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Evaluating microalgae productivity when cultivated on ammonia-rich anaerobic digester effluent for commercial products and industrial applications

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Agricultural and Environmental Chemistry

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA, DAVIS

Approved:

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Dr. Annaliese K. Franz, Chair

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Dr. Dirk Holstege

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Dr. Thomas Young

ABSTRACT

This dissertation focuses on microalgae cultivation on nutrient-dense food waste permeate (FWP) for biofuel feedstock production and wastewater remediation. My work identifies methods for microalgae cultivation on nutrient-dense waste streams and the effect of this concentrated waste stream on microalgae growth and productivity and their resultant remediation capacity.

Chapter 1 discusses the commercial value of microalgae-derived natural products, the current economic challenges of producing these algae products, specifically biofuels. This chapter describes the use of wastewater, specifically anaerobic digester effluent and its byproducts, as a potential method to reduce the cost of microalgae cultivation. Further, the inherent benefits and challenges of anaerobic digestion technology and the use of its nutrient-dense waste streams as an alternative nutrient source for microalgae cultivation are discussed.

Chapter 2 discusses the microplate screening methodology that was implemented to evaluate ten different microalgae strains and their ability to be cultivated on various permeate conditions. Strains and conditions identified in the microplate studies (10-mL) were scaled up to larger batch cultures (250-mL), and a baseline culturing method was established. Out of the 10 strains evaluated at microplate scale (10-mL), only four demonstrated robust growth on the FWP used throughout this research. Resultant nutrient concentration changes were monitored alongside microalgae growth, and the final analyses demonstrate the feasibility of using the waste biogas from anaerobic digestion in a microalgae cultivation system.

Chapter 3 describes methods to optimize biomass productivity of microalgae on a stripped and unstripped FWP by focusing on two possible inhibitory characteristics of FWP: (1)

the high ammonia concentrations and (2) the low phosphorus concentrations. This chapter discusses the use of nitrifying bacteria to reduce the high ammonia concentrations while maintaining the high nitrogen concentrations. This chapter also discusses altering N/P ratios in FWP using phosphorus supplementations to address the relatively low initial phosphorus concentrations.

Chapter 4 discusses methods to optimize lipid production in *C. sorokiniana* and *C. vulgaris* by implementing a nitrogen deplete phase. An additional application of an H_2O_2 stressor to further enhance lipid production is discussed. Analysis of these experiments suggests a nitrogen deplete phase should enhance lipid productivity in both *C. sorokiniana* and *C. vulgaris*, but the additional H_2O_2 stressor is only effective in enhancing lipid accumulation in *C. sorokiniana*. This chapter also details my work in identifying, evaluating, and optimizing both extraction and quantification methodologies for neutral lipid quantification in harvested microalgae biomass. Further discussion regarding necessary future investigations is presented.

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TABLE OF CONTENTS

TABLE OF FIGURES ix

LIST OF TABLES xiv

CHAPTER 1 – Using Wastewater as a Source of Nutrients to Enhance the Economic Feasibility of Microalgae-Derived Biofuels and Other Valuable Commercial Products 1

1.1. Microalgae are Biofactories Capable of Producing Commercially Valuable Products 1

1.2. Prohibitive Factors in Microalgae Cultivation for Industrial and Commercial Applications 6

1.3. Integrating the use of Anaerobic Digester Wastewater for a More Sustainable Microalgae Cultivation Process 8

DISSERTATION SUMMARY 13

REFERENCES 15

CHAPTER 2 – Using a Novel Three Stage Screening Method to Evaluate Growth of Microalgae on Food Waste Permeate 19

2.1. INTRODUCTION 19

2.2. MATERIALS AND METHODS 21

2.2.1. Microalgae Culture Conditions 21

2.2.2. Anaerobic Digester Wastewater 23

2.2.4. Experimental Setup for Three-Tier Growth Studies 24

2.2.5. Using Recycled Wastewater as a Diluent for FWP Dilutions 26

2.2.6. Ammonia Concentration Changes with CO_2 and Air Supplementation 27

2.2.7. Testing CO_2 Supplementation Effects on *C. sorokiniana* Growth 28

2.2.8. Scaling Up *C. sorokiniana* Cultivation to 2-L Scale 29

2.2.9. Biomass Determination 30

2.2.10. Lipid Extraction 30

2.2.11. Bleach-Assisted Nile Red Microplate Assay for Neutral Lipid Quantification 30

2.2.12. Statistical Analysis 32

2.3. RESULTS AND DISCUSSION 32

2.3.1. Nutrient Analysis of FWP 32

2.3.2. Microplate Screening 33

2.3.4 Monitoring Ammonia Concentrations and pH Over Time 36

2.3.5. Evaluating CO_2 Supplementation of *C. sorokiniana* Cultures at Batch Scale (250 mL) 40

2.3.6. Microalgae Cultivation using FWP and SFWP Media at Batch Scale (250 mL) 43

2.3.7. Nutrient Remediation 47

2.3.8. Evaluating Recycled Wastewater as a Diluent for Microalgae Cultivation 49

2.3.9. Cultivation of *C. sorokiniana* using 10% FWP at 2-L Scale 51

2.4. CONCLUSIONS 54

2.5. REFERENCES 56

CHAPTER 3 – Addressing Nutrient Inhibition to Increase Microalgae Growth when Cultivated on Food Waste Permeates 60

3.1. INTRODUCTION 60

3.2. MATERIALS AND METHODS 64

3.2.1. Microalgae Culture Conditions 64

3.2.2. Anaerobic Digester Wastewater 64

3.2.3. *C. sorokiniana* Cultivation on Biochar-Treated FWP Dilutions at Microplate Scale.. 66

3.2.4. Nutrient Supplementation of FWP and SFWP using Jaworski's Medium Stock Nutrients 70

3.2.5. Nitrifying Bacteria Pretreatments to Address Inhibitory Ammonia Concentrations 71

3.2.6. Adjusting N:P Ratios using Phosphorus Supplementation 75

3.2.7. Evaluating Excess Phosphorus Supplementation of SFWP for *C. sorokiniana* Cultivation 77

3.2.8. Biomass Determination Using Gravimetric Analysis ... 78

3.2.9. Bleach-Assisted Nile Red Microplate Assay for Neutral Lipid Quantification ... 78

3.2.10. Statistical Analysis ... 79

3.3. RESULTS AND DISCUSSION ... 80

3.3.10. Cultivation of Microalgae on Biochar-Filtered FWP at Microplate Scale ... 80

3.3.11. Phosphorus Supplementation and Adjusting the Nitrogen-to-Phosphorus Ratio ... 90

3.3.12. Nitrifying Bacteria Treatment of FWP ... 97

3.3.13. Cultivation of *C. sorokiniana* on Nitrifying Bacteria-Treated FWP ... 103

3.4. CONCLUSIONS ... 107

3.5. REFERENCES ... 110

CHAPTER 4 – Using Nitrogen and Oxidative Stress to Increase Lipid Productivity in *C. sorokiniana* and *C. vulgaris* and Addressing Lipid Quantification Limitation ... 113

4.1. INTRODUCTION ... 113

4.2. MATERIALS AND METHODS ... 116

4.2.1. Microalgae Culture Conditions ... 116

4.2.2. Anaerobic Digester Wastewater ... 116

4.2.3. Two-Phase Microalgae Cultivation ... 117

4.2.4. Biomass Determination ... 121

4.2.5. Lipid Extraction ... 122

4.2.6. Bleach-Assisted Nile Red Microplate Assay for Neutral Lipid Quantification ... 122

4.2.7. Evaluating Pigment Interference in the Bleach-Assisted Nile Red Assay ... 123

4.2.8. Comparing the Sulfo-Phospho-Vanillin Assay Versus the Bleach-Assisted Nile Red Assay for Lipid Quantification of Highly Pigmented Extracts ... 124

4.2.9. Using Biochar Versus Graphitized Carbon for Pigment Removal ... 125

4.2.10. Graphitized Carbon Pretreatment of Lipid Extracts ... 126

4.2.11. Bead Beating and Sonication Extraction Methods 128

4.2.12. Evaluating Different Solvent Systems for Microwave-Assisted Extractions of Lipids 131

4.2.13. Evaluating Lipid Recovery After Microwave-Assisted Extraction from Microalgae using a 2:1 $CHCl_3$/MeOH Solvent System 133

4.2.14. Statistical Analysis 134

4.3. RESULTS AND DISCUSSION 134

4.3.1. Two-Phase Cultivation of *C. sorokiniana* 134

4.3.2. Two-Phase Cultivation of *C. vulgaris* 143

4.3.3. Pigment interference in the Bleach-Assisted Nile Red Assay 148

4.3.4. Optimizing Graphitized Carbon Treatments of Lipid Extracts for lipid quantification 153

4.3.5. Using Biochar versus Graphitized Carbon to Remove Pigments from Lipid Extracts 157

4.3.6. Evaluating Bleach-Assisted Nile Red Lipid Assay Precision Between Both Individuals and Replicate Assays 159

4.3.7. Evaluating Bead-Beating Versus Sonication Methods for Neutral Lipid Extraction from Microalgae Biomass 163

4.3.8. Microwave-Assisted Extraction (MAE) of Neutral Lipids from Microalgae Biomass 170

4.4. CONCLUSIONS 176

4.5. REFERENCES 179

APPENDIX 181

TABLE OF FIGURES

Figure 1. The summary flow of the inputs and valuable outputs from microalgae cultivation. Photo source: CEC Project Report, Franz et al. (2020)[5] 2

Figure 2. Valuable co-products of microalgae, including examples of chlorophyll, carotenoids, and lipids. 3

Figure 3. Diagram of biogas production, permeate, and stripped permeate by the anaerobic digestion of organic waste. The permeates in this project are referred to as food waste permeate (FWP) and stripped food waste permeate (SFWP). Algae Image: https://botany.natur.cuni.cz/algo/CAUP/H1986_Chlorella_sorokiniana.htm. 9

Figure 4. Schematic of the three-level screening approach used to screen multiple microalgae strains for both FWP and SFWP dilutions. The different dilutions are identified by the letters A-F (A=10% FWP, B=30% FWP, C=50% FWP, D=10% SFWP, E=30% SFWP, F=50% SFWP). The working volume for the microplate studies and batch-scale studies are 10- and 250-mL volumes, respectively. 24

Figure 5. Representative microplate for *C. sorokiniana* (left) and microplate shaker setup (right) for the level two microplate screenings of *C. sorokiniana*, *C. vulgaris*, *S. acuminatus*, and *S. obliquus*. Blank permeate samples were maintained in a separate plate and accounted for in the absorbance of the microalgae wells. 34

Figure 6. Changes in pH and ammonia concentrations over time in 10% FWP or 30% SFWP with either CO_2 or air bubbling at batch scale (250-mL). (a) 10% FWP + air, (b) 10% FWP + 6% CO_2/air, (c) 30% SFWP + air, (d) 30% SFWP + 6% CO_2/air 37

Figure 7. Growth curves for *C. sorokiniana* cultivated in (a) 10% FWP and (b) 30% SFWP with pumped air versus a 6% CO_2/air mixture at batch scale (250-mL). FWP = food waste permeate; SFWP = stripped food waste permeate; Syn media = synthetic media, BG-11. 41

Figure 8. Growth curves for (a) *C. sorokiniana*, (b) *C. vulgaris*, (c) *S. acutus f. alternans*, and (d) *S. obliquus* cultured in different experimental conditions at 250-mL batch scale, as measured by optical density. FWP = food waste permeate; SFWP = stripped food waste permeate; JW = Jaworski's Medium. 44

Figure 9. Growth curves for *C. sorokiniana* cultivated on (a) 10% FWP and (b) 30% SFWP diluted using deionized water (DI), recycled wastewater (RWW), or a 1.7:1 DI/RWW mixture at microplate scale (10-mL). FWP = food waste permeate; SFWP = stripped food waste permeate; JW = Jaworski's medium 51

Figure 10. Growth curves for *C. sorokiniana* cultivated on 10% FWP in August 2019 and August 2020 at 1.75 and 1.5-L scales, respectively. FWP = food waste permeate 53

Figure 11. Biochar-treated food waste permeate (FWP) before and after treatment with 1 g of walnut-derived biochar per 40 mL FWP. Photo credit: Tyler Barzee. 66

Figure 12. Microplate layout for evaluating *C. sorokiniana* growth on biochar-treated FWP. All conditions were tested with N=5, with the sixth well in the plate being the blank control. 67

Figure 13. (a) Growth curves from preliminary studies evaluating *C. sorokiniana* cultivation on several dilutions of biochar-filtered 10% food waste permeate (10% CF-FWP). Cultivation was performed at a microplate scale (10 mL) with N=5. (b) Growth curves for *C. sorokiniana* cultivation on various dilutions of 10% CF-FWP and 10% CF-SFWP. Cultivation was performed at a microplate scale (10 mL) and N=3. 81

Figure 14. *C. sorokiniana* growth on 10% biochar-filtered food waste permeate (CF-FWP) with various supplementations. Cultivation was performed at 10-mL microplate scale with N=5. 85

Figure 15. Transmission electron microscopy images of *C. sorokiniana* grown on a) Jaworski's Medium, b) bicohar-filtered 10% FWP (10% CF-FWP), c) 10% CF-FWP + JW nutrients, and d) 10% CF-FWP with 100% phosphate supplementation. Ultrastructure specificiation is described in the Appendix (Figure A1). 87

Figure 16. Microplate setup demonstrating the pigment variation between *C. sorokiniana* cultured on various 10% CF-FWP conditions versus a Jaworski's Medium (JW) control. The JW control is indicated, and the remaining plates contain *C. sorokiniana* cultivated on 10% CF-FWP with different supplementations. 88

Figure 17. Treatment of undiluted (a) FWP and (b) SFWP with different amounts of biochar. Treatment effectiveness was determined by ocular inspection. The conditions are as follows from left to right: 20 mg/mL, 10 mg/ml, 5 mg/mL, 2 mg/mL. 89

Figure 18. Growth curves for *C. sorokiniana* cultivation on (a) 10% FWP, (b) 10% SFWP, and (c) 50% SFWP supplemented with different volumes of Jaworski's Medium (JW) nutrients. Cultivation was performed at microplate scale (10-mL), and values are the average of duplicates. 91

Figure 19. *C. sorokiniana* growth on 10% FWP adjusted to have (a) 3:1 N/P ratio, (b) 6:1 N/P and 15:1 N/P ratios (c) 23:1 N/P ratio, and (d) 33:1 N/P ratio. Experiments were performed with the following culture voluems: (a), (b), and (c) = 250 mL scale and (c) was performed at 100 mL scale. 93

Figure 20. Growth curves for *C. sorokiniana* cultivated at batch scale (250 mL) on 10% and 30% SFWP with and without phosphorus supplementation. SFWP = stripped food waste permeate, JW = Jaworski's Medium (synthetic medium) 96

Figure 21. Total nitrogen (TN) and ammonia nitrogen concentration changes in 10% food waste permeate over time with and without nitrifying bacteria (NB) as a pretreatment method and cultivated with either air or CO_2 bubbling. (a) no NB + CO_2; (b) with NB + CO_2; (c) no NB + air; (d) with NB + air........ 99

Figure 22. Compositional change in nitrogen species during the treatment of 100% food waste permeate (FWP) using nitrifying bacteria (NB) at 2-L scale. (a) 100% FWP+NB in June 2018, (b) 100% FWP-NB in April 2019, (c) 100% FWP+NB in April 2019. 102

Figure 23. Growth curves for *C. sorokiniana* on nitrifying bacteria (NB) treated 10% FWP, untreated 10% FWP from the NB treatment experiment (untreated), and a "fresh" 10% FWP control prepared from the stock container (unadjusted). All conditions were tested in triplicate in 100-mL volumes. FWP = food waste permeate. 104

Figure 24. Growth curves for *C. sorokiniana* on nitrifying bacteria (NB) treated and untreated 10% FWP along with an unadjusted control prepared with fresh permeate. All conditions were performed at 250 mL batch culture scale. FWP = food waste permeate; B = bubbled; +/- = with/without. 106

Figure 25. Growth curves for *C. sorokiniana* cultivated in 1.5-L of 10% FWP and 30% SFWP followed by a 125-mL scale second phase of either freshwater (FW), filtered seawater (SW), or the original media performed in (a) 2018 and (b) 2019. The first phase was performed at N=1, and the second phase conditions were performed at N=3. FWP = food waste permeate; SFWP = stripped food waste permeate. 138

Figure 26. Growth curves for *C. sorokiniana* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide. All conditions were performed in triplicate. FWP = food waste permeate; SFWP = stripped food waste permeate 141

Figure 27. Comparison of neutral lipid content in *C. sorokiniana* when cultivated using two-phase methodology followed by treatment with or without hydrogen peroxide as a metabolic stressor. FW = tap water; SW = filtered seawater; FWP = food waste permeate........ 142

Figure 28. Growth curves for *C. vulgaris* cultivated in 1.5-L of 10% FWP and 30% SFWP followed by a 125-mL scale second phase of either freshwater (FW), filtered seawater (SW), or the original media. The first phase was performed at N=1, and the second phase conditions were performed at N=3. FWP = food waste permeate; SFWP = stripped food waste permeate....... 144

Figure 29. Growth curves for *C. vulgaris* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide. All conditions were performed in triplicate. FWP = food waste permeate; SFWP = stripped food waste permeate 147

Figure 30. Comparison of neutral lipid content in *C. vulgaris* when cultivated using two-phase methodology followed by treatment with or without hydrogen peroxide as a metabolic stressor. FW = tap water; SW = filtered seawater; FWP = food waste permeate...................... 148

Figure 31. Graph demonstrating the neutral lipid content in 1 mL of *C. sorokiniana* lipid extracts quantified by bleach-assisted Nile Red assay. The "standard" was a 100 µL aliquot of 1 mg/mL lipid standard. 150

Figure 32. The concentration of lipids in *C. sorokiniana* lipid extracts as determined by a bleach-assisted Nile red assay (NRLA) and sulfo-phospho-vanillin assay (SPV) demonstrating pigment inference in the NRLA. All conditions were performed in triplicate. 152

Figure 33. Comparison between chlorophyll pigments measured at 680 nm and neutral lipid content as measured by bleach-assisted Nile Red assay. The conditions evaluated the mass of graphitized carbon (GC) used to reduce pigment concentrations in lipid extracts of *C. sorokiniana* cultivated on (a) 10% FWP and (b) BG-11 synthetic media. 155

Figure 34. Comparison of the color changes observed of *C. sorokiniana* lipid extracts treated with varying amounts of graphitized carbon (GC). 156

Figure 35. Comparison between graphitized carbon (GC) treatment durations using 25 mg GC to treat lipid extracts from *C. sorokiniana* cultivated on (a) 10% FWP and (b) BG-11 synthetic medium. FWP = food waste permeate. 157

Figure 36. Comparison between biochar versus graphitized carbon as a pretreatment for highly pigmented *C. sorokiniana* lipid extracts being analyzed by a bleach-assisted Nile Red assay. The assay was performed two times with a triplicate design. 158

Figure 37. Neutral lipids by weight as determined by bleach-assisted Nile Red assay. Individual assays were performed by: CWY = Cody Yothers, KMR 1 & 2 = Kayla Rude. Samples were of the same algae extract with and without graphitized carbon (GC) treatment. Standard was 100 µL of a 1 mg/mL lipid standard in $CHCl_3$. 160

Figure 38. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by sonication, bead-beating using 250 mg beads, or filling the extraction tube with beads up to the 0.75 mL mark (0.75 mL beads). 164

Figure 39. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by bead-beating, two rounds of sonication (sonication x2), or three rounds of sonication (sonication x3). 166

Figure 40. Summary of both (a) lipid concentrations and (b) neutral lipid content extracted from microalgae using microwave-assisted extraction (MAE) with either $CHCl_3$ or 2:1 $CHCl_3$/MeOH solvent to evaluate lipid content. A standard was added to evaluate lipid recovery using MAE. 171

Figure 41. Comparison between neutral lipid quantification and pigment removal in *C. sorokiniana* lipid extracts after being treated with 50 mg graphitized carbon (GC), 25 mg GC, or 25 mg GC twice, consecutively. This comparison was performed on samples extracted with either (a) $CHCl_3$ or (b) 2:1 $CHCl_3$/MeOH. 173

Figure 42. extracts from *C. sorokiniana* biomass extracted using microwave-assisted extraction with a 2:1 $CHCl_3$/MeOH solvent system. The images are in procedural order: (a) extract before MAE (left) and after MAE (right), (b) extracts at N=3 after MAE, (c) extracts after shaking, and (d) extracts after graphitized carbon treatment. 176

LIST OF TABLES

Table 1. Oil yields produced from popular biofuel feedstock crops: soybean and sunflower. These crops are compared to the estimated algal oil yield. Table and associated notes adapted from Darzins et al. and Chisti. 4

Table 2. Microalgae strains investigated for cultivation on food waste permeate (FWP) and their sources. UT = University of Texas; NCMA = Bigelow National Center for Marine Algae 22

Table 3. Summary of nutrient concentrations (mg/L) in anaerobic digester permeates before and after the stripping process. FWP = food waste permeate; SFWP = stripped food waste permeate. 23

Table 4. Monitored parameters comparing 10% FWP and 30% SFWP supplemented with air versus a 6% CO_2/air mixture at a 250-mL scale. Final measurements were taken on day 13. Ammonia-nitrogen speciation was determined using the Henderson-Hasselbalch equation and the total NH_3-N concentrations. FWP = food waste permeate; SFWP = stripped food waste permeate 39

Table 5. Ratios of NH_4^+/NH_3 over time in 10% FWP and 30% SFWP supplemented with air or 6% CO_2/air mixture at 250-mL scale. 39

Table 6. Growth characteristics and remediation capacity for *C. sorokiniana* tested at 250-mL scale with air versus a 6% CO_2/air mixture at batch scale (250-mL). FWP = food waste permeate; SFWP = stripped food waste permeate; TN = total nitrogen 42

Table 7. Growth characteristics of four microalgae strains tested at 250-mL scale. 45

Table 8. Initial concentrations of total nitrogen (TN), ammonia-nitrogen (NH_3-N), nitrates (NO_3^--N), and phosphates (PO_4^{3-}) in 10% FWP and 30% SFWP along with removal efficiencies of each nutrient by *C. sorokiniana*, *C. vulgaris*, *S. acutus f. alternans*, and *S. obliquus*. 48

Table 9. Total nitrogen (TN) and ammonia-nitrogen (NH_3-N) concentrations in the 10% FWP RWW and 30% SFWP RWW that were used to prepare the various 10% FWP and 30% SFWP *C. sorokiniana* cultures evaluated in this microplate scale (10-mL) study. RWW = recycled wastewater; FWP = food waste permeate; SFWP = stripped food waste permeate 50

Table 10. Growth characteristics of *C. sorokiniana* cultivated on 10% FWP in August of 2019 and August 2020, respectively. 53

Table 12. *C. sorokiniana* growth characteristics when cultivated on 10% food waste permeate (FWP) with differing nitrogen-to-phosphorus ratios and a synthetic media control.[a] JW = Jaworski's Medium 94

Table 13. Growth characteristics for *C. sorokiniana* grown on 10% and 30% SFWP with and without phosphorus supplementation at 250 mL scale. Included are the nitrogen and phosphorus concentrations and the associated N/P ratio of the initial growth media. 96

Table 14. Concentrations of total nitrogen (TN) and ammonia-nitrogen (NH_3-N) when treated with or without nitrifying bacteria (NB) and a 6% CO_2/air versus air supplementation method. FWP = food waste permeate 100

Table 15. Change in nitrogen composition over time with and without nitrifying bacteria (NB) for two separate treatments performed in April 2018 and April 2019. 103

Table 16. Growth characteristics for *C. sorokiniana* cultivated on nitrifying bacteria (NB) treated and untreated 10% FWP at 100 mL scale. 105

Table 17. Growth characteristics for *C. sorokiniana* on nitrifying bacteria (NB) treated and untreated 10% FWP. All conditions were performed at 250 mL batch culture scale. 107

Table 19. Growth characteristics for *C. sorokiniana* tested at 125-mL scale using a two-phase nutrient depletion method. FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater. 139

Table 20. Nutrient composition of initial FWP/SFWP and subsequent final resuspension media (125-mL volumes) for *C. sorokiniana* cultivated using a two-phase method. FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater 139

Table 21. Growth characteristics for *C. sorokiniana* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide. FWP = food waste permeate; SFWP = stripped food waste permeate 142

Table 22. Growth characteristics for *C. vulgaris* tested at 125-mL scale using a two-phase nutrient depletion method. FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater. 144

Table 23. Nutrient composition of initial FWP/SFWP and subsequent final resuspension media (125-mL volumes) for *C. vulgaris* cultivated using a two-phase method. FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater 145

Table 24. Growth characteristics for *C. vulgaris* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide. FWP = food waste permeate; SFWP = stripped food waste permeate 147

Table 25. Neutral lipid content in 1.1 mL of *C. sorokiniana* lipid extracts as quantified by bleach-assisted Nile Red assay. The analysis was performed between relative conditions and their comparison to the theoretical value. 150

Table 26. The concentration of lipids in *C. sorokiniana* lipid extracts as determined by a bleach-assisted Nile red assay (NRLA) and sulfo-phospho-vanillin assay (SPV). 152

Table 27. Percent neutral lipids by weight as determined by bleach-assisted Nile Red assay. Individual assays were performed by: CWY = Cody Yothers, KMR 1 & 2 = Kayla Rude. Samples were of the same algae extract with and without graphitized carbon (GC) treatment. Standard was 100 µL of a 1 mg/mL lipid standard in $CHCl_3$. 160

Table 28. Comparisons between the spiked and unspiked conditions to determine the accuracy and evaluate the precision of the bleach-assisted Nile Red assay. Individual assays were performed by: CWY = Cody Yothers, KMR 1 & 2 = Kayla Rude. Samples were of the same *C. sorokiniana* extract with and without graphitized carbon (GC) treatment. Standard was 100 µL of a 1 mg/mL lipid standard in $CHCl_3$. 162

Table 29. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by sonication, bead-beating using 250 mg beads, or filling the extraction tube with beads up to the 0.75 mL mark (0.75 mL beads). 164

Table 30. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by bead-beating, two rounds of sonication (sonication x2), or three rounds of sonication (sonication x3). 166

Table 31. Summary of sonication and bead-beating extractions of microalgae extracts to evaluate lipid detection and recovery. 169

Table 32. Summary of sonication and bead-beating extractions of a lipid standard without algal biomass to evaluate lipid detection and recovery. 169

Table 33. Summary of neutral lipid content extracted from microalgae using microwave and either $CHCl_3$ or 2:1 $CHCl_3$/MeOH solvent to evaluate lipid content. A standard was added to evaluate lipid recovery using MAE. Extract = 4 mL $CHCl_3$ 171

Table 34. Comparison between neutral lipid content in *C. sorokiniana* lipid extracts extracted with either $CHCl_3$ or 2:1 $CHCl_3$/MeOH solvent and treated with 50 mg graphitized carbon (GC), 25 mg GC, or 25 mg GC twice, consecutively. 174

