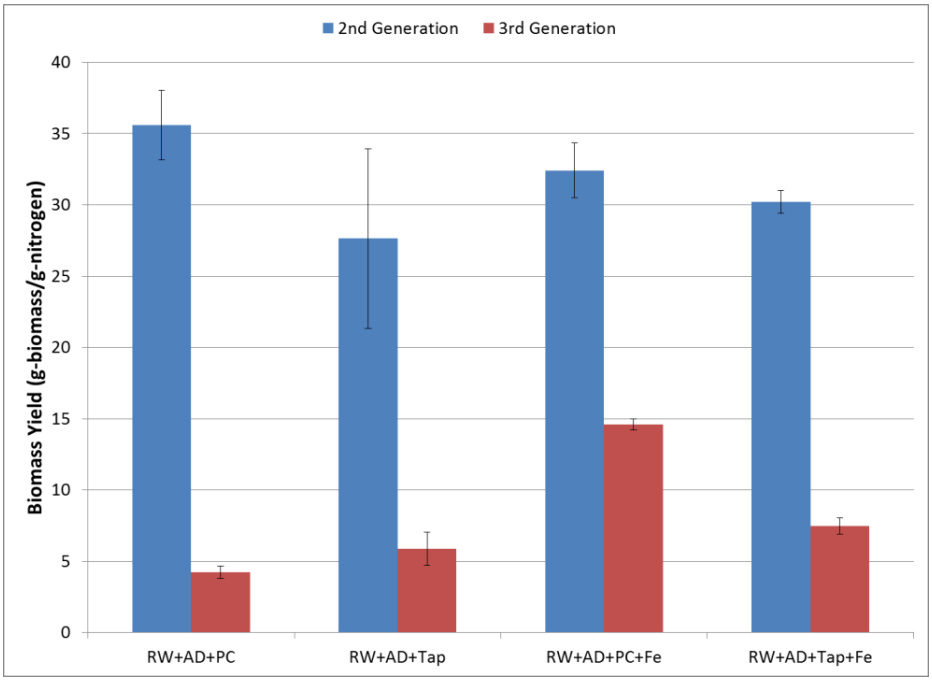


Figure 18. Biomass yields (g biomass/g nitrogen) of AD-amended WC-1 cultures at the time of harvest for 2nd and 3rd generation growth. 3rd generation yields experienced a significant decrease likely due to ammonia volatilization.

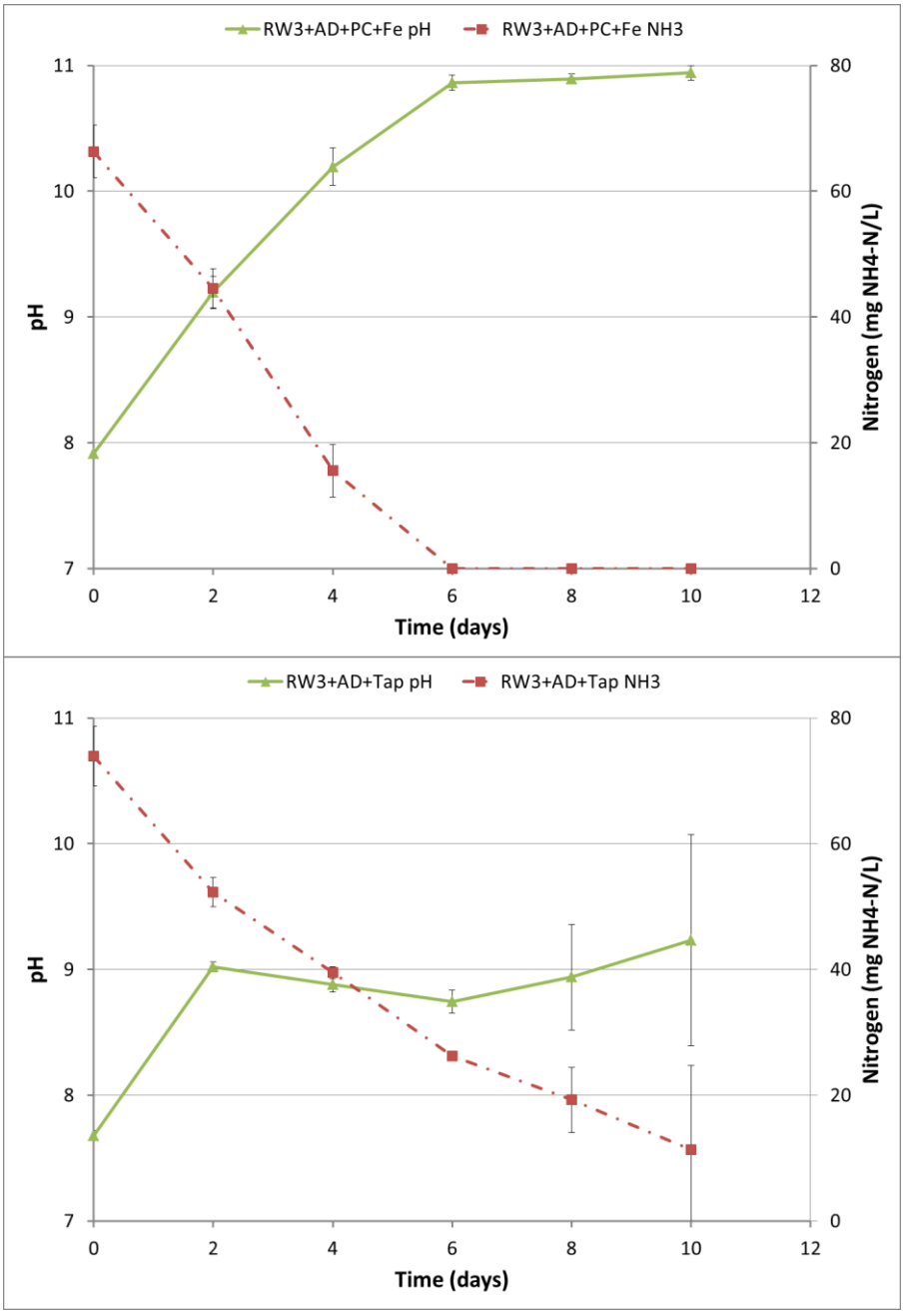


Figure 19. pH (primary vertical axis) and nitrogen concentration (secondary vertical axis) over time for RW3+AD+PC+Fe (top) and RW3+AD+Tap (bottom). The pH of the iron-amended condition (top) increased above the pKa of ammonium/ammonia (pKa=9.25), and nitrogen was depleted by day 6. The pH of the condition without iron (bottom) remained below 9.25 for the majority of the experiment, and nitrogen was present at the time of harvest (day 10).

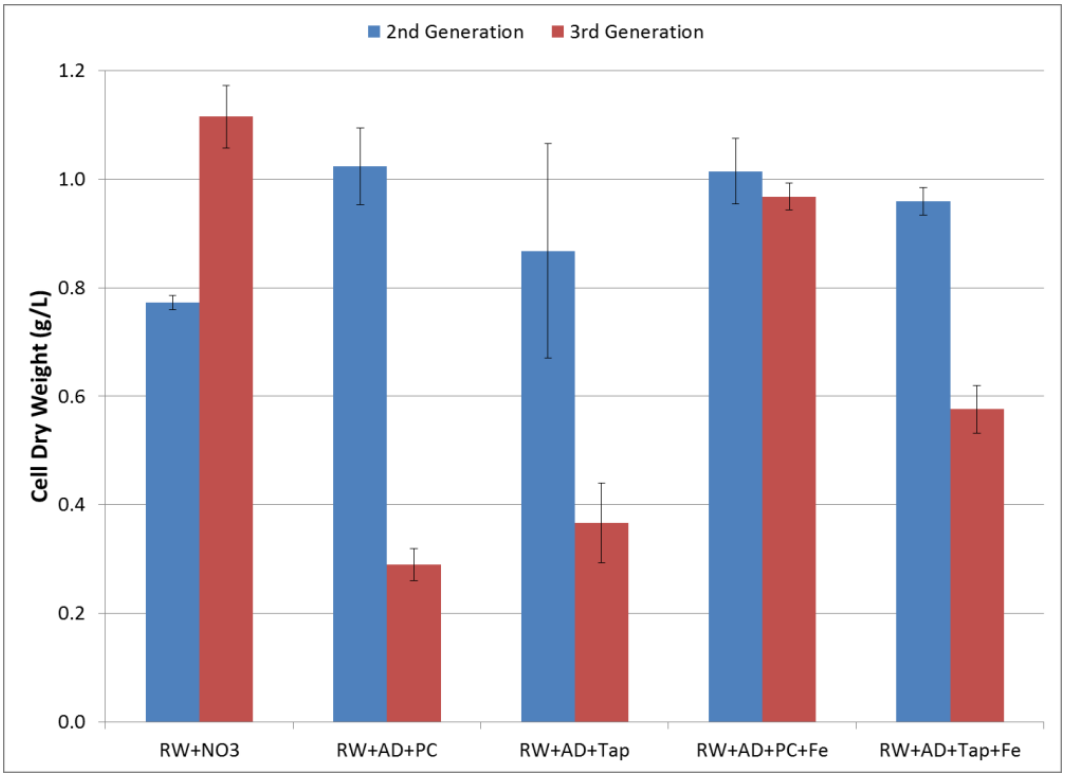


Figure 20. Cell dry weight values of WC-1 at the time of harvest for 2nd and 3rd generation growth. 3rd generation cell dry weights generally decreased for anaerobic digestate cultures with the exception of RW+AD+PC+Fe.

2nd and 3rd Generation Lipid Content of WC-1

Lipid extractions were performed at the time of harvest on the 2nd and 3rd generations of growth of WC-1 (Figures 21 and 22). The total extractable lipid contents of WC-1 cultures amended with iron were significantly higher than for cultures without iron in the 2nd generation experiment (Figure 21). The condition with the highest lipid content (RW2+AD+PC+Fe) reached roughly 15% (w/w) total lipid which is comparable to the lipid content achieved by WC-1 grown in fresh primary clarifier effluent. Although the recycled harvest water condition reached similar total lipid content compared to growth in freshly collected primary clarifier effluent, the triacylglyceride

(TAG) content was much higher in the fresh primary clarifier effluent. The difference is likely due to the time of harvest for each condition. The fresh primary clarifier effluent cultures were harvested roughly 4 days after nitrogen depletion while the recycled harvest water cultures were harvested directly after nitrogen depletion.

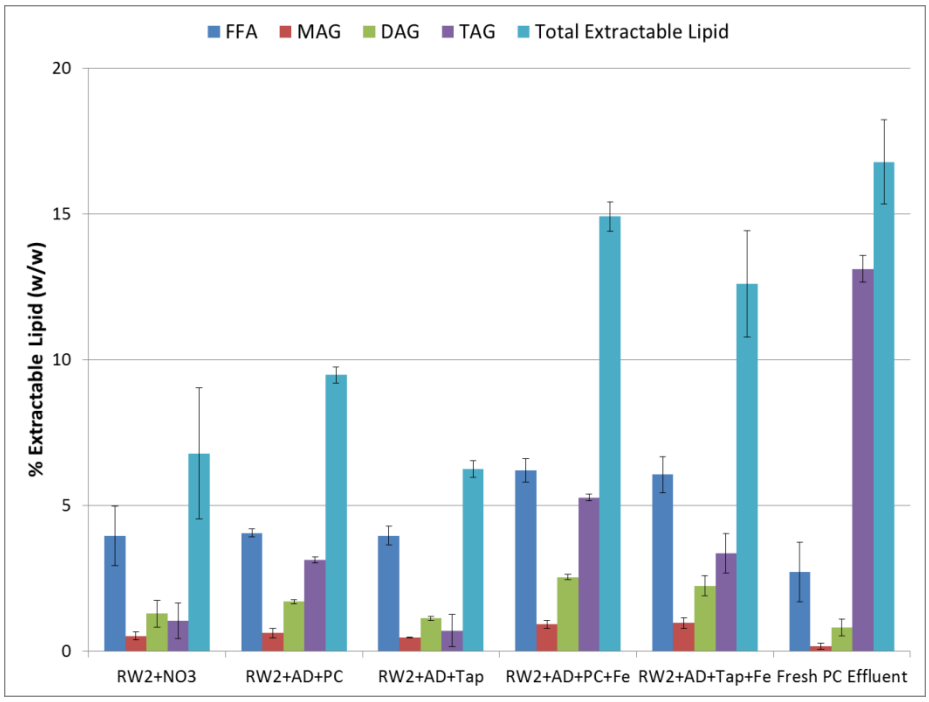


Figure 21. Extractable lipid content (% w/w) of WC-1 at the time of harvest of the 2nd generation growth experiment (t=8 days). Graph shows free fatty acids (FFA), monoacylglycerides (MAG), diacylglycerides (DAG), triacylglycerides (TAG) and total extractable lipid (TOTAL) contents of each condition. The lipid profile of WC-1 grown in freshly collected primary clarifier effluent is also shown. Recycled harvest water conditions were harvested just after nitrogen depletion, and the "Fresh PC Effluent" cultures were harvested roughly 5 days after nitrogen depletion.

The inhibition of growth in the 3rd generation resulted in a large decrease in lipid content (Figure 22). Cultures amended with anaerobic digestate reached a maximum of roughly 5% (w/w) total lipid with the poorest lipid accumulating condition (RW3+AD+PC) reaching 2.4% (w/w) total lipid (Figure 22). However, the 3rd

generation cultures grown on nitrate increased in extractable lipid compared to 2nd generation cultures (cf. Figures 21 & 22). This increase in lipid content could be due to extended nitrogen deplete conditions experienced in the 3rd generation where cultures were harvested 10 days after inoculation compared to 8 days after inoculation in 2nd generation cultures.

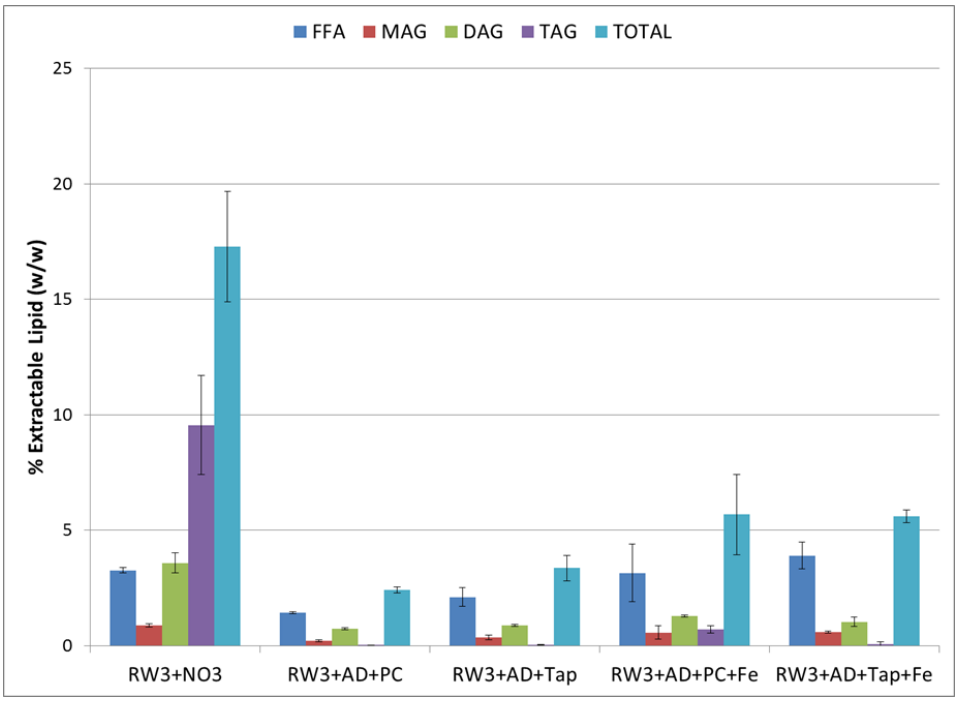


Figure 22. Extractable lipid content (% w/w) of WC-1 at the harvest of the 3rd generation growth experiment (t=10 days). Graph shows free fatty acids (FFA), monoacylglycerides (MAG), diacylglycerides (DAG), triacylglycerides (TAG) and total extractable lipid (TOTAL) contents of each condition.