Table 35. Comparison of *C. sorokiniana* MAE extracts pretreated with graphitized carbon (GC) the associated percent recoveries and percent neutral lipids by weight. 175

CHAPTER 1 – Using Wastewater as a Source of Nutrients to Enhance the Economic Feasibility of Microalgae-Derived Biofuels and Other Valuable Commercial Products

1.1. Microalgae are Biofactories Capable of Producing Commercially Valuable Products

Microalgae, a unicellular microscopic form of algae, can produce many different commercially valuable products such as animal feed, nutraceuticals, pharmaceuticals, and biofuels.[1,2] The constant technological developments in today's society have provided opportunities for applying microalgae as a sustainable alternative in a variety of industries, including consumer products and wastewater treatment. Microalgae products have become a billion-dollar market in the last decade due to increased awareness regarding the health benefits of microalgae and the increasing demand for more sustainable, plant-derived products and production systems.[2] A recent market analysis report by Data Bridge Market Research reported that this increased awareness and increasing product demand is the driving force behind the expected annual growth rate of over 6% in the microalgae market between 2021 and 2028.[3] By 2031, the microalgae-based commercial product market is estimated to reach $4.2 billion.[4]

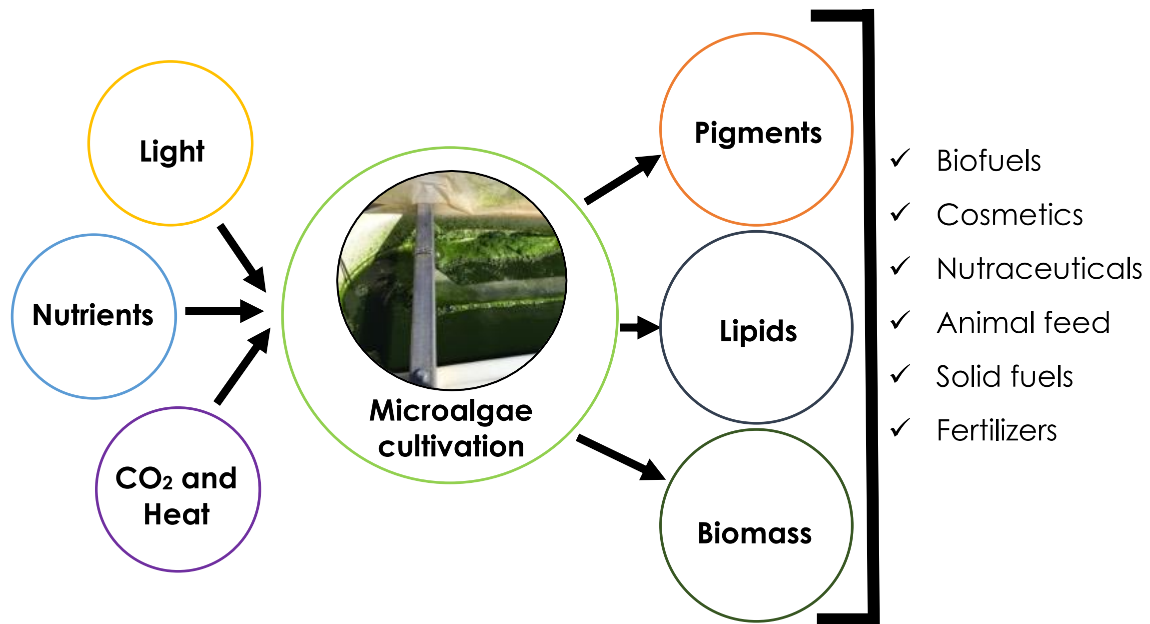

Figure 1. The summary flow of the inputs and valuable outputs from microalgae cultivation. Photo source: CEC Project Report, Franz et al. (2020)[5]

Although microalgae products and technology are rising in popularity, the value of microalgae has been recognized for centuries.[6] *Chlorella* sp. is one microalgae species whose nutritional benefits have been exploited for decades as a health food.[2] *Spirulina platensis* has become a popular nutritional supplement and is well-known for its bright green color.[6] Significant value is derived from the pigments produced by microalgae, including chlorophyll, carotenoids, and phycobiliproteins (Figures 1 and 2). Chlorophyll is used as a nutraceutical and natural pigment for feed, pharmaceuticals, and cosmetics.[7] Carotenoids are commonly found as popular nutritional supplements in the form of β-carotene, astaxanthin, lutein, etc. The β-carotene market alone is worth over an estimated $500 million globally, and in 2015, algae-derived β-carotene accounted for over 35% of the total revenue.[8] In addition to nutraceuticals, the relatively recent shift towards algae-derived biofuels has gained traction as a sustainable

biofuel alternative, and the production of these biofuels has developed into a large field of research of its own in recent years.

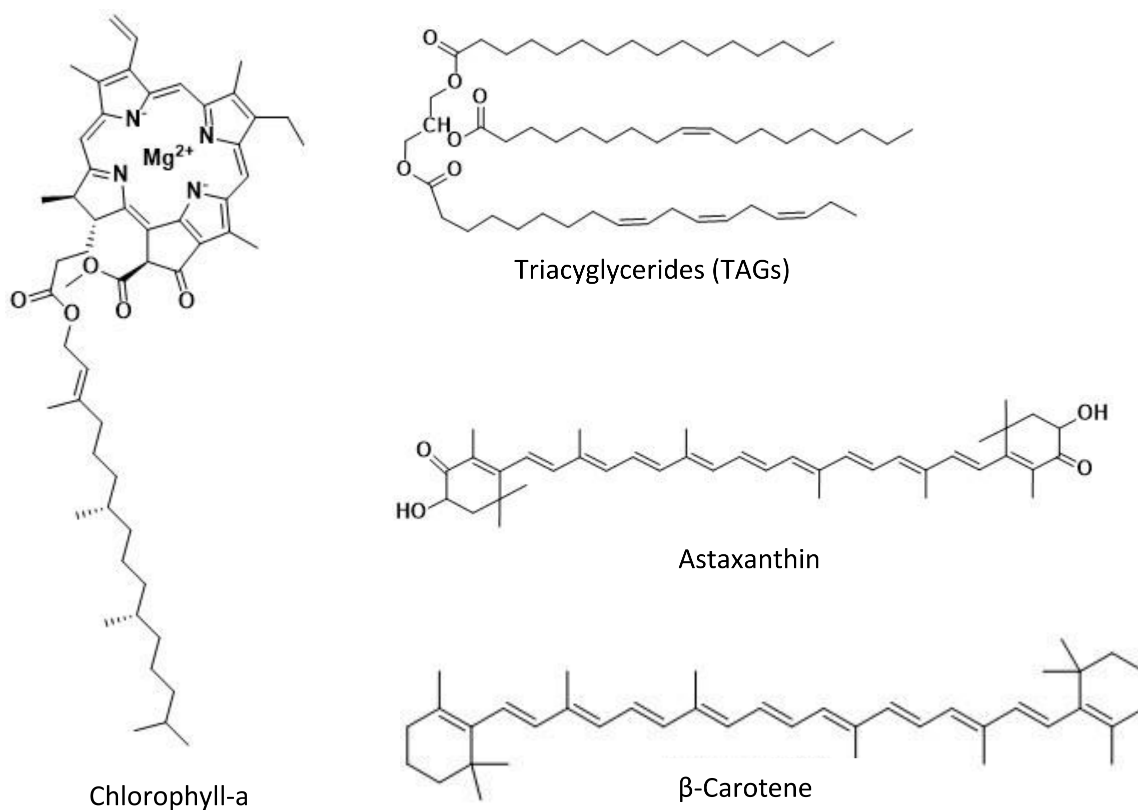

Figure 2. Valuable co-products of microalgae, including examples of chlorophyll, carotenoids, and lipids.

Today's fuel market is dependent on a diminishing volume of environmentally detrimental fossil fuels, and in response, there are increased efforts to find alternative fuel feedstocks. Common biofuel feedstocks such as corn and soybean require significant usage of arable land and afford relatively low lipid yields (Table 1). Alternatively, certain microalgae strains have been shown to accumulate large quantities of lipids, making them a potential alternative for biofuel production (Figure 1 and 2).[9,10] When considering microalgae-derived

biofuel production combined with the production of other microalgae-derived products and technologies (i.e., wastewater treatment and environmental remediation), the value of the microalgae market further increases.[5]

Table 1. Oil yields produced from popular biofuel feedstock crops: soybean and sunflower. These crops are compared to the estimated algal oil yield. Table and associated notes adapted from Darzins et al. and Chisti.[11,12]

	Darzins et al. (2010)	Chisti (2008)		
Crop	Oil Yield (L/ha/yr)	Oil Yield (L/ha)	Land area needed (M ha)[a]	Percent of existing US cropping area[a]
Corn	na	172	1540	846
Soybean	450	446	594	326
Microalgae[b]	3800 – 50800[c]	58700	4.5	2.5

na = not available
[a] For meeting 50% of all transport fuel needs of the United States
[b] 30% oil (w/w) in biomass
[c] These are estimated yields from the IEA Bioenergy Task 39 report.[11]

The choice to use microalgae as a biofuel feedstock is further strengthened by microalgae's ability to fix carbon dioxide and uptake surrounding nutrients (Figure 1) without extensive use of arable land (Table 1). Microalgae also surpass terrestrial crops in their carbon fixation rates because the increased growth rate of microalgae increases the overall carbon fixation efficiency compared to terrestrial plants.[13] In fact, microalgae are responsible for an estimated 50% of the total carbon fixation on the planet, and microalgae contribute significantly to the overall oxygen makeup in the atmosphere.[14] Carbon fixation through microalgae cultivation, in turn, would mitigate greenhouse gas emissions, benefitting the overall sustainability of the cultivation system. In addition, the uptake of surrounding nutrients,

specifically nitrogen and phosphorus, is vital in the metabolic processes responsible for producing biomolecules such as DNA and proteins. These elements also have structural applications, such as in the thylakoid phospholipid bilayer, the membrane across which photosynthesis occurs. Therefore, limiting the availability of these nutrients plays a significant role in cultivation strategies to increase the production of lipids and other valuable co-products.

Unlike other terrestrial biofuel feedstock crops, the uptake of surrounding nutrients provides the potential for the microalgae to act as a remediation strategy for eutrophic or contaminated waters. Eutrophication is commonly associated with excessive nitrogen and phosphorus concentrations resulting from agricultural runoff. Phosphorus fertilizer use has experienced an estimated 25% increase since 1964 and is widely used for corn and soybean cultivars, two popular biofuel feedstock crops; so, rather than reducing the risks of eutrophication, corn and soybean cultivation could be contributing to it.[15] So, unlike corn and soybean cultivation, microalgae cultivation has the potential to lower the risk of eutrophication of our resources by preventing excessive nutrient loading from anthropogenic activities (e.g., farming, industry).

Eutrophic waters are discharged into other water bodies that make their way into our coastal waters, causing widespread eutrophication throughout our coastal ecosystems. The National Oceanic and Atmospheric Administration (NOAA) estimates that over 65% of estuaries and coastal waters in the contiguous United States are moderate to severely degraded by excessive nutrient inputs.[16] Eutrophication leads to algal blooms, and these algal blooms consume dissolved oxygen, create an oxygen-deprived environment, block sunlight essential for photosynthesis, and killing plants, fish, and the habitats in which these fish reside. The

decomposition of plants and other organic matter produces carbon dioxide, which transforms into carbonic acid, lowering the pH of seawater and creating an acidic environment (i.e., ocean acidification). Ocean restoration projects and water treatment methods address the harmful effects of eutrophication, but methods preventing nutrient runoff and eutrophication should be implemented rather than only reacting to the existing problem.[16]

1.2. Prohibitive Factors in Microalgae Cultivation for Industrial and Commercial Applications

The main issue prohibiting large-scale, widespread biofuel production via microalgae is the cost of microalgae cultivation, constituting up to 85% of the total cost of biofuel production.[17] Two main culturing methods are used: raceway ponds and photobioreactors. Raceway ponds are more practical at large scale because they are significantly less expensive to operate than photobioreactors.[18] However, raceways ponds commonly encounter bacterial contamination, extended growth periods, and environmental influences such as temperature and precipitation, which can significantly decrease microalgae productivity.[5] Photobioreactors, while more expensive, allow for a lot more control in the cultivation procedure. The balance between lowering the cost of operation and affecting the microalgae outputs is a prominent issue in industrial systems and scientific research. On average, the high cultivation costs result in overall biodiesel costs between $5-10/gal, which is not competitive with the $3/gal diesel prices currently seen in today's fuel market.[17] To lower the cost of cultivation, a combination of technologies and markets can be considered to lower production costs and increase the sustainability of the microalgae cultivation systems. Specifically, wastewater has been proposed

as an alternative growth medium due to its abundant availability, nutrient content, and low cost.

Diverting wastewater resources to properly equipped treatment facilities has the potential to reduce the transfer of excessive nutrient concentrations into our freshwater resources. A timely potential hazard of untreated or poorly treated wastewater includes eutrophication of lakes and rivers leading to algal blooms, which can further lead to ecosystem disruption and loss of biodiversity.[19] So, coupling microalgae cultivation and wastewater remediation could provide a sustainable, economically feasible alternative source of nutrients while mitigating the risk of surface water eutrophication. Although microalgae cultivation has been reported using various municipal wastewater streams, such wastewater produced in multistage treatment plants is generally characterized by having relatively low nutrient concentrations.[9,10,20] An additional drawback to using municipal wastewater for microalgae cultivation is that the wastewater source is unsuitable for products produced for human and animal consumption.

United States law prohibits using municipal wastewater streams to be used as cultivation media for microalgae-derived consumable products, such as products for the cosmetics and nutraceuticals markets, because these wastewaters result from human waste.[21] Dairy farms are also a common source of wastewater for microalgae production. Dairy manure waste streams and anaerobically digested dairy manure waste are readily available and are high in the essential nutrients: nitrogen, phosphorus, and potassium; therefore, microalgae cultivation on dairy waste streams is common and well-studied.[22,23] However, because this dairy manure wastewater inherently contains biological hazards, its use to cultivate microalgae

for human or animal consumption must follow strict regulations regarding its use as dictated by the Code of Federal Regulations (CFR).

So, while wastewater is a promising, inexpensive source of nutrients for microalgae cultivation, it is imperative to identify alternative wastewater streams derived from non-human and non-animal waste sources for microalgae cultivation to avoid the strict limitations associated with these potential cultivation media. The alternative streams must contain high nutrient concentrations beneficial for microalgae growth and be readily available at a low cost. In section 1.3, I describe the use of wastewater streams produced via the anaerobic digestion process of food waste. These streams have both the benefit and challenge of having high nutrient concentrations, yet they are derived from a non-prohibited source (i.e., food waste).

1.3. Integrating the use of Anaerobic Digester Wastewater for a More Sustainable Microalgae Cultivation Process

In recent years, there has been increased interest in anaerobic digestion technology due to the many environmental benefits it provides, such as renewable energy and reduced carbon emissions.[24] The US alone has over 2200 operational facilities operating at water treatment plants, landfills, and farms. However, there are also environmental risks associated with the use of anaerobic digestates. Two areas of concern that arise when applying anaerobic digestate as fertilizer are: (1) their effect on soil health due to metal accumulation and (2) the possible increase in NH_3 emissions after application.[25] So, finding alternative applications of this anaerobic digestate is vital to fully use the benefits of anaerobic digestion technology while also avoiding the potential environmental risk with digestate disposal.

Anaerobic digestion of food waste generates a nutrient-dense wastewater stream and biogas composed of methane and carbon dioxide (Figure 3). The wastewater stream produced after ultrafiltration of the food waste digestate is called "food waste permeate" (FWP) (Figure 3). FWP has been characterized as having high ammonia-nitrogen (NH_3-N) concentrations, high chemical oxygen demand (COD), and relatively low phosphorus concentrations.[26] Due to the complex nature and dark color of FWP, it is essential to consider possible stressors such as nutrient toxicity, nutrient deficiency, and light penetration, all of which can be both a benefit and a challenge to microalgae cultivation.[27–29]

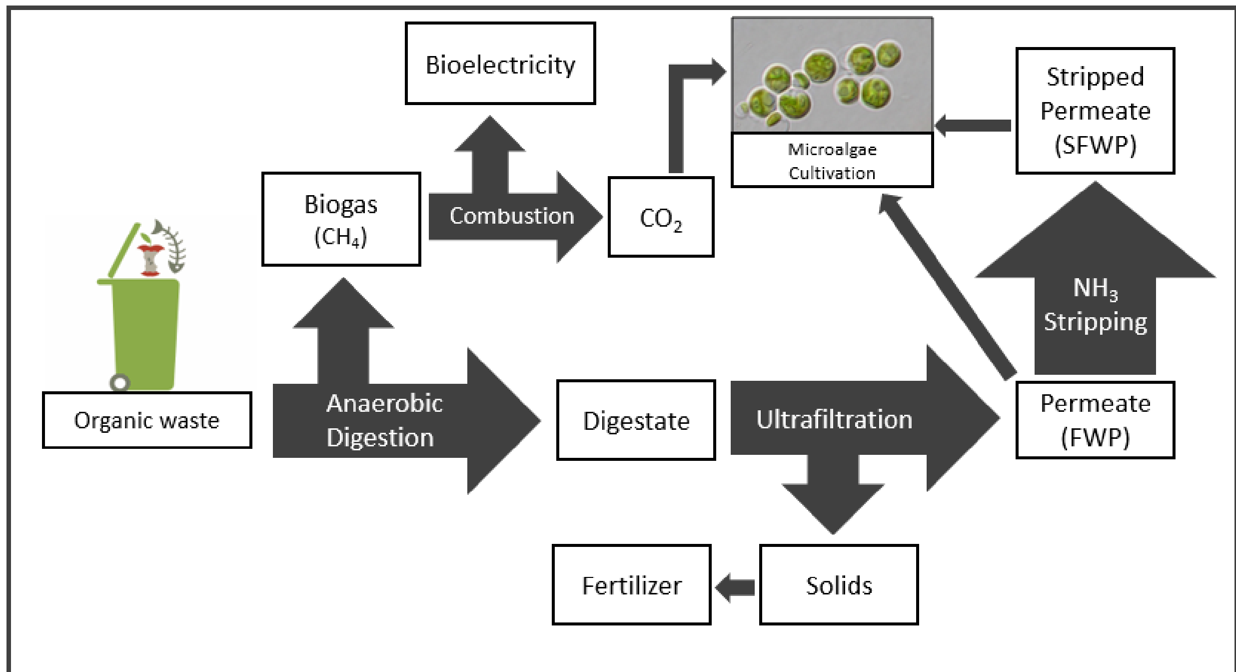

Figure 3. Diagram of biogas production, permeate, and stripped permeate by the anaerobic digestion of organic waste. The permeates in this project are referred to as food waste permeate (FWP) and stripped food waste permeate (SFWP). Algae Image: https://botany.natur.cuni.cz/algo/CAUP/H1986_Chlorella_sorokiniana.htm.

Stressors such as salt stress, light intensity, nutrient deficiency, oxidative stress, and temperature are valuable tools to manipulate microalgae metabolism to produce a favored product.[27,30,31] Nutrient deficiency and other oxidative stressors are two relatively inexpensive and scalable methods to increase lipid productivity in microalgae. Nutrient deficiency is one of the most common techniques to increase lipid productivity by affecting the metabolic processes responsible for cell growth and replication. Limiting nitrogen, for example, prevents the synthesis of new nitrogen-containing biomolecules (e.g., DNA, proteins) and is well known to signal a metabolic shift towards the production of lipid and starch molecules instead of growing and replicating. The same growth inhibition is observed in the case of phosphorus and phosphate deficiency.[32] Our lab observed a greater than 100% increase in lipid production when nitrogen deficiency and hydrogen peroxide treatments were used in combination during the cultivation of *Phaeodactylum tricornutum*.[30] A factor that is incredibly stressful to microalgae when cultivated on permeate is the high concentrations of nitrogen, specifically ammonia. At the high concentrations seen in permeate, microalgae growth would be inhibited entirely, not even allowing the opportunity to produce lipids.

As a method to reduce the high ammonia concentrations in FWP, the permeate can be put through an ammonia-stripping process using the addition of sodium carbonate to remove ammonia, yielding a stripped FWP (SFWP) containing around 10% of the initial ammonia concentrations.[33] While ammonia-stripping is possible, the cost of the energy, time, and inputs must be considered in the overall efficiency of the process for microalgae cultivation and the production of valuable co-products.

Nutrient-dense waste streams such as anaerobic digester permeate create a problem for wastewater treatment because they cannot be efficiently and effectively treated.[34] Traditional treatment stages, such as chlorination, are not equipped to handle such a high nutrient load, creating an upset in the treatment process step(s).[35] Alternative treatment methods must be developed to ensure safe environmental discharge. Previous studies have shown that microalgae cultivation is capable of remediating other wastewater streams.[36,37] These previous remediation studies demonstrate the potential of microalgae to utilize the nitrogen and other nutrients in anaerobic digestate resulting in a remediated wastewater stream and significant biomass accumulation.[38,39] However, there is little precedent in current literature evaluating methods of cultivating microalgae on such a complex, high strength wastewater stream such as anaerobic digester permeate for industrial-scale biofuel production applications.

One significant benefit of anaerobic digestion technology is the biogas generation and biogas combustion that produces CO_2, heat, and bioelectricity (Figure 3). The biogas produced during anaerobic digestion of food waste at the Renewable Energy Anaerobic Digester (UC Davis READ) and the South Area Transfer Station (SATS) digester is primarily CH_4 and CO_2 at approximately a 50/50 CH_4/CO_2 ratio.[5] The CH_4 is combusted, producing bioelectricity, CO_2, and heat in addition to other trace compounds such as CO, NO_x, and SO_x, and VOC side-products. Bioelectricity has the potential to be sold to outside consumers, or it can be routed into the microalgae cultivation setup itself. After combustion, the biogas is composed of around 6% CO_2 blended air. This CO_2 mixture can be piped into microalgae cultivation systems as an additional carbon source and a means of mixing and aeration. Utilizing the CO_2 rather than blowing it off

after combustion is a more environmentally friendly, sustainable alternative that increases the feasibility of microalgae cultivation on anaerobic digestate for biofuel production.[40]

Microalgae sequester CO_2 during the photosynthetic process, which is the driving force behind research into using microalgae for CO_2 sequestration in flue gasses. Some studies have shown the potential of *Chlorella sorokiniana* to reduce CO_2 concentrations in industrial flue gas, but there was also observed inhibition of microalgae growth at the higher concentrations tested.[41] The flue gas used in the study by Kumar et al. varied in composition and strength with a composition of primarily CO_2 and H_2S at 15.6% v/v and 120 mg/L, respectively.[41] This flue gas composition is comparably different to that of the biogas produced at the READ and SATS facilities mentioned previously to be primarily a 6% CO_2/air mixture. Carbon dioxide concentration has a significant impact on microalgae growth, and in general, microalgae perform better at lower (~ 5%) CO_2 concentrations; however, microalgae tolerance to CO_2 varies by species and conditions being used.[41,42]

When considered in combination, modern technologies, consumer demand, and the need for sustainable alternative feedstocks and commercial products are the driving force behind research into different microalgae technologies and applications for the growing market. Microalgae are valuable in various industries such as biofuels and wastewater treatment as low cost, sustainable alternative feedstocks and treatment systems. In addition, the harnessing of additional value-added products (e.g., solid fuels, animal feed, nutraceuticals) have the potential to further offset cultivation costs making microalgae technologies more economically feasible.

DISSERTATION SUMMARY

This dissertation focuses on microalgae cultivation on nutrient-dense food waste permeate for biofuel feedstock production and wastewater remediation. My work identifies methods for microalgae cultivation on nutrient-dense waste streams and the effect of this concentrated waste stream on microalgae growth and productivity and their resultant remediation capacity. Chapter 1 discusses the commercial value of microalgae-derived natural products, the current economic challenges of producing these algae products, specifically biofuels. This chapter describes the use of wastewater, specifically anaerobic digester effluent and its byproducts, as a potential method to reduce the cost of microalgae cultivation. Further, the inherent benefits and challenges of anaerobic digestion technology and the use of it nutrient-dense waste streams as an alternative nutrient source for microalgae cultivation are discussed.

Chapter 2 discusses the microplate screening methodology that was implemented to evaluate ten different microalgae strains and their ability to be cultivated on various permeate conditions. Strains and conditions identified in the microplate studies (10 mL) were scaled up to larger batch cultures (250 mL), and a baseline culturing method was established. Out of the 10 strains evaluated at microplate scale (10 mL), only four demonstrated robust growth on the FWP used throughout this research with *C. sorokiniana* having the highest growth potential. The use of a 6% CO_2/air mixture mimicking the CO_2 concentrations in the anaerobic digester biogas was supplemented during microalgae cultivation at batch scale. Resultant nutrient concentration changes were monitored alongside microalgae growth and the final analyses demonstrate the feasibility of using the waste biogas from anaerobic digestion in a microalgae

cultivation system. Finally, this chapter describes increasing *C. sorokiniana* cultivation to a 2-L scale and the effects on microalgae growth.

Chapter 3 describes methods to optimize biomass productivity of microalgae on a stripped and unstripped FWP by focusing on two possible inhibitory characteristics of FWP: (1) the high ammonia concentrations and (2) the low phosphorus concentrations. This chapter discusses the use of nitrifying bacteria as a method to reduce initial ammonia concentrations to a more tolerable level for microalgae cultivation while maintaining the high nitrogen concentrations. This chapter also discusses altering N/P ratios in FWP using phosphorus supplementations to address the relatively low initial phosphorus concentrations. These experiments demonstrate no increase in microalgae growth, suggesting another factor, or a combination of factors, is responsible for the low nitrogen and high phosphorus remediation capacities observed in Chapter 2.

Chapter 4 discusses methods to optimize lipid production in *C. sorokiniana* and *C. vulgaris* by implementing a nitrogen deplete phase. An additional application of an H_2O_2 stressor to further enhance lipid production is discussed. Analysis of these experiments suggests a nitrogen deplete phase should enhance lipid productivity in both *C. sorokiniana* and *C. vulgaris*, but the additional H_2O_2 stressor is only effective in enhancing lipid accumulation in *C. sorokiniana*. This chapter also details my work in identifying, evaluating, and optimizing both extraction and quantification methodologies for neutral lipid quantification in harvested microalgae biomass. These experiments show prevalent interference in highly pigmented lipid extracts and that carbon treatment is a feasible method to reduce these pigments. Further discussion regarding necessary future investigations is presented.