SLA-04 Growth in Recycled Harvest Water

The average cell concentrations and pH values of each condition during three generations of SLA-04 growth in recycled harvest water are shown in Figure 23. The first and second generation experiments were carried out for 8 days and third generation

growth was evaluated for 6 days. Recycling harvest water appears to have no inhibitory effect on the growth of SLA-04 over three generations. Cell concentrations reached approximately 6 x 10^7 cells/mL in each generation. A slight decrease in the number of cell doublings occurred in the 2nd generation compared to the 1st generation, but the average number of cell doublings actually increased for each condition from the second to the third generation (Table 6). Cultures supplemented with $NaNO_3$ (RW+NO3), which had no make-up water added during any generation, experienced a decrease of 1.34 cell doublings between the 1st and 2nd generation. Third generation cell doublings increased in this treatment back to similar levels achieved during the first generation. Third generation cultures amended with primary clarifier make-up water and anaerobic digestate (RW3+PC+AD) exhibited 2.39 more cell doublings than the second generation (Table 6), the largest cell doubling increase of any condition.

Table 6. Average number of cell doublings (± standard deviation, n=3 (1st generation), ± range, n=2 (2nd and 3rd generation)) for each condition in 1st, 2nd and 3rd generation growth of SLA-04.

	RW+NO3	**RW+AD**	**RW+AD+Fe**		
1st Generation	6.06 ± 1.00	7.55 ± 0.58	7.93 ± 0.32		
	RW+NO3	**RW+PC+AD**	**RW+TAP+AD**	**RW+PC+AD+Fe**	**RW+TAP+AD+Fe**
2nd Generation	4.72 ± 0.04	6.16 ± 0.31	5.81 ± 0.63	6.17 ± 0.05	6.25 ± 0.07
3rd Generation	5.76 ± 0.39	8.55 ± 0.05	7.43 ± 0.06	8.07 ± 0.50	8.54 ± 0.40

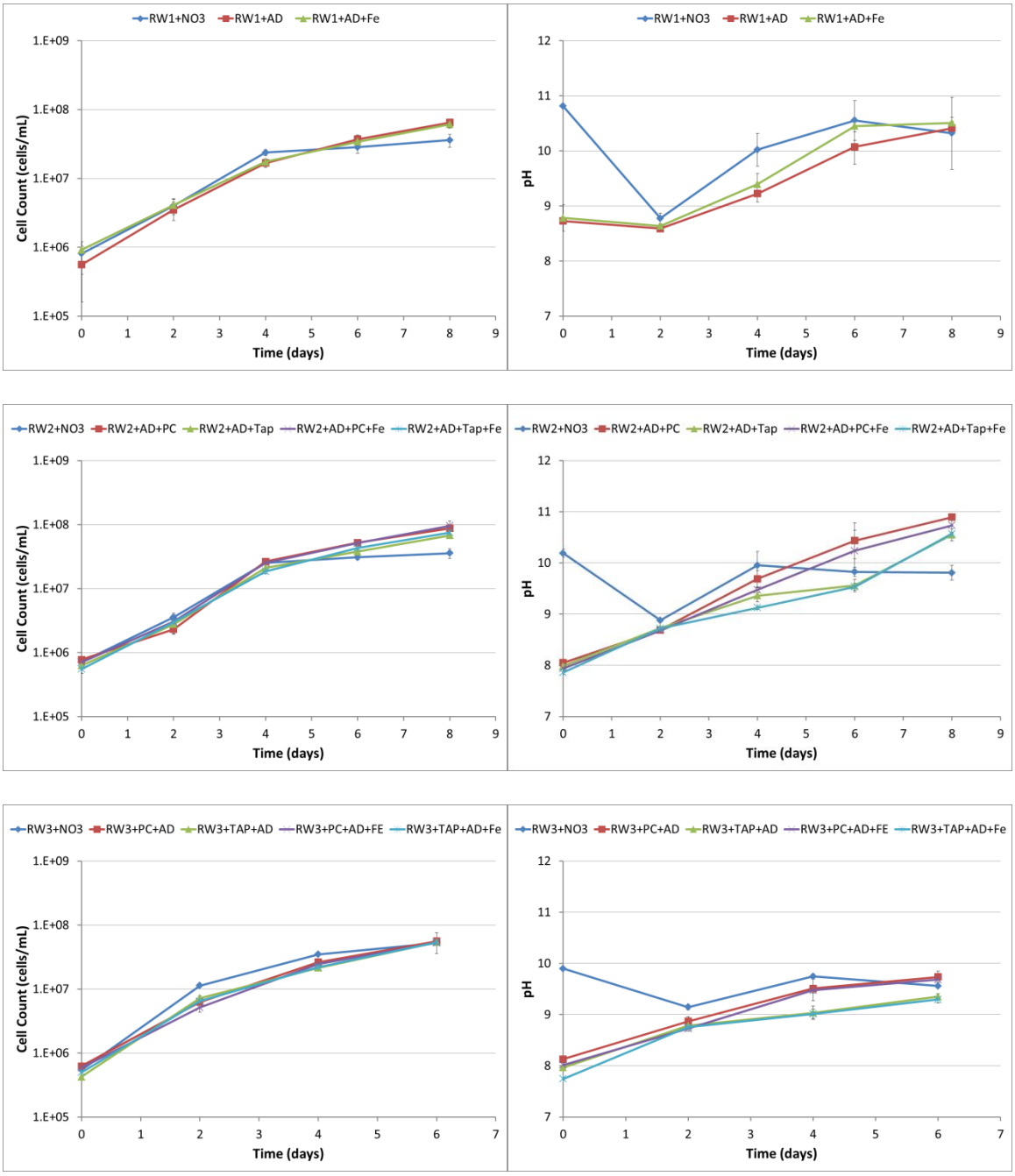


Figure 23. Average cell density and pH values of triplicate treatments during 1st generation growth (±standard deviation) and duplicate treatments during 2nd and 3rd generation growth (± range) for SLA-04.

Conclusions

The work presented in this chapter evaluated the ability to grow strains WC-1 and SLA-04 in recycled harvest water. Each alga was grown in recycled harvest water for three generations to determine if growth inhibition occurred in the reused water. WC-1 showed no inhibition after two generations of growth, but excess ammonia present in cultures amended with anaerobic digestate during the 3rd generation caused a decrease in cell doublings, growth rates and cell dry weights. Although growth decreased in cultures supplemented with anaerobic digestate, WC-1 grew without inhibition during each generation for cultures amended with $NaNO_3$. SLA-04 showed no inhibition after three generations of growth in recycled harvest water. Cell concentrations and cell doublings were very similar in each generation. Further analyses need to be performed to compare cell dry weights and lipid contents achieved in each condition for SLA-04.

CHAPTER 6

CONCLUSIONS

Project Rationale

Microalgae are continually being studied as a biofuel feedstock, but many technical hurdles must be overcome to ensure these organisms can be used as a sustainable source of fuel. Along with finding robust strains of algae, adequate nutrient and water sources must be identified so that the production of algal biofuels does not put further stress on dwindling resources. Optimizing production methods will ensure that nutrients and water are efficiently used and costs are minimized.

This thesis summarizes the work completed to optimize the growth and lipid production of two isolated, alkaliphilic microalgae in various municipal wastewater streams, and demonstrates how nutrients and water can be recycled in the process. Two strains, *Scenedesmus* sp. WC-1 and *Chlorella* sp. SLA-04, were initially grown in primary clarifier effluent and diluted anaerobic digestate. *Navicula* sp. RGd-1 was also screened, but the organism was unable to sustain growth. WC-1 and SLA-04 were then cultured in primary clarifier effluent using several inorganic carbon addition strategies to evaluate the effects that different forms and concentrations of inorganic carbon had on growth and lipid production. Finally, harvest water was collected and amended with anaerobic digestate to elucidate whether WC-1 and SLA-04 could be grown using recycled water and nutrients.

Attainment of Project Aims

The project goals were to:

(i) Identify robust microalgae that are capable of sustaining growth in untreated municipal wastewater.

(ii) Vary the species and concentration of the inorganic carbon supplied to algal cultures in order to optimize growth and lipid production.

(iii) Integrate recycle streams in order to decrease the water footprint and nutrient demand required by large-scale algal growth.

(i) Identification of Robust Microalgae

Scenedesmus sp. WC-1 and *Chlorella* sp. SLA-04 each grew in primary clarifier effluent (PC) and diluted anaerobic digestate (AD). WC-1 was able to grow in PC to cell concentrations similar to those in the Bold's basal medium control cultures for each of the three generations of growth. SLA-04 grown in PC was unable to reach similar cell concentrations achieved in Bold's 8.7 without the addition of supplemental nitrogen, so nitrogen likely needs to be added either as a laboratory or industrial grade compound or as a recycling stream to reach optimal cell density. The amount of supplementary nitrogen likely depends on the nitrogen concentration initially present in PC.

Undiluted AD and 1:5 diluted AD cultures did not sustain growth likely due to ammonium inhibition and/or increased light attenuation due to high turbidity. Other dilutions of AD, 1:10, 1:25 and 1:50, were able to sustain growth of WC-1 and SLA-04. Growth inhibition in AD cultures was found to be due to a deficiency in iron, an

important element in nitrogen assimilation and photosynthetic processes (Cai et al. 2013; Greene et al. 1992; Terry and Abadía 1986). Cultures were able to grow better when iron was supplemented to cultures at inoculation. The addition of iron may have stimulated chlorophyll synthesis, enabling those cultures to fix inorganic carbon more efficiently than cultures without added iron.

(ii) Growth on Varying Inorganic Carbon Sources

The study presented here evaluated the growth, nutrient utilization, lipid production and biodiesel potential of two freshwater microalgae grown in municipal wastewater. Experiments were conducted in tubular bioreactors supplemented with several regimes of inorganic carbon to test what effect the form and concentration of inorganic carbon present in the medium had on growth and lipid accumulation of strains WC-1 and SLA-04. Both strains achieved similar growth rates and cell dry weights in wastewater whether given continuous 5% CO_2 sparge or under periodic sparges to control the culture pH. This result suggests that cultures can grow while using less carbon dioxide which may be very important for industrial applications, because large-scale algal biofuel facilities may require an unattainable amount of CO_2 from stationary emission sources (Pate et al. 2011). The two strains were able to utilize the ammonium, nitrate, and phosphate present in the wastewater, and high removal efficiencies of nitrogen and phosphorus were achieved. Nitrogen and phosphorus must be removed from wastewater to meet EPA regulations and prevent the eutrophication of ecosystems downstream from wastewater treatment plants. The efficient nitrogen and phosphorus removal by strains WC-1 and SLA-04 suggests these organisms may be able to be utilized for wastewater

remediation while also producing a biofuel feedstock. The 50 mM bicarbonate addition did not result in increased TAG content (% w/w) in either WC-1 or SLA-04, and the lipid productivity (g/L) decreased for each strain following the addition of 50 mM HCO_3^- due to decreased cell dry weights in those cultures. Although the addition of bicarbonate generally had unexpected results with respect to TAG synthesis, considerable FAME production was achieved by culturing both strains in domestic wastewater.

(iii) Nutrient and Water Recycling

WC-1 was able to grow in recycled water supplemented with anaerobic digestate without inhibition for two generations. Growth after the third harvest water recycle displayed inhibition in cultures supplemented with anaerobic digestate, but an analysis error involving collected anaerobic digestate caused the initial ammonium concentration in 3rd generation cultures to be nearly twice as high as intended. The high level of ammonium likely caused the inhibition due to the amount of free ammonia in the cultures. Similar to results discussed in (i), the addition of iron resulted in increased chlorophyll concentrations of WC-1 cultures.

SLA-04 was able to grow without inhibition over three generations of growth in recycled harvest water. The number of cell doublings and maximum growth rates actually increased in the 3rd generation of growth, which suggests that inhibitory compounds released in the medium either had no effect on SLA-04 or were diluted to non-inhibitory levels upon the addition of make-up water.

Future Work

Several issues can be resolved through future work to advance algal biofuels closer to commercialization. Exploring additional methods of water and nutrient recycling would be one of the most pertinent areas to explore. In addition to studying whether water can be reused multiple times, developing low-energy methods of biomass separation would be beneficial for reducing process costs. Investigation into nutrient recycling through hydrothermal liquefaction and pyrolysis may elucidate whether these processes are able to recycle nitrogen and phosphorus not incorporated into biofuels and other bioproducts. Determining the nutrient recovery efficiencies of each process may provide insight for large-scale facilities in the future.

In addition to exploring the feasibility of process integration, bench-top experiments involving algal growth in wastewater would need to be scaled-up to pilot-scale and full-scale applications to investigate how selected microalgae perform in environments more similar to "real world" industrial production conditions. Massive amounts of algal biomass are needed to offset a significant percentage of transportation fuels, so further knowledge of how productive algae can be in non-sterile, outdoor conditions must be obtained.

REFERENCES

Amarasinghe, Upali, Tushaar Shah, Hugh Turral, and Bal Anand. 2007. *India's Water Future to 2025-2050: Business-as-Usual Scenario and Deviations*. Inernational Water Management Institute.

Aresta, Michele, Angela Dibenedetto, and Grazia Barberio. 2005. "Utilization of Macro-Algae for Enhanced CO_2 Fixation and Biofuels Production: Development of a Computing Software for an LCA Study." *Fuel Processing Technology* 86(14-15):1679–93.

Batan, Liaw, Jason Quinn, Bryan Willson, and Thomas Bradley. 2010. "Net Energy and Greenhouse Gas Emission Evaluation of Biodiesel Derived from Microalgae." *Environmental Science & Technology* 44(20):7975–80.

Behrenfeld, Michael J., Kirby Worthington, Robert Sherrell, Francisco Chavez, Peter Struton, Michael McPhaden, and Donald Shea. 2006. "Controls on Tropical Pacific Ocean Productivity Revealed through Nutrient Stress Diagnostics." *Nature* 442(7106):1025–28.

Bekheet, I. A., and P. J. Syrett. 1977. "Urea-Degrading Enzymes in Algae." *British Phycological Journal* 12(2):137–43.

Belkhodja, R., F. Morales, A. Abadia, J. Gomez-Aparisi, and J. Abadia. 1994. "Chlorophyll Fluorescence as a Possible Tool for Salinity Tolerance Screening in Barley (*Hordeum Vulgare L.*)." *Plant Physiology* 104(2):667–73.

Bhatnagar, Ashish, Monica Bhatnagar, Senthil Chinnasamy, and K. C. Das. 2010. "Chlorella Minutissima--a Promising Fuel Alga for Cultivation in Municipal Wastewaters." *Applied Biochemistry and Biotechnology* 161(1-8):523–36.

Biller, P., A. Ross, S. Skill, A. lea-Langton, B. Balasundaram, C. Hall, R. Riley, and C. Llewellyn. 2012. "Nutrient Recycling of Aqueous Phase for Microalgae Cultivation from the Hydrothermal Liquefaction Process." *Algal Research* 1(1):70–76.

Cai, Ting, Stephen Y. Park, and Yebo Li. 2013. "Nutrient Recovery from Wastewater Streams by Microalgae: Status and Prospects." *Renewable and Sustainable Energy Reviews* 19:360–69.

Chisti, Yusuf. 2007. "Biodiesel from Microalgae." *Biotechnology Advances* 25(3):294–306.

Chowdhury, Raja, Sridhar Viamajala, and Robin Gerlach. 2012. "Reduction of Environmental and Energy Footprint of Microalgal Biodiesel Production through Material and Energy Integration." *Bioresource Technology* 108:102–11.

Clarens, Andres F., Hagai Nassau, Eleazer P. Resurreccion, Mark A. White, and Lisa M. Colosi. 2011. "Environmental Impacts of Algae-Derived Biodiesel and Bioelectricity for Transportation." *Environmental Science & Technology* 45(17):7554–60.

Collet, Pierre, Arnaud Helias, Laurent Lardon, Monique Ras, Romy-Alice Goy, and Jean-Philippe Steyer. 2011. "Life-Cycle Assessment of Microalgae Culture Coupled to Biogas Production." *Bioresource Technology* 102(1):207–14.

Cordell, Dana, Jan-Olof Drangert, and Stuart White. 2009. "The Story of Phosphorus: Global Food Security and Food for Thought." *Global Environmental Change* 19(2):292–305.

Darley, W. M. 1982. "Algal Biology: A Physiological Approach." P. 168 pp. in *Basic Microbiology*. Oxford, UK: Blackwell Scientific Publications.

Davis, Ryan, Andy Aden, and Philip T. Pienkos. 2011. "Techno-Economic Analysis of Autotrophic Microalgae for Fuel Production." *Applied Energy* 88(10):3524–31.

Dismukes, G. Charles, Damian Carrieri, Nicholas Bennette, Gennady M. Ananyev, and Matthew C. Posewitz. 2008. "Aquatic Phototrophs: Efficient Alternatives to Land-Based Crops for Biofuels." *Current Opinion in Biotechnology* 19(3):235–40.

DOE, US. 2010. "National Algal Biofuels Technology Roadmap." *US Department of Energy, Office of Energy Efficiency and Renewable Energy*.

Eustance, Everett Robert Gardner, Karen Moll, Joseph Menicucci, Robin Gerlach, and Brent Peyton. 2013. "Growth, Nitrogen Utilization and Biodiesel Potential for Two Chlorophytes Grown on Ammonium, Nitrate or Urea." *Journal of Applied Phycology* 25(6):1663–77.

Fernández, Galván, and Quesada. 2004. "Nitrogen Assimilation and Its Regulation. The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*." *Advances in Photosynthesis and Respiration* 7:637–59.

Frank, Edward D., Amgad Elgowainy, Jeongwoo Han, and Zhichao Wang. 2012. "Life Cycle Comparison of Hydrothermal Liquefaction and Lipid Extraction Pathways to Renewable Diesel from Algae." *Mitigation and Adaptation Strategies for Global Change* 18(1):137–58.

Fricke, Klaus, Heike Santen, Rainer Wallmann, Axel Hüttner, and Norbert Dichtl. 2007. "Operating Problems in Anaerobic Digestion Plants Resulting from Nitrogen in MSW." *Waste Management (New York, N.Y.)* 27(1):30–43.

Fuggi, Amodio, V. Di Martino Rigano, Vincenza Vona, and C. Rigano. 1981. “Nitrate and Ammonium Assimilation in Algal Cell-Suspensions and Related pH Variations in the External Medium, Monitored by Electrodes.” *Plant Science Letters* 23(2):129–38.

Gardner, Robert, Keith Cooksey, Florence Mus, Richard Macur, Karen Moll, Everett Eustance, Ross Carlson, Robin Gerlach, Matthew Fields, and Brent Peyton. 2012. “Use of Sodium Bicarbonate to Stimulate Triacylglycerol Accumulation in the Chlorophyte *Scenedesmus* Sp. and the Diatom *Phaeodactylum Tricornutum*.” *Journal of Applied Phycology* 24(5):1311–20.

Gardner, Robert D., Egan Lohman, Robin Gerlach, Keith E. Cooksey, and Brent M. Peyton. 2013. “Comparison of CO2 and Bicarbonate as Inorganic Carbon Sources for Triacylglycerol and Starch Accumulation in *Chlamydomonas Reinhardtii*.” *Biotechnology and Bioengineering* 110(1):87–96.

Gardner, Robert, Patrizia Peters, Brent Peyton, and Keith E. Cooksey. 2010. “Medium pH and Nitrate Concentration Effects on Accumulation of Triacylglycerol in Two Members of the Chlorophyta.” *Journal of Applied Phycology* 23(6):1005–16.

Greene, R. M., R. J. Geider, Z. Kolber, and P. G. Falkowski. 1992. “Iron-Induced Changes in Light Harvesting and Photochemical Energy Conversion Processes in Eukaryotic Marine Algae.” *Plant Physiology* 100(2):565–75.

Greene, Richard M., Richard J. Geider, and Paul G. Falkowski. 1991. “Effect of Iron Limitation on Photosynthesis in a Marine Diatom.” *Limnology and Oceanography* 36:1772–82.

Greenwell, H. C., L. M. L. Laurens, R. J. Shields, R. W. Lovitt, and K. J. Flynn. 2010. “Placing Microalgae on the Biofuels Priority List: A Review of the Technological Challenges.” *Journal of the Royal Society, Interface / the Royal Society* 7(46):703–26.

Griffiths, M. J., R. P. van Hille, and S. T. L. Harrison. 2010. “Selection of Direct Transesterification as the Preferred Method for Assay of Fatty Acid Content of Microalgae.” *Lipids* 45(11):1053–60.

Groom, Martha J., Elizabeth M. Gray, and Patricia a Townsend. 2008. “Biofuels and Biodiversity: Principles for Creating Better Policies for Biofuel Production.” *Conservation Biology : The Journal of the Society for Conservation Biology* 22(3):602–9.

Guieysse, Benoit, Quentin Béchet, and Andy Shilton. 2013. “Variability and Uncertainty in Water Demand and Water Footprint Assessments of Fresh Algae Cultivation Based on Case Studies from Five Climatic Regions.” *Bioresource Technology* 128:317–23.

Hill, Jason, Erik Nelson, David Tilman, Stephen Polasky, and Douglas Tiffany. 2006. “Environmental, Economic, and Energetic Costs and Benefits of Biodiesel and Ethanol Biofuels.” *Proceedings of the National Academy of Sciences of the United States of America* 103(30):11206–10.

Hu, Qiang, Milton Summerfeld, Eric Jarvis, Maria Ghirardi, Matthew Posewitz, and Michael Seibert. 2008. “Microalgal Triacylglycerols as Feedstocks for Biofuel Production: Perspectives and Advances.” *The Plant Journal : For Cell and Molecular Biology* 54(4):621–39.

Ip, SY, JS Bridger, CT Chin, WRB Martin, and WGC Raper. 1982. “Algal Growth in Primary Settled Sewage: The Effects of Five Key Variables.” *Water Research* 16(ii).

Ivanov, A., Y. Park, E. Miskiewicz, J. Raven, N. Huner, and G. Oquist. 2000. “Iron Stress Restricts Photosynthetic Intersystem Electron Transport in *Synechococcus* Sp. PCC 7942.” *FEBS Letters* 485(2-3):173–77.

Ji, Min-Kyu, R. Abou-Shanab, S. Kim, E. Salama, S. Lee, A. Kabra, Y. Lee, S. Hong, and B. Jeon. 2013. “Cultivation of Microalgae Species in Tertiary Municipal Wastewater Supplemented with CO_2 for Nutrient Removal and Biomass Production.” *Ecological Engineering* 58:142–48.

Kuenzler, EJ. 1965. “Glucose-6-Phosphate Utilization by Marine Algae.” *Journal of Phycology* 31(17):156–64.

Lackner, Klaus S., S. Brennan, J. Matter, A. Park, A. Wright, and B. van der Zwaan. 2012. “The Urgency of the Development of CO_2 Capture from Ambient Air.” *Proceedings of the National Academy of Sciences of the United States of America* 109(33):13156–62.

Lam, Man Kee, and Keat Teong Lee. 2012. “Potential of Using Organic Fertilizer to Cultivate *Chlorella Vulgaris* for Biodiesel Production.” *Applied Energy* 94:303–8.

Lau, P., N. Tam, and Y. Wong. 1995. “Effect of Algal Density on Nutrient Removal from Primary Settled Wastewater.” *Environmental Pollution* 89(1):59–66.

Leftley, J. W., and P. J. Syrett. 1973. “Urease and ATP: Urea Amidolyase Activity in Unicellular Algae.” *Journal of General Microbiology* 77(1):109–15.

Li, Yecong, Y. Chen, P. Chen, M. Min, W. Zhou, B. Martinez, J. Zhu, and R. Ruan. 2011. "Characterization of a Microalga *Chlorella* Sp. Well Adapted to Highly Concentrated Municipal Wastewater for Nutrient Removal and Biodiesel Production." *Bioresource Technology* 102(8):5138–44.

Liu, Xiaowei, B. Saydah, P. Eranki, L. Colosi, M. Greg, J. Rhodes, and A. Clarens. 2013. "Pilot-Scale Data Provide Enhanced Estimates of the Life Cycle Energy and Emissions Profile of Algae Biofuels Produced via Hydrothermal Liquefaction." *Bioresource Technology* 148:163–71.

Lohman, Egan J., Robert Gardner, Luke Halverson, Richard Macur, Brent Peyton, and Robin Gerlach. 2013. "An Efficient and Scalable Extraction and Quantification Method for Algal Derived Biofuel." *Journal of Microbiological Methods* 94(3):235–44.

Lohman, Egan J., Robert Gardner, Todd Pederson, Brent Peyton, Keith Cooksey, and Robin Gerlach. 2014. "Optimized Inorganic Carbon Regime for Enhanced Growth and Lipid Accumulation in *Chlorella Vulgaris*." *Biotechnology for Biofuels* [In Review.

Maestrini, Serge Y., Jean-Michel Robert, John W. Leftley, and Yves Collos. 1986. "Ammonium Thresholds for Simultaneous Uptake of Ammonium and Nitrate by Oyster-Pond Algae." *Journal of Experimental Marine Biology and Ecology* 102(1):75–98.

Martin, C., J. De la Noüe, and G. Picard. 1985. "Intensive Cultivation of Freshwater Microalgae on Aerated Pig Manure." *Biomass* 7:245–59.

Martinez, ME, JM Jimenez, and F. El Yousfi. 1999. "Influence of Phosphorus Concentration and Temperature on Growth and Phosphorus Uptake by the Microalga *Scenedesmus Obliquus*." *Bioresource Technology* 67:233–40.

Moroney, JV, and Aravind Somanchi. 1999. "How Do Algae Concentrate CO_2 to Increase the Efficiency of Photosynthetic Carbon Fixation?" *Plant Physiology* 119(January):9–16.

Ördög, Vince, Wendy a. Stirk, Péter Bálint, Johannes Staden, and Csaba Lovász. 2011. "Changes in Lipid, Protein and Pigment Concentrations in Nitrogen-Stressed *Chlorella Minutissima* Cultures." *Journal of Applied Phycology* 24(4):907–14.

Pate, Ron, Geoff Klise, and Ben Wu. 2011. "Resource Demand Implications for US Algae Biofuels Production Scale-Up." *Applied Energy* 88(10):3377–88.

Pratt, R., and J. Fong. 1940. “Studies on *Chlorella Vulgaris* II. Further Evidence That *Chlorella* Cells Form a Growth-Inhibiting Substance.” *American Journal of Botany* 27(6):431–36.

Radmer, R., and O. Ollinger. 1980. “Light-Driven Uptake of Oxygen , Carbon Dioxide , and Bicarbonate by the Green Alga *Scenedesmus* ".” *Plant Physiology* 65(4):723–29.

Resurreccion, Eleazer P., Lisa M. Colosi, Mark a White, and Andres F. Clarens. 2012. “Comparison of Algae Cultivation Methods for Bioenergy Production Using a Combined Life Cycle Assessment and Life Cycle Costing Approach.” *Bioresource Technology* 126:298–306.

Reynolds, C. S. 1984. *The Ecology of Freshwater Phytoplankton*. Cambridge University Press.

Rhine, ED, and RL Mulvaney. 1998. “Improving the Berthelot Reaction for Determining Ammonium in Soil Extracts and Water.” *Soil Science Society of America* 62(2):473–80.

Richmond, Amos, Sabine Karg, and Samy Boussiba. 1982. “Effects of Bicarbonate and Carbon Dioxide on the Competition between *Chlorella Vulgaris* and *Spirulina Platensis*.” *Plant and Cell Physiology* 23(8):1411–17.

Roche, J., P. Boyd, R. McKay, and R. Geider. 1996. “Flavodoxin as an in Situ Marker for Iron Stress in Phytoplankton.” *Nature* 802–5.

Sandmann, G. 1985. “Consequences of Iron Deficiency on Photosynthetic and Respiratory Electron Transport in Blue-Green Algae.” *Photosynthesis Research* 6(3):261–71.

Sheehan, J., T. Dunahay, J. Benemann, and P. Roessler. 1998. “Look Back at the U.S. Department of Energy’s Aquatic Species Program: Biodiesel from Algae; Close-Out Report.”

Sialve, Bruno, Nicolas Bernet, and Olivier Bernard. 2009. “Anaerobic Digestion of Microalgae as a Necessary Step to Make Microalgal Biodiesel Sustainable.” *Biotechnology Advances* 27(4):409–16.

Singh, Anoop, and Stig Irving Olsen. 2011. “A Critical Review of Biochemical Conversion, Sustainability and Life Cycle Assessment of Algal Biofuels.” *Applied Energy* 88(10):3548–55.

Terry, Norman, and Javier Abadía. 1986. “Function of Iron in Chloroplasts.” *Plant Nutrition* 9:609–46.

Vassiliev, I. R., Z. Kolber, K. Wyman, D. Mauzerall, V. Shukla, and P. Falkowski. 1995. “Effects of Iron Limitation on Photosystem II Composition and Light Utilization in *Dunaliella Tertiolecta*.” *Plant Physiology* 109(3):963–72.

Vijverberg, Jacobus, DF Kalf, and M. Boersma. 1996. “Decrease in *Daphnia* Egg Viability at Elevated pH.” *Limnology and Oceanography* 1627–32.

Wang, Liang, M. Min, Y. Li, P. Chen, Y. Chen, Y. Liu, Y. Wang, and R. Ruan. 2010. “Cultivation of Green Algae *Chlorella* Sp. in Different Wastewaters from Municipal Wastewater Treatment Plant.” *Applied Biochemistry and Biotechnology* 162(4):1174–86.

Xin, Li, Hu Hong-Ying, and Yang Jia. 2010. “Lipid Accumulation and Nutrient Removal Properties of a Newly Isolated Freshwater Microalga, *Scenedesmus* Sp. LX1, Growing in Secondary Effluent.” *New Biotechnology* 27(1):59–63.

Yang, Jia, M. Xu, X. Zhang, Q. Hu, M. Sommerfeld, and Y. Chen. 2011. “Life-Cycle Analysis on Biodiesel Production from Microalgae: Water Footprint and Nutrients Balance.” *Bioresource technology* 102(1):159–65.

Yu, Guo, Yuanhui Zhang, Lance Schideman, Ted Funk, and Zhichao Wang. 2011. “Distributions of Carbon and Nitrogen in the Products from Hydrothermal Liquefaction of Low-Lipid Microalgae.” *Energy & Environmental Science* 4(11):4587.

Yu, Yin, H. Hu, X. Li, Y. Wu, X. Zhang, and S. Jia. 2012. “Accumulation Characteristics of Soluble Algal Products (SAP) by a Freshwater Microalga *Scenedesmus* Sp. LX1 during Batch Cultivation for Biofuel Production.” *Bioresource technology* 110:184–89.

Zhu, L. D., J. Takala, E. Hiltunen, and Z. M. Wang. 2013. “Recycling Harvest Water to Cultivate *Chlorella Zofingiensis* under Nutrient Limitation for Biodiesel Production.” *Bioresource technology* 144:14–20.

Zhu, Liandong, and Tarja Ketola. 2012. “Microalgae Production as a Biofuel Feedstock: Risks and Challenges.” *International Journal of Sustainable Development & World Ecology* 19(3):268–74.

APPENDICES

APPENDIX A

CO-AUTHOR PUBLICATION ABSTRACTS

Title

An Efficient and Scalable Extraction and Quantification Method for Algal Derived Biofuel

Authors

Egan J. Lohman, Robert D. Gardner, Luke Halverson, Richard E. Macur, Brent M. Peyton, Robin Gerlach.

Journal

Journal of Microbiological Methods, 2013. 94(3):235–244.

Abstract

Microalgae are capable of synthesizing a multitude of compounds including biofuel precursors and other high value products such as omega-3-fatty acids. However, accurate analysis of the specific compounds produced by microalgae is important since slight variations in saturation and carbon chain length can affect the quality, and thus the value, of the end product. We present a method that allows for fast and reliable extraction of lipids and similar compounds from a range of algae, followed by their characterization using gas chromatographic analysis with a focus on biodiesel-relevant compounds. This method determines which range of biologically synthesized compounds is likely responsible for each fatty acid methyl ester (FAME) produced; information that is fundamental for identifying preferred microalgae candidates as a biodiesel source. Traditional methods of analyzing these precursor molecules are time intensive and prone to high degrees of variation between species and experimental conditions. Here we detail a new method which uses microwave energy as a reliable, single-step cell disruption technique to extract lipids from live cultures of microalgae. After extractable lipid characterization (including lipid type (free fatty acids, mono-, di- or tri-acylglycerides) and carbon chain length determination) by GC-FID, the same lipid extracts are transesterified into FAMEs and directly compared to total biodiesel potential by GC-MS. This approach provides insight into the fraction of total FAMEs derived from extractable lipids compared to FAMEs derived from the residual fraction (i.e. membrane bound phospholipids, sterols, etc.). This approach can also indicate which extractable lipid compound, based on chain length and relative abundance, is responsible for each FAME. This method was tested on three species of microalgae; the marine diatom Phaeodactylum tricornutum, the model Chlorophyte Chlamydomonas reinhardtii,

and the freshwater green alga Chlorella vulgaris. The method is shown to be robust, highly reproducible, and fast, allowing for multiple samples to be analyzed throughout the time course of culturing, thus providing time-resolved information regarding lipid quantity and quality. Total time from harvesting to obtaining analytical results is less than 2h.