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CHAPTER 2 – Using a Novel Three Stage Screening Method to Evaluate Growth of Microalgae on Food Waste Permeate

2.1. INTRODUCTION

Microalgae are efficient “biofactories” capable of producing commercially valuable products such as biofuels, nutraceuticals, pharmaceuticals, and animal feed; however, the application of microalgae for these production purposes, specifically in the biofuel market, are not economically feasible due to high cultivation and harvesting costs which account for up to 85% of total biofuel production cost.[1,2] Value-added products such as fertilizer may help offset microalgae cultivation and biofuel production costs, but further considerations need to be made to address the upfront costs of the cultivation process, such as those associated with nutrient sourcing.[13]

To lower the cost of cultivation, wastewater has been proposed as an alternative growth medium due to its abundant availability, nutrient content, and low cost. In addition, diverting this wastewater also reduces the hazards to freshwater sources that result if wastewater is not appropriately treated, such as algal blooms that can lead to ecosystem disruption and loss of biodiversity.[3] Coupling microalgae cultivation and wastewater remediation can provide an economically feasible alternative while also lowering the risk of surface water eutrophication. This coupling can also circumvent the high costs associated with the transport and treatment of wastewater streams.[4] Although microalgae cultivation has been reported using various municipal wastewater streams, such wastewater produced in multistage treatment plants is generally characterized by relatively low nutrient levels.[5–7] Herein, I describe the use of

wastewater from anaerobically digested food waste, referred to as food waste permeate (FWP), for microalgae cultivation and wastewater remediation. The FWP provides both benefits and challenges with its nutrient-dense composition.

In recent years, there has been increased interest in anaerobic digestion technology due to the many environmental benefits, such as renewable energy, increased soil health, and reduced carbon emissions.[8] A byproduct of anaerobic digestion is a wastewater stream characterized by extremely high nitrogen concentrations, specifically ammonia. Nutrient-dense waste streams such as these create a problem for wastewater treatment because they may not be efficiently and effectively treated.[9] Traditional treatments, especially biological treatments, are not equipped for high nutrient loading, creating an upset in the treatment process step(s).[10] That said, alternative treatment methods must be developed to ensure safe environmental discharge. Microalgae can utilize nitrogen and other nutrients in anaerobic digestate resulting in a remediated wastewater stream and significant biomass accumulation.[11,12]

In addition to the nutrient-rich anaerobic digestate, another side product of the anaerobic digestion process is biogas rich in methane and carbon dioxide. This biogas provides a source of electricity, heat, and a 6% CO_2/air mixture when combusted. These three products are typically costly inputs for microalgae cultivation. However, in the case of electricity, it can also be sold, making it a valuable co-product to offset the costs of microalgae cultivation.[13] Funneling the CO_2 mixture into the microalgae cultivation process mitigates the costs of outside carbon sources and increases the sustainability of the production process overall.[14] The use of CO_2 as a supplementation method for microalgae cultivation is well-studied and has been shown to increase microalgae productivity in various microalgae strains, including those studied

in this research.[15–17] Carbon dioxide supplementation has the additional benefit of lowering pH due to the formation of carbonic acid. The pH of the anaerobic digestate used in this research has a pH of around 8.5 – 9. This pH is not optimal for many microalgae species, and it affects the speciation of important nutrients, specifically ammonia, so the relationship between pH and ammonia is vital to monitor in this research.

This research investigates and identifies microalgae production and wastewater remediation methods using nutrient-dense wastewater characterized by excessive nitrogen and ammonia levels. In this study, qualitative and quantitative microplate screening and quantitative batch culture methods were used to identify four microalgae species capable of growing on this highly concentrated wastewater stream. Initial microplate screenings involved testing different wastewater dilutions (10%, 30%, and 50% wastewater/water) with ten different microalgae species. Subsequently, batch culture experiments were performed to test selected wastewater dilutions (narrowed down based on microplate screenings) and microalgae strains exhibiting quantitative growth at microplate scale. In addition, nutrient assays and chromatography were performed on initial and final anaerobic digester permeates to determine the remediation capacity of viable microalgae species.

2.2. MATERIALS AND METHODS

2.2.1. Microalgae Culture Conditions

The microalgae species used for these studies are summarized in Table 2 and were chosen based on: demonstrative growth on other waste streams, ammonia tolerance, growth rate, and high potential lipid yields. Freshwater microalgae species were inoculated and

maintained in BG-11 liquid culture (Sigma-Aldrich, St. Louis, MO), and marine microalgae species were inoculated and maintained in F/2 liquid culture. All cultures were inoculated in 1-L Corning Pyrex media bottles (Corning 1395-1L) using stir bars with air bubbling (Petco Air Pump). Incoming air is sterile filtered by Polyvent 4 disposable filters (Whatman, Kent, UK). The culture suspensions were maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All equipment and materials were autoclaved and maintained under sterile conditions. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA).

Table 2. Microalgae strains investigated for cultivation on food waste permeate (FWP) and their sources. UT = University of Texas; NCMA = Bigelow National Center for Marine Algae

Microalgae	Strain	Source	Xenic/ Axenic?	Environment
Chlorella sorokiniana	UTEX 1230	UT—Austin	Axenic	Freshwater
Chlorella vulgaris	UTEX 2714	UT—Austin	Axenic	Freshwater
Parachlorella kesserli	UTEX 262	UT—Austin	Axenic	Freshwater
Phaeodactylum tricornutum	UTEX 646	UT—Austin	Axenic	Marine
Scenedesmus dimorphous	UTEX 1237	UT—Austin	Axenic	Freshwater
Scenedesmus acuminatus	UTEX 415	UT—Austin	Axenic	Freshwater
Scenedesmus obliquus	UTEX 1450	UT—Austin	Axenic	Freshwater
Scenedesmus acutus f. alternans	UTEX B 72	UT—Austin	Xenic	Freshwater
Tetraselmis suecica	UTEX B 2286	UT—Austin	Xenic	Marine
Nannochloropsis salina	CCMP 537	NCMA	Axenic	Marine

2.2.2. Anaerobic Digester Wastewater

Ultra-filtered food waste permeates (FWPs) were obtained from the thermophilic Sacramento South Area Transit Station (SATS).[18] An additional ammonia-stripping process using sodium carbonate was performed on a subset of this FWP to remove high ammonia concentrations, yielding a stripped FWP (SFWP).[19] Initial characterization was performed externally by Denele Analytical, Inc (Table 3). Throughout the experiments, permeate was stored at room temperature in air-tight containers to mimic wastewater storage at larger scale operations. Containers were minimally disturbed when setting up a new experiment. Any compositional analysis performed after the initial characterization by Denele Analytical Inc was determined with in-house analytical methods, including ion chromatography using a Dionex™ Aquion™ Ion Chromatography (IC) System equipped with a Dionex™ IonPac™ IC AS23 4x250 mm capillary column and Dionex™ IonPac™ IC AG23 4x50 guard column (ThermoFisher, San Jose, CA) and Hach test kits.

Table 3. Summary of nutrient concentrations (mg/L) in anaerobic digester permeates before and after the stripping process. FWP = food waste permeate; SFWP = stripped food waste permeate.

Nutrient	Nutrient Concentrations (mg/L)	
	FWP	SFWP
TKN	2710 ± 100	278 ± 10
NH_3-N	2340 ± 100	207 ± 20
P	18 ± 2	47 ± 2
K	1710 ± 30	1155 ± 7
Ca	6.85 ± 0	n/a
Mg	8 ± 4	2.7 ± 0.6
Na	819.50 ± 0.09	3340 ± 40
TOC	20750 ± 6000	21250 ± 6000
pH	8.4	8.6

* Measurements were performed by Denele Analytical, Inc.

2.2.4. Experimental Setup for Three-Tier Growth Studies

This study was performed using a three-level screening approach, including microplate studies (10mL cultures) and larger, 250-mL batch culture studies, as demonstrated in Figure 4.

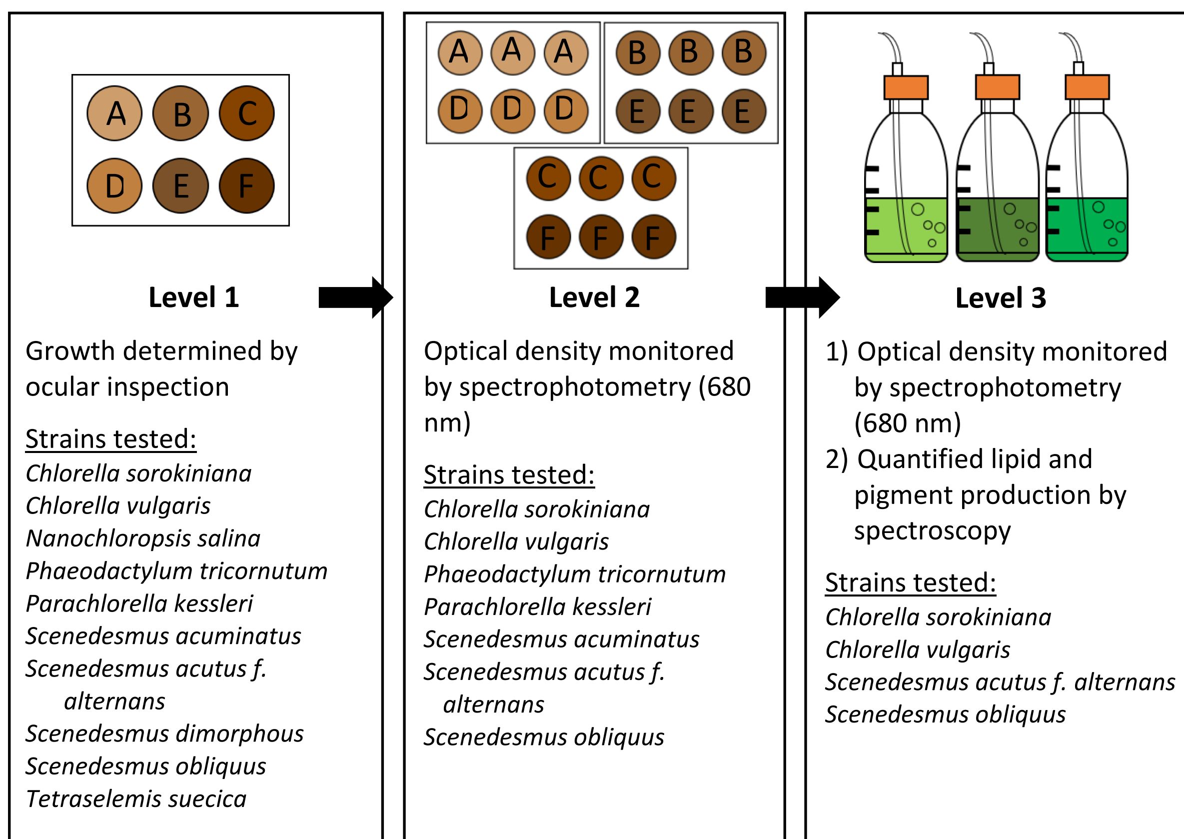

Figure 4. Schematic of the three-level screening approach used to screen multiple microalgae strains for both FWP and SFWP dilutions. The different dilutions are identified by the letters A-F (A=10% FWP, B=30% FWP, C=50% FWP, D=10% SFWP, E=30% SFWP, F=50% SFWP). The working volume for the microplate studies and batch-scale studies are 10- and 250-mL volumes, respectively.

2.2.4.1. Microplate Studies

The first two screening levels utilized 6-well Falcon Tissue Culture Plates (Fisher Scientific, Chicago, IL). Liquid stock cultures were grown to early stationary phase based on

absorbance measurements at 680 nm with the same culturing methods as those described for stock culture maintenance. Culture plates were prepared to test different FWP concentrations for each of the microalgae strains, with the final conditions being 10% FWP, 30% FWP, and 50% FWP. Dilutions were performed using ultrapure water. Three additional conditions were tested using stripped-food waste permeate (SFWP) to investigate the effect of ammonia concentration on microalgae productivity. The dilutions use to cultivate microalgae on SFWP were the same as those tested with the FWP (i.e., 10% SFWP, 30% SFWP, and 50% SFWP) and utilized ultrapure water as the diluent. The first level followed a single replicate microplate design for rapid screening. In this initial screening, growth was qualitatively determined using visual observation of pigments and cell density. The second level followed a microplate design with three biological replicates for each condition with each strain. For this second screening level, growth was quantitatively measured by monitoring cell density within each well using a Synergy HI Multi-Mode Plate Reader (Biotek, Winooski, VT) at a wavelength of 680 nm. Blanks were utilized to account for matrix interference. All plates were covered and shaken on a ThermoScientific MaxQ2000 shaker at 100 rpm (ThermoFisher, San Jose, CA) without additional air or CO_2 bubbling.

2.2.4.2. Batch Culture Studies

The third screening level utilized a batch-culture design with at least two replicates for each strain. The conditions evaluated included 250-mL volumes of 10% FWP, 30% SFWP, and a synthetic media control. Liquid stock cultures were grown to early stationary phase based on absorbance measurements at 680 nm with the same culturing methods as those described for

stock culture maintenance. All cultures were inoculated (10% inoculation v/v) into 500-mL Corning Pyrex media bottles (Corning 1395-500) using magnetic stir bars and a 6% CO_2/air mixture for mixing and aeration. All culture flasks were autoclaved before preparation to mitigate the possibility of outside contamination. The CO_2 concentration was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). Evaporative loss was replaced using ultrapure water to maintain the appropriate culture volume (250 mL), and sterile conditions were not maintained to closer mimic larger-scale operations. Cell density was measured spectrophotometrically at 680 nm to monitor growth. The growth rate was determined by using Equation 2.1.

$$\text{Growth rate } (\mu) = \frac{\ln\left(A_f/A_0\right)}{t_f - t_0} \qquad [2.1]$$

where A_f is the absorbance of the culture at 680 nm on the day that the culture entered stationary phase, and A_0 is the absorbance of the culture at 680 nm on the day that the culture entered exponential phase. The t_f is the day the culture entered stationary phase, and t_0 is the day the culture entered exponential phase. Biomass productivity was calculated using Equation 2.2.

$$\text{Biomass productivity} = (m)(v)(\mu) \qquad [2.2]$$

where m is the mass of harvested algae biomass, v is the volume of culture when harvesting, and μ is the growth rate.

2.2.5. Using Recycled Wastewater as a Diluent for FWP Dilutions

The evaluation of recycled wastewater (RWW) utilized 6-well Falcon Tissue Culture Plates (Fisher Scientific, Chicago, IL). Recycled wastewater was obtained from the harvested

supernatant of previous microalgae growth studies. Nitrogen and ammonia concentrations in the 10% FWP and 30% SFWP RWW diluents were quantified using Hach testing kits. Liquid stock cultures were grown to early stationary phase based on absorbance measurements at 680 nm with the same culturing methods as those described for stock culture maintenance. Six different conditions were evaluated alongside a synthetic media control. Both 10% FWP and 30% SFWP conditions were prepared using the following diluent conditions: deionized (DI) water, 10% FWP RWW, or 30% SFWP RWW, respectively. A final condition derived from pilot-scale studies utilized a 1.7:1 mixture of DI water and each respective recycled wastewater (RWW) as a diluent. The ratio was determined based on pilot scale studies performed concurrently by collaborators (Zhang lab at UC Davis). Conditions were performed in triplicate. *C. sorokiniana* was inoculated using a 10% inoculation (v/v), and growth was quantitatively measured by monitoring cell density within each well using a Synergy HI Multi-Mode Plate Reader (Biotek, Winooski, VT) at a wavelength of 680 nm. Blanks were utilized to account for matrix interference. All plates were shaken on a ThermoScientific MaxQ2000 shaker at 100 rpm (ThermoFisher, San Jose, CA) without additional air or CO_2 bubbling.

2.2.6. Ammonia Concentration Changes with CO_2 and Air Supplementation

A 10% dilution of FWP and a 30% dilution of SFWP were prepared in a 500-mL Corning Pyrex media bottle equipped with a magnetic stir bar and a 6% CO_2/air mixture or pumped air for mixing and aeration. The CO_2 concentration was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). Gas flows were metered at 200 mL/min to assure equal aeration between all sample conditions. Samples were placed on a stir plate under a 16:8 light/dark

cycle and monitored for pH and ammonia concentrations. Ammonia concentrations were measured using Hach test kits, and pH was monitored using an AlgaeConnect system. Samples were taken at the following timepoints: 0 min, 30 min, 1 h, 2 h, 4 h, 24 h, 72 h, 96 h, 144 h, 216 h, 312 h. The Henderson-Hasselbach equation (Equation 2.3) was used to determine the predominant ammonia species at individual time points.

$$pH = pKa + \log\left(\frac{[A^-]}{[HA]}\right) \quad [2.3]$$

2.2.7. Testing CO_2 Supplementation Effects on *C. sorokiniana* Growth

The conditions evaluated included 250-mL volumes of 10% FWP, 30% SFWP, and synthetic media controls. The evaluation of CO_2 supplementation was performed through two separate experiments due to time and space constraints. The first experiment evaluated *C. sorokiniana* growth on 10% FWP with 6% CO_2/air versus pumped air, and the second experiment evaluated *C. sorokiniana* growth on 30% SFWP with 6% CO_2/versus pumped air. For both experiments, all cultures were prepared in 500-mL Corning Pyrex media bottles (Corning 1395-500) equipped with magnetic stir bars and either a 6% CO_2/air mixture or pumped air for mixing and aeration. All culture flasks were autoclaved before preparation to mitigate the possibility of outside contamination; however, sterile conditions were not maintained in order to closer mimic larger-scale operations. A 10% inoculation (v/v) of stock culture was used to inoculate the experimental cultures, and cell density was measured spectrophotometrically using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA) at 680 nm. The growth rate and biomass productivity were determined using Equations 2.1 and

2.2. The CO_2 concentration was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). All conditions were performed in triplicate.

2.2.8. Scaling Up *C. sorokiniana* Cultivation to 2-L Scale

A 1.75 L culture of *C. sorokiniana* in 10% FWP dilution was prepared in a 2-L Corning Pyrex media bottle by combining 175 mL of 100% FWP stock, 1400 mL of industrial tap water (ITW), and 175 mL of stock *C. sorokiniana* culture (10% inoculation v/v). The 10% FWP experimental condition was performed in triplicate. A control flask was created by combining 1575 mL of BG-11 media and 175 mL of stock *C. sorokiniana* culture (10% inoculation v/v). The control flask was to confirm microalgae viability for inoculation and was therefore prepared as a single replicate. All flasks were equipped with a magnetic stir bar and a continuous stream of 6% CO_2/air mixture for mixing and aeration. The CO_2 concentration was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). Evaporative loss was replaced using tap water to maintain the appropriate culture volume, and sterile conditions were not maintained in order to closer mimic larger-scale operations. Cell density was measured spectrophotometrically at 680 nm to monitor growth. This experiment was performed two times, once in August 2019 and again in August 2020. The growth rate and biomass productivity were determined using Equations 2.1 and 2.2.

2.2.9. Biomass Determination

Upon reaching and maintaining stationary phase for at least three days (as determined based on optical density at 680 nm), the microalgae cultures (250 mL) were each pelleted by centrifugation and washed with deionized water. The algae pellets were flash-frozen in liquid nitrogen and lyophilized to dryness (Labconco Freezone 6, Kansas City, MO). Lyophilized biomass was weighed and recorded as biomass per liter of culture. Biomass was stored at –20 °C under argon for analysis.

2.2.10. Lipid Extraction

Lipids were extracted from freeze-dried biomass using a modified Folch method.[20] Microalgae biomass was suspended in chloroform and sonicated for 1 min using a hand sonicator (Fisher Scientific Model 120 Sonic Dismembrator, ThermoFisher, San Jose, CA). After sonication, a 2:1 methanol/chloroform mixture was added to each sample and sonicated for 1 min. Sonication was repeated one more time for a total of 2 x 1 min sonication cycles following the addition of the methanol/chloroform mixture. After the final sonication, 0.1 M PBS was added to each sample, mixed, and centrifuged for layer separation. The chloroform layer was transferred to a glass vial and stored at –20 °C for analysis.

2.2.11. Bleach-Assisted Nile Red Microplate Assay for Neutral Lipid Quantification

2.2.11.1. Graphitized Carbon Pretreatment

Extracts were treated with graphitized carbon to remove interfering pigments using a method adapted from Cao et al. (2019).[21] In a 2-mL Eppendorf tube, 25 mg of graphitized

carbon (< 500 nm DLS; Sigma Aldrich, St. Louis, MO) was added to 1 mL of lipid extract and vortexed for 15 sec. The tubes were placed on a benchtop shaker and shaken for 30 min at room temperature. After shaking, the extract was passed through a 0.22 µm syringe filter and stored at –20 °C for analysis. All analyses were performed within 24 hs of treatment.

2.2.11.2. Neutral Lipid Assay

Neutral lipids in extracts were quantified using a bleach-enhanced Nile red microplate assay adapted from Higgins et al. (2014).[22] A 1 mg/mL algae oil standard in chloroform was prepared using Thrive® Culinary Algae Oil. The lipid extracts and the 1 mg/mL algae oil standard in chloroform were diluted 2:1 with methanol. Diluted samples and standards were added to a 96-well polypropylene microplate (Greiner, Millipore Sigma, St. Louis, MO) in quadruplicate and triplicate, respectively. Lipid standard was added to the microplate to achieve a range of 0 – 100 µg per well. The plate was heated at 55 °C to evaporate solvent then cooled to room temperature on the benchtop. Lipids were resuspended in 2-propanol followed by adding a Nile Red solution in DMSO (final concentration of Nile Red ~ 160 µg/mL) and incubated in obscurity for 5 min. Next, a 3% aqueous sodium hypochlorite solution was added to each cell and incubated in obscurity for 30 min. After bleaching, fluorescence measurements were taken using a Synergy HI Multi-Mode Plate Reader at excitation wavelengths of 530/40 nm and emission wavelengths of 590/40 nm. The neutral lipid content of the lipid extract was quantified using a standard curve derived from the lipid standards.

2.2.12. Statistical Analysis

All results are reported as mean values ± standard deviation. Student's t-test, one-way ANOVA, and post hoc Tukey's HSD tests were used to determine significant differences between microalgae strains and between experimental conditions of the growth data at a 5% significance level.

2.3. RESULTS AND DISCUSSION

2.3.1. Nutrient Analysis of FWP

Nutrient characterization assays of the FWP and SFWP showed the presence of essential nutrients needed to promote microalgae growth. Nitrogen concentrations are exceedingly high (>2000 mg/L), especially considering that over 80% of the total nitrogen is in the form of ammonia (see Table 3). In FWP, nitrogen concentrations are in excess with over 2000 mg N/L. After stripping, the resulting SFWP has around 200 mg N/L. Phosphorus concentrations are relatively low compared to nitrogen concentrations, so it was hypothesized that phosphorus would be a limiting factor in microalgae growth. Phosphorus supplementation experiments are covered in Chapter 3 of this dissertation. Variation in the permeate composition over time is attributed to the fact that cultivation experiments were performed over two years using room temperature conditions to model wastewater storage at a larger scale operation. Storage at room temperature is more energy and cost-efficient than storage at low temperature in a cold room (or something similar) and is therefore what this experiment utilized. The compositional changes and variation are attributed to bacteria, settling, and other natural processes present in the permeate throughout its storage.

2.3.2. Microplate Screening

In microplate screenings, a total of 10 microalgae strains were initially screened, with four strains showing robust growth on at least one of the three FWP dilutions. This initial growth supports the hypothesis that multiple microalgae strains can be cultivated on ammonia-rich anaerobic digester effluent upon dilution. In the first level of microplate screening, seven strains showed visible growth on at least 10% FWP: *C. sorokiniana*, *C. vulgaris*, *P. kessleri*, *P. triconutum, S. acuminatus, S. acutus f. alternans*, and *S. obliquus.* The ammonia-rich anaerobic digestate proved to be toxic or otherwise unsuitable to support growth for *N. salina*, *T. suecica*, and *S. dimorphous,* which were therefore eliminated from future screening experiments.

In level two microplate screenings, four of these seven microalgae strains showed quantifiable growth on at least one of the three FWP dilutions, as measured by optical density. The general setup of these microplates is demonstrated in Figure 5. The overall trend demonstrated that lower concentrations of FWP afforded higher biomass production for most strains compared to those grown at higher concentrations. The 10% FWP dilution proved to be most suitable in supporting higher microalgae growth than growth on the 30% and 50% FWP dilutions, as measured by optical density. The higher biomass growth was attributed to the reduced nutrient concentrations, specifically ammonia.

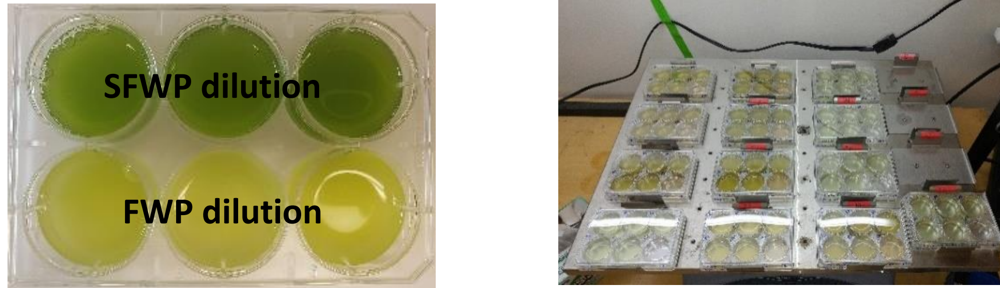

Figure 5. Representative microplate for *C. sorokiniana* (left) and microplate shaker setup (right) for the level two microplate screenings of *C. sorokiniana*, *C. vulgaris*, *S. acuminatus*, and *S. obliquus*. Blank permeate samples were maintained in a separate plate and accounted for in the absorbance of the microalgae wells.

It is well known that microalgae are sensitive to ammonia.[23–25] Ammonia toxicity in microalgae is well studied and has been found to vary greatly between microalgae classes. [23–25] Chlorophytes have been found to have EC_{50} values ranging anywhere from 68.5 – 1013 mg/L NH_4^+-N.[26] With a pH > 8, much of the ammonia in FWP and SFWP is in the unionized form, and the unionized form of ammonia has been identified as more toxic.[27] Bubbling the permeate with air to reduce toxic ammonia concentrations would result in ammonia being lost to the atmosphere as a harmful gas in addition to losing it as a source of nitrogen that the microalgae would have otherwise used. Therefore, reducing the initial ammonia concentrations in a manner that does not volatilize it would enhance microalgae growth, and this hypothesis was investigated using the SFWP.

Cultivation on the SFWP resulted in higher growth potentials compared to those grown on 10% FWP. Seven of the ten strains showed visible growth on both 10% and 30% SFWP, with *C. sorokiniana* also growing on the 50% SFWP dilution. This visible difference in growth between microalgae cultivated on 10%/30% FWP versus 10%/30% SFWP supports the

hypothesized harmful effect of ammonia on microalgae growth. With ammonia concentrations over 200 mg/L in 10% FWP and over 60 mg/L in 30% SFWP, the culture medium is quite toxic to the growing microalgae.[26] Overall, microalgae show increased growth when cultivated on the 30% SFWP dilution compared to 10% FWP, as determined by cell density. This growth increase would indicate that a lower ammonia concentration would benefit microalgae growth when cultivated on FWP.

Not only does this study demonstrate the possible effect ammonia concentration has on microalgae growth, but it also demonstrates that SFWP requires less dilution compared to FWP in order to achieve similar or better microalgae growth. The decreased dilution factor required for cultivation on SFWP should reduce the overall demand for freshwater resources and therefore be more sustainable.

Based on cell density measurements in microplate screening, *C. sorokiniana* was identified as one of the most productive strains for cultivation using SFWP. Although *C. sorokiniana* growth was observed for 10% SFWP, it was determined that the energy and resources required to prepare 10% SFWP made that condition less ideal for larger-scale operations. Additionally, while *C. sorokiniana* growth was also observed on 50% SFWP, no other species tested here showed growth on 50% SFWP; therefore, 50% SFWP was not considered in future screenings. Therefore, only 10% FWP and 30% SFWP conditions were further explored in level three batch culture testing. *C. vulgaris* showed limited to no growth at microplate scale in either condition; however, the success of *C. vulgaris* reported in a wide array of previous literature prompted its inclusion at the batch culture level experiments in this study [28–30].

The microplate screening experiments proved that some microalgae strains should grow on ammonia-rich FWP with dilution and pretreatments (i.e., ammonia stripping). Limitations to microplate cultivation (e.g., reduced air exchange, evaporation, limited mixing) were always considered when analyzing and evaluating microalgae growth at both levels of microplate screening. Using microplate cultivation allowed for successful rapid parallel screening of various algae species and strains.

The fact that only four out of the ten species showed measurable growth highlights that only select microalgae strains are tolerant of anaerobic digester permeate as a cultivation media, including species known to grow on other wastewater streams, such as *S. dimorphous*, *N. salina*, and *P. tricornutum*.[31–33] Furthermore, while *C. sorokiniana*, *C. vulgaris*, and *S. obliquus* are commonly studied microalgae strains, *S. acutus f. alternans* is not well-documented. Much of the literature available on *S. acutus f. alternans* focuses on metal tolerance, with very few studies on the cultivation of *S. acutus f. alternans* using nutrient-dense wastewater.[34,35] These findings provide valuable insight into the wastewater/nutrient tolerance and its effect on the overall biomass productivity of microalgae.

2.3.4 Monitoring Ammonia Concentrations and pH Over Time

The pH of 10% FWP significantly affected ammonia speciation and volatilization (Figure 6). Ammonia nitrogen has a pK_a of 9.24, so nitrogen exists primarily as NH_4^+ below the pH of 9.24 (Table 5). Of the two forms of ammonia nitrogen (i.e., ammonia/ammonium), the ammonium is the less toxic and less volatile of the two species; therefore, the optimal pH of the cultivation medium should be below that 9.24 pK_a threshold.[36] The initial pH of the 10% FWP

was 8.35, and the initial pH of the 30% SFWP was 8.65 (Table 4). The use of aeration with pumped air versus a 6% CO_2/air mixture had a significant effect on the pH of the medium when tested at batch scale (250 mL) (Figure 6).

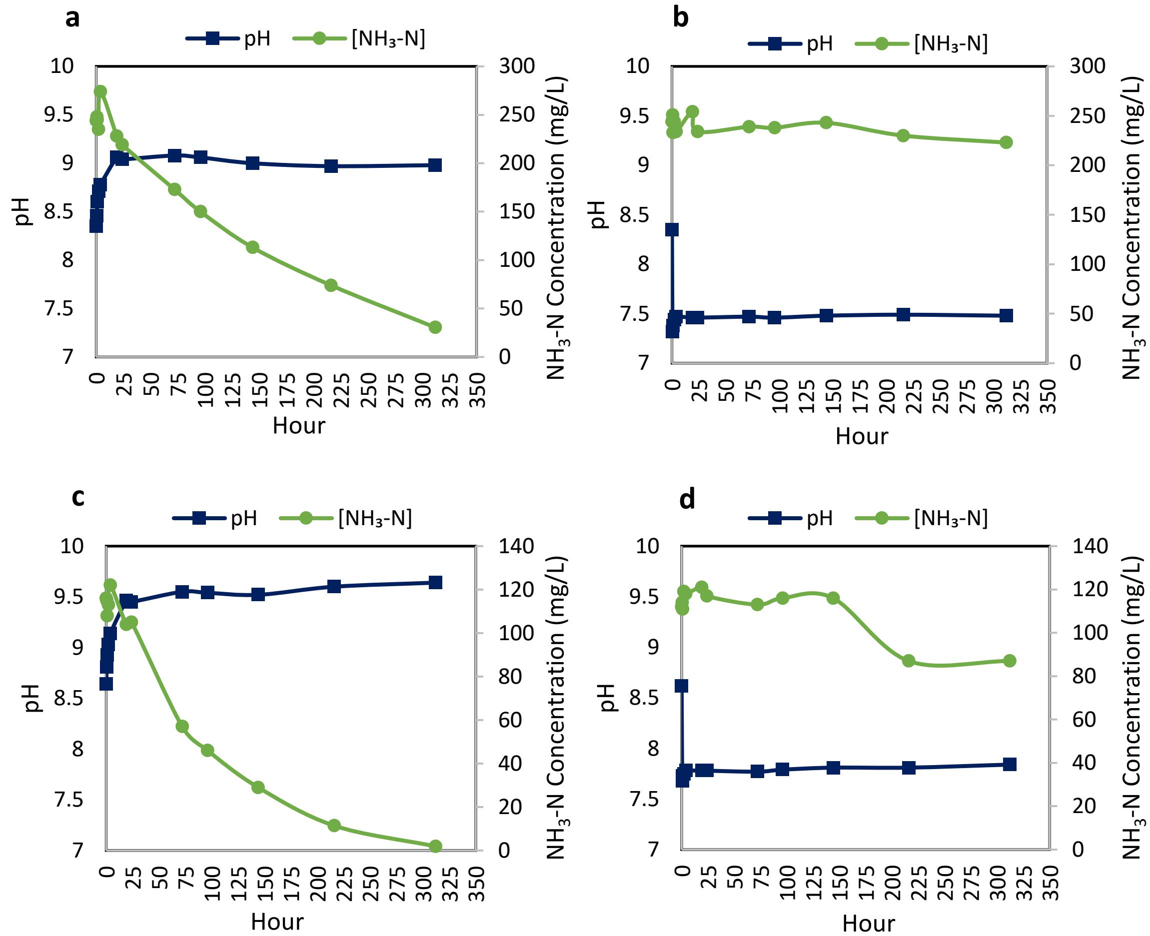

Figure 6. Changes in pH and ammonia concentrations over time in 10% FWP or 30% SFWP with either CO_2 or air bubbling at batch scale (250 mL). (a) 10% FWP + air, (b) 10% FWP + 6% CO_2/air, (c) 30% SFWP + air, (d) 30% SFWP + 6% CO_2/air

With pumped air, the pH of the 10% FWP rose to above pH 9, and the pH of 30% SFWP quickly rose above 9.5, both increases happening within less than 36 hours (Figure 6). This increase in pH was followed by an 89% reduction in ammonia nitrogen concentration in the

10% FWP and a 98% reduction of ammonia nitrogen in the 30% SFWP due to volatilization. When aerated with the 6% CO_2/air mixture, the pH of the 10% FWP decreased to pH 7.5, and the pH of 30% SFWP quickly decreased to pH 7.8, both decreases happening within less than 30 minutes (Figure 6). This decrease in pH can be attributed to the formation of bicarbonate (HCO_3^-; pk_a=10.33) and carbonic acid (H_2CO_3; pK_a=6.35) upon bubbling CO_2 into the pH-basic permeate cultures. This increase in carbonic acid would then decrease the pH. After reaching a constant pH between 7.5 and 8, significantly less ammonia loss was observed throughout the experiment.

Table 4. Monitored parameters comparing 10% FWP and 30% SFWP supplemented with air versus a 6% CO_2/air mixture at a 250-mL scale. Final measurements were taken on day 13. Ammonia-nitrogen speciation was determined using the Henderson-Hasselbalch equation and the total NH_3-N concentrations. FWP = food waste permeate; SFWP = stripped food waste permeate

Sample	pH		NH_3-N Concentration (mg/L)			NH_3-N Speciation					
						NH_4^+ Concentration (mg/L)			NH_3 Concentration (mg/L)		
	Initial	Final	Initial	Final	% Change	Initial	Final	% Change	Initial	Final	% Change
10% FWP + air	8.35	8.98	244	31	-88%	216	20	-91%	28	11	-61%
10% FWP + CO_2	8.35	7.48	244	223	-9%	216	219	1%	28	3.8	-86%
30% SFWP + air	8.64	9.64	116	1.9	-98%	93	0.5	-99%	23	1.4	-94%
30% SFWP + CO_2	8.62	7.84	112	87	-22%	90	84	-7%	22	3.3	-85%

Table 5. Ratios of NH_4^+/NH_3 over time in 10% FWP and 30% SFWP supplemented with air or 6% CO_2/air mixture at 250-mL scale.

Sample	Hour										
	0	0.5	1	2	4	24	72	96	144	216	312
10% FWP + air	8	6	4	3	3	2	1	2	2	2	2
10% FWP + CO_2	8	83	72	63	59	60	59	60	58	56	58
30% SFWP + air	4	3	2	2	1.3	0.6	0.5	0.5	0.5	0.4	0.4
30% SFWP + CO_2	4	32	36	31	29	29	30	28	27	27	25

Using the Henderson-Hasselbalch equation, the predominant species of ammonia in both 10% FWP and 30% SFWP cultures aerated with 6% CO_2 was determined to be NH_4^+ (Table 5). Due to the decreased volatility of NH_4^+, the concentration of ammonia is solubilized and not lost through volatilization. The prevention of volatilization is desirable in environmental health and sustainability and in maintaining the nitrogen content needed for microalgae cultivation.

The concentration of NH_4^+ stays high relative to the concentration of NH_3 in the sample aerated with CO_2, whereas the ratio of NH_4^+/NH_3 slowly decreases in the cultures bubbled with pumped air (Table 5). This decreased ratio is consistent with a continual increase in the concentration of NH_3. The gradually decreasing NH_4^+ to NH_3 ratio, in addition to the continued measurable loss of NH_3 due to volatilization, demonstrates the importance of pH in nitrogen speciation for wastewater treatment. That said, all future experiments were performed using 6% CO_2/air to maintain the high nitrogen concentrations hypothesized to increase microalgae growth.

2.3.5. Evaluating CO_2 Supplementation of *C. sorokiniana* Cultures at Batch Scale (250 mL)

In these experiments, it was observed that when using a 6% CO_2/air mixture to mix and aerate 30% SFWP cultures, the CO_2 supplementation increased *C. sorokiniana* growth significantly (Figure 7). The 6% CO_2/air mixture was used to represent the composition of biogas produced during the anaerobic digestion process. This biogas can then be utilized for microalgae cultivation creating a more sustainable microalgae cultivation process. Carbon dioxide has been studied thoroughly as a method to increase microalgae growth.[16,37] Considering this previous literature, CO_2 supplementation would significantly increase

microalgae productivity in general; however, this significant increase was not observed in the 10% FWP conditions. The hypothesis for using CO_2 supplementation is that increased carbon availability results in increased carbon usage and increased biomass productivity. This trend may not have been observed here due to the already high carbon concentration found in FWP, which is around 20,000 mg C/L. Additionally, the negative impact of the high ammonia concentrations may have mitigated the benefits of carbon supplementation.

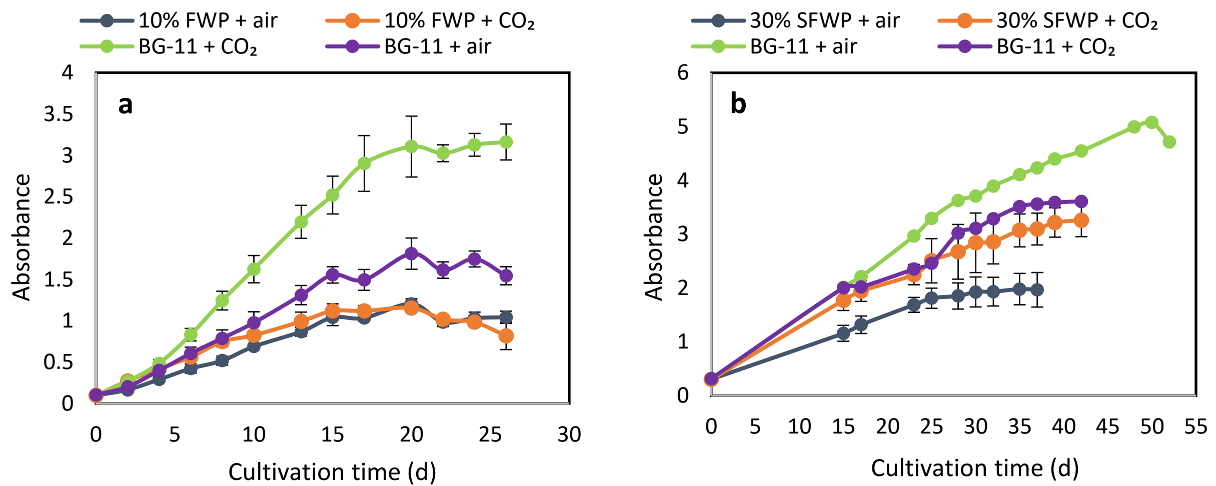

Figure 7. Growth curves for *C. sorokiniana* cultivated in (a) 10% FWP and (b) 30% SFWP with pumped air versus a 6% CO_2/air mixture at batch scale (250 mL). FWP = food waste permeate; SFWP = stripped food waste permeate; Syn media = synthetic media, BG-11.

Table 6. Growth characteristics and remediation capacity for *C. sorokiniana* tested at 250-mL scale with air versus a 6% CO_2/air mixture.[a] FWP = food waste permeate; SFWP = stripped food waste permeate; TN = total nitrogen

Condition	Harvest day	Harvested Biomass[b] (mg)	Growth rate (d^{-1})	Biomass productivity ($mgL^{-1}d^{-1}$)	Percent Remediation (%)	
					TN[c]	NH_3-N
10% FWP + air	26	68 ± 5	0.126 ± 0.003	38 ± 4	86 ± 4	98 ± 2
10% FWP + CO_2	26	66 ± 7	0.162 ± 0.007	48 ± 3	29 ± 8	41 ± 10
BG-11 + CO_2	26	184± 31	0.116 ± 0.002	163 ± 33	10 ± 7	na
BG-11 + air	26	96 ± 7	0.095 ± 0.006	67 ± 6	-11 ± 1	na
30% SFWP + air	37	170 ± 23	0.071 ± 0.004	52 ± 10	65 ± 7	99.5 ± 0.3
30% SFWP + CO_2	42	225 ± 24	0.075 ± 0.007	72 ± 13	32 ± 7	91 ± 8
BG-11 + air	52	267	0.03	32	-117	na
BG-11 + CO_2	42	284	0.03	37	-153	na

[a] Values are presented as mean ± SD of triplicates except the 30% SFWP experimental synthetic media controls done with a single replicate due to space availability.
[b] Biomass based on harvesting at stationary phase; strains reached stationary phase at different days throughout the experiments and were harvested on different days.
[c] Negative values are likely due to contamination, possibly from residual biomass in the supernatant.

Significant differences in remediation capacities were observed between *C. sorokiniana* cultures bubbled with air and cultures bubbled with 6% CO_2/air (Table 6). A 99 ± 17% difference in TN removal and an 82 ± 16% difference in NH_3-N removal were observed between *C. sorokiniana* cultures grown on 10% FWP bubbled with air versus CO_2. Differences of 68 ± 22% and 9 ± 8% were observed for TN and NH_3-N removal, respectively, when *C. sorokiniana* was cultured on 30% SFWP with air versus CO_2. Both 30% SFWP conditions observed high ammonia remediation capacity, and, therefore, the difference between their remediation capacities was small. The apparent high remediation capacities observed for *C. sorokiniana* cultivated on 10% FWP and 30% SFWP with air is actually due to ammonia volatilization rather than remediation, as described in section 2.3.4 of this dissertation.

Other anaerobic digestion processes may produce varying concentrations of CO_2 blended with air, which, in turn, would affect microalgae growth and microalgae remediation capacities. Therefore, future studies must evaluate the effect of different CO_2 concentrations on microalgae growth when cultivated on both FWP and SFWP dilutions. The CO_2 supplementation of *C. sorokiniana* cultures resulted in the same if not better growth on FWP/SFWP. Because of this, it should be concluded that the 6% CO_2/air mixture is an adequate replacement for pumped air, and the sustainability benefits of the cyclic anaerobic digestion/microalgae cultivation process can be harnessed.

2.3.6. Microalgae Cultivation using FWP and SFWP Media at Batch Scale (250 mL)

The cultivation of four strains (*C. sorokiniana*, *C. vulgaris*, *S. acutus f. alternans*, and *S. obliquus*) was compared at 250-mL scale culture volumes where growth was observed on the 10% FWP and 30% SFWP conditions based on optical density as measured by UV/Vis spectroscopy (Figure 8). All species in all conditions exhibited a short lag phase (0-3 days); the exponential growth rates varied considerably across species and conditions, ranging between 6 and 38 days.

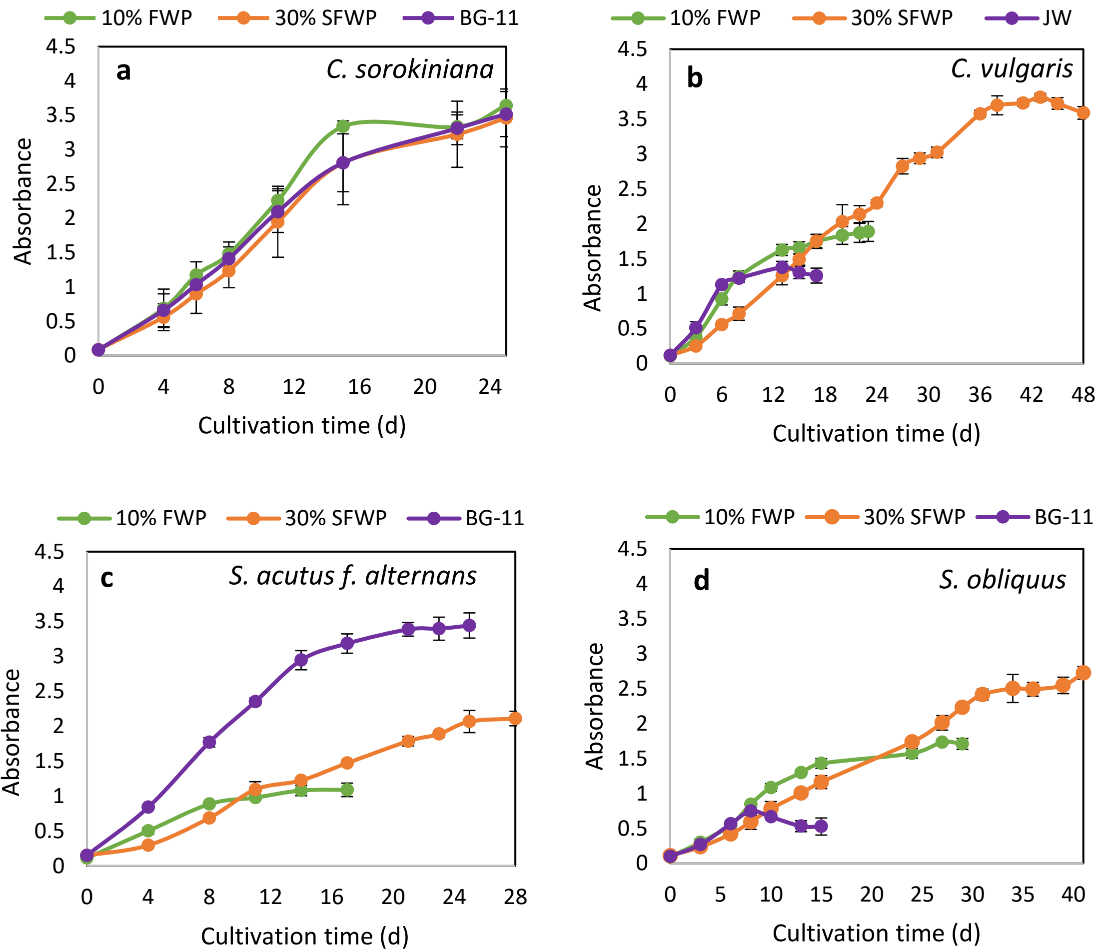

Figure 8. Growth curves for (a) *C. sorokiniana*, (b) *C. vulgaris*, (c) *S. acutus f. alternans*, and (d) *S. obliquus* cultured in different experimental conditions at 250-mL batch scale, as measured by optical density. FWP = food waste permeate; SFWP = stripped food waste permeate; JW = Jaworski's Medium.

Table 7. Growth characteristics of four microalgae strains tested at 250-mL scale.[a]

Microalgae	Growth media	Biomass harvested[b] (mg)	Growth rate (d^{-1})	Biomass productivity ($mgL^{-1}d^{-1}$)	Lipids (%)
C. sorokiniana	10% FWP	274 ± 22	0.2459 ± 0.0003	290 ± 20	13 ± 2
	30% SFWP	235 ± 42	0.15 ± 0.02	150 ± 30	12 ± 2
	BG-11	201 ± 86	0.13 ± 0.03	120 ± 70	12 ± 2
C. vulgaris	10% FWP	139 ± 6	0.15 ± 0.01	80 ± 10	8 ± 3
	30% SFWP	397 ± 17	0.09 ± 0.01	160 ± 20	13 ± 5
	JW	121 ± 10	0.377 ± 0.004	190 ± 20	26 ± 4
S. acutus f. alternans	10% FWP	144 ± 8	0.26 ± 0.01	155 ± 11	n/a
	30% SFWP	231 ± 20	0.106 ± 0.003	104 ± 10	n/a
	BG-11	347 ± 8	0.179 ± 0.001	265 ± 8	n/a
S. obliquus	10% FWP	158 ± 15	0.147 ± 0.002	100 ± 10	n/a
	30% SFWP	206 ± 12	0.083 ± 0.004	74 ± 7	n/a
	BG-11	75 ± 8	0.25 ± 0.01	78 ± 5	n/a

[a] Values are presented as mean ± SD of triplicates
[b] Biomass based on harvesting at stationary phase; strains reached stationary phase at different days throughout the experiments and were harvested on different days. See Table A1 in the Appendix for more details.

C. sorokiniana, *C. vulgaris*, and *S. obliquus* had comparable or increased overall biomass accumulation when cultivated on permeate compared to the synthetic media (Table 7). Conversely, *S. acutus f. alternans* showed significantly reduced growth when cultivated using permeate and compared to synthetic media (Table 7). However, compared to the other three strains, the growth characteristics of *S. acutus f. alternans* were similar. This growth discrepancy suggests that *S. acutus f. alternans* has higher growth potential overall and may benefit more from further media supplementations to mimic the composition of synthetic media. *S. obliquus* and *C. sorokiniana* showed the highest biomass growth when cultivated on 10% FWP, even when compared to *C. sorokiniana* grown on synthetic media.

With the exception of *S. acutus f. alternans* species, all strains cultivated using lower ammonia conditions (i.e., 30% SFWP conditions) exhibited biomass productivities similar to that found using the synthetic media control experiments (Table 7). The most prolonged exponential growth phases were observed for *S. obliquus* and *C. vulgaris* (30 and 35 days, respectively) when cultivated on 30% SFWP while *C. sorokiniana* and *S. acutus f. alternans* had exponential growth phases less than 15 days across all conditions (Figure 8c). The highest biomass accumulation was observed for microalgae cultivated on 30% SFWP for all species except *C. sorokiniana,* where the accumulation was about equal to that on 10% FWP (Table 7). The substantial differences in biomass accumulation and growth rates contribute to the significant differences in biomass productivities between species and experimental conditions.

The results from batch-scale studies suggest that *C. sorokiniana* and *S. acutus f. alternans* may be preferred for biomass cultivation over *C. vulgaris* and *S. obliquus,* which have longer exponential growth periods and lower biomass productivity overall considering milligrams of biomass produced per milliliter of culture per day (Table 7). These longer exponential phases may reduce microalgae cultivation efficacy as an industrially viable process. Additionally, the identified growth trends and permeate analyses indicate that microalgae may benefit from further media supplementations to mimic the composition of synthetic media, specifically with *S. acutus f. alternans*. Other treatments to address certain inhibitory factors such as low phosphorus concentrations and high ammonia concentrations can benefit microalgae productivity.

2.3.7. Nutrient Remediation

C. sorokiniana and *S. obliquus* afforded the highest nitrogen removal efficiencies of 64% and 31%, respectively, and greater than 95% phosphorus removal. While remediation efficiency varied considerably between species and conditions, nearly all experimental conditions resulted in the complete remediation of phosphates (Table 8). Aside from phosphates, the highest removal efficiencies were measured with ammonia when *C. sorokiniana* and *S. obliquus* were cultivated on 30% SFWP. These conditions afforded between 80% and 90% remediation of ammonia nitrogen. It is important to note that SFWP had lower initial ammonia concentrations and less nitrogen to remediate, contributing to the higher ammonia remediation percentages. Nonetheless, the remediation efficiency of ammonia was consistently higher than that of total nitrogen. The 10% FWP had lower remediation efficiencies across all microalgae species indicating lower nitrogen uptake during growth. The reduced nitrogen uptake is attributed to the higher ammonia concentrations causing inhibition in overall productivity in microalgae. Reduced nitrogen uptake should also be correlated to the increased growth of microalgae species on SFWP compared to FWP. Nitrate/nitrite concentrations commonly increased in FWP and SFWP samples. These increases can likely be attributed to bacteria present in the cultures and nitrogen fixation.[38] For these experiments, sterile conditions were not maintained due to the consideration that such conditions are unfeasible for larger-scale operations, so the potential of bacterial contamination is higher.

Table 8. Initial concentrations of total nitrogen (TN), ammonia-nitrogen (NH_3-N), nitrates (NO_3^--N), and phosphates (PO_4^{3-}) in 10% FWP and 30% SFWP along with removal efficiencies of each nutrient by *C. sorokiniana*, *C. vulgaris*, *S. acutus f. alternans*, and *S. obliquus*.[a,f]

Microalgae	Growth media	Initial concentrations [b]				% removal [b,c,d]			
		TN (mg N/L)	NH_3-N (mg/L)	NO_3^--N (mg/L)	PO_4^{3-} (mg/L)	TN	NH_3-N	NO_3^--N	PO_4^{3-}
C. sorokiniana	10% FWP	228 ± 16	186 ± 13	2 ± 3	11 ± 4	64 ± 21	75 ± 25	-209 ± 36	> 95
	30% SFWP	108 ± 3	67 ± 17	9 ± 10	57 ± 1	83 ± 3	99.1 ± 0.5	16 ± 7	> 95
	Syn media	268 ± 79	n/a	1273 ± 165	61 ± 4	43 ± 3	n/a	> 95	> 95
C. vulgaris	10% FWP	201 ± 4	172 ± 6	4 ± 4	13 ± 8	26 ± 21	27 ± 3	16 ± 9	> 95
	30% SFWP	113 ± 29	28 ± 4	15 ± 6	26 ± 14	> 95	80 ± 13	39 ± 5	> 95
	Syn media	15.7 ± 0.6	n/a	62 ± 4	17 ± 1	89 ± 10	n/a	> 95	> 95
S. acutus f. alternans	10% FWP	263 ± 34	237 ± 33	9.0 ± 0.2	8 ± 1	4 ± 4	17.0 ± 0.8	-1138 ± 59	> 95
	30% SFWP	76 ± 28	81 ± 7	13 ± 9	40 ± 28	9 ± 8	82 ± 1	-809 ± 30	93 ± 7
	Syn media[e]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
S.obliquus	10% FWP	220 ± 53	161 ± 10	nd	8.8 ± 0.2	31 ± 3	45 ± 2	n/a	> 95
	30% SFWP	135 ± 7	82 ± 3	17 ± 7	51 ± 8	61 ± 5	92 ± 7	-817 ± 50	61 ± 5
	Syn media	270 ± 18	n/a	1317 ± 147	53 ± 36	34 ± 1	n/a	36.2 ± 0.7	74 ± 2

n/a = not present/below detection limit; nd = below detection limit.