Title

Carbon Partitioning in Lipids Synthesized by *Chlamydomonas reinhardtii* when Cultured Under Three Unique Inorganic Carbon Regimes

Authors

Egan J. Lohman, Robert D. Gardner, Luke Halverson, Brent M. Peyton, Robin Gerlach

Journal

Algal Research. Ms. Ref. No.: ALGAL-D-14-00005R1. Accepted August 06, 2014

Abstract

Inorganic carbon is a fundamental component for microalgal lipid biosynthesis. Understanding how the concentration and speciation of dissolved inorganic carbon (DIC) influences lipid metabolism in microalgae may help researchers optimize the production of these high value metabolites. Using relatively straight forward methods for quantifying free fatty acids (FFA), mono- (MAG), di- (DAG), tri-acylglycerides (TAG), and total cellular fatty acids (FAME), lipid profiles over time were established for *Chlamydomonas reinhardtii* when grown under three unique inorganic carbon regimes. Specifically, cultures sparged with atmospheric air were compared to cultures which were sparged with 5% CO_2 (v/v) and cultures supplemented with 50 mM $NaHCO_3$ just prior to medium nitrogen depletion. All three conditions exhibited similar lipid profiles prior to nitrogen depletion in the medium, with FFA and MAG being the predominant lipid metabolites. However, these precursors were quickly reallocated into DAG and subsequently TAG after nitrogen depletion. C_{16} DAG did not accumulate significantly in any of the treatments, whereas the C_{18} DAG content increased throughout both exponential and stationary growth. C_{16} and C_{18} TAG began to accumulate after nitrogen depletion, with C_{16} TAG contributing the most to overall TAG content. C_{16} fatty acids exhibited a shift towards saturated C_{16} fatty acids after nitrogen depletion. Results provide insight into inorganic carbon partitioning into lipid compounds and how the organism's lipid metabolism changes due to N-deplete culturing and inorganic carbon source availability. The methodologies and findings presented here may be adapted to other organisms with high industrial relevance.

APPENDIX B

TABULATED DATA

B.1 1st Generation Growth of WC-1 in Wastewater and Bold's 8.7

Scenedesmus sp. WC-1 was grown in primary clarifier effluent (PC), three dilutions of anaerobic digestate (AD3-5) and in Bold's 8.7 basal medium (B8.7) for the 1st generation of growth.

Table B.1. Average Cell Concentrations for 1st Generation WC-1 Growth

Time (days)	B8.7	AD1	AD2	AD3	AD4	AD5	PC
0	6.00E+04	6.00E+04	6.00E+04	6.00E+04	6.00E+04	6.00E+04	6.00E+04
1	7.32E+05	2.92E+04	4.92E+04	1.57E+05	8.03E+05	8.90E+05	1.23E+05
2	1.74E+06	5.25E+04	4.67E+04	3.37E+05	3.34E+06	2.93E+06	6.30E+05
3	6.02E+06	3.33E+04	3.33E+04	7.03E+05	3.70E+06	3.65E+06	3.70E+06
4	1.07E+07	3.50E+04	3.25E+04	2.07E+06	5.33E+06	4.10E+06	7.77E+06
5	1.40E+07	3.42E+04	2.42E+04	3.90E+06	6.98E+06	5.12E+06	1.48E+07
8	2.34E+07	0.00E+00	0.00E+00	6.28E+06	9.55E+06	5.28E+06	2.58E+07
10	2.45E+07	0.00E+00	0.00E+00	7.47E+06	1.11E+07	4.82E+06	2.86E+07

Table B.2. Standard Deviation of Cell Concentrations for 1st Generation WC-1 Growth

Time (days)	B8.7	AD1	AD2	AD3	AD4	AD5	PC
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	2.95E+05	1.81E+04	1.84E+04	3.55E+04	1.50E+05	3.63E+05	3.16E+04
2	1.85E+05	6.61E+03	2.32E+04	1.42E+05	8.94E+05	2.52E+05	5.29E+04
3	1.97E+06	1.61E+04	3.82E+03	1.59E+05	4.00E+05	8.66E+04	3.61E+05
4	1.47E+06	5.00E+03	1.15E+04	5.13E+05	2.52E+05	1.00E+05	1.16E+06
5	2.36E+06	8.04E+03	2.90E+04	4.48E+05	1.14E+06	2.57E+05	2.09E+06
8	1.37E+06	0.00E+00	0.00E+00	1.53E+06	4.77E+05	5.01E+05	3.41E+06
10	3.50E+06	0.00E+00	0.00E+00	7.78E+05	1.89E+06	5.20E+05	1.91E+06

Table B.3. Average pH for 1st Generation WC-1 Growth

Time (days)	B8.7	AD1	AD2	AD3	AD4	AD5	PC
1	8.91	9.31	9.08	8.97	9.10	9.15	9.09
2	9.98	9.32	9.05	8.91	9.32	9.43	9.16
3	11.02	9.31	9.00	8.98	9.84	9.36	10.68
4	11.21	9.30	9.00	9.16	9.64	9.04	11.37
5	10.69	9.30	9.01	9.15	8.60	8.30	10.26
8	11.52	9.18	8.91	9.00	7.93	8.31	10.97
10	11.56	9.08	8.87	8.56	8.06	7.92	10.36

Table B.4. Standard Deviation of pH for 1st Generation WC-1 Growth

Time (days)	B8.7	AD1	AD2	AD3	AD4	AD5	PC
1	0.29	0.02	0.02	0.02	0.08	0.11	0.02
2	0.12	0.01	0.03	0.01	0.19	0.07	0.04
3	0.06	0.02	0.03	0.01	0.02	0.04	0.29
4	0.05	0.03	0.03	0.07	0.12	0.09	0.05
5	0.12	0.01	0.03	0.07	0.07	0.02	0.17
8	0.01	0.01	0.06	0.15	1.15	0.03	0.06
10	0.04	0.01	0.05	0.10	0.03	0.03	0.20

B.2 2nd Generation Growth of WC-1 in Wastewater and Bold's 8.7

Scenedesmus sp. WC-1 was grown in primary clarifier effluent (PC), three dilutions of anaerobic digestate (AD3-5) and in Bold's 8.7 basal medium (B8.7) for the 2nd generation of growth.

Table B.5. Average Cell Concentrations for 2nd Generation WC-1 Growth

Time (days)	B8.7	AD3	AD4	AD5	PC
0	6.00E+04	6.00E+04	6.00E+04	6.00E+04	2.30E+05
4	2.55E+06	2.97E+05	2.14E+06	2.37E+06	6.83E+06
5	5.43E+06	6.30E+05	3.30E+06	2.37E+06	1.51E+07
7	1.17E+07	3.18E+06	4.16E+06	2.75E+06	2.17E+07
9	1.57E+07	5.40E+06	4.94E+06	2.90E+06	2.83E+07
12	2.02E+07	5.90E+06	4.95E+06	3.05E+06	2.62E+07
13	2.34E+07	1.01E+07	4.80E+06	3.17E+06	3.62E+07
15	2.73E+07	1.01E+07	5.57E+06	3.67E+06	3.00E+07
18	3.87E+07	1.02E+07	N/A	N/A	3.40E+07

Table B.6. Standard Deviation of Cell Concentrations for 2nd Generation WC-1 Growth

Time (days)	B8.7	AD3	AD4	AD5	PC
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4	3.64E+05	1.40E+05	4.41E+05	2.39E+05	1.64E+06
5	3.21E+05	2.44E+05	5.35E+05	2.08E+05	2.21E+06
7	1.58E+06	5.92E+05	9.35E+05	2.78E+05	6.81E+05
9	1.97E+06	3.03E+06	1.29E+06	2.65E+05	2.39E+06
12	3.39E+06	2.00E+06	1.45E+06	3.32E+05	4.04E+05
13	3.27E+06	2.82E+06	1.42E+06	1.53E+05	7.64E+05
15	5.46E+06	1.14E+06	1.72E+06	2.08E+05	3.46E+06

B.3 Microelement and Iron Addition to WC-1 in 2nd Generation Growth

A microelement solution and an iron solution were added to 2nd generations AD4 cultures both separately and together to test for a nutrient deficiency.

Table B.7. Cell Density of 2nd Generation AD4 Cultures Given a Microelement and Iron Solution

Time (days)	Iron Addition	Microelement Addition	Both Additions
0	6.00E+04	6.00E+04	6.00E+04
4	2.10E+06	2.54E+06	1.70E+06
5	3.70E+06	2.20E+06	3.50E+06
7	4.70E+06	2.50E+06	4.70E+06
9	6.25E+06	3.00E+06	4.90E+06
12	6.40E+06	3.36E+06	4.95E+06
13	6.40E+06	3.30E+06	4.30E+06
14	N/A	N/A	6.20E+06
15	7.00E+06	3.50E+06	1.00E+07
18	1.70E+07	5.60E+06	1.50E+07
19	2.02E+07	6.10E+06	1.40E+07

B.4 Iron Addition to 3rd Generation WC-1 Cultures

Iron was added to 3rd generation AD4 cultures at the time of inoculation and at seven days after inoculation. A control culture with no added iron addition was also grown.

Table B.8. Cell Density of 3rd Generation WC-1 Cultures Grown in 1:25 Diluted Anaerobic Digestate with Iron Added at Inoculation or at Seven Days into Growth

Time (Days)	Fe at Inoculation	Fe at Day 7	No Fe
0	6.00E+04	6.00E+04	6.00E+04
2	4.50E+05	4.45E+05	4.30E+05
4	4.10E+06	3.70E+06	3.30E+06
7	1.70E+07	3.40E+06	4.50E+06
9	2.40E+07	1.06E+07	5.40E+06
12	2.03E+07	1.40E+07	5.30E+06
15	2.05E+07	1.16E+07	6.30E+06
17	2.70E+07	1.44E+07	7.70E+06

B.5 1st Generation Growth of SLA-04 in Wastewater and Bold's 8.7

Chlorella sp. SLA-04 was grown in primary clarifier effluent (PC), anaerobic digestate diluted 1:25 with added iron (AD4) and Bold's 8.7 basal medium for the 1st generation of growth

.

Table B.9. Average Cell Concentrations for 1st Generation SLA-04 Growth

Time (days)	B8.7	AD4	PC
0	4.24E+05	3.93E+05	3.96E+05
3	1.46E+07	1.63E+07	1.38E+07
4	2.31E+07	2.00E+07	1.93E+07
6	3.84E+07	4.45E+07	5.32E+07
10	8.71E+07	5.05E+07	7.25E+07

Table B.10. Standard Deviation of Cell Concentrations for 1st Generation SLA-04 Growth

Time (days)	B8.7	AD4	PC
0	6.54E+04	3.80E+04	4.77E+04
3	4.73E+05	3.60E+06	3.54E+05
4	9.07E+05	1.38E+06	1.41E+06
6	1.87E+06	3.51E+06	1.39E+07
10	3.21E+05	5.00E+05	7.07E+05

Table B.11. Average pH for 1st Generation SLA-04 Growth

Time (days)	B8.7	AD4	PC
0	7.87	7.99	8.15
3	10.14	9.17	9.48
4	10.93	9.39	10.14
6	10.65	8.86	10.25
10	11.22	6.25	10.84

Table B.12. Standard Deviation of pH for 1st Generation SLA-04 Growth

Time (days)	B8.7	AD4	PC
0	0.05	0.01	0.08
3	0.14	0.06	0.02
4	0.06	0.06	0.06
6	0.17	0.10	0.04

10	0.04	0.23	0.06

B.6 2nd Generation Growth of SLA-04 in Wastewater and Bold's 8.7

Again, *Chlorella* sp. SLA-04 was grown in primary clarifier effluent (PC), anaerobic digestate diluted 1:25 with added iron (AD4) and Bold's 8.7 basal medium for the 2nd generation of growth.

Table B.13. Average Cell Concentrations for 2nd Generation SLA-04 Growth

Time (days)	B8.7	AD4	PC
0	1.10E+05	1.78E+05	1.37E+05
2	8.75E+05	1.71E+06	1.32E+06
5	2.65E+07	2.46E+07	2.65E+07
7	4.43E+07	3.41E+07	5.27E+07
9	7.01E+07	4.78E+07	7.73E+07
12	1.23E+08	5.07E+07	6.70E+07
14	1.36E+08	5.25E+07	6.70E+07
16	1.47E+08	N/A	N/A
19	1.65E+08	N/A	N/A
21	1.47E+08	N/A	N/A

Table B.14. Standard Deviation of Cell Concentrations for 2nd Generation SLA-04 Growth

Time (days)	B8.7	AD4	PC
0	2.07E+04	6.29E+03	1.15E+04
2	5.50E+05	3.39E+05	1.20E+05
5	5.42E+06	4.89E+06	2.29E+06
7	3.47E+06	8.02E+06	9.57E+06
9	2.91E+06	3.96E+06	1.05E+07
12	1.13E+07	2.08E+06	6.08E+06
14	1.39E+07	1.04E+07	6.24E+06
16	3.61E+06	N/A	N/A
19	1.33E+07	N/A	N/A
21	1.76E+07	N/A	N/A

B.7 Iron and Nitrogen Addition to 2nd Generation SLA-04 Cultures

Iron and Nitrogen were added separately to 2nd generation PC and AD4 cultures to test for a nutrient deficiency.

Table B.15. Cell Concentration of 2nd Generation SLA-04 Cultures Given an Iron Addition at Day 16

Time (days)	B8.7	AD+Fe	PC+Fe
0	1.10E+05	1.73E+05	1.30E+05
2	8.75E+05	1.95E+06	1.40E+06
5	2.65E+07	2.20E+07	2.90E+07
7	4.43E+07	2.68E+07	6.36E+07
9	7.01E+07	4.50E+07	8.80E+07
12	1.23E+08	5.00E+07	7.40E+07
14	1.36E+08	4.30E+07	7.20E+07
16	1.47E+08	5.60E+07	8.20E+07
19	1.65E+08	4.60E+07	8.70E+07
21	1.47E+08	4.70E+07	8.00E+07

Table B.16. Cell Concentration of 2nd Generation SLA-04 Cultures Given a Nitrogen Addition at Day 16

Time (days)	B8.7	AD4+NH4	PC+NH4
0	1.10E+05	1.78E+05	1.50E+05
2	8.75E+05	4.38E+05	5.51E+04
5	2.65E+07	2.15E+07	2.45E+07
7	4.43E+07	3.29E+07	4.58E+07
9	7.01E+07	4.60E+07	6.70E+07
12	1.23E+08	4.90E+07	6.40E+07
14	1.36E+08	5.10E+07	6.00E+07
16	1.47E+08	4.60E+07	6.90E+07
19	1.65E+08	4.40E+07	1.36E+08
21	1.47E+08	5.20E+07	1.46E+08
22			1.46E+08
23			1.47E+08
26			1.50E+08

B.8 Generational Comparison for WC-1 and SLA-04

Each generation of primary clarifier effluent growth was compared for WC-1 and SLA-04 to determine whether each strain grows consistently between generations.