[a] Values are presented as a mean ± SD of triplicates.

[b] Synthetic media was not tested for ammonia as it is not a component in the media stocks or MilliQ water.

[c] A negative percent removal value indicates an increase in nutrient concentration.

[d] Percent removal values of > 95 were used when the respective nutrient was not detected in the final permeates when analyzed.

[e] Supernatant was lost, so analyses could not be performed; therefore, the data is not shown.

[f] More nutrient remediation data, including final concentrations and additional nutrients, can be found in the Appendix (Table A2)

The microalgae growth experiments demonstrated an average nitrogen remediation efficiency of 49 ± 3% with variations for different species (Table 8). *C. sorokiniana* exhibited the highest nitrogen remediation efficiency while *C. vulgaris* and *S. acutus f. alternans* generally showed nitrogen removal efficiencies below 50%, with some as low as 5%. Induction of stationary phase is attributed to phosphorus depletion/limitation.[39] While phosphorus limitation should induce stationary phase, there may also be a combination of factors given the complex composition of the FWP and SFWP. Nitrogen limitation is widely known to increase microalgae lipid production in multiple oleaginous microalgae species, so reaching nitrogen-depleted conditions would benefit biofuel production.[39,40] The effects of nitrogen deficiency on lipid accumulation in *C. sorokiniana* and *C. vulgaris* are discussed in Chapter 4 of this dissertation.

2.3.8. Evaluating Recycled Wastewater as a Diluent for Microalgae Cultivation

The 10% FWP and 30% SFWP cultures prepared using recycled wastewater (RWW)/DI water diluent afforded *C. sorokiniana* growth consistent with those cultures diluted with only DI water at microplate scale (10-mL) (Figure 9). An overall increase in microalgae growth on 30% SFWP conditions was consistent with previous *C. sorokiniana* growth studies, as determined by optical density. This decreased growth on 10% FWP is attributed to the high concentrations of nitrogen—specifically ammonia—present in the media and diluent (Table 9). Additionally, a failure to achieve nitrogen depleted conditions was observed in the 10% FWP conditions upon reaching stationary phase.

Table 9. Total nitrogen (TN) and ammonia-nitrogen (NH_3-N) concentrations in the 10% FWP RWW and 30% SFWP RWW that were used to prepare the various 10% FWP and 30% SFWP *C. sorokiniana* cultures evaluated in this microplate scale (10 mL) study. RWW = recycled wastewater; FWP = food waste permeate; SFWP = stripped food waste permeate

Permeate Diluent [a]	TN (mg/L)	NH_3-N (mg/L)	Concentration in 1.7:1 DI/RWW diluent (mg/L)	
			TN	NH_3-N
10% FWP RWW	125	104	74	61
30% SFWP RWW	0	0	0	0

[a] Permeate was supernatant from previous cultivation experiments evaluating microalgae cultivation on 10% FWP and 30% SFWP.

Because nitrogen was still present in the 10% FWP RWW, the nitrogen is available to the new cultures prepared using recycled wastewater (Table 9). The additional nitrogen available in the cultures diluted with only RWW explains the lower microalgae growth observed compared to those diluted with DI water or the 1.7:1 DI/RWW mixture. On the other hand, the DI/RWW mixture seemed to benefit the microalgae, possibly contributing additional nutrients while maintaining overall low concentrations. This slight supplementation with the diluted RWW should explain the slight increase in biomass accumulation in cultures cultivated with the 1.7:1 DI/RWW mixture. The differences in growth trends observed in the 10% FWP were not observed in the 30% SFWP cultures. Unlike with the 10% FWP, the low ammonia-nitrogen concentrations in 30% SFWP allow the culture to reach nitrogen depletion upon reaching stationary phase (Table 9). So, the harvested supernatant used as the recycled wastewater in this study contributed little to no additional ammonia resulting in the observed increase in biomass accumulation compared to biomass accumulation in the 10% FWP cultures.

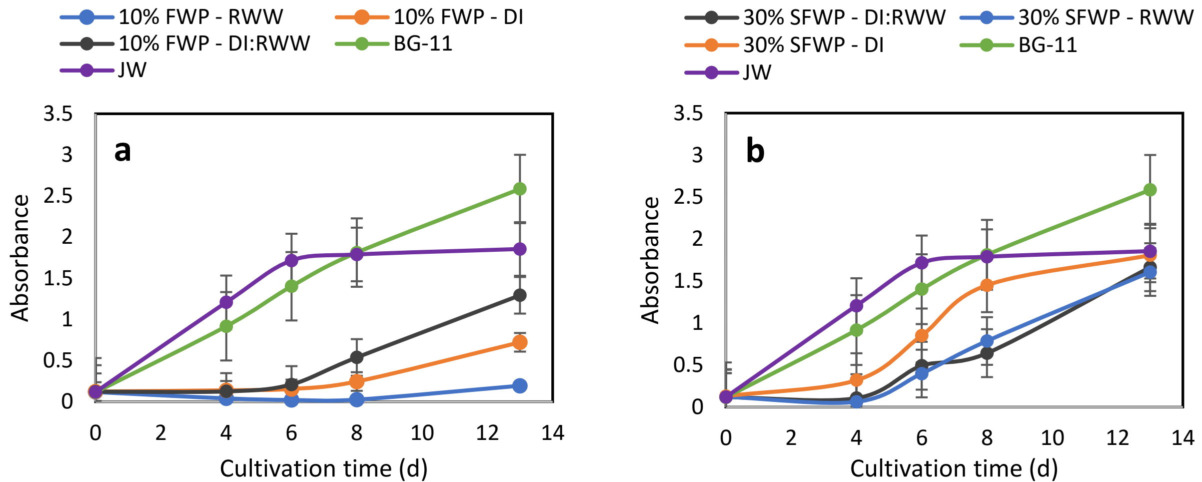

Figure 9. Growth curves for *C. sorokiniana* cultivated on (a) 10% FWP and (b) 30% SFWP diluted using deionized water (DI), recycled wastewater (RWW), or a 1.7:1 DI/RWW mixture at microplate scale (10 mL). FWP = food waste permeate; SFWP = stripped food waste permeate; JW = Jaworski's medium

This study demonstrates the capability of using recycled wastewater as a diluent for microalgae cultivation. These initial data are promising, and these studies should be repeated at a larger scale beyond the microplate scale used in this study to better understand the possible effects of recycled wastewater versus freshwater. Using RWW would allow for a more sustainable cultivation process, lowering the overall cost of microalgae cultivation. In addition to scaling up, an evaluation should be performed to monitor the RWW composition over time and with different microalgae cultivars.

2.3.9. Cultivation of *C. sorokiniana* using 10% FWP at 2-L Scale

Significant *C. sorokiniana* growth was observed when cultured on 10% FWP during the 2019 and 2020 growth periods and measured by optical density (Figure 10). The smaller amount of harvested biomass from the 2020 experiment is likely due to the smaller volume of

culture (1.5 L) compared to the 1.75-L volume cultures used during the 2019 experiment (Table 10). It is also important to note that due to extenuating circumstances, the August 2020 cultivation process was monitored less regularly than the experiment performed in August 2019.

The unavoidable monitoring limitation may have contributed to the difference in growth rate and biomass productivity because more frequent measurements would have provided a more exact time point for the start of stationary phase. Specifically, based on the observed growth curve of *C. sorokiniana* (Figure 10), the August 2020 experiment should have been continued for a few more days to fully establish stationary phase and reach its maximum biomass potential. The start of stationary phase was likely between day 19 and day 24, but measurements could not be taken during that time. Additional biomass loss was observed when volume from the third replicate was lost during the harvesting process. Not only was the biomass accumulation greater in August of 2019 compared to that of August 2020, but the growth rate and biomass productivity were increased during the August 2019 experiment as well (Table 10).

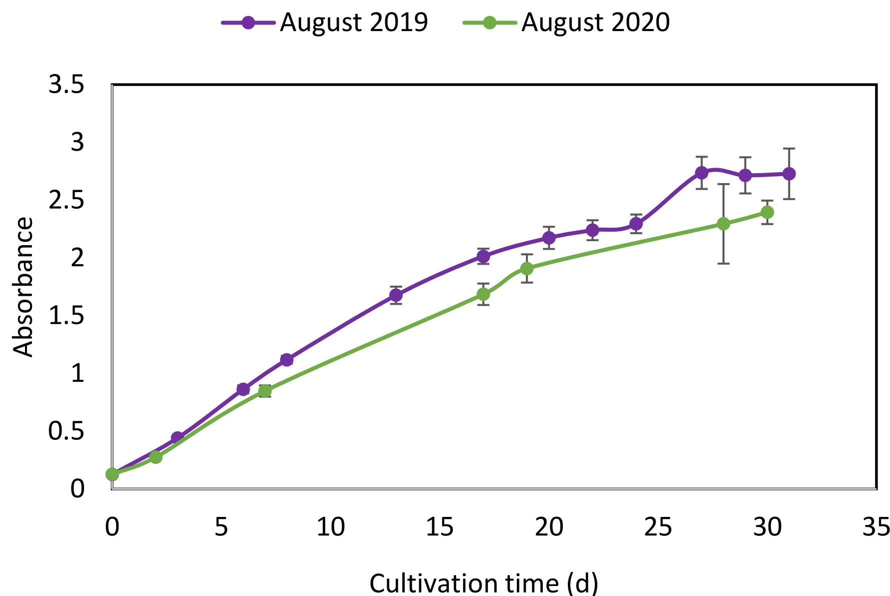

Figure 10. Growth curves for *C. sorokiniana* cultivated on 10% FWP in August 2019 and August 2020 at 1.75 and 1.5-L scales, respectively. FWP = food waste permeate

Table 10. Growth characteristics of *C. sorokiniana* cultivated on 10% FWP in August of 2019 and August 2020, respectively.[b]

Experiment	Condition	Culture volume (L)	Biomass harvested[a] (mg)	Growth rate (d^{-1})	Biomass productivity ($mgL^{-1}d^{-1}$)	Neutral Lipids (%)
August 2019	10% FWP	1.75	1338 ± 114	0.143 ± 0.002	116 ± 9	6 ± 1
August 2020	10% FWP	1.5	997 ± 56	0.116 ± 0.004	76 ± 7	na

na = not available
[a] Biomass based on harvesting at stationary phase; strains reached stationary phase at different days throughout the experiments and were harvested on different days.
[b] Values presented as mean ± SD of triplicates

The lower growth rate observed in the August 2020 experiment may be due to inconsistent conditional parameters (i.e., CO_2 concentration variation) and less monitoring capability (Table 10). As stated previously, a more reliable timepoint for the beginning and end of exponential phase should have been determined with closer monitoring during the August

2020 experiment. Nevertheless, the variations in growth rate and biomass accumulation result in calculated biomass productivity around 50% higher in August 2019 than in August 2020.

2.4. CONCLUSIONS

The three-tier growth experiments demonstrate that four of the ten microalgae species investigated (*C. sorokiniana*, *C. vulgaris*, *S. acutus f. alternans*, and *S. obliquus*) have potential as an alternative remediation method for food waste permeate (FWP) and other nutrient-dense waste streams. *C. sorokiniana* and *S. acutus f. alternans* demonstrated shorter growth periods and overall higher biomass productivity, as determined by cell density. Nutrient remediation efficiency varied widely across the four species. The highest remediation capacity was observed for phosphates with a removal efficiency of greater than 90% in nearly all conditions; total nitrogen remediation efficiencies were significantly lower overall. The 30% SFWP conditions afforded higher percent remediation values overall, albeit highly variable. *C. sorokiniana* demonstrated the highest TN remediation capacity with removal efficiencies above 60% and 80% in the FWP and SFWP conditions, respectively. *C. sorokiniana* also demonstrated the highest remediation capacities for NH_3-N (> 70%) and phosphorus (> 95%) compared to the other three strains tested.

The use of 6% CO_2/air, a mixture meant to mimic biogas produced by the anaerobic digestion process, mixes and aerates microalgae cultures with little to no adverse effects on microalgae productivity. In the case of *C. sorokiniana* cultivation on 30% SFWP, CO_2 supplementation increased growth significantly, as determined by optical density. In addition to CO_2 supplementation, the use of recycled wastewater (RWW) for diluting FWP and SFWP

benefits microalgae productivity when added to water in a 1.7:1 DI/RWW ratio. The results of the CO_2 supplementation and recycled wastewater studies support the application of alternative cultivation inputs to increase the overall sustainability of the microalgae cultivation process.

Overall, the discrepancies between our remediation efficiencies and growth trends compared to those described in previous literature demonstrate a knowledge gap that must be investigated further. Increasing the pool of research for microalgae cultivation on nutrient-dense waste streams such as FWP will continue to increase the prospective use in wastewater treatment and as a feedstock for other valuable co-products. Wastewater is a lower-cost alternative compared to synthetic media and therefore lowers cultivation costs to be more economically feasible in the biofuels and natural products markets.

Further research should investigate the possible inhibitory characteristics of FWP (i.e., phosphorus limitation, ammonia toxicity, nitrogen depletion), which should maximize wastewater treatment efficiency and produce other valuable co-products. Optimizing other nutrients may also increase the effectiveness of carbon supplementation with CO_2 by reducing the inhibitory effect of limiting nutrient concentrations. In addition to addressing the hypothesized limiting factors, it may be valuable to address the pH variations observed in the studies described here. Additional studies should evaluate the hypothesis that pH maintenance at a more optimal pH for each strain may increase microalgae growth and therefore be more industrially and commercially desirable.

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CHAPTER 3 – Addressing Nutrient Inhibition to Increase Microalgae Growth when Cultivated on Food Waste Permeates

3.1. INTRODUCTION

Microalgae cultivation on anaerobic digester permeate has proven advantageous, increasing microalgae cell density while also remediating the highly concentrated wastewater as demonstrated in Chapter 2. Cultivation of microalgae on food waste permeate (FWP) supports microalgae with productivity equal to or better than synthetic media controls due to the high concentrations of nitrogen and the presence of other valuable nutrients such as phosphorus and potassium. These nutrients are essential in microalgae metabolic processes, acting as the building blocks for structural components such as proteins, lipid membranes, and genetic material.[1] So, the high nutrient concentrations in FWP, specifically nitrogen, should result in increased biomass productivity. While biomass productivity is increased, the complete depletion of nutrients, specifically nitrogen, is never achieved.

Nitrogen depletion is a well-known initiator for lipid production; therefore, failure to reach nitrogen depleted conditions should severely affect lipid accumulation and affect the applicability of microalgae as a biofuel feedstock.[2] The premature induction of stationary phase, in addition to the presence of excess nitrogen, supports the hypothesis that increased biomass accumulation can be achieved with a supplementation/pretreatment method to address inhibitory factors such as nutrient depletion and nutrient toxicity. In addition to nutrient concentrations, other physical characteristics such as light penetration should also be considered to reach optimal growth potential.[3–6]

Light interference in microalgae cultivation is caused by inadequate penetration of light past the surface of the growth media, inhibiting photosynthetic activity. High levels of turbidity (e.g., opaqueness), color depth, and different wavelengths of light have been demonstrated to affect microalgae productivity.[3–6] Given the dark brown color of both the FWP and its ammonia-stripped counterpart, stripped food waste permeate (SFWP), it is hypothesized that the color of the permeates is inhibiting microalgae growth resulting in a longer lag phase, prolonged exponential phase, and overall lower biomass accumulation. Although light penetration is crucial and considered in this chapter, the effect of nutrient composition on microalgae growth inhibition will be the primary focus of this chapter.

Phosphorus is an essential nutrient for microalgae cultivation. At the low phosphorus concentrations found in FWP, the theoretical biomass density achieved during microalgae cultivation is limited. Previous studies have demonstrated that conditions with higher phosphorus concentrations yielded a higher biomass density.[7] These results support the hypothesis that phosphorus supplementation of FWP has the potential to increase the overall biomass productivity of microalgae.

The ratio of nitrogen concentration to phosphorus concentration (N/P) is another important consideration in optimizing microalgae cultivation. The Redfield ratio of N/P = 16:1 is often referred to as the "optimal" nitrogen to phosphorus ratio; however, this ratio has been shown to be less than optimal for freshwater microalgae and under varied cultivation conditions.[8–10] For example, Klausmeier et al. performed experiments on 29 species of freshwater and marine phytoplankton to determine the optimal nitrogen to phosphorus ratio for each species.[8] From this data, they reported that the optimal ratio N/P lies between 8.2 and

45.0, with a large percentage falling between 6 and 24. Within that range, chlorophytes preferred an N/P ratio around 20. Optimal values likely differ even more so when considering a wastewater environment due to the ability of microalgae to adapt their internal nutrient ratio to that of their surrounding environment.[8]

Nitrogen in the form of ammonia has the potential to inhibit microalgae growth rather than promote growth.[11,12] At high concentrations, ammonia is toxic to microalgae cells, which is attributed to inhibition of the photosynthetic pathway.[12,13] Although ammonia toxicity varies with microalgae species and the surrounding environment, all species will have a toxicity threshold.[11,14] With concentrations higher than 2000 mg/L in FWP, ammonia concentrations exceed toxicity thresholds for many microalgae species. Because of high ammonia concentrations, ammonia stands to be a severe inhibitor of microalgae growth in these studies. This project aims to lower the initial ammonia concentrations to a non-toxic level so microalgae can proliferate. Separate from the stripping process discussed in Chapter 2, we have proposed two methods to lower/remove ammonia in FWP: (1) dilution of FWP and (2) pretreatment of FWP with nitrifying bacteria (NB).

Dilution is a standard method to reduce toxic nutrient concentrations and increase light penetration in wastewater and was thoroughly studied and discussed in Chapter 2 of this manuscript.[3,4,15] The dilution of FWP will lower the overall nitrogen concentration to avoid toxicity. While dilution is common and effective, reducing the need for freshwater is desired to maintain a more sustainable system.

The ability of nitrifying bacteria (NB) to convert ammonia into nitrites and nitrates makes it a possible pretreatment method that can be used to enhance microalgae growth when

cultivated on the treated FWP. Using NB as a treatment for wastewater is a well-studied technique used in many treatment facilities.[16,17] A recent studies have demonstrated the use of bacterial pretreatment of anaerobic digestate to increase microalgae growth.[18] However, to our knowledge, pretreatment of FWP with NB has not yet been used as a wastewater pretreatment coupled with microalgae cultivation as a method to reduce toxic ammonia concentrations and maintain high nitrogen concentrations. Our hypothesis states that reducing ammonia concentrations while maintaining an overall high nitrogen content will allow for more biomass accumulation and increase nutrient remediation capacity.

Considering these possible physical and chemical limitations, we hypothesize that the three major factors contributing to inhibited microalgae growth on FWP are alteration of light intensity, nutrient depletion, and nutrient toxicity. More specifically, this research investigates two strategies to increase biomass productivity by adjusting nutrient concentrations to levels previously shown in the literature to benefit microalgae cultivation: 1) phosphorus supplementation to increase biomass productivity by preventing premature induction of stationary phase due to low phosphorus concentrations in the permeate. 2) Pretreatment with nitrifying bacteria to lower the toxic concentrations of ammonia while maintaining the nitrogen content for microalgae to use. In a smaller capacity, this research also investigates the hypothesis that reducing the pigments of the permeate using biochar will afford a clarified FWP cultivation media and thus will increase microalgae growth by decreasing light attenuation.

3.2. MATERIALS AND METHODS

3.2.1. Microalgae Culture Conditions

Axenic cultures of *C. sorokiniana* (UTEX 1230) and *C. vulgaris* (UTEX 2714) were obtained from UTEX (University of Texas, Austin, TX). For experiments, *C. sorokiniana* was inoculated and maintained in BG-11 liquid culture (Sigma-Aldrich, St. Louis, MO), and *C. vulgaris* was inoculated and maintained in Jaworski's medium liquid culture (CCAP, Argyll, Scottland, UK). All cultures were inoculated in 1-L Corning Pyrex media bottles (Corning 1395-1L) using stir bars with air bubbling (Petco Air Pump). Incoming air is sterile filtered by Polyvent 4 disposable filters (Whatman, Kent, UK). The culture suspensions were maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All equipment and materials were autoclaved and maintained under sterile conditions. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA).

3.2.2. Anaerobic Digester Wastewater

Ultra-filtered food waste permeates (FWPs) were obtained from the thermophilic Sacramento South Area Transit Station (SATS).[18] An additional ammonia-stripping process using sodium carbonate was performed on a subset of this FWP to remove high ammonia concentrations, yielding a stripped FWP (SFWP).[19] Initial characterization was performed externally by Denele Analytical, Inc (Table 11). Over the course of the experiments, permeate was stored at room temperature in air-tight containers to mimic wastewater storage at larger

scale operations. Containers were minimally disturbed when setting up a new experiment. Any compositional analysis performed after the initial characterization by Denele Analytical Inc was determined with in-house analytical methods, including ion chromatography using a Dionex™ Aquion™ Ion Chromatography (IC) System equipped with a Dionex™ IonPac™ IC AS23 4x250 mm capillary column and Dionex™ IonPac™ IC AG23 4x50 guard column (ThermoFisher, San Jose, CA) and Hach test kits.

Table 11. Summary of nutrient concentrations (mg/L) in anaerobic digester permeates before and after the stripping process. FWP = food waste permeate; SFWP = stripped food waste permeate.

Nutrient	Nutrient Concentrations (mg/L)	
	FWP	SFWP
TKN	2710 ± 100	278 ± 10
NH_3-N	2340 ± 100	207 ± 20
P	18 ± 2	47 ± 2
K	1710 ± 30	1155 ± 7
Ca	6.85 ± 0	n/a
Mg	8 ± 4	2.7 ± 0.6
Na	819.50 ± 0.09	3340 ± 40
TOC	20750 ± 6000	21250 ± 6000
pH	8.4	8.6

* Measurements were performed by Denele Analytical, Inc.

For the preliminary biochar-treatment experiments (Section 3.2.3.1.), the biochar-treated FWP was obtained from the Zhang lab in the Biological and Agricultural Engineering Department at UC Davis. In the Zhang lab, this biochar-treated permeate was treated using 1 g walnut shell-derived biochar per 40 mL of permeate, shaken at 250 rpm for 24 hours, and then passed through a 0.45 µm filterr to remove the biochar; the resulting permeate is shown in Figure 11. For the experiments described in section 3.2.3.3., the biochar filtered permeate was

obtained from the experiment evaluating the effect of different amounts of biochar on permeate pigment (Section 3.3.2.2.).

Figure 11. Biochar-treated food waste permeate (FWP) before and after treatment with 1 g of walnut-derived biochar per 40 mL FWP. Photo credit: Tyler Barzee.

3.2.3. *C. sorokiniana* Cultivation on Biochar-Treated FWP Dilutions at Microplate Scale

3.2.3.1. Preliminary Evaluation of *C. sorokiniana* Growth on Biochar-Filtered FWP

The evaluation of the effects of biochar-treatment of FWP on *C. sorokiniana* growth utilized 6-well sterile Falcon Tissue Culture Plates (Fisher Scientific, Chicago, IL). Liquid stock cultures of *C. sorokiniana* were grown to late exponential phase based on absorbance measurements at 680 nm with the same culturing methods as those described for stock culture maintenance. Culture plates were prepared to test 10% FWP, 30% FWP, and 50% FWP dilutions

of biochar-treated FWP obtained from the Zhang lab in the Biological and Agricultural Engineering Department at UC Davis (see Section 3.2.2). A plate with Jaworski's Medium (JW) was prepared to act as a control. Each condition was prepared in quintuplicate with a sixth well containing only the media to act as a blank (see Figure 12). A 10% Inoculation (v/v) was used to inoculate each well to achieve a final volume of 10 mL. Microalgae growth was quantitatively measured by monitoring cell density within each well using a Synergy HI Multi-Mode Plate Reader (Biotek, Winooski, VT) at a wavelength of 680 nm. The blank controls were utilized to account for matrix interference. All plates were shaken on a ThermoScientific MaxQ2000 shaker at 100 rpm (ThermoFisher, San Jose, CA).

Figure 12. Microplate layout for evaluating *C. sorokiniana* growth on biochar-treated FWP. All conditions were tested with N=5, with the sixth well in the plate being the blank control.

3.2.3.2. Preparation of Biochar-Treated FWP using Increasing Biochar Concentrations

The walnut shell-derived biochar used in this experiment was sourced from the Zhang lab at the University of California-Davis. In 500-mL Corning glass bottles, 50 mL of 100% FWP was added along with 1000 mg, 500 mg, 250 mg, or 100 mg of biochar and capped. Bottles were placed on a shaker plate at 250 rpm for 1 hour and then filtered through a 0.22 µm filter to remove particulates. Pigment removal by the biochar was observed by ocular inspection, and permeates were stored at 4 °C for use in future experiments.

3.2.3.3. Evaluating *C. sorokiniana* Growth on Biochar-Treated FWP and SFWP

The evaluation of the effects of biochar-treatment of both FWP and SFWP on *C. sorokiniana* growth utilized 6-well sterile Falcon Tissue Culture Plates (Fisher Scientific, Chicago, IL). Liquid stock cultures of *C. sorokiniana* were grown to late exponential phase based on absorbance measurements at 680 nm with the same culturing methods as those described for stock culture maintenance. The cultures utilizing biochar treated FWP were labeled as follows: 10% FWP biochar, 30% FWP biochar, 30% SFWP biochar, and 50% SFWP biochar. Cultures utilizing untreated FWP were labeled as only 10% FWP, 30% FWP, 30% SFWP, and 50% SFWP. Each condition was prepared by dispensing 1 mL, 3 mL, or 5 mL of either treated or untreated FWP/SFWP into each well for 10% FWP, 30% SFWP/SFWP, and 50% SFWP, respectively. A 10% inoculation (v/v) was used to inoculate each culture, and the volume was brought to 10 mL with deionized water. A plate with Jaworski's Medium (JW) and a plate with BG-11 media were prepared to act as control plates. Each condition was prepared in triplicate, and a separate "blank plate" was prepared to avoid contamination of the wells used to account for matrix

interference. A 10% inoculation (v/v) was used to inoculate each well, achieving a final volume of 10 mL. Microalgae growth was quantitatively measured by monitoring cell density within each well using a Synergy HI Multi-Mode Plate Reader (Biotek, Winooski, VT) at a wavelength of 680 nm. All plates were shaken on a ThermoScientific MaxQ2000 shaker at 100 rpm (ThermoFisher, San Jose, CA).