Table B.17. Average Cell Concentrations for Three Generations of WC-1 Growth in Primary Clarifier Effluent

Time (days)	1st Gen	2nd Gen	3rd Gen
0	6.00E+04	2.30E+05	6.00E+04
1	1.23E+05	N/A	N/A
2	6.30E+05	N/A	1.70E+05
3	3.70E+06	N/A	N/A
4	7.77E+06	6.83E+06	4.53E+06
5	1.48E+07	1.51E+07	N/A
6	N/A	N/A	1.90E+07
7	N/A	2.17E+07	N/A
8	2.58E+07	N/A	N/A
9	N/A	2.83E+07	2.39E+07
10	2.86E+07	N/A	N/A
12	N/A	2.62E+07	2.50E+07
13	N/A	3.62E+07	N/A
15	N/A	3.00E+07	2.71E+07
18	N/A	3.40E+07	N/A

Table B.18. Standard Deviation of Cell Concentrations for Three Generations of WC-1 Growth in Primary Clarifier Effluent

Time (days)	1st Gen	2nd Gen	3rd Gen
0	0.00E+00	0.00E+00	0.00E+00
1	3.16E+04	N/A	N/A
2	5.29E+04	N/A	2.65E+04
3	3.61E+05	N/A	N/A
4	1.16E+06	1.64E+06	7.77E+05
5	2.09E+06	2.21E+06	N/A
6	N/A	N/A	1.73E+06
7	N/A	6.81E+05	N/A
8	3.41E+06	N/A	N/A
9	N/A	2.39E+06	4.75E+06
10	1.91E+06	N/A	N/A
12	N/A	4.04E+05	3.21E+05
13	N/A	7.64E+05	N/A
15	N/A	3.46E+06	2.39E+06
18	N/A	4.27E+06	N/A

Table B.19. Average Cell Concentrations for Two Generations of SLA-04 Growth in Primary Clarifier Effluent

Time (days)	1st Gen	2nd Gen
0	3.96E+05	1.37E+05
2	N/A	1.32E+06
3	1.38E+07	N/A
4	1.93E+07	N/A
5	N/A	2.65E+07
6	5.32E+07	N/A
7	N/A	5.27E+07
9	N/A	7.73E+07
10	7.25E+07	N/A
12	N/A	6.70E+07
14	N/A	6.70E+07

Table B.20. Standard Deviation of Cell Concentrations for Two Generations of SLA-04 Growth in Primary Clarifier Effluent

Time (days)	1st Gen	2nd Gen
0	4.77E+04	1.15E+04
2	N/A	1.20E+05
3	3.54E+05	N/A
4	1.41E+06	N/A
5	N/A	2.29E+06
6	1.39E+07	N/A
7	N/A	9.57E+06
9	N/A	1.05E+07
10	7.07E+05	N/A
12	N/A	6.08E+06
14	N/A	6.24E+06

B.9 Growth of WC-1 and SLA-04 in Primary Clarifier Effluent

Scenedesmus sp. WC-1 and *Chlorella* sp. SLA-04 were both grown in primary clarifier effluent (PC) under several inorganic carbon regimes.

Scenedesmus sp. WC-1

Table B.21. Average Cell Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
0	3.83E+05	4.30E+05	3.83E+05	3.83E+05	4.30E+05	3.83E+05
2	1.05E+06	1.34E+06	1.08E+06	1.33E+06	1.65E+06	1.20E+06
3	8.63E+06	8.35E+06	9.20E+06	8.50E+06	8.60E+06	8.85E+06
4	2.38E+07	3.91E+07	2.78E+07	2.68E+07	9.10E+06	2.37E+07
5	2.43E+07	3.80E+07	2.53E+07	2.68E+07	9.70E+06	2.17E+07
6	2.39E+07	3.67E+07	2.93E+07	2.81E+07	8.90E+06	2.46E+07
8	2.28E+07	3.60E+07	2.35E+07	2.59E+07	8.90E+06	2.03E+07

Table B.22. Standard Deviation of Cell Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
0	4.04E+04	5.66E+04	4.04E+04	4.04E+04	5.66E+04	4.04E+04
2	1.40E+05	4.95E+04	1.61E+05	1.15E+05	7.07E+04	3.52E+05
3	1.17E+06	2.12E+05	7.21E+05	4.92E+05	5.66E+05	7.09E+05
4	1.89E+06	6.36E+05	6.15E+06	6.81E+05	1.27E+06	5.20E+06
5	2.34E+06	5.66E+06	3.60E+06	5.25E+06	1.84E+06	2.31E+06
6	2.40E+06	3.75E+06	4.73E+06	2.66E+06	2.55E+06	1.58E+06
8	1.75E+06	7.07E+06	8.66E+05	4.19E+06	5.66E+05	5.86E+06

Table B.23. Average pH for WC-1 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
0	8.40	6.43	8.39	6.90	6.38	6.90
2	8.48	6.65	8.24	7.10	6.75	7.03
3	8.43	7.27	8.00	7.20	7.33	7.55
4	9.88	6.98	9.16	6.85	10.20	9.86
5	10.22	7.18	9.49	7.09	10.69	10.32
6	10.12	7.02	9.30	6.99	10.79	10.52
8	10.07	7.00	9.38	6.88	10.69	10.66

Table B.24. Standard Deviation of pH for WC-1 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
0	0.00	0.00	0.00	0.00	0.00	0.00
2	0.10	0.03	0.03	0.04	0.07	0.02
3	0.08	0.06	0.13	0.07	0.08	0.09
4	0.03	0.17	0.72	0.03	0.01	0.02
5	0.03	0.01	0.65	0.09	0.01	0.09
6	0.05	0.02	0.60	0.05	0.00	0.08
8	0.09	0.03	0.51	0.03	0.03	0.04

Table B.25. Average Chlorophyll a + b Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
2	2.05	1.85	2.84	2.82	2.19	2.51
3	4.28	8.79	3.84	4.93	8.56	4.62
4	3.85	7.78	3.38	4.05	8.27	3.95
5	3.65	7.87	2.90	3.64	7.27	3.95
6	3.56	7.45	2.36	3.28	6.50	3.73
8	2.87	5.74	2.34	2.75	4.63	3.13

Table B.26. Standard Deviation of Chlorophyll a + b Concentrations for WC-1 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
2	0.02	0.18	0.37	0.23	0.58	0.52
3	0.37	0.19	0.14	0.46	1.48	0.35
4	0.28	0.70	0.33	0.59	0.70	0.30
5	0.35	0.22	0.22	0.69	1.27	0.30
6	0.50	0.79	0.28	0.29	0.21	0.28
8	0.24	0.11	0.78	0.40	0.48	0.08

Chlorella sp. SLA-04

Table B.27. Average Cell Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
0	5.10E+05	4.20E+05	4.95E+05	5.00E+05	5.50E+05	4.50E+05

2	7.91E+06	1.75E+06	4.65E+06	1.75E+06	2.70E+06	1.75E+06
3	5.27E+07	4.25E+07	4.45E+07	2.70E+07	2.55E+07	3.17E+07
4	1.76E+08	1.08E+08	1.59E+08	1.44E+08	7.10E+07	1.67E+08
5	1.86E+08	2.56E+08	1.98E+08	1.26E+08	1.47E+08	1.77E+08
6	1.95E+08	2.04E+08	1.88E+08	1.87E+08	1.60E+08	1.97E+08
7	1.98E+08	2.30E+08	2.09E+08	2.00E+08	1.75E+08	2.01E+08
8	1.58E+08	2.30E+08	2.16E+08	1.95E+08	1.86E+08	1.53E+08

Table B.28. Standard Deviation of Cell Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
0						
2	8.31E+05	6.36E+05	3.14E+05	4.58E+05	9.90E+05	
3	4.04E+06	6.36E+06	4.77E+06	3.61E+06	2.12E+06	4.16E+06
4	5.57E+06	2.47E+07	4.58E+06	3.00E+07	5.66E+06	2.34E+07
5	7.94E+06	6.43E+07	2.17E+07	1.22E+07	1.41E+06	2.07E+07
6	5.77E+06	2.83E+06	3.54E+07	2.99E+07	1.41E+07	2.03E+07
7	7.21E+06	7.07E+05	1.67E+07	2.65E+07	2.12E+07	1.28E+07
8	8.50E+06	1.41E+07	1.71E+07	3.51E+07	3.25E+07	1.10E+07

Table B.29. Average pH for SLA-04 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bold 8.7 + HCO3	5% CO2 + HCO3
0	8.11	6.28	8.17	6.88	6.30	6.76
2	8.46	6.70	8.34	7.23	6.63	7.11
3	8.76	6.88	7.83	7.13	6.92	6.99
4	10.14	7.19	8.18	7.21	8.58	8.56
5	10.34	7.36	8.18	7.13	9.97	9.85
6	10.37	7.17	7.98	7.15	10.53	10.29
7	10.57	7.10	7.84	6.90	10.76	10.55
8	10.45	7.11	8.01	7.14	10.84	10.64

Table B.30. Standard Deviation of pH for SLA-04 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bold 8.7 + HCO3	5% CO2 + HCO3
0						
2	0.04	0.01	0.03	0.05	0.02	0.02
3	0.00	0.00	0.08	0.07	0.01	0.06

4	0.01	0.01	0.05	0.06	0.00	0.04
5	0.02	0.18	0.03	0.02	0.01	0.00
6	0.05	0.01	0.23	0.02	0.00	0.00
7	0.06	0.04	0.19	0.00	0.04	0.01
8	0.07	0.00	0.12	0.04	0.06	0.02

Table B.31. Average Chlorophyll a+b Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
2	1.99	1.13	1.47	0.97	0.62	1.36
3	8.46	5.80	7.80	5.03	3.49	7.40
4	8.89	11.25	8.02	8.64	10.33	9.12
5	7.04	14.03	8.11	8.78	12.26	8.26
6	6.97	11.76	9.03	9.24	9.42	9.40
7	6.68	10.93	7.76	8.03	8.31	8.06
8	5.82	8.44	7.44	7.46	8.40	7.60

Table B.32. Standard Deviation of Chlorophyll a+b Concentrations for SLA-04 Grown in PC under Each Inorganic Carbon Regime

Time (days)	pH Control + HCO3	Bolds 8.7	pH Control	5% CO2	Bolds 8.7 + HCO3	5% CO2 + HCO3
2	0.40	0.32	0.44	0.20	0.01	0.16
3	0.27	0.39	0.82	1.58	0.33	0.45
4	2.56	0.72	0.36	2.18	1.03	0.35
5	0.31	0.02	0.53	1.44	0.28	0.26
6	0.23	0.52	0.09	1.25	1.28	1.05
7	0.09	0.11	0.26	0.16	1.47	0.65
8	0.60	2.01	0.24	0.27	0.81	0.70

B.10 Lipid and FAME Content of WC-1

Lipid extractions and direct transesterifications were performed on *Scenedesmus* sp. WC-1 after cultures were harvested.

Table B.33. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for WC-1

	Bolds 8.7	Bolds 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	8.14	2.73	2.71	3.67	4.12	3.59
MAG	0.45	0.45	0.16	0.46	0.27	0.28

DAG	2.09	1.35	0.81	1.61	1.10	1.37
TAG	9.67	9.57	13.11	15.25	11.09	15.07
Total Extractable	20.35	14.09	16.78	20.99	16.58	20.31
Total FAME	30.72	30.71	31.62	35.06	31.40	36.91

Table B.34. Standard Deviation of FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for WC-1

	Bolds 8.7	Bolds 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	1.21	0.00	1.02	0.57	1.37	0.81
MAG	0.03	0.00	0.10	0.09	0.14	0.15
DAG	0.23	0.00	0.28	0.05	0.21	0.38
TAG	0.29	0.00	0.46	0.04	1.18	0.68
TOTAL	1.76	0.00	1.45	0.64	2.86	2.01
Total FAME	0.00	0.37	0.77	3.61	0.22	1.05

Table B.35. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for WC-1

	Bolds 8.7	Bolds 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	0.18	0.03	0.02	0.05	0.07	0.04
MAG	0.01	0.01	0.00	0.01	0.00	0.00
DAG	0.05	0.02	0.01	0.02	0.02	0.01
TAG	0.22	0.11	0.10	0.20	0.17	0.16
Total Extractable	0.45	0.17	0.13	0.27	0.26	0.21
Total FAME	0.69	0.36	0.24	0.45	0.48	0.39

Table B.36. Standard Deviation FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for WC-1

	Bolds 8.7	Bolds 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	0.03	0.00	0.01	0.01	0.02	0.01
MAG	0.00	0.00	0.00	0.00	0.00	0.00
DAG	0.01	0.00	0.00	0.00	0.00	0.00
TAG	0.01	0.00	0.00	0.00	0.02	0.01
Total Extractable	0.04	0.00	0.01	0.01	0.04	0.02
Total FAME	0.00	0.00	0.01	0.05	0.00	0.01

B.11 Lipid and FAME Content of SLA-04

Again, lipid extractions and direct transesterifications were performed on *Chlorella* sp. SLA-04 after cultures were harvested.

Table B.37. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for SLA-04

	Bold's 8.7	Bold's 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	5.06	4.97	4.55	3.79	5.01	4.51
MAG	0.11	0.11	0.08	0.10	0.11	0.09
DAG	2.22	1.61	1.75	1.43	1.85	1.69
TAG	20.29	11.65	18.28	9.92	19.58	12.58
Total Extractable	27.68	18.33	24.66	15.24	26.55	18.88
Total FAME	49.56	34.40	48.99	30.72	49.41	35.71

Table B.38. Standard Deviation FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME content (% w/w) for SLA-04

	Bold's 8.7	Bold's 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	0.26	0.32	1.57	0.09	0.05	0.35
MAG	0.02	0.02	0.03	0.05	0.06	0.02
DAG	0.14	0.14	0.60	0.11	0.29	0.12
TAG	0.20	2.51	6.90	0.92	1.19	0.66
TOTAL	0.22	2.98	9.09	0.68	0.94	1.01
Total FAME	0.41	1.00	1.80	2.34	3.15	2.66

Table B.39. Average FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for SLA-04

	Bold's 8.7	Bold's 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	0.09	0.06	0.06	0.05	0.07	0.06
MAG	0.00	0.00	0.00	0.00	0.00	0.00
DAG	0.04	0.02	0.02	0.02	0.03	0.02
TAG	0.34	0.15	0.25	0.12	0.29	0.17
Total Extractable	0.47	0.23	0.34	0.18	0.39	0.25
Total FAME	0.83	0.44	0.67	0.37	0.72	0.47

Table B.40. Standard Deviation of FFA, MAG, DAG, TAG, Total Extractable Lipid and FAME concentration (g/L) for SLA-04

	Bold's 8.7	Bold's 8.7 + HCO3	pH Control	pH Control + HCO3	5% CO2	5% CO2 + HCO3
FFA	0.00	0.00	0.02	0.00	0.00	0.00
MAG	0.00	0.00	0.00	0.00	0.00	0.00
DAG	0.00	0.00	0.01	0.00	0.00	0.00
TAG	0.00	0.03	0.09	0.01	0.02	0.01
TOTAL	0.00	0.04	0.12	0.01	0.01	0.01
Total FAME	0.01	0.01	0.02	0.03	0.05	0.04

B.12 Growth of WC-1 in Recycled Harvest Water

WC-1 was grown in recycled harvest water supplemented with anaerobic digestate for three generations. For the second and third generations, make-up water was added to cultures containing anaerobic digestate in the form of either tap water or primary clarifier effluent.

The initial nitrogen concentration in the 3rd generation of WC-1 growth in recycled harvest water was nearly twice as high as the intended concentration of 41 mg N/L.