3.2.3.4. Phosphate Supplementation of Biochar-Treated FWP for *C. sorokiniana* Cultivation at Microplate Scale

The evaluation of the effects of phosphate supplementation of biochar-treated FWP (CF-FWP) on *C. sorokiniana* growth utilized 6-well sterile Falcon Tissue Culture Plates (Fisher Scientific, Chicago, IL). Liquid stock cultures of *C. sorokiniana* were grown to late exponential phase based on absorbance measurements at 680 nm with the same culturing methods as those described for stock culture maintenance. Culture plates were prepared to test 10% CF-FWP with all Jaworski's Medium (JW) nutrient stocks solutions (CF-FWP + JW nutrients), two different volumes of only JW phosphate-containing stock solutions (CF-FWP + 100% phosphates, CF-FWP + 50% phosphates), and a control plate utilizing no additional nutrient supplementations (CF-FWP + no nutrients). The biochar-treated FWP was obtained from the Zhang lab at UC Davis. The volume of JW phosphate stock added in each condition was determined based on the preparation protocol for JW media (1 mL nutrient stock/1 L media). The "100% phosphates" and "50% phosphates" cultures were prepared by adding 100 µL or 50 µL, respectively, of each phosphate-containing JW nutrient stock to each appropriate microplate well. These supplements were equivalent to 6 µg phosphorus and 3 µg phosphorus

for the "100% phosphates" and "50% phosphates" conditions, respectively. For the "JW nutrients" condition, stocks containing nitrogen were not added to avoid adding additional nitrogen to the permeate. Each condition was performed in quintuplicate alongside a blank control. Microalgae growth was quantitatively measured by monitoring cell density within each well using a Synergy HI Multi-Mode Plate Reader (Biotek, Winooski, VT) at a wavelength of 680 nm. The blank controls were utilized to account for matrix interference. All plates were shaken on a ThermoScientific MaxQ2000 shaker at 100 rpm (ThermoFisher, San Jose, CA). Upon reaching stationary phase, aliquots were taken to be analyzed using transmission electron microscopy (TEM) imaging.

3.2.4. Nutrient Supplementation of FWP and SFWP using Jaworski's Medium Stock Nutrients

Evaluation of JW nutrient supplementation utilized 6-well Falcon Tissue Culture Plates (Fisher Scientific, Chicago, IL). Liquid stock cultures of *C. sorokiniana* were grown to late exponential phase based on absorbance measurements at 680 nm with the same culturing methods as those described for stock culture maintenance. Culture plates were prepared to test 10% FWP, 10% SFWP, and 50% SFWP dilutions with different volumes of Jaworski's Medium (JW) nutrient stocks. The volume of JW stock to add in each condition was determined based on the preparation protocol for JW media (1 mL nutrient stock/1L media). There are nine total stock solutions to prepare JW media, so nine different nutrient stocks were added to each condition. When preparing the controls, no additional nutrients were added to the FWP/SFWP, and these conditions were labeled: 10% FWP – No JW, 10% SFWP – No JW, and 50% SFWP – No JW. The "100% JW" cultures were prepared by adding 100 µL of each JW nutrient stock to each

appropriate microplate well, and these wells were labeled: 10% FWP – 100% JW, 10% SFWP – 100% JW, and 50% SFWP – 100% JW. The "50% JW" cultures were prepared by adding 50 µL of each JW nutrients stock to the microplate wells, and these wells were labeled: 10% FWP – 50% JW, 10% SFWP – 50% JW, and 50% SFWP – 50% JW. Each condition was performed in duplicate alongside a blank control. Microalgae growth was quantitatively measured by monitoring cell density within each well using a Synergy HI Multi-Mode Plate Reader (Biotek, Winooski, VT) at a wavelength of 680 nm. The blank controls were utilized to account for matrix interference. All plates were shaken on a ThermoScientific MaxQ2000 shaker at 100 rpm (ThermoFisher, San Jose, CA).

3.2.5. Nitrifying Bacteria Pretreatments to Address Inhibitory Ammonia Concentrations

3.2.5.1. Preliminary Evaluation of Nitrifying Bacteria Treatment Efficacy on FWP Bubbled with CO_2 versus Air

For all conditions, 250-mL volumes of diluted FWP with and without nitrifying bacteria (NB) were dispensed into 500-mL Corning Pyrex media bottles equipped with a magnetic stir bar and bubbled with either a 6% CO_2/air mixture or pumped air for mixing and aeration. The CO_2 concentration was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). The activity of nitrifying bacteria was monitored with periodic measurements of total nitrogen, ammonia, nitrates, and nitrite concentrations. Total nitrogen and ammonia were measured using total nitrogen and ammonia Hach kits, respectively. Nitrate and nitrite concentrations were measured using a Dionex™ Aquion™ Ion Chromatography (IC) System equipped with a Dionex™ IonPac™ IC AS23 4x250 mm capillary column and Dionex™ IonPac™ IC

AG23 4x50 guard column (ThermoFisher, San Jose, CA). When ammonia and nitrite concentrations remained constant, the permeate was filtered through a Whatman No. 5 filter, and the treated permeate was stored at 4° C for future use.

3.2.5.2. Preliminary Evaluation of *C. sorokiniana* Growth on Nitrifying Bacteria-Treated FWP

Treated and untreated, filtered permeates from the preliminary NB treatment of 10% FWP with CO_2 versus air study were used as the growth medium for this experiment (see 3.2.5.1.). A 90 mL aliquot of both the treated and untreated 10% FWP were transferred to separate 500-mL Corning Pyrex media bottles equipped with a magnetic stir bar and bubbled with a 6% CO_2/air mixture for mixing and aeration. Additional cultures were prepared with "fresh" 10% FWP (i.e., from the stock bucket and not the previous experiment) were prepared to use as a control. A 10-mL aliquot of late exponential phase *C. sorokiniana* stock culture was dispensed into each flask to achieve a final volume of 100 mL. All three conditions were performed in triplicate. Cultures were placed on stir plates and maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All culture flasks were autoclaved before preparation to minimize the possibility of outside contamination. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA). The CO_2 mixture used to aerate the samples was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA).

3.2.5.3. Preliminary Inoculation and Treatment of FWP with Nitrifying Bacteria at 2-L Scale (June 2018)

A 1.2 L volume of undiluted food waste permeate was inoculated with 300 mL of NB culture into a 2-L Corning Pyrex media bottle equipped with a magnetic stir bar and bubbled with 6% CO_2/air mixture for mixing and aeration. The CO_2 concentration was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). The activity of nitrifying bacteria was monitored with periodic measurements of total nitrogen, ammonia, nitrates, and nitrite concentrations. Total nitrogen and ammonia were measured using total nitrogen and ammonia Hach kits, respectively. Nitrates and nitrite concentrations were measured on a Dionex™ Aquion™ Ion Chromatography (IC) System equipped with a Dionex™ IonPac™ IC AS23 4x250 mm capillary column and Dionex™ IonPac™ IC AG23 4x50 guard column (ThermoFisher, San Jose, CA). When ammonia concentrations reached a constant level, the permeate was filtered through a Whatman No. 5 filter, and the treated permeate was stored at 4° C for future use.

3.2.5.4. Inoculation and Treatment of FWP with Nitrifying Bacteria at 2-L Scale (August 2018)

A 1.2 L volume of diluted food waste permeate was inoculated with 300 mL of stock nitrifying bacteria culture into 2-L Corning Pyrex media bottles equipped with a magnetic stir bar and bubbled with a 6% CO_2/air mixture for mixing and aeration. Another 1.2 L volume of undiluted FWP was transferred to a separate 2-L Corning Pyrex media bottle along with 300 mL of sterile-filtered NB stock medium. The CO_2 concentration was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). The activity of nitrifying bacteria was monitored with periodic measurements of total nitrogen, ammonia, nitrates, and nitrite concentrations. Total

nitrogen and ammonia were measured using total nitrogen and ammonia Hach kits, respectively. Nitrate and nitrite concentrations were measured on the same ion chromatography system as described above. When ammonia and nitrite concentrations reached a constant level, the permeate was filtered through a Whatman No. 5 filter, and the treated permeate was stored at 4° C for future use.

3.2.5.5. Evaluating *C. sorokiniana* Growth on Nitrifying Bacteria-Treated FWP

The filtered permeate from the August 2018 NB-treatment period was used as the growth medium for this experiment (see 3.2.5.4.). Three conditions (and a synthetic media control, BG-11) were considered with this experiment: 1) NB-treated, bubbled 10% FWP (August 2018), 2) Untreated, bubbled 10% FWP (August 2018), and 3) untreated, unaerated 10% FWP (new from the stock bucket). For each condition, 25-mL aliquots of each type of undiluted FWP were transferred to individual 500-mL Corning Pyrex media bottles equipped with a magnetic stir bar and bubbled with a 6% CO_2/air mixture for mixing and aeration. A 25-mL aliquot of late exponential phase *C. sorokiniana* stock culture was dispensed into each flask, and industrial tap water was added to reach a final volume of 250 mL and final FWP concentration of 10%. All three experimental conditions were performed in triplicate. The synthetic media culture was performed with a single replicate as it was used only as a gauge of stock microalgae viability. Cultures were placed on stir plates and maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All culture flasks were autoclaved before preparation to mitigate the possibility of outside contamination.

Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA). The CO_2 mixture used to aerate the samples was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA). The growth rate and biomass productivity were determined using equations 3.1 and 3.2, respectively.

$$\text{Growth rate } (\mu) = \frac{\ln\left(A_f/A_0\right)}{t_f - t_0} \qquad [3.1]$$

where A_f is the absorbance of the culture at 680 nm on the day that the culture entered stationary phase, and A_0 is the absorbance of the culture at 680 nm on the day the culture entered exponential phase. The t_f is the day that the culture entered stationary phase, and t_0 is the day the culture entered exponential phase. Biomass productivity was calculated using Equation 2.2.

$$\text{Biomass productivity} = (m)(v)(\mu) \qquad [3.2]$$

where m is the mass of harvested algae biomass, v is the volume of culture when harvesting, and μ is the growth rate.

3.2.6. Adjusting N:P Ratios using Phosphorus Supplementation

3.2.6.1. Preparing the 3:1, 6:1, and 15:1 N/P Conditions

For those cultures testing 3:1, 6:1, and 15:1 N/P nutrient ratios, 250-mL volumes of a 10% FWP dilution were dispensed into 500-mL Corning Pyrex media bottles equipped with magnetic stir bars and bubbled with a 6% CO_2/air mixture for mixing and aeration. Dipotassium phosphate was added to each prepared flask to achieve the ratios of 6:1 and 15:1 and mixed thoroughly to dissolve. For the 3:1 N/P condition, aliquots of a 25 mg/L NaH_2PO_4 solution were

transferred to each prepared flask to achieve the desired 3:1 dilution. Upon complete dissolution of K_2HPO_4 for the 6:1 And 15:1 N/P conditions and NaH_2PO_4 for the 3:1 N/P condition solution, *C. sorokiniana* (10% inoculation v/v) was added to each flask to achieve the final FWP dilutions. All conditions were performed in triplicate.

3.2.6.2. Preparing the 23:1 N/P Condition

For the 23:1 N/P samples, 100-mL volumes of a 10% FWP dilution were dispensed into 500-mL Corning Pyrex media bottles equipped with magnetic stir bars and bubbled with 6% CO_2/air mixture for mixing and aeration. Dipotassium phosphate was added to each prepared flask to achieve the 23:1 N/P ratio and mixed thoroughly to dissolve. After dissolving the K_2HPO_4, a 10% inoculation (v/v) of *C. sorokiniana* was added to each flask to achieve the final FWP dilutions. All conditions were performed in triplicate.

3.2.6.3. Preparing the 33:1 N/P Condition for Preliminary Study

For the 33:1 N/P samples, 250-mL volumes of a 10% FWP dilution were dispensed into a 500-mL Corning Pyrex media bottle using magnetic stir bars and 6% CO_2/air mixture for mixing and aeration. Aliquots of a 6.5 mg/mL $Ca(H_2PO_4)_2$ solution were transferred to each prepared flask to achieve the desired 33:1 dilution and mixed thoroughly. After adding the phosphate solution, a 10% inoculation (v/v) of *C. sorokiniana* was added to each flask to achieve the final FWP dilutions. This preliminary study was performed with a single replicate due to time and space constrictions.

3.2.6.4. Testing the N/P Conditions

Cultures were placed on stir plates and maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All culture flasks were autoclaved before preparation to mitigate the possibility of contamination. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA). The growth rate and biomass productivity were determined using Equations 3.1 and 3.2. The CO_2 mixture used to aerate the samples was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA).

3.2.7. Evaluating Excess Phosphorus Supplementation of SFWP for *C. sorokiniana* Cultivation

For the cultures testing excess phosphorous supplementation, 225-mL volumes of 10% SFWP and 30% SFWP dilutions were dispensed into autoclaved 500-mL Corning Pyrex media bottles using magnetic stir bars and 6% CO_2/air mixture for mixing and aeration. Aliquots of a 25 mg/L NaH_2PO_4 stock solution were transferred to each prepared flask to achieve the desired N/P ratio. After dissolving the NaH_2PO_4 solution, *C. sorokiniana* (10% inoculation v/v) was added to each flask to achieve the final FWP dilutions. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA). Growth rate and biomass productivity was determined by using Equation 3.1 and 3.2, respectively.

3.2.8. Biomass Determination Using Gravimetric Analysis

Upon reaching and maintaining stationary phase for the specified duration of time, the microalgae cultures (250 mL) were each pelleted by centrifugation and washed with deionized water. The algae pellets were flash-frozen in liquid nitrogen and lyophilized to dryness (Labconco Freezone 6, Kansas City, MO). Lyophilized biomass was weighed and recorded as biomass per liter of culture. Biomass was stored at –20 °C under argon until analysis.

3.2.9. Bleach-Assisted Nile Red Microplate Assay for Neutral Lipid Quantification

3.2.9.1. Graphitized Carbon Pretreatment

Extracts were treated with graphitized carbon to remove interfering pigments. In a 2-mL Eppendorf tube, 25 mg of graphitized carbon (Sigma Aldrich, St. Louis, MO) was added to 1 mL of lipid extract and vortexed for 15 sec. The tubes were placed on a benchtop shaker and shaken for 30 min at room temperature. After shaking, the extract was passed through a 0.22 µm syringe filter and stored at –20 °C for analysis. All analyses were done within 24 hours of treatment.

3.2.9.2. Neutral Lipid Assay

Neutral lipids in extracts were quantified using a bleach-enhanced Nile red microplate assay adapted from Higgins et al. (2014).[22] A 1 mg/mL algae oil standard in chloroform was prepared using Thrive® Culinary Algae Oil. The lipid extracts and the 1 mg/mL algae oil standard in chloroform were diluted 2:1 with methanol. Diluted samples and standards were added to a 96-well polypropylene microplate (Greiner, Millipore Sigma, St. Louis, MO) in quadruplicate and

triplicate, respectively. Lipid standard was added to the microplate to achieve a range of 0 – 100 µg per well. The plate was heated at 55 °C to evaporate solvent then cooled to room temperature on the benchtop. Lipids were resuspended in 2-propanol followed by adding a Nile Red solution in DMSO (final concentration of Nile Red ~ 160 µg/mL) and incubated in obscurity for 5 min. Next, a 3% aqueous sodium hypochlorite solution was added to each cell and incubated in obscurity for 30 min. After bleaching, fluorescence measurements were taken using a Synergy HI Multi-Mode Plate Reader at excitation wavelengths of 530/40 nm and emission wavelengths of 590/40 nm. The neutral lipid content of the lipid extract was quantified using a standard curve derived from the lipid standards.

3.2.10. Statistical Analysis

All results are reported as mean values ± standard deviation. Student's t-test, one-way ANOVA, and post hoc Tukey's HSD tests were used to determine significant differences between microalgae strains and between experimental conditions of the growth data at a 5% significance level.

3.3. RESULTS AND DISCUSSION

3.3.10. Cultivation of Microalgae on Biochar-Filtered FWP at Microplate Scale

3.3.10.1. Cultivation *of C. sorokiniana* on Biochar-Treated FWP

C. sorokiniana exhibited lower growth potential when cultivated on biochar-filtered 10% FWP and 30% SFWP (10% CF-FWP or 10% CF-SFWP) compared to microalgae cultivated on untreated permeate, based on cell density (Figure 13b). Color has been shown to impact microalgae growth, so the effect of biochar filtration was evaluated as a pretreatment method to remove color in the FWP and SFWP. The experiments were designed based on our hypothesis that the light interference due to the dark color of permeate would be mitigated and would increase microalgae growth.[20] The application of biochar filtration as a pretreatment method was found to have no positive impact on overall biomass production (Figure 13).

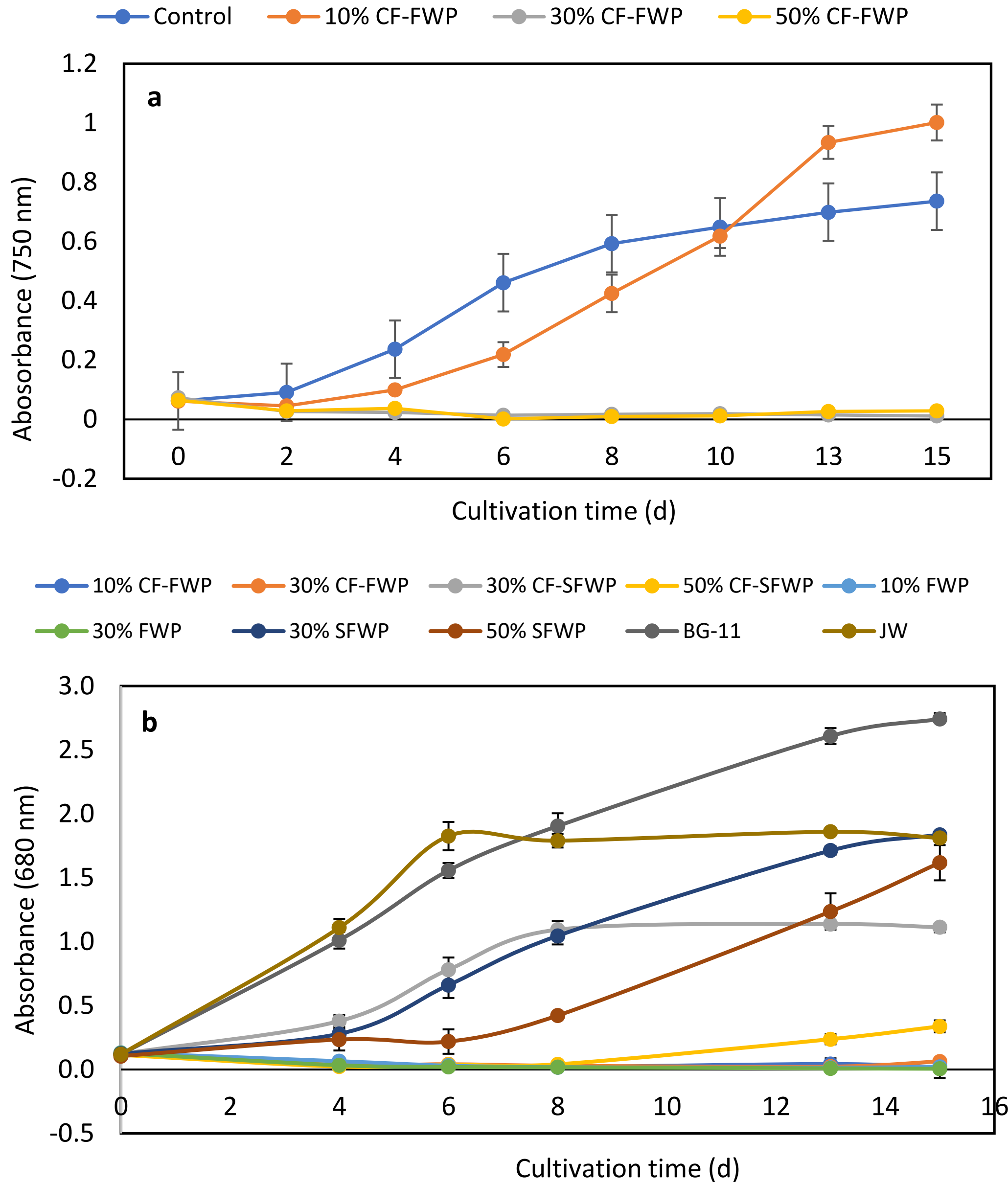

Figure 13. (a) Growth curves from preliminary studies evaluating *C. sorokiniana* cultivation on several dilutions of biochar-filtered 10% food waste permeate (10% CF-FWP). Cultivation was performed at a microplate scale (10 mL) with N=5. (b) Growth curves for *C. sorokiniana* cultivation on various dilutions of 10% CF-FWP and 10% CF-SFWP. Cultivation was performed at a microplate scale (10 mL) and N=3.

C. sorokiniana cell density on 10% biochar-filtered FWP (10% CF-FWP) in the initial studies (Figure 13a) was similar to that seen when cultivated on Jaworski's Medium. Conversely, *C. sorokiniana* did not grow on either the 30% CF-FWP or 50% CF-FWP. These observations are consistent with the cell density measurements of *C. sorokiniana* on untreated 10%, 30%, and 50% FWP described in Chapter 2. However, when the experiment was repeated (Figure 13b), there was no measured growth on either the 10% FWP or 10% CF-FWP, and the highest growth was observed on untreated 30% SFWP and 50% SFWP. These comparisons suggest that charcoal filtration of FWP has no positive impact on microalgae growth at microplate scale.

It is important to note that the initial results were measured at 750 nm (Figure 13a), and those of the repeated study (Figure 13b) were measured at 680 nm. A change in measurement procedures for microplate studies was established between these two studies, hence the change in wavelength used between experiments. The microalgae growth observed on the 10% CF-FWP as measured by cell density (Figure 13a) may be due to some other factor, such as bacterial contamination. Additionally, the permeate used in the repeat study (Figure 13) utilized FWP and SFWP that were treated in-house using biochar obtained from the Zhang lab at UC Davis (see Section 3.2.3.). The difference in biochar treatment would also introduce error if the treatment of FWP used in-house differed significantly compared to the treatments performed in the Zhang lab. Nonetheless, the varying results between the preliminary and repeated study (Figure 13a and Figure 13b) are hypothesized to mainly derive from the difference in wavelength used to measure biomass density. Therefore, this experiment should

be repeated using measurements at 680 nm to understand the impact of biochar filtration on microalgae growth.

It is known that the treatment of wastewater with activated carbon can remove various volatile organic compounds (VOCs) in addition to the pigment molecules.[21] This form of carbon (i.e., biochar from organic waste) is ineffective in removing inhibitory chemical compounds associated with limited microalgae growth. There are many forms of activated carbon, and some variations are optimized for specific target compounds. Upon further evaluating the permeate composition, a more suitable charcoal variation should be chosen to treat food waste permeate and increase microalgae biomass density. For example, specific activated carbons are developed to remove high molecular weight organic compounds such as pigments (e.g., chlorophyll, carotenoids), chemical oxygen demand, and other possible organic micropollutants. Another example would be activated carbon that facilitates BOD, COD, and TOC adsorption, which are very high in FWP (> 20000 mg C/L). Reduction of high oxygen demand should promote microalgae growth by providing a more oxygen-rich environment resulting in shorter lag phases and increased overall biomass productivity. While the CF-FWP microplate studies results suggest no positive effect on microalgae growth, future experiments should evaluate different carbon substrates.[22] Evaluating different, specific forms of carbon will better establish if the removal of pigments does not affect microalgae growth.

3.3.10.2. Cultivation of *C. sorokiniana* on 10% CF-FWP with Phosphate Supplementation

Cultivation of *C. sorokiniana* on 10% CF-FWP supplemented with higher phosphorus concentrations resulted in increased biomass (Figure 14). These results support the hypothesis that permeate is limiting in phosphorus and that increasing phosphorus concentrations would result in higher biomass accumulation. However, the results seen here differ from what was expected when considering the JW nutrient condition and the 100% phosphates condition. These conditions contain the same concentrations of supplemented phosphorus, with the only difference between them being that those supplemented with all JW stocks were also supplemented with other trace nutrients. Because of this, equivalent growth trends and biomass accumulation were expected. This deviation from the anticipated outcome can be due to additional trace nutrients incompatible with microalgae growth on FWP. Further testing should be performed at a larger scale to verify that phosphorus supplementation leads to increased biomass yield. Testing with increasing phosphorus concentrations should also be evaluated to support the data shown here, demonstrating that a higher concentration of phosphorus results in higher biomass accumulation.

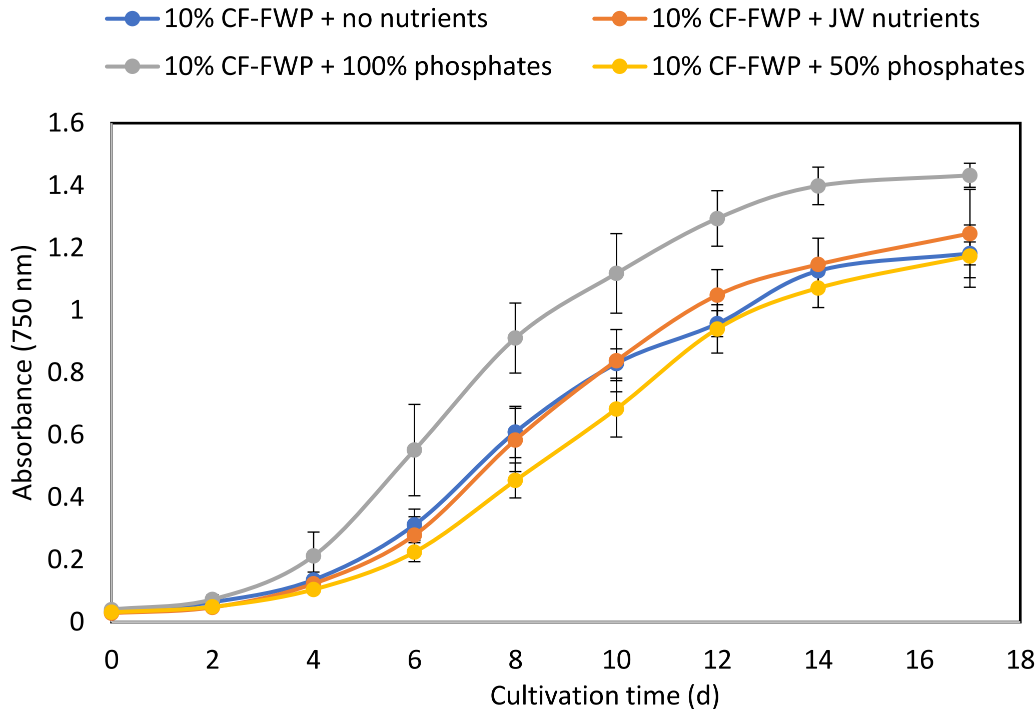

Figure 14. *C. sorokiniana* growth on 10% biochar-filtered food waste permeate (CF-FWP) with various supplementations. Cultivation was performed at 10-mL microplate scale with N=5.

3.3.10.3. Transmission Electron Microscopy of *C. sorokiniana* Cultivated on CF-FWP with and without Nutrient Supplementation

Transmission electron microscopy (TEM) imaging shows a significant increase in starch production in microalgae cultivated on 10% CF-FWP. Microalgae grown in any of the 10% CF-FWP conditions showed accumulation of numerous starch bodies and very few lipid bodies, compared to cultivation in Jaworski's Medium (JW). When cultivated on the Jaworski's Medium (JW) synthetic media, microalgae accumulated more lipid bodies than starch bodies. An increase in lipid yield can be attributed to the nutrient depletion afforded when microalgae reach stationary phase in JW. Nutrient depletion, specifically nitrogen depletion, is well-known to induce lipid accumulation due to metabolic stress induced by the surrounding

environment.[2,23] Nitrogen depletion is not observed in the 10% CF-FWP conditions. While nitrogen concentrations do decrease over time, they never reach the concentrations necessary to promote lipid accumulation. The lack of lipid accumulation for cultivation in 10% CF-FWP conditions is consistent with other experiments throughout this dissertation that were performed using different concentrations and forms of FWP and SFWP. As observed in Figure 15, there is a slight variation in the structural characteristics observed between the 10% CF-FWP conditions. Some variation in starch bodies is observed between (Figure 15) where an increased number of starch bodies is observed in 10% CF-FWP (Figure 15b) compared to the number of starch bodies observed in 10% CF-FWP + 100% phosphates (Figure 15d). Considering starch synthesis is a stress response in microalgae, the increase in starch bodies in *C. sorokiniana* cultivation on 10% CF-FWP (Figure 15b) would support the hypothesis that those microalgae cultivated on 10% CF-FWP were under more stress than those cultured on 10% CF-FWP with phosphate supplementation. Chloroplast size and density increased in the 10% CF-FWP cultures compared to the synthetic media control. Chloroplasts house pigments such as chlorophyll and carotenoids, and the increase in the chloroplast would be consistent with an increase in observable pigment (Figure 16).

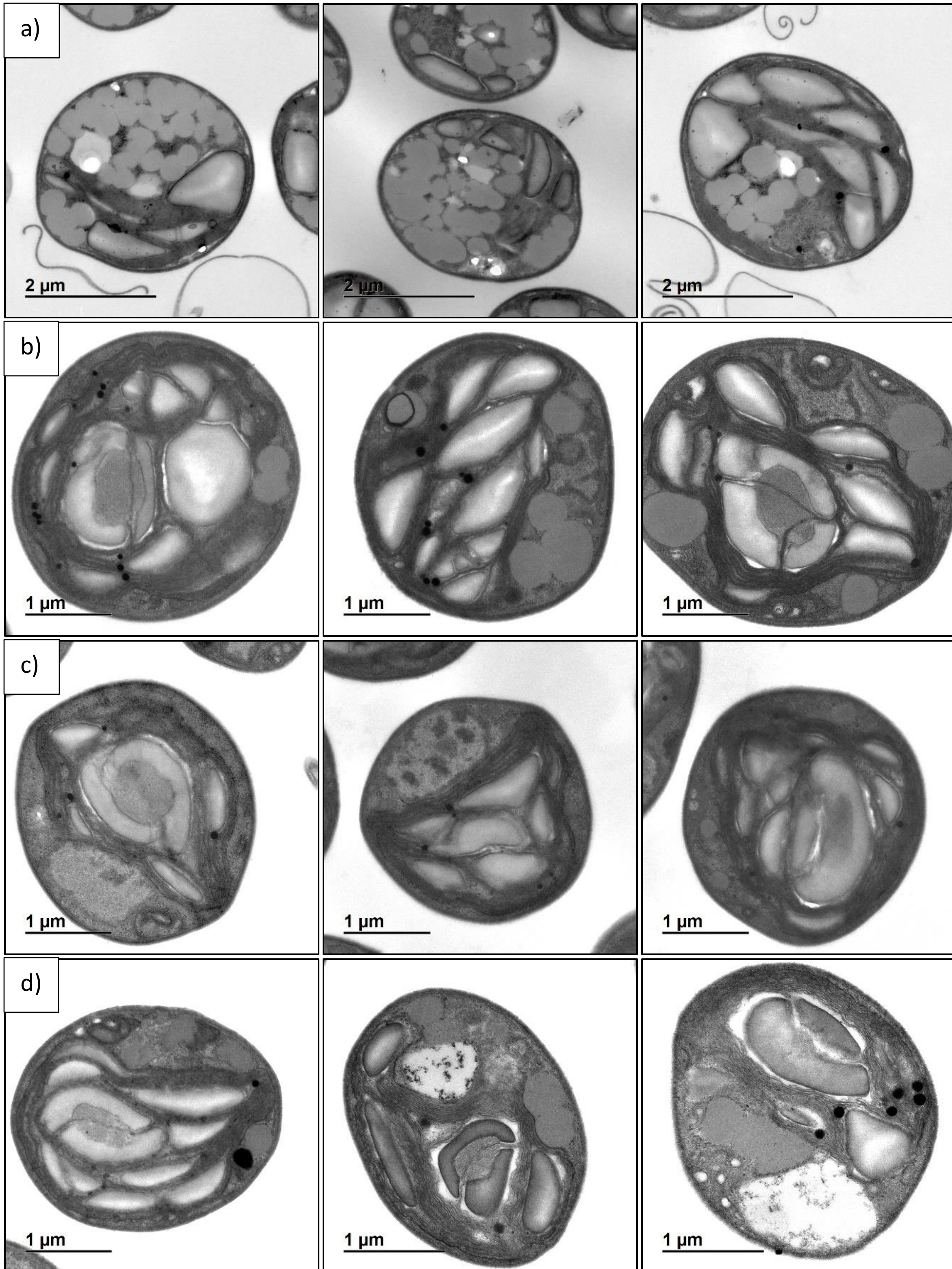

Figure 15. Transmission electron microscopy images of *C. sorokiniana* grown on a) Jaworski's Medium, b) bicohar-filtered 10% FWP (10% CF-FWP), c) 10% CF-FWP + JW nutrients, and d) 10% CF-FWP with 100% phosphate supplementation. Ultrastructure specificiation is described in the Appendix (Figure A1).

Figure 16. Microplate setup demonstrating the pigment variation between *C. sorokiniana* cultured on various 10% CF-FWP conditions versus a Jaworski's Medium (JW) control. The JW control is indicated, and the remaining plates contain *C. sorokiniana* cultivated on 10% CF-FWP with different supplementations.

3.3.10.4. Evaluating the Treatment of FWP with Various Biochar Concentrations

Biochar treatment results in a less pigmented food waste permeate stock solution (Figure 17). Conditions having higher amounts of biochar had the most effective removal of pigments than those treated with lower biochar concentrations (see Figure 17), which was expected considering the higher total surface area available with increased biochar content. This trend was observed in both SFWP and FWP when treated with the same concentrations of biochar. Initial 100% FWP conditions were lighter in color than that of the 100% SFWP, so this would explain why after treatment, the FWP appeared lighter overall than the SFWP conditions.

The 1000 mg/L FWP and SFWP treated permeate (Figure 17b) were used to cultivate microalgae.

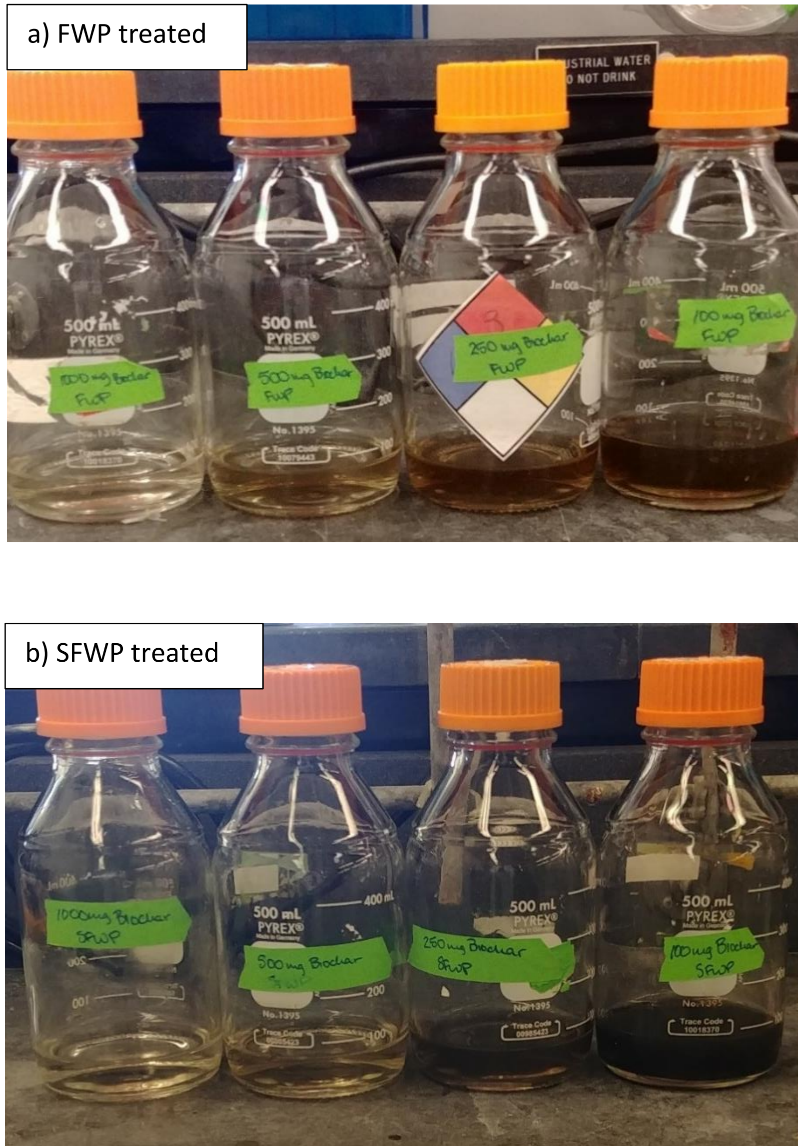

Figure 17. Treatment of undiluted (a) FWP and (b) SFWP with different amounts of biochar. Treatment effectiveness was determined by ocular inspection. The conditions are as follows from left to right: 20 mg/mL, 10 mg/ml, 5 mg/mL, 2 mg/mL.

3.3.11. Phosphorus Supplementation and Adjusting the Nitrogen-to-Phosphorus Ratio

3.3.11.1. Jaworski's Medium Supplementation of FWP at Microplate Scale (10 mL)

The supplementation of synthetic media nutrients resulted in insignificant changes in cell density within each growth media (Figure 18). Those cultures supplemented with additional nutrients had slightly lower growth on both SFWP dilutions and a slightly higher growth on the FWP dilution compared to cultures without supplementation. It was hypothesized that the additional nutrients, especially those used in synthetic media optimized for microalgae cultivation, would support higher microalgae growth than the slight increase observed in the 10% FWP with 50% JW nutrient concentrations and 100% JW nutrient supplementation (Figure 18a). Overall, the 10% SFWP produced the highest cell density, as determined by optical density at 680 nm, among the three dilutions tested. The higher growth on 10% SFWP is consistent with observations discussed in Chapter 2 regarding microplate screenings of *C. sorokiniana* on FWP and SFWP dilutions. All but the 10% FWP without JW nutrients showed increased growth on FWP and SFWP dilutions with and without supplementation compared to the BG-11 synthetic media control (Figure 18). Conditions with the lowest concentrations of ammonia (i.e., the SFWP conditions) had larger cell densities and shorter lag phases than microalgae cultured on higher concentrations of ammonia. The observed difference in growth supports the hypothesis that the high ammonia concentrations in FWP is a major inhibitory factor in microalgae cultivation and is in large part the cause for the low growth observed for many microalgae strains (see Chapter 2).

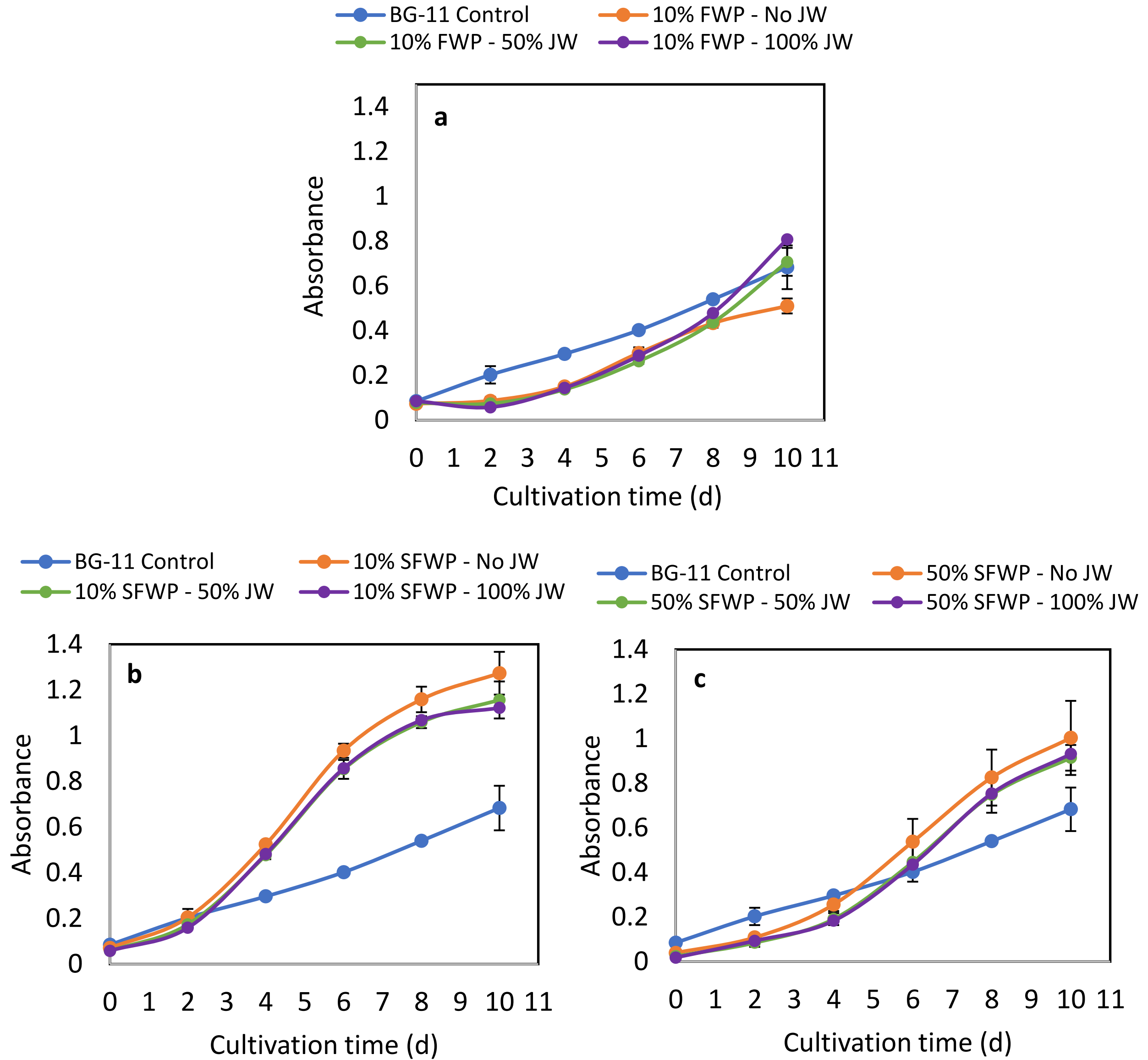

Figure 18. Growth curves for *C. sorokiniana* cultivation on (a) 10% FWP, (b) 10% SFWP, and (c) 50% SFWP supplemented with different volumes of Jaworski's Medium (JW) nutrients. Cultivation was performed at microplate scale (10 mL), and values are the average of duplicates.

3.3.11.2. Evaluating Various Nutrient Ratios and Their Effects on Microalgae Cultivation at Batch Scale (250 mL)

The various N/P experimental ratios achieved with phosphorus supplementation performed similarly to their respective unsupplemented conditions as determined by optical density (Figure 19 and Table 12). Any possible differences that were seen were statistically insignificant ($p < 0.05$). The 3:1 and 33:1 N/P ratios were chosen because they are the N/P ratios seen in Jaworski's Medium (JW) and BG-11, respectively. *C. sorokiniana* has increased growth when cultivated on BG-11 compared to JW, but this relationship between the N/P ratios of synthetic media and *C. sorokiniana* growth was not observed in the conditions evaluated here. It is important to note that after reevaluation of the BG-11 media composition, it was determined that the N/P ratio is 44:1 instead of 33:1, so future experiments should evaluate the 44:1 N/P ratio instead of 33:1 tested here. When evaluating *C. sorokiniana* growth on FWP with 3:1 N/P, the apparent growth potential, as determined by optical density, indicates no significant difference between the supplemented and unsupplemented conditions. The samples from the 3:1 N/P ratio experiment were not harvested due to the insignificant growth differences between conditions as determined by optical density and certain time constraints. That said, the 3:1 N/P experiment is still valuable in investigating the effect of various N/P ratios on *C. sorokiniana* growth, especially on lower N/P ratios. Previous literature found that 6:1, 15:1, and 23:1 were optimal N/P ratios for various freshwater microalgae strains, but the results show that adjusting N/P to those specific ratios is not optimal for *C. sorokiniana*, the strain of interest in this research.[8] Other microalgae strains, such as those in the Scenedesmusceae family (e.g., *Scenedesmus acutus f. alternans*, *Scenedesmus obliquus*), should

be evaluated. Other microalgae strains in the Chlorophyta phylum should also be considered as previous literature supports the hypothesis that the ratios evaluated here are optimal for many Chlorophyte species.

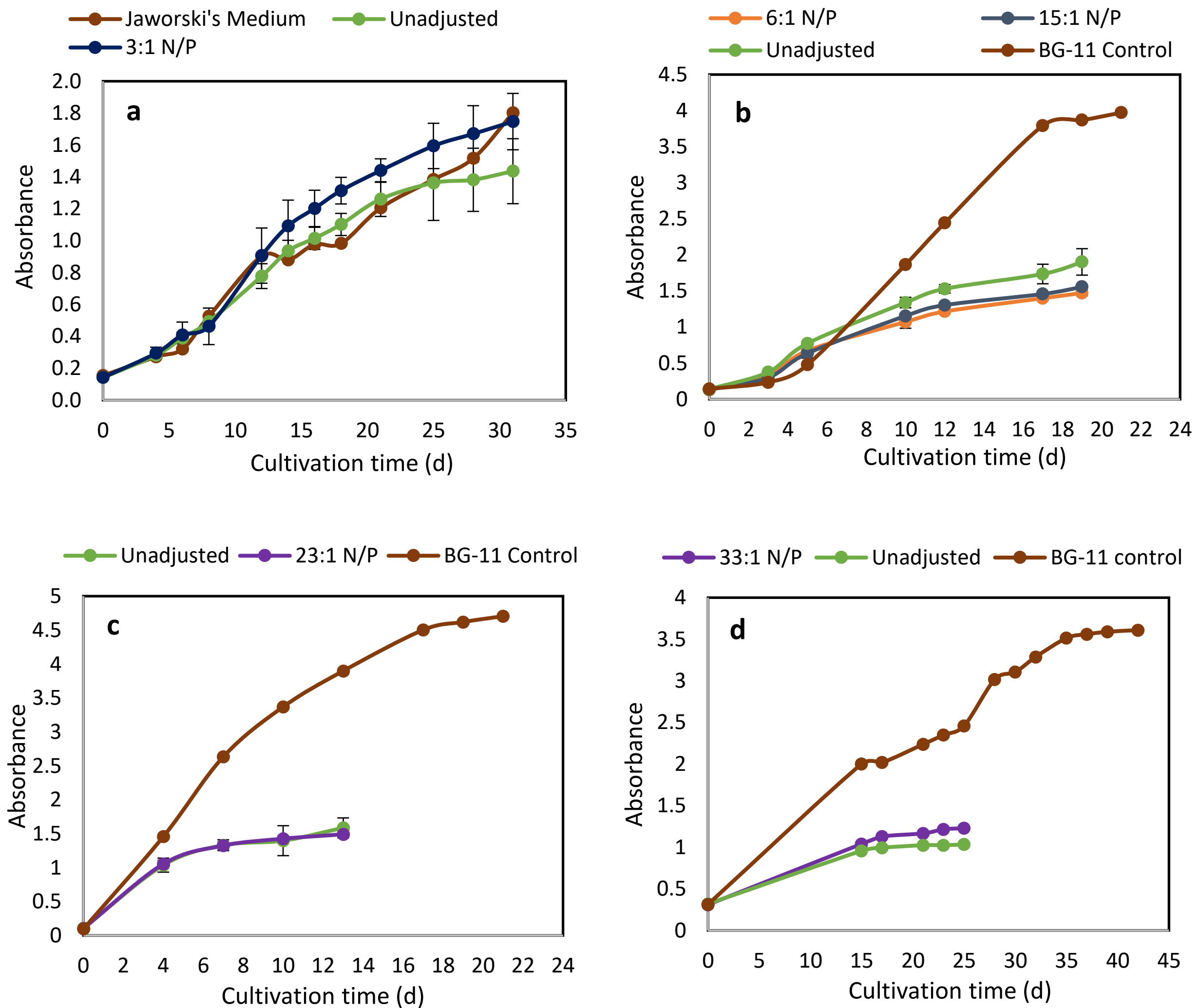

Figure 19. *C. sorokiniana* growth on 10% FWP adjusted to have (a) 3:1 N/P ratio, (b) 6:1 N/P and 15:1 N/P ratios (c) 23:1 N/P ratio, and (d) 33:1 N/P ratio. Experiments were performed with the following culture voluems: (a), (b), and (c) = 250 mL scale and (c) was performed at 100 mL scale.

Table 11. *C. sorokiniana* growth characteristics when cultivated on 10% food waste permeate (FWP) with differing nitrogen-to-phosphorus ratios and a synthetic media control.[a] JW = Jaworski's Medium

Condition	N:P (~)	Culture volume (mL)	Harvest day[b]	Harvested Biomass (mg)	Biomass productivity ($mgL^{-1}day^{-1}$)	Percent neutral lipid content (w/w)
3:1 N/P 10% FWP	3:1	250	31[c]	n/a	n/a	n/a
JW Control	3:1	250	31[c]	n/a	n/a	n/a
Unadjusted 10% FWP	26:1	250	19	145 ± 10	95 ± 9	7.3 ± 0.3
6:1 N/P 10% FWP	6:1	250	19	111 ± 4	68 ± 5	6.2 ± 0.2
15:1 N/P 10% FWP	23:1	250	19	121 ± 2	83 ± 7	6.1 ± 0.2
BG-11 Control[c]	44:1	250	21	289	210	4.5 ± 0.2
Unadjusted 10% FWP	33:1	100	13	50 ± 6	189 ± 26	9.0 ± 0.7
23:1 N/P 10% FWP	23:1	100	13	48 ± 3	185 ± 13	8.3 ± 0.4
BG-11 Control	44:1	100	21	156 ± 16	392 ± 47	6.8 ± 0.6
Unadjusted 10% FWP[d]	147:1	250	25	82	25	n/a
33:1 N/P 10% FWP[d]	33:1	250	25	101	34	n/a
BG-11 Control[d]	44:1	250	42	284	84	n/a

[a] Values presented at mean ± SD of triplicates
[b] Biomass based on harvesting at stationary phase; strains reached stationary phase at different days throughout the experiments and were therefore harvested on different days
[c] Not actually harvested but ended the experiment at day 31
[d] Performed with N=1 replicates due to lack of space

Variation is observed between the unadjusted controls of the separate experiments. While this variation can be attributed to culture volume since the volumes do vary (Table 12), it is more likely due to the method optimization occurring over the course of this project. Any variation could also be due to slight compositional change in the FWP over time, specifically ammonia. With the opening and closing of the lid, in addition to the slight disruption during experimental preparations, initial ammonia concentrations changed slightly over time which may affect overall microalgae growth in the later experiments. Further analysis should be performed to investigate these differences in more depth.

The N/P ratio may not cause the insignificant difference observed between the different conditions evaluated here (Table 12). As discussed in Chapter 2, FWP contains inhibitory ammonia concentrations, lessening any benefit associated with increasing the N/P ratio. Future studies should explore the combination of treatments addressing high ammonia concentrations followed by the adjustment of the N/P ratio. By doing this, the inhibitory levels of ammonia would have less of an impact on overall growth, and therefore the increase in phosphorus would have a more significant effect on microalgae growth.

3.3.11.3. Evaluating Excess Phosphorus Supplementation of SFWP at Batch Scale (250 mL)

The supplementation of SFWP with phosphorus to achieve excess phosphorus concentrations relative to nitrogen concentrations resulted in increased microalgae growth, specifically in the 30% SFWP conditions (Figure 20 and Table 13). Both 10% FWP and 30% SFWP dilutions were chosen based on the growth observed in the microplate studies described in Chapter 2. The addition of phosphorus shortened the lag phase and increased the growth rate for the 30% SFWP conditions, but neither the optical density nor lag phase was significantly different in the 10% SFWP conditions (Figure 20). The increased growth of *C. sorokiniana* on 30% SFWP compared to the 10% SFWP is consistent with previous microplate studies. Increased growth on 30% SFWP is likely due to the increased initial concentration of nitrogen, allowing for a higher growth potential overall. The growth of *C. sorokiniana* cultivated on 10% SFWP only differed near the end of the stationary phase right before harvesting. The increase in microalgae growth on 30% SFWP with phosphorus supplementation combined with the insignificant differences observed in the FWP phosphorus supplementation experiment

suggests that phosphorus may be increasing microalgae growth; however, an increase is not observed in FWP conditions because the toxic ammonia concentrations supersede the benefits provided through phosphorus supplementation.