Table B.41. Average Cell Concentrations for 1st Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW1+NO3	RW1+AD	RW1+AD+Fe
0	8.800E+05	6.417E+05	8.733E+05
2	2.797E+06	2.420E+06	2.627E+06
4	1.157E+07	7.783E+06	7.950E+06
6	1.673E+07	1.637E+07	1.650E+07
8	2.033E+07	2.173E+07	2.370E+07

Table B.42. Standard Deviation of Cell Concentrations for 1st Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW1+NO3	RW1+AD	RW+AD+Fe
0	4.000E+04	4.649E+05	2.517E+04
2	1.973E+05	1.375E+05	1.447E+05
4	2.388E+06	3.753E+05	1.308E+06
6	2.639E+06	7.572E+05	5.196E+05
8	5.925E+06	1.848E+06	2.961E+06

Table B.43. Average pH for 1st Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW1+NO3	RW1+AD	RW+AD+FE
0	9.16	8.20	8.02
2	10.42	9.10	9.42
4	10.50	9.75	10.29
6	11.26	10.38	10.80
8	10.69	8.99	10.80

Table B.44. Standard Deviation of pH for 1st Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW1+NO3	RW1+AD	RW1+AD+Fe
0	4.041E-02	1.528E-02	3.464E-02
4	4.212E-01	4.359E-02	1.000E-02
5	1.266E-01	3.287E-01	3.215E-02
6	1.350E-01	1.528E-02	2.082E-02
7	7.379E-01	6.807E-02	4.509E-02

Table B.45. Average Cell Concentrations for 2nd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
0	4.50E+05	5.10E+05	4.80E+05	3.85E+05	3.70E+05
2	1.77E+06	2.39E+06	2.16E+06	2.39E+06	2.00E+06
4	8.35E+06	8.95E+06	9.03E+06	9.60E+06	1.04E+07
6	1.20E+07	2.20E+07	2.10E+07	1.98E+07	2.16E+07
8	2.06E+07	2.93E+07	2.80E+07	2.25E+07	2.34E+07

Table B.46. Range of Cell Concentrations for 2nd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
0	1.80E+05	2.00E+04	8.00E+04	3.00E+04	2.00E+04
2	1.60E+05	5.41E+05	1.50E+05	3.00E+04	6.00E+04
4	4.40E+06	1.30E+06	5.00E+04	1.30E+06	7.00E+05
6	5.70E+06	2.10E+06	4.00E+06	7.10E+06	6.20E+06
8	3.40E+06	6.00E+05	2.40E+06	1.60E+06	9.80E+06

Table B.47. Average pH for 2nd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe

0	10.29	8.01	7.76	8.09	7.80
2	10.24	9.23	9.07	9.31	9.24
4	11.39	10.86	10.80	10.97	10.85
6	10.94	11.00	10.48	11.09	10.90
8	10.50	10.66	9.92	11.25	10.86

Table B.48. Range of pH for 2nd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
0	0.66	0.14	0.12	0.37	0.19
2	0.27	0.08	0.08	0.18	0.05
4	0.12	0.28	0.06	0.06	0.03
6	0.30	0.04	0.84	0.04	0.04
8	0.60	0.25	1.35	0.14	0.04

Table B.49. Average Cell Concentrations for 3rd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+AD+PC	RW3+AD+Tap	RW3+AD+PC+Fe	RW3+AD+Tap+Fe
0	4.70E+05	2.80E+05	3.60E+05	4.75E+05	4.25E+05
2	3.24E+06	1.96E+06	1.82E+06	2.12E+06	1.71E+06
4	1.19E+07	4.33E+06	4.50E+06	4.13E+06	4.03E+06
6	2.01E+07	9.75E+06	4.68E+06	1.03E+07	6.85E+06
8	1.82E+07	8.20E+06	6.45E+06	2.24E+07	9.55E+06
10	2.49E+07	8.20E+06	1.37E+07	2.98E+07	1.60E+07

Table B.50. Range of Cell Concentrations for 3rd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+AD+PC	RW3+AD+Tap	RW3+AD+PC+Fe	RW3+AD+Tap+Fe
0	0.00E+00	4.00E+04	1.20E+05	7.00E+04	1.00E+04
2	2.00E+04	2.00E+05	5.20E+05	2.00E+05	2.00E+04
4	2.65E+06	6.50E+05	2.30E+06	1.25E+06	1.15E+06
6	1.00E+06	2.70E+06	8.50E+05	2.00E+05	1.30E+06
8	2.40E+06	1.60E+06	7.00E+05	2.80E+06	3.00E+05
10	2.10E+06	1.40E+06	9.50E+06	4.30E+06	2.30E+06

Table B.51. Average pH for 3rd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+AD+PC	RW3+AD+Tap	RW3+AD+PC+Fe	RW3+AD+Tap+Fe
0	10.01	8.00	7.68	7.92	7.63

2	10.57	9.05	9.02	9.20	9.08
4	11.10	9.04	8.88	10.20	9.13
6	11.65	8.72	8.75	10.86	9.00
8	11.65	8.74	8.94	10.89	10.38
10	11.20	8.12	9.23	10.94	10.49

Table B.52. Range of pH for 3rd Generation Growth of WC-1 in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+AD+PC	RW3+AD+Tap	RW3+AD+PC+Fe	RW3+AD+Tap+Fe
0	0.06	0.02	0.04	0.03	0.06
2	0.34	0.06	0.04	0.13	0.03
4	0.66	0.02	0.06	0.15	0.15
6	0.11	0.07	0.09	0.06	0.20
8	0.03	0.00	0.42	0.04	0.10
10	0.39	0.22	0.84	0.06	0.02

B.13 Nitrogen Concentration in Recycled Harvest Water Experiment

Table B.53. Average Ammonium Nitrogen Concentration in 3rd Generation WC-1 Recycled Harvest Water Experiment

Time (days)	RW3+AD+PC	RW3+AD+Tap	RW3+AD+PC+Fe	RW3+AD+Tap+Fe
0	71.26	73.90	66.29	77.29
2	43.61	52.30	44.56	50.25
4	31.00	39.50	15.55	33.70
6	20.78	26.21	0.00	18.52
8	17.48	19.27	0.00	2.67
10	2.73	11.36	0.00	0.00

Table B.54. Range of Ammonium Nitrogen Concentration in 3rd Generation WC-1 Recycled Harvest Water Experiment

Time (days)	AD+PC	AD+Tap	AD+PC+Fe	AD+Tap+Fe
0	3.55	4.74	4.16	7.51
2	1.30	2.35	3.12	0.63
4	2.92	0.95	4.15	0.42
6	0.30	0.34	0.00	4.18
8	1.24	5.19	0.00	3.19
10	4.08	13.35	0.00	0.00

B.14 WC-1 Growth Rate Comparison

The average growth rates of 2nd and 3rd generations WC-1 growth in recycled harvest water were compared to see if inhibition could be seen.

Table B.55. Average Growth Rates of WC-1 in 2nd and 3rd Generation Recycled Harvest Water Experiment

	RW+NO3	RW+AD+PC	RW+AD+Tap	RW+AD+PC+Fe	RW+AD+Tap+Fe
2nd Generation	0.48	0.51	0.51	0.51	0.52
3rd Generation	0.63	0.34	0.36	0.41	0.36

Table B.56. Range of Growth Rates of WC-1 in 2nd and 3rd Generation Recycled Harvest Water Experiment

	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
2nd Generation	0.03	0.00	0.01	0.00	0.06
3rd Generation	0.01	0.00	0.04	0.03	0.01

B.15 Chlorophyll Content of WC-1

3rd generation chlorophyll concentrations were determined for WC-1 grown in recycled harvest water.

Table B.57. Average Chlorophyll a+b Concentrations for WC-1 Grown in 3rd Generation Recycled Harvest Water

Time (days)	RW3+NO3	RW3+AD+PC	RW3+AD+Tap	RW3+AD+PC+Fe	RW3+AD+Tap+Fe
0	0.00	0.00	0.00	0.00	0.00
2	1.02	0.56	0.59	1.06	0.89
4	3.26	0.99	0.78	3.74	1.70
6	6.32	1.22	1.03	7.31	2.59
8	7.10	1.59	1.48	10.07	5.78
10	6.25	1.26	2.07	9.66	8.16

Table B.58. Range of Chlorophyll a+b Concentrations for WC-1 Grown in 3rd Generation Recycled Harvest Water

Time (days)	RW3+NO3	RW3+AD+PC	RW3+AD+Tap	RW3+AD+PC+Fe	RW3+AD+Tap+Fe
0	0.00	0.00	0.00	0.00	0.00
2	0.53	0.14	0.13	0.14	0.08
4	1.44	0.11	0.19	0.66	0.19

6	2.95	0.30	0.31	1.15	0.17
8	2.46	0.48	1.11	2.32	0.05
10	1.76	0.05	1.56	2.10	1.63

B.16 pH and. Nitrogen Concentration in 3rd Generation WC-1 Cultures

The medium pH and nitrogen concentrations are shown over time for 3rd generation cultures with and without added iron.

Table B.59. Average medium pH and Nitrogen Concentration in RW3+AD+PC+Fe Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1

Time (days)	pH	Nitrogen Conc.
0	7.92	66.29
2	9.20	44.56
4	10.20	15.55
6	10.86	0
8	10.89	0
10	10.94	0

Table B.60. Range of medium pH and Nitrogen Concentration in RW3+AD+PC+Fe Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1

Time (days)	pH	Nitrogen Conc.
0	0.03	4.16
2	0.13	3.12
4	0.15	4.15
6	0.06	0
8	0.04	0
10	0.06	0

Table B.61. Average medium pH and Nitrogen Concentration in RW3+AD+Tap Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1

Time (days)	pH	Nitrogen Conc.
0	7.68	73.90
2	9.02	52.30
4	8.88	39.50
6	8.75	26.21
8	8.94	19.27
10	9.23	11.36

Table B.62. Range of medium pH and Nitrogen Concentration in RW3+AD+Tap Cultures during 3rd Generation Recycled Harvest Water Experiment for WC-1

Time (days)	pH	Nitrogen Conc.
0	0.04	4.74
2	0.04	2.35
4	0.06	0.95
6	0.09	0.34
8	0.42	5.19
10	0.84	13.35

B.17 2nd and 3rd Generation Cell Dry Weight Comparison for WC-1

Table B.63. Average Cell Dry Weight of WC-1 Cultures during 2nd and 3rd Generation Recycled Harvest Water Experiment

	RW+NO3	RW+AD+PC	RW+AD+Tap	RW+AD+PC+Fe	RW+AD+Tap+Fe
2nd Generation	0.77	1.02	0.87	1.01	0.96
3rd Generation	1.12	0.29	0.37	0.97	0.58

Table B.64. Range of Cell Dry Weight of WC-1 Cultures during 2nd and 3rd Generation Recycled Harvest Water Experiment

	RW+NO3	RW+AD+PC	RW+AD+Tap	RW+AD+PC+Fe	RW+AD+Tap+Fe
2nd Generation	0.01	0.07	0.20	0.06	0.03
3rd Generation	0.06	0.03	0.07	0.02	0.04

B.18 Lipid Content of 2nd and 3rd Generation WC-1 Cultures

Table B.65. Average Lipid content (% w/w) of WC-1 Cultures during 2nd Generation Recycled Harvest Water Experiment

	FFA	MAG	DAG	TAG	Total Extractable Lipid
RW2+NO3	3.95	0.52	1.28	1.03	6.78
RW2+AD+PC	4.05	0.61	1.69	3.12	9.47
RW2+AD+Tap	3.96	0.47	1.12	0.70	6.25
RW2+AD+PC+Fe	6.20	0.91	2.53	5.26	14.91
RW2+AD+Tap+Fe	6.06	0.96	2.24	3.35	12.60
Fresh Primary Effluent	2.71	0.16	0.81	13.11	16.78

Table B.66. Range of Lipid content (% w/w) of WC-1 Cultures during 2nd Generation Recycled Harvest Water Experiment

	FFA	MAG	DAG	TAG	Total Extractable Lipid
RW2+NO3	1.03	0.14	0.46	0.61	2.24
RW2+AD+PC	0.14	0.15	0.08	0.10	0.27
RW2+AD+Tap	0.32	0.02	0.07	0.56	0.29
RW2+AD+PC+Fe	0.40	0.14	0.09	0.12	0.51
RW2+AD+Tap+Fe	0.62	0.18	0.35	0.67	1.82
Fresh Primary Effluent	1.02	0.10	0.28	0.46	1.45

Table B.67. Average Lipid content (% w/w) of WC-1 Cultures during 3rd Generation Recycled Harvest Water Experiment

	FFA	MAG	DAG	TAG	Total Extractable Lipid
RW3+NO3	3.26	0.87	3.58	9.55	17.27
RW3+AD+PC	1.43	0.21	0.74	0.02	2.40
RW3+AD+Tap	2.10	0.35	0.88	0.03	3.36
RW3+AD+PC+Fe	3.14	0.55	1.28	0.70	5.67
RW3+AD+Tap+Fe	3.90	0.58	1.03	0.07	5.59

Table B.68. Range of Lipid content (% w/w) of WC-1 Cultures during 3rd Generation Recycled Harvest Water Experiment

	FFA	MAG	DAG	TAG	Total Extractable Lipid
RW3+NO3	0.11	0.07	0.42	2.15	2.39
RW3+AD+PC	0.05	0.05	0.04	0.00	0.13
RW3+AD+Tap	0.40	0.10	0.04	0.01	0.55
RW3+AD+PC+Fe	1.25	0.29	0.05	0.16	1.75
RW3+AD+Tap+Fe	0.59	0.04	0.20	0.08	0.27

B.19 Growth of SLA-04 in Recycled Harvest Water

SLA-04 was grown in recycled harvest water supplemented with anaerobic digestate for three generations. For the second and third generations, make-up water was added to cultures containing anaerobic digestate in the form of either tap water or primary clarifier effluent.

Table B.69. Average Cell Concentrations for 1st Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW1+NO3	RW1+AD	RW1+AD+Fe

0	8.07E+05	5.60E+05	9.13E+05
2	3.99E+06	3.50E+06	4.10E+06
4	2.38E+07	1.67E+07	1.75E+07
6	2.84E+07	3.72E+07	3.42E+07
8	3.60E+07	6.51E+07	6.13E+07

Table B.70. Standard Deviation of Cell Concentrations for 1st Generation Growth of SLA-04in Recycled Harvest Water

Time (days)	RW1+NO	RW1+AD	RW1+AD+Fe
0	1.45E+05	3.99E+05	1.26E+05
2	1.11E+06	1.05E+06	8.58E+05
4	1.50E+06	2.33E+06	1.64E+06
6	6.22E+06	5.28E+06	1.18E+06
8	1.22E+07	7.70E+06	7.77E+06

Table B.71. Average pH for 1st Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW1+NO3	RW1+AD	RW1+AD+Fe
0	10.81	8.73	8.78
2	8.78	8.59	8.64
4	10.02	9.22	9.40
6	10.55	10.07	10.45
8	10.32	10.41	10.51

Table B.72. Standard Deviation of pH for 1st Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW1+NO3	RW1+AD	RW1+AD+Fe
0	0.03	0.02	0.24
2	0.10	0.05	0.02
4	0.29	0.15	0.19
6	0.36	0.32	0.10
8	0.66	0.13	0.10

Table B.73. Average Cell Concentrations for 2nd Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
0	7.10E+05	7.75E+05	6.30E+05	7.05E+05	5.55E+05
2	3.55E+06	2.32E+06	2.78E+06	3.06E+06	2.97E+06

4	2.54E+07	2.66E+07	2.10E+07	2.52E+07	1.85E+07
6	3.08E+07	5.22E+07	3.79E+07	5.19E+07	4.30E+07
8	3.57E+07	8.76E+07	6.79E+07	9.60E+07	7.52E+07

Table B.74. Range of Cell Concentrations for 2nd Generation Growth of SLA-04in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
0	8.00E+04	1.10E+05	1.60E+05	3.00E+04	3.00E+04
2	5.60E+05	3.80E+05	1.00E+05	7.40E+05	9.80E+05
4	2.05E+06	1.80E+06	2.75E+06	7.50E+05	1.40E+06
6	2.60E+06	1.00E+05	1.40E+06	2.00E+05	3.60E+06
8	6.20E+06	2.00E+06	4.20E+06	1.68E+07	5.20E+06

Table B.72. Average pH for 2nd Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
0	10.19	8.05	7.99	7.94	7.86
2	8.88	8.69	8.73	8.68	8.72
4	9.96	9.69	9.36	9.48	9.13
6	9.83	10.44	9.56	10.24	9.54
8	9.81	10.90	10.55	10.74	10.57

Table B.73. Range of pH for 2nd Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW2+NO3	RW2+AD+PC	RW2+AD+Tap	RW2+AD+PC+Fe	RW2+AD+Tap+Fe
0	0.08	0.02	0.01	0.06	0.04
2	0.04	0.04	0.03	0.02	0.04
4	0.27	0.16	0.12	0.05	0.03
6	0.09	0.35	0.12	0.40	0.07
8	0.14	0.01	0.12	0.13	0.02

Table B.74. Average Cell Concentrations for 3rd Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+PC+AD	RW3+TAP+AD	RW3+PC+AD+FE	RW3+TAP+AD+Fe
0	5.45E+05	6.25E+05	4.25E+05	5.85E+05	5.00E+05
2	1.14E+07	6.28E+06	7.23E+06	5.15E+06	6.50E+06
4	3.49E+07	2.64E+07	2.15E+07	2.45E+07	2.20E+07
6	5.26E+07	5.61E+07	5.40E+07	5.28E+07	5.37E+07

Table B.75. Range of Cell Concentrations for 3rd Generation Growth of SLA-04in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+PC+AD	RW3+TAP+AD	RW3+PC+AD+FE
9.00E+04	1.00E+04	1.00E+04	5.00E+04	8.00E+04
7.00E+05	1.05E+06	7.50E+05	8.00E+05	4.00E+05
1.80E+06	1.70E+06	1.60E+06	8.00E+05	2.80E+06
2.00E+06	2.02E+07	4.40E+06	8.00E+05	2.00E+05

Table B.76. Average pH for 3rd Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+PC+AD	RW3+TAP+AD	RW3+PC+AD+FE	RW3+TAP+AD+Fe
0	9.90	8.13	7.97	8.02	7.75
2	9.15	8.87	8.78	8.73	8.76
4	9.75	9.51	9.04	9.48	9.02
6	9.56	9.74	9.35	9.69	9.30

Table B.77. Range of pH for 3rd Generation Growth of SLA-04 in Recycled Harvest Water

Time (days)	RW3+NO3	RW3+PC+AD	RW3+TAP+AD	RW3+PC+AD+FE	RW3+TAP+AD+Fe
0	0.02	0.04	0.03	0.01	0.03
2	0.05	0.09	0.04	0.05	0.03
4	0.05	0.24	0.13	0.05	0.09
6	0.02	0.11	0.06	0.01	0.07

APPENDIX C

ORGANISM SELECTION

C.1 Selected Strains

The strains that were chosen for further investigation were *Scenedesmus* sp. WC-1, *Chlorella* sp. SLA-04 and *Navicula* sp. RGd-1. These three strains were chosen due to their alkaliphilic nature and high growth rates and lipid contents. The three strains were also outlined in an awarded DOE grant that funded the majority of the work performed in this thesis.