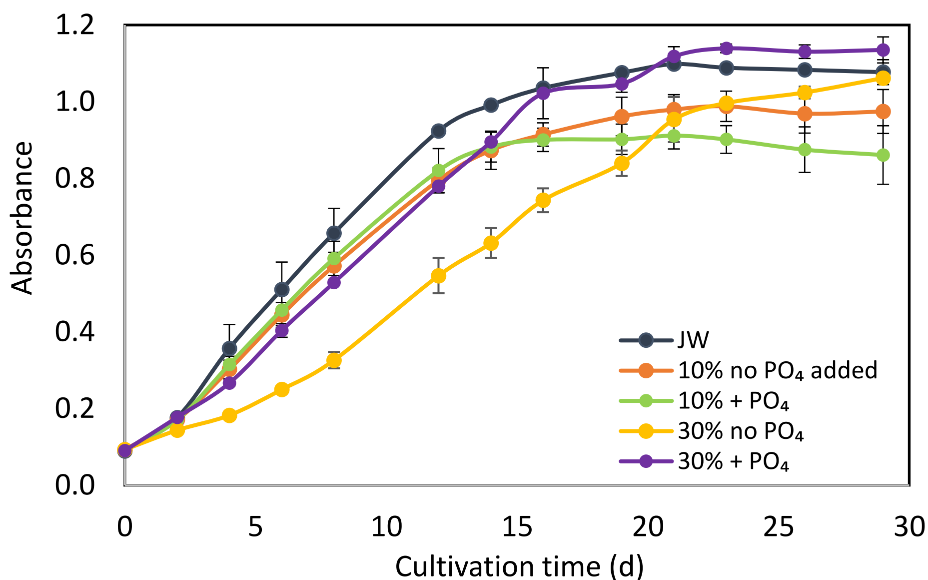

Figure 20. Growth curves for *C. sorokiniana* cultivated at batch scale (250 mL) on 10% and 30% SFWP with and without phosphorus supplementation. SFWP = stripped food waste permeate, JW = Jaworski's Medium (synthetic medium)

Table 12. Growth characteristics for *C. sorokiniana* grown on 10% and 30% SFWP with and without phosphorus supplementation at 250 mL scale. Included are the nitrogen and phosphorus concentrations and the associated N/P ratio of the initial growth media.[a]

Condition	Harvest day	Harvested Biomass (mg)	Biomass productivity ($mgL^{-1}d^{-1}$)	Initial [TN] (mg/L)	Initial [P] (mg/L)	N:P (~)
10% SFWP no PO_4	29	91.1 ± 0.7	47 ± 1	28	4.7	6:1
10% SFWP + PO_4	29	85 ± 2	43.2 ± 0.6	28	93	1:3
30% SFWP no PO_4	29	110 ± 1	46 ± 2	83	28	6:1
30% SFWP + PO_4	29	147 ± 3	78 ± 5	83	280	1:3
Jaworski's Medium	29	102 ± 5	55 ± 4	16	6	3:1

[a] Values presented as mean ± SD of duplicates

Using excess phosphorus supplementation may also hinder the economic feasibility of using FWP/SFWP for microalgae cultivation by increasing the input costs. It is suggested that economic assessments should be performed to determine if this would be the case. Because of the uncertainty in economic feasibility and for the purposes of the project, further experimentation was not pursued, but the potential of phosphorus supplementation with FWP should still be explored further in future projects. One such study should evaluate the relationship between ammonia and phosphorous, as both are hypothesized to have a compounded effect on microalgae growth on FWP.

3.3.12. Nitrifying Bacteria Treatment of FWP

3.3.12.1. Compositional Changes with Nitrifying Bacteria Treatment of 10% FWP with Either CO_2 or Air Supplementation at Batch Scale (250 mL)

Aeration using a 6% CO_2/air mixture during nitrifying bacteria (NB) pretreatments allows for the maintenance of high total nitrogen (TN) concentrations while decreasing the total ammonia concentrations in 10% FWP (Figure 21). Cultures without NB and aerated with 6% CO_2/air afforded a 23 ± 12% reduction in TN and a 16 ± 7% reduction in total ammonia (Table 14). Comparatively, there was an 84% ± 37% reduction of TN and 91% ± 26% reduction in total ammonia when cultured without NB and aerated with pumped air (Table 14). The decrease in the cultures aerated with CO_2 can be attributed to ammonia volatilization. While CO_2 lowers the pH and shifts the chemical equilibrium to favor the NH_4^+ ammonium species, some ammonia is still in the volatile NH_3 form that can be lost when bubbling gas through the liquid. These equilibrium changes are demonstrated in Chapter 2 of this dissertation. Nonetheless, the

comparison between the TN and ammonia reduction in cultures aerated with CO_2 versus air demonstrates the significant effect CO_2 has on nitrogen concentrations in FWP.

The NB cultures bubbled with air and those bubbled with 6% CO_2/air demonstrated similar changes in ammonia concentration over time (Figures 21b and 21d). The similarity in ammonia concentration supports the hypothesis that the functional capacity of NB to convert ammonia to nitrite and nitrate is not significantly affected by the supplementation of CO_2. Still, there was a significant decrease in TN when bubbled with air. This difference in TN is still not as significant as the decrease of TN observed in the cultures without NB and bubbled with air. Decreased total nitrogen loss in the NB cultures with air is attributed to the nitrogen species changing upon nitrification by NB. Nitrite and nitrate are both soluble and ionized in water, meaning the loss of either form due to volatilization is unlikely. Therefore, the initial TN reduction observed in the NB cultures bubbled with air can be attributed to the NB lag phase, when the nitrification process is not yet fully established, and the chemical speciation of ammonia is still changing.

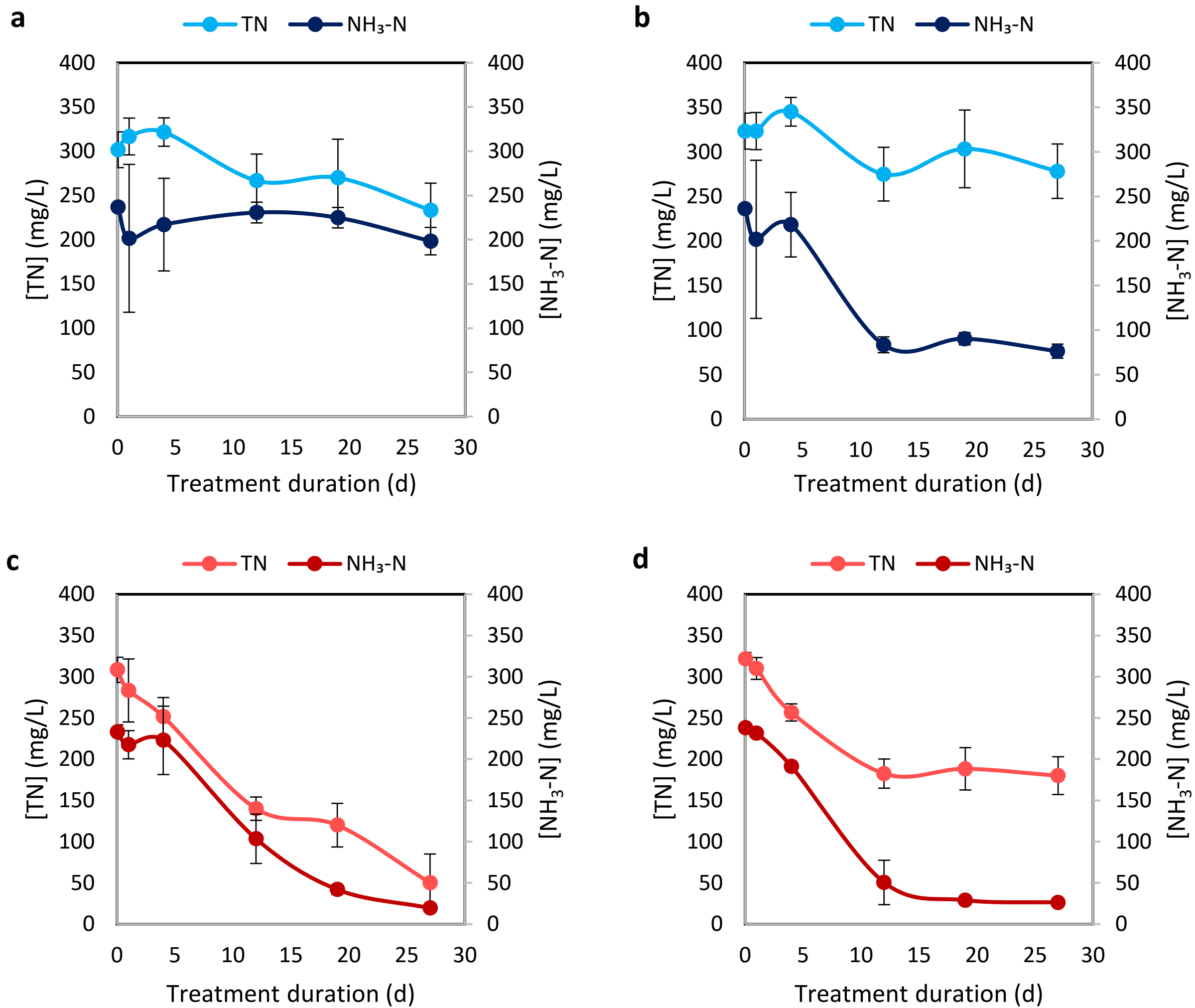

Figure 21. Total nitrogen (TN) and ammonia nitrogen concentration changes in 10% food waste permeate over time with and without nitrifying bacteria (NB) as a pretreatment method and cultivated with either air or CO_2 bubbling. (a) no NB + CO_2; (b) with NB + CO_2; (c) no NB + air; (d) with NB + air.

Table 13. Concentrations of total nitrogen (TN) and ammonia-nitrogen (NH_3-N) when treated with or without nitrifying bacteria (NB) and a 6% CO_2/air versus air supplementation method.[a] FWP = food waste permeate

Parameter		Condition	Treatment duration (d)					
			0	1	4	12	19	27
+ CO_2	TN	10% FWP	301 ± 20	316 ± 21	321 ± 16	266 ± 30	270 ± 46	233 ± 31
		10% FWP + NB	323 ± 14	323 ± 25	345 ± 35	275 ± 18	303 ± 8	278 ± 4
	NH_3-N	10% FWP	237 ± 6	201 ± 84	217 ± 52	230 ± 12	224 ± 12	198 ± 15
		10% FWP + NB	236 ± 1	201 ± 89	218 ± 36	83 ± 9	90 ± 7	76 ± 8
+ air	TN	10% FWP	308 ± 15	283 ± 38	251 ± 23	140 ± 14	120 ± 26	50 ± 35
		10% FWP + NB	321 ± 8	310 ± 13	257 ± 10	183 ± 18	188 ± 26	180 ± 23
	NH_3-N	10% FWP	233 ± 9	218 ± 17	223 ± 41	103 ± 30	42 ± 6	20 ± 1
		10% FWP + NB	238 ± 2	232 ± 4	191 ± 6	51 ± 27	29 ± 4	26 ± 4

[a] Values presented as mean ± SD of triplicates

This study demonstrates that the addition of NB is effective in remediating high ammonia concentrations while still maintaining the high TN concentrations. Both air and CO_2 can be used during the pretreatment without affecting NB growth, but using CO_2 is recommended to minimize any nitrogen volatilization during the establishment period of the NB. Additionally, the 6% CO_2/air mixture can be sourced from the anaerobic digestion process that provides the FWP, so utilizing it during NB treatment would increase the sustainability of the treatment process.

3.3.12.2. Preliminary Nitrifying Bacteria Pretreatment of 100% FWP at 2-L Scale

The addition of nitrifying bacteria significantly decreased ammonia concentration in 100% FWP by 75% while maintaining the nitrogen in the form of nitrite, a product of the nitrification performed by the nitrifying bacteria (Figure 22c). A reduction in ammonia concentration was also observed in the culture without NB. This ammonia loss can be

attributed to a loss of ammonia through gas volatilization over time and is not unexpected considering the results of the previous study (Figure 21 and Table 14) showing this loss. The ammonia reduction in the control samples without NB is also observed in the samples with NB, further supporting the idea of normalized loss of ammonia through gas volatilization. Hence, the change in total nitrogen can be attributed solely to the loss of ammonia over time.

The treatment duration of this experiment was significantly longer (48 days) than that of the preliminary 2-L study (26 days) (Figure 22). The preliminary study (Figure 22a) utilized TN and NH_3-N to determine the cessation of NB activity and treatment completion point. Upon reaching a constant concentration of ammonia, the media was removed and filtered for storage. In this study, instead of using only TN and NH_3-N to determine the treatment endpoint, ion chromatography was also used to measure nitrite/nitrate concentrations. Upon reaching constant nitrite and ammonia concentrations, the samples were filtered for storage. Using nitrite and ammonia concentrations to determine NB treatment duration resulted in slightly longer treatment times (~ 30 days) than the preliminary study (~ 20 days). This time difference can be attributed to the NB used in each experiment being from different bottles. The longer treatment duration can also be due to variations in CO_2 concentrations and therefore changes in pH and ammonia speciation. The experiment was continued after the abatement of NB activity to evaluate other possible changes caused by the NB treatment over a long duration of time. It is important to note that these conditions were only performed with a single replicate due to space constraints. Future work to follow up on these preliminary results should include a design with experiments performed in triplicate.

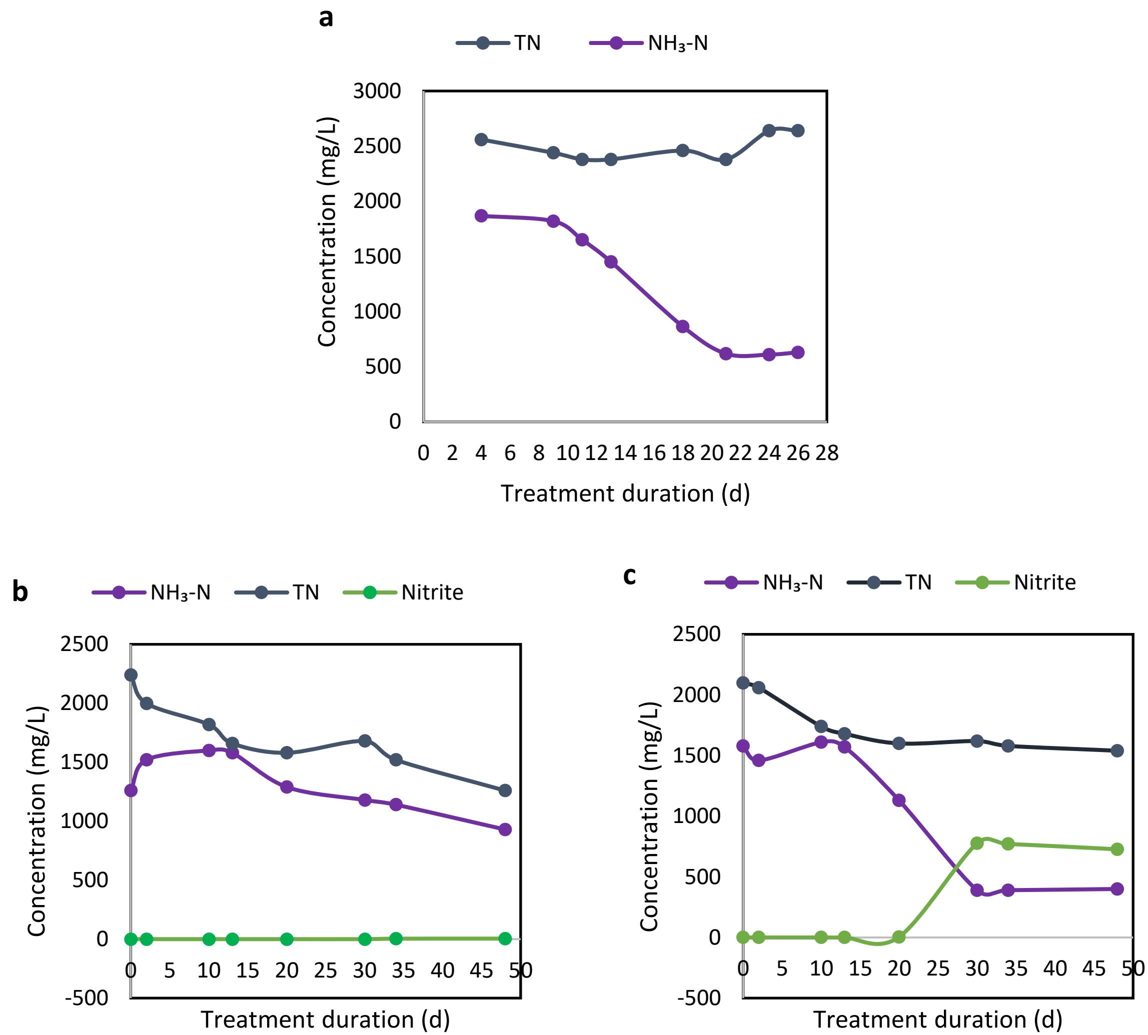

Figure 22. Compositional change in nitrogen species during the treatment of 100% food waste permeate (FWP) using nitrifying bacteria (NB) at 2-L scale. (a) 100% FWP+NB in June 2018, (b) 100% FWP-NB in April 2019, (c) 100% FWP+NB in April 2019.

Table 14. Change in nitrogen composition over time with and without nitrifying bacteria (NB) for two separate treatments performed in April 2018 and April 2019.[a]

Experiment		Parameter (mg/L)	Treatment duration (d)	Initial concentration[a] (mg/L)	Final concentration (mg/L)	Remediation capacity[b]
June 2018	+NB	TN	26	2560	2640	3%
		NH_3-N	26	1868	630	66%
April 2019	+NB	TN	48	2100	1540	36%
		NH_3-N	48	1580	400	75%
		NO_2^-	48	nd	727	n/a
	-NB	TN	48	2240	1260	44%
		NH_3-N	48	1260	930	34%
		NO_2^-	48	nd	6	n/a

n/a = not available
nd = not detected; below detection threshold
[a] Values presented as the average ± SD of triplicates

3.3.13. Cultivation of *C. sorokiniana* on Nitrifying Bacteria-Treated FWP

3.3.13.1. Preliminary *C. sorokiniana* Cultivation on Nitrifying Bacteria-Treated FWP at Small Scale (100 mL)

Preliminary studies demonstrated that unadjusted, untreated permeate (i.e., FWP from the stock container) provided an environment supporting higher microalgae growth (Figure 23). Microalgae cultivated on NB-treated 10% FWP demonstrated a 67 ± 16% reduction in biomass productivity compared to microalgae cultivated in “fresh” 10% FWP obtained from the stock permeate container (Table 16). These results were unexpected considering the lower ammonia concentrations in the NB-treated permeate cultures. The low biomass productivity demonstrated by microalgae cultivated on NB-treated FWP is attributed to competition between NB and microalgae. The NB-treated permeate was only filtered through a Whatman No. 5 filter (2.5 µm pore size) to filter out as many NB cells as possible without the loss of

ammonia due to vacuum. A larger filter pore size would result in NB-containing media being carried into the microalgae cultivation experiment. The low inoculation volume of microalgae (10% v/v) and the faster growth cycle of NB compared to the microalgae would result in NB outcompeting microalgae for the nutrients from the media, therefore inhibiting microalgae growth. Further studies need to be performed to establish the relationship between the residual NB cells in the filtered, NB-treated 10% FWP and *C. sorokiniana*.

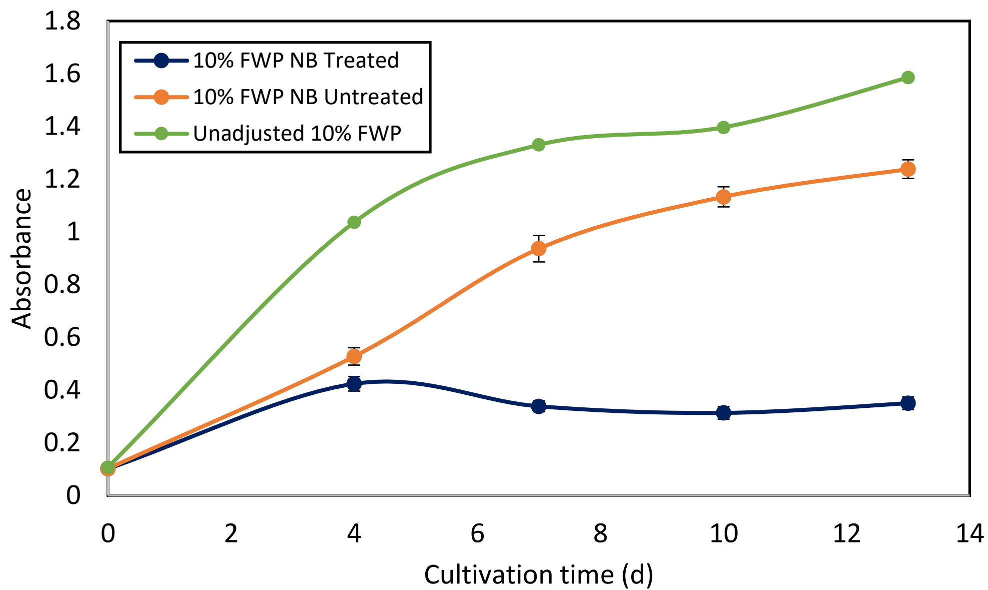

Figure 23. Growth curves for *C. sorokiniana* on nitrifying bacteria (NB) treated 10% FWP, untreated 10% FWP from the NB treatment experiment (untreated), and a “fresh” 10% FWP control prepared from the stock container (unadjusted). All conditions were tested in triplicate in 100-mL volumes. FWP = food waste permeate.

Table 15. Growth characteristics for *C. sorokiniana* cultivated on nitrifying bacteria (NB) treated and untreated 10% FWP at 100 mL scale.[a]

Condition[b]	Harvest Day	Harvested Biomass (mg)	Growth rate (d^{-1})	Biomass productivity ($mgL^{-1}d^{-1}$)
10% FWP NB-treated	13	17 ± 1	0.36 ± 0.01	63 ± 6
10% FWP NB-untreated	13	47 ± 3	0.316 ± 0.006	158 ± 13
10% FWP Unadjusted	13	50 ± 6	0.359 ± 0.007	189 ± 26

[a] Values are presented mean ± SD of triplicates
[b] The "10% FWP NB-untreated" permeate was obtained from the NB treatment experiment described in Section 3.3.3.1. The "10% FWP Unadjusted" control was prepared from the stock container.

3.3.13.2. Cultivation of *C. sorokiniana* on Nitrifying Bacteria-Treated FWP at Batch Scale (250 mL)

Cultivating *C. sorokiniana* on nitrifying bacteria (NB)-treated 10% FWP at a larger (250 mL) scale resulted in significantly higher optical densities when compared to the preliminary, small volume (100 mL) experimental results (Figure 24). When comparing these results to that of the preliminary study, the overall harvested biomass is significantly higher for all conditions in the present study. The increase in harvested biomass is attributed to the increased volume, affording longer cultivation times before physical space becomes a limiting factor. Although the overall optical density and harvested biomass was higher in this study than that of the preliminary study (Figure 23), the NB treatment of FWP still had minimal effect on growth rate and biomass productivity compared to those cultivated on the 10% FWP without NB treatment and bubbled with 6% CO_2/air (Figure 24).

The microalgae growth observed on the 10% FWP without NB treatment and bubbled with 6% CO_2/air was similar to the microalgae growth observed on 10% FWP without NB and not bubbled (Table 17). However, the steeper exponential phase observed for *C. sorokiniana* cultivated on 10% FWP without NB and not bubbled resulted in increased biomass productivity by increasing the growth rate (Table 17). Nonetheless, the similarities in optical density and final harvested biomass between the two 10% FWP without NB conditions support the conclusion that bubbling with 6% CO_2/air has no significant effect on microalgae growth.

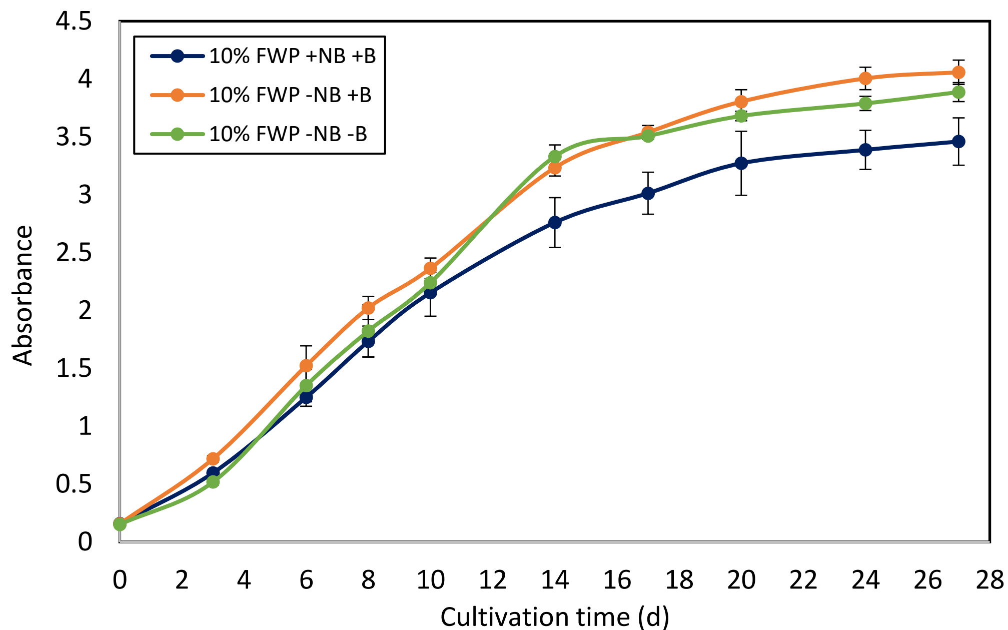

Figure 24. Growth curves for *C. sorokiniana* on nitrifying bacteria (NB) treated and untreated 10% FWP along with an unadjusted control prepared with fresh permeate. All conditions were performed at 250 mL batch culture scale. FWP = food waste permeate; B = bubbled; +/- = with/without.

Table 16. Growth characteristics for *C. sorokiniana* on nitrifying bacteria (NB) treated and untreated 10% FWP. All conditions were performed at 250 mL batch culture scale.[a]

Condition	Harvest Day	Harvested biomass (mg)	Growth rate (d^{-1})	Biomass productivity ($mgL^{-1}d^{-1}$)
10% FWP +NB +B[b]	27	243 ± 8	0.151 ± 0.004	147 ± 9
10% FWP -NB +B[b]	27	261 ± 4	0.159 ± 0.002	166 ± 4
10% FWP -NB -B[c]	27	266 ± 20	0.1877 ± 0.0007	200 ± 10

[a] Values are presented as an average ± SD of triplicates
[b] The "10% FWP +NB +B" and "10% FWP -NB +B" were obtained from the April 2019 NB treatment experiment where cultures were bubbled (+/- B) using 6% CO_2/air with or without NB (+/- NB); these permeates are described in Section 3.3.3.2.
[c] The "10% FWP -NB -B" control was prepared from the stock container, so it was not treated with NB or bubbled previous to its use.

3.4. CONCLUSIONS

Cultivation of *C. sorokiniana* has been demonstrated in various conditions utilizing food waste permeate (FWP) and stripped food waste permeate (SFWP). Microalgae cell density remained relatively consistent throughout the cultivation experiments varying the nitrogen-to-phosphorus ratio, using nitrifying bacteria (NB) pretreatment