C.2 *Scenedesmus* sp. WC-1 Description

Scenedesmus sp. WC-1 is a green alga shown in Figure C.1. WC-1 was isolated from an alkaline stream in the Heart Lake area in Yellowstone National Park, USA. The organism grows mainly as a single cell throughout exponential growth, but clusters of 2, 4, or 8 begin to appear when growth approaches the stationary phase. Cultures of WC-1 grow to a cell density of roughly 4E7 cells/mL in as little as four days after inoculation (Figure C.2).

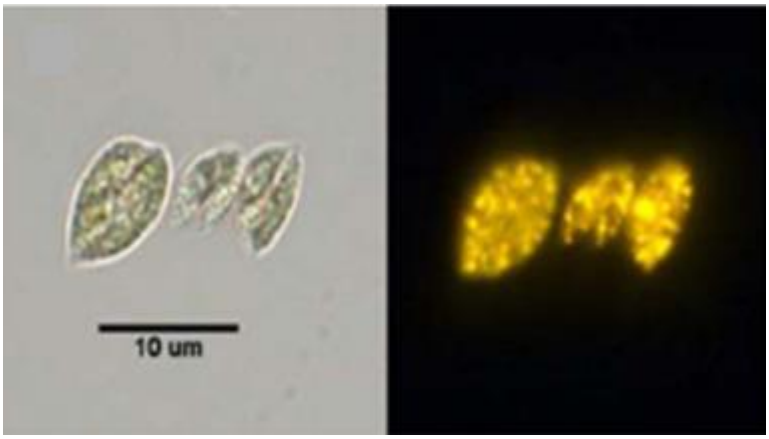


Figure C.1. Transmitted light micrograph (left) and Nile Red fluorescence image (right) of Scenedesmus sp. WC-1.

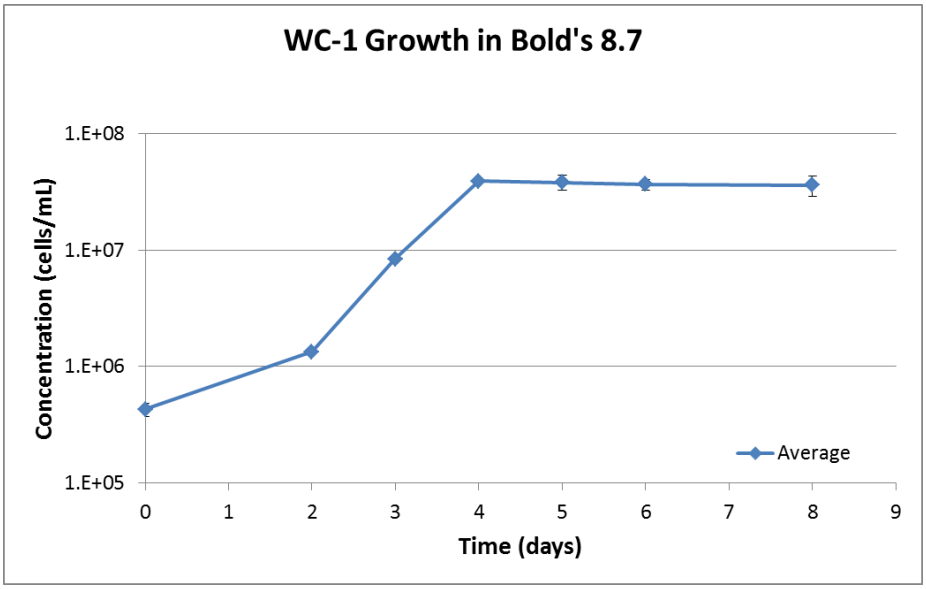


Figure C.2. Average growth curve for WC-1 grown in triplicate in Bold's 8.7 basal medium on 5% CO_2.

C.3 *Chlorella* sp. SLA-04 Description

Chlorella sp. SLA-04 is a green alga shown in Figure C.3. SLA-04 was isolated from Soap Lake in Washington State, USA. The organism grows as small, round cells until nutrient depletion when many cells become oblong. SLA-04 cultures reach a cell density of approximately 2E8 cells/mL after four to five days of growth (Figure C.4).

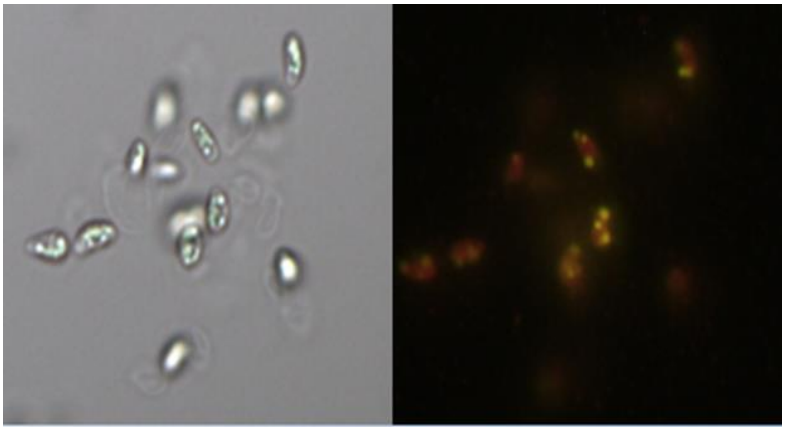


Figure C.3. Transmitted light micrograph (left) and Nile Red fluorescence image (right) of *Chlorella* sp. SLA-04.

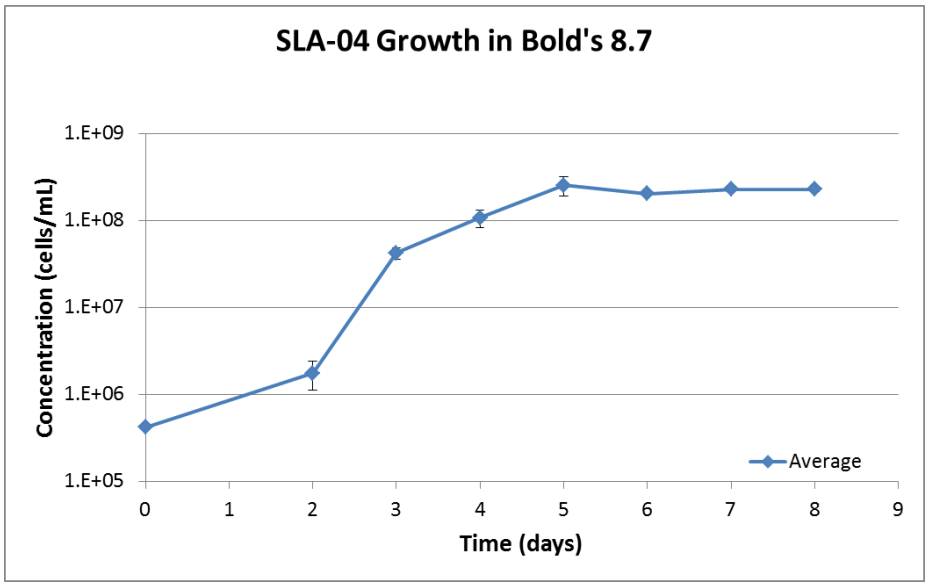


Figure C.4. Average growth curve for SLA-04 grown in triplicate in Bold's 8.7 basal medium on 5% CO2.

C.4 *Navicula* sp. RGd-1 Description

Navicula sp. RGd-1 is a diatom (shown in Figure C.5) that was isolated from an alkaline stream in Yellowstone National Park, USA. Although this strain showed promising growth rates and lipid production in a defined basal medium, RGd-1 was unable to grow in municipal wastewater, and the organism was not considered in the experiments outlined in this thesis.

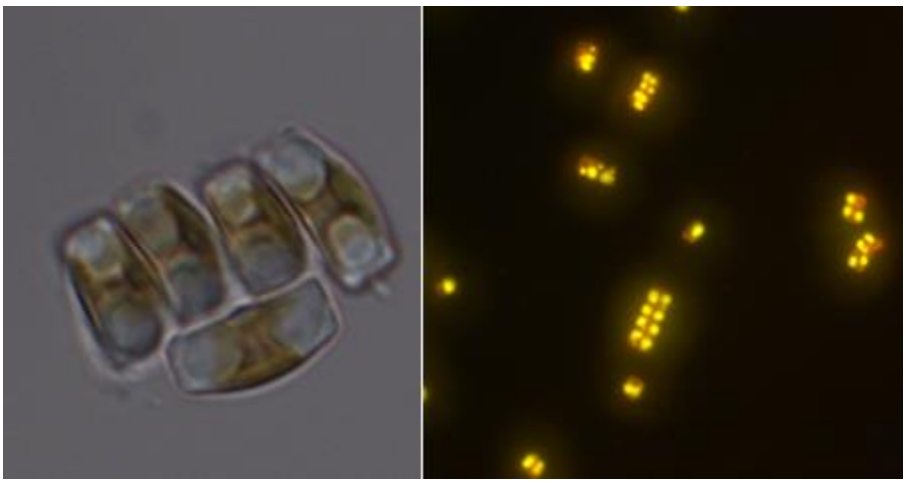


Figure C.3. Transmitted light micrograph (left) and Nile Red fluorescence image (right) of *Navicula* sp. RGd-1.

APPENDIX D

COAL BED METHANE WATER STUDY

The following report details work completed during the spring of 2012.

D.1 Introduction

Over the past decade, coal bed methane (CBM) has become one of the nation's most popular energy sources. Methane, or natural gas, is one of the cleanest burning hydrocarbons, making it a good alternative to heavier gases, oil, and coal. Natural gas power plants have a much smaller carbon footprint than a coal fired plant, because methane produces less than half the carbon dioxide as coal during combustion. In order to sustain natural gas power, vast reserves of natural gas must be located and developed.

The relatively recent discovery of large reserves of CBM could satisfy the high demand for the methane needed to generate electricity at current consumption rates. CBM is generated during the formation of coal. First, aerobic bacteria use free oxygen in the plant material to generate methane. When oxygen has been depleted, anaerobic archaea reduce carbon dioxide in a process called methanogenesis. This type of methane is referred to as biogenic methane. The produced methane remains in the coal bed as a gas in open pockets, dissolved in water, adsorbed to the surface of coal, or absorbed within the coal. A coal bed may contain up to seven times more methane than conventional rock beds.

The methane remains in the coal bed due to high pressures in the bed. Huge amounts of water are held in these coal beds, which contributes to the high pressure. In order to release the natural gas, the water in the coal beds must be removed to alleviate the pressure. Mining companies drill down into the coal bed, reinforce the bore hole, and lower a pump into the well to bring the water to the surface. As more and more water is pumped from the well, methane is released from the coal bed and is collected. An active well can produce up to 17,000 gallons of water each day, and a well may need to be pumped for several years before maximum methane production is reached. The water being removed is saline and alkaline. This huge volume of unusable waste water is politically and environmentally controversial in regions where CBM is being produced. The water is either discharged into evaporation ponds or nearby river drainages, sprayed on dirt roads for dust control, or sent to a water treatment facility. The use of evaporation ponds is the most widely used method of disposal, but the ponds require a lot of land, creating a breeding ground for mosquitoes and potentially an eyesore for residents and tourists.

Using the produced water from CBM wells to produce algal biofuels may prove to be a practical solution to this water problem. A cheap, renewable water source is one of the biggest hurdles that must be overcome for algal biofuels to become commercially viable. Large scale algal bioreactors are typically designed as an array of open ponds. These facilities could be placed near CBM wells, and produced water would be dispersed to each pond where algae are grown and cultivated for biofuel.

In order for this to be possible, a number of problems need to be addressed. The chemical composition of the produced water must be evaluated to see if it can be used to make media in which algae can grow. An algal strain that can grow in high salinity,

alkaline conditions must be found, and the strain must produce high levels of lipid per cell weight. If the water can be used and a successful organism is found, the use of produced water in an algal biofuel facility may be a viable option.

Montana State University is a good place to conduct research looking for a suitable organism to grow in produced water. MSU has been working with algal species that are found in harsh conditions, namely Yellowstone National Park and Soap Lake, WA. Several strains have been isolated from these locations, and they may be able to grow in media made with produced water.

D.2 Produced Water Chemistry

The chemistry of the produced water is a major concern that must be evaluated prior to attempting to grow algae. Not all water pumped from coal bed methane wells is the same. The location, depth, and type of coal bed impact the chemical make-up of the produced water. The depth of the well plays a large role in terms of water quality. In general, shallow beds contain the "cleanest" water, and the salinity of the water increases as coal beds get deeper.

Coal bed mining methane production wells are located in the Powder River Basin. The Powder River Basin is located in southeastern Montana and northwestern Wyoming, and it is one of the largest reservoirs of methane in the United States. Thousands of wells have already been drilled in the region, and many more are projected to be drilled in the next ten years. The coal beds located in the Powder River Basin are fairly shallow (300-800ft deep), which means the water is much cleaner than water from deep beds found in other parts of the country.

The following analyses were conducted using CBM water collected by Elliot Barnhart, a PhD student in microbiology, from a well in the southeastern Montana section of the Powder River Basin. The pH of the system was 7.90-8.00 with high salinity. Filtered samples were analyzed for inorganic carbon on a Skalar Formax TOC/TN analyzer. Total dissolved inorganic carbon was roughly 23mM-C. The pH and salinity of the system dictate the carbon species to be mostly sodium bicarbonate.

Remaining water properties were taken from field reports on the Montana Groundwater Information Center website. These reports show major ion concentrations, trace element concentrations, and pH levels. The website reported a pH of 8.15 and a bicarbonate concentration of around 28mM, which are both slightly higher than the values obtained in the lab. The website also reported a sodium ion concentration of around 26mM.

The water chemistry was then compared to common medium used for Chlorophyte cultures, such as Bolds basal medium (Nichols and Bold 1965) and AM6. The components of each medium were broken down into ionic concentrations, and the medium concentrations were compared to that of produced water. The comparison showed that the produced water had only a small fraction of most medium components, so the produced water would need to be supplemented with necessary salts and solutions.

The Bolds medium would also need to be titrated with sodium hydroxide to raise the pH to the required level of 8.7.

Table D.1. Chemical constituency data for CBM water and Bolds basal medium. Specific ion concentrations are presented for both, and the percent difference is calculated. Positive percent differences show produced water has a higher concentration of an ion in the standard Bolds medium, and a negative value means the ion needs to be supplemented. For the most part, the produced water was deficient of most compounds.

Bolds		**CBM**				
Ion	Conc (mM)	Ion	Conc. (mg/L)	MW	Conc. (mM)	% Diff (CBM:BOLDS)
Na^{+}	3.37	Na	590	22.99	25.66	661.69
Mg^{2+}	0.30	Mg	2.35	24.31	0.10	-68.22
Ca^{2+}	0.17	Ca	10.7	40.08	0.27	56.98
K^{+}	0.86	K	5.76	39.10	0.15	-82.90
NO_3^{-}	2.94	NO3	0.257	62.00	0.00	-99.86
Cl^{-}	0.77	Cl	21.32	35.45	0.60	-21.69
SO_4^{2-}	0.30	SO3	12.5	80.06	0.16	-48.70
PO_4^{3-}	1.72	PO3	0.25	78.97	0.00	-99.82

D.3 Organism Screen

A growth screen was completed to see what organisms, if any, would grow in produced water. Five organisms, two green algae and three diatom strains, were tested, and growth was measured visually. The organisms were grown in both pure CBM water and supplemented water.

The two green algae strains observed were *Scenedesmus sp.* WC-1 and another chlorophyte referred to as GK-6 G3. The three diatoms used in the screen were RGd-1, RGd-2, and GK-1S D1. WC-1, RGd-1, and RGd-2 were isolated from Witch Creek in Yellowstone National Park. Witch Creek has a pH of around 9.3. GK-6 and GK-1S were isolated from Little Soap Lake in Washington where the pH ranges from 8.5 to 10.0.

The organisms' specific medium was made, and 100ml of each medium was filter-sterilized into a 250mL shaker flask using a syringe and a 0.2μm filter. The media could not be autoclaved, because the autoclaving process would convert the bicarbonate to carbon dioxide gas. The composition of the media would no longer be representative of true produced water, and the results of the experiment would be uncharacteristic.

When each medium was separated into the flasks, the organisms could be transferred into their specific media. A five milliliter inoculum was placed into each flask for a total of ten transfers, five produced water and five supplemented flasks. The

flasks were then placed in an incubator, where they were subjected to 25˚C and a 14/10 light-dark cycle.

All of the organisms except RGd-1 grew in the supplemented media, but none grew on pure CBM water. The organisms grew at different rates, with WC-1 and RGd-2 achieving the fastest rate. Two additional transfers were made once the culture had reached stationary phase. The subsequent transfers were completed to make sure the organisms were not growing on nutrients from the initial transfer alone.

The organism screen proved that multiple strains of algae could be grown in supplemented produced water, so a particular species needed to be chosen to be further studied. Each organism was considered. The two diatoms that grew formed a biofilm on the inside of the flasks, which would be difficult to sample and analyze. Each of the green algae grew well, but GK-1S grew as stringy, fibrous clumps. The clumps could not easily be pulled into a pipette. A sonication bath was used in an attempt to break up the clumps, but they would not separate. For these reasons, it was decided that GK-1S, RGd-2, and GK-6 would be too difficult to effectively study.

D.4 *Scendesmus* sp. strain WC-1

The other green alga, WC-1, was chosen for further study. WC-1 belongs to the genus, *Scendesmus*, in the Chlorophyceae class of green algae. WC-1 was one of the best growing strains in the organism screen, and it did not form a biofilm in the shaker flask. Having a fast growth organism which can be easily sampled was an ideal choice.

WC-1 has also been previously studied at Montana State University. The other organisms used in the screen were isolated, but no work has been performed on them. Growth studies have been done on WC-1, and growth curves could be referenced during the produced water experiment. This information would be useful in comparing ideal growth conditions to produced water growth conditions.

Previous studies have also shown that WC-1 can produce significant amounts of lipid, and the strain can be triggered with bicarbonate. The bicarbonate trigger introduces an inorganic source of carbon when algal cells deplete the nitrogen in their media (Gardner et al., 2010). The introduction of bicarbonate causes the cell to undergo a metabolic shift from free fatty acid synthesis to storage molecule (TAG) synthesis (Gardner et al., 2011). The bicarbonate trigger can significantly increase the amount of lipid in the algae, which increases the biofuel potential.

D.5 Media Matrix

An experimental design needed to be created for growing WC-1 in multiple conditions. WC-1 grows in Bolds basal medium 8.7, which is the standard Bolds basal medium titrated to a pH of 8.7. The media recipe consists of ten additions, seven salts

and three solutions. The media comparison detailed earlier showed the water would have enough $CaCl_2$ and NaCl, so those two salts would not be added during the experiment.

It was decided the organism would be grown in nine separate growth conditions, referred to as the growth matrix. The first transfer of the matrix was pure, filtered coal bed methane water, and from there, a salt or solution would be added for the next transfer. The components of the medium were added, and 100mL of medium were put into shaker flasks until the final transfer had fully supplemented Bolds 8.7.

Many different combinations of Bolds components were possible, so the ingredients of the medium were added in the order they appeared in the original recipe. After the first salt of the medium was added, sodium hydroxide was used to titrate to a pH of around 8.7 for each transfer. The medium did not need to be titrated after each salt addition, because the pH remained mostly unchanged. If a change in pH of 0.1 or more was observed, the medium was titrated back to 8.7.

Table D.2. Each transfer of the growth matrix is detailed below. The medium began with pure coal bed methane water, and the components of Bolds medium were added one-by-one, pulling out 100mL between additions. The medium was titrated to a pH close to 8.7 for each transfer starting with the second one.

Starting with 1 L CBM	**Weight**	**Units**
Transfer 1 (100mL)		
Pure CBM		
Transfer 2 (100mL)		
KH_2PO_4	0.1575	g
Transfer 3 (100mL)		
$MgSO_4$	0.06	g
Transfer 4 (100mL)		
$NaNO_3$	0.175	g
Transfer 5 (100mL)		
K_2HPO_4	0.045	g
Transfer 6 (100mL)		
H_3BO_3	0.0125	g
Transfer 7 (100mL)		
Soln 1	400	µL
Transfer 8 (100mL)		
Soln 2	300	µL
Transfer 9 (100mL)		
Microelement	200	µL

After each transfer was prepared and filtered into its respective shaker flask, the medium was inoculated. The matrix was inoculated from a working stock of WC-1 growing on Bolds 8.7 in nanopure water.

The cell concentration of the working stock was first counted to accurately assess the proper volume of inoculum. Once the cell concentration was determined, the

working stock was pelletized via centrifugation and then resuspended in DI to insure the inoculum did not contain additional nutrients.

Once the cells were suspended in DI water, the proper amount was pulled out in a micropipette and placed in each of the nine matrix flasks. The desired initial concentration of each transfer was 1.0E5 cells per milliliter, which could be achieved using known concentrations and volumes. The nine transfers were placed in the 25˚C incubator and allowed to grow. A sample would be pulled from each transfer every day, so cell concentration and Nile Red fluorescence could be tracked. Cell concentrations were determined using a hemocytometer. Samples were taken until the cells either reached stationary phase or started to die off.

D.6 Growth Results

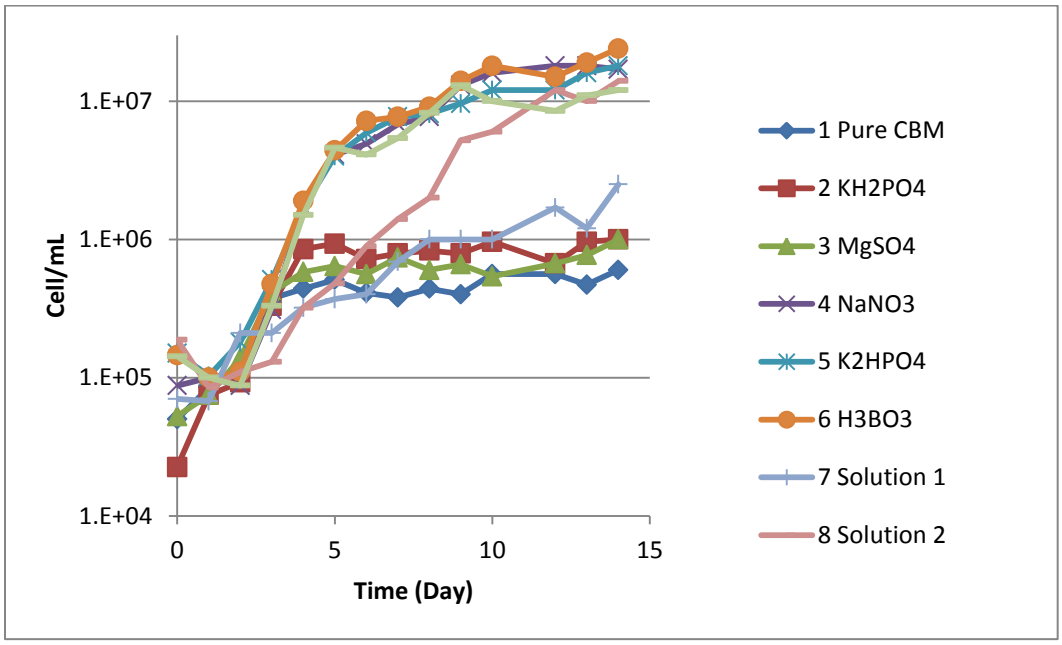


Figure D.1. The growth chart showing how each of the nine cultures grew over a two week span.

The organism grew in each of the nine transfer flasks. Some transfers grew better than others, but each showed an increase in cell concentration. On average, the flasks containing media supplemented with more Bolds components grew better. Although cell counts at t=0 were inconsistent, the cell concentrations merged in the following two days, and the initial cell count variation was contributed to human error.

After a two day lag phase, each of the transfers started growing. Transfers 1, 2, and 3 grew until Day 4, at which time the culture entered stationary phase. Each of these

cultures achieved a cell concentration of around 5.0E5 cells/mL. Previous growth studies of WC-1 have shown cultures reaching 3.0E7 cells/mL before becoming stationary. Cultures lacking Bolds components grew to 1E5-1E6 cells /mL, roughly two orders of magnitude less than fully supplemented Bolds medium.

Transfers 4, 5, 6, and 9 each grew better than the first three. These transfers each grew until Day 8, where they reached stationary at around 1.0E7 cells/mL. This cell concentration is much closer to the previous growth study results. These results indicate that nitrate, sulfate, and/or phosphate need to be present for WC-1 to successfully grow.

Transfers 7 and 8 showed interesting results. These transfers tracked with the first three transfers at around 5.0E5 cells/mL until the sixth day. After Day 6, transfer 8 continued to slowly grow until Day 12. Transfer 8 took about four days longer than transfers 4, 5, 6, and 9 to reach stationary phase at 1.0E7 cells/mL. Transfer 7 grew to around 1.0E6, but it did not continue to grow to a higher concentration.

These results are difficult to explain. Transfers 7 and 8 received Bolds Solution 1 and 2, respectively. Solution 1 contains sodium EDTA and potassium hydroxide, and Solution 2 contains iron sulfate and sulfuric acid. Neither of these significantly changed the pH upon addition, and yet transfers 7 and 8 grew much differently than transfers 4, 5, 6, and 9. One would expect the organism to grow similarly starting with transfer 4. Perhaps the two solutions need the microelement solution to effectively grow, or perhaps the flasks had a contaminant that hindered growth. The experiment needs to be repeated to see whether similar results are obtained.

D.7 Nile Red Results

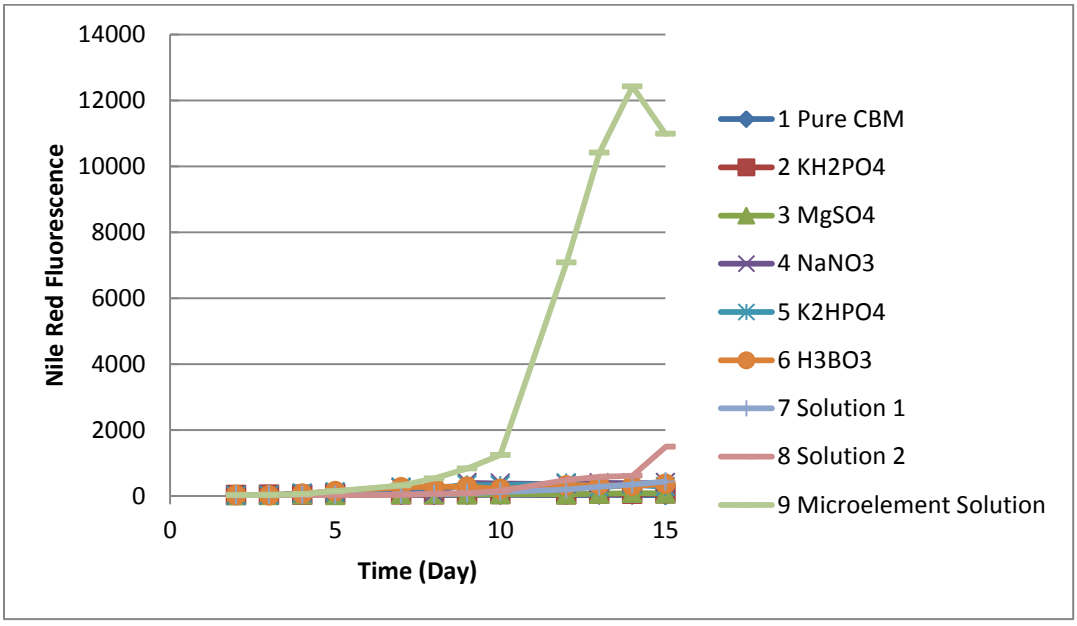


Figure D.2. The Nile Red Fluorescence values taken from Day 2 to Day 15.

The Nile Red Fluorescence was also recorded over a two week period. The values for each transfer were very similar until Day 9. At this point, transfer 9 began rising sharply until it reached its peak value at Day 14. The other transfers remained relatively low and approximately equal to one another. Transfer 9 reached a peak fluorescence of 12,420, while transfers 4, 5, 6, 8, and 9 each reached values of around 400. Transfers 1, 2, and 3 resulted in values of 30 to 50.

The organism was not able to accumulate significant amounts of lipid until the final transfer which introduced Bolds Microelement Solution. The solution contains small amounts of metals including: zinc, manganese, molybdenum, copper and cobalt. This result suggests that the microelement solution might be necessary for the accumulation of lipid within the cell. Many of the trace metals in the microelement solution are necessary cofactors for certain metabolic reactions. It is possible that one or more of the reactions involved in TAG synthesis require these trace metals to proceed. This finding deserves additional study.

D.8 Conclusion/Future Plans

The research completed over the past semester has been very interesting. It has shown that coal bed methane produced water can be used to make media for algal growth. Multiple organisms are able to grow in supplemented produced water. *Scendesmus sp.* strain WC-1 grows particularly well in the Bolds supplemented medium, and the organism is able to accumulate high levels of lipid in fully supplemented media. These results may prove to be an important stepping stone in converting coal bed methane wells into a source of water for algal biofuel production facilities.

Future research includes performing a factorial growth experiment, studying one of the diatoms that grew in the produced water media, and scaling up previous experiments. A factorial growth experiment will allow many combinations of media to be tested simultaneously, which would show exactly what is needed and what can be excluded in the media. Studying one of the diatoms may be interesting, as well. The diatoms could possibly grow better, grow in a wider range of media, or produce more lipid. In order to evaluate the growth of a diatom, an effective sampling technique will need to be created. Once the ideal growth conditions and organism has been determined, experiments will be scaled up from flasks and test tubes to large photobioreactors where more in depth experiments can be performed. All of the future experiments will be run in triplicate to see whether results are consistent.

D.9 References

Gardner R, Cooksey K, Mus F, Macur R, Moll K, Eustance E, Carlson R, Gerlach R, Fields M,Peyton B. 2012. Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the Chlorophyte *Scenedesmus* sp. and the diatom *Phaeodactylum tricornutum*. J Appl Phycol:1-10. doi:10.107/s10811-011-9782-0.

Gardner R, Peters P, Peyton B, Cooksey K. 2011. Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the Chlorophyta. J Appl Phycol 26:1005-1016.

Nichols H, Bold H (1965) Trichosarcina polymorpha Gen. et Sp. Nov. J Phycol 1:34–38

APPENDIX E

BOLD'S BASAL MEDIUM

Component	Amt per 1 Liter Medium	Final Medium Concentration
KH2PO4	175 mg	1.29 x 10-3 M
CaCl2*2H2O	25 mg	1.70 x 10-4 M
MgSO4*7H2O	75 mg	3.04 x 10-4 M
NaNO3	250 mg	2.94 x 10-3 M
K2HPO4	75 mg	4.31 x 10-4 M
NaCl	25 mg	4.28 x 10-4 M
H3BO3	11.42 mg	4.62 x 10-4 M
Trace Metal Solution	1 mL	
Solution 1	1 mL	
Solution 2	1 mL	

Trace Metal Solution

Component	Amt per 1 Liter Medium	Final Medium Concentration
ZnSO4*7H2O	8.82 g	7.67 x 10-5 M
MnCl2*4H2O	1.44 g	1.82 x 10-5 M
MoO3	.71 g	1.23 x 10-5 M
CuSO4*5H2O	1.57 g	1.57 x 10-5 M
Co(NO3)2*6H2O	.49 g	4.21 x 10-6 M

Solution 1

Component	Amt per 1 Liter Medium	Final Medium Concentration
EDTA	50.0 g	4.28 x 10-4 M
KOH	31.0 g	1.38 x 10-3 M

Solution 2

Component	Amt per 1 Liter Medium	Final Medium Concentration
FeSO4*7H2O	4.89 g	4.48 x 10-5 M
H2SO4	1.0 mL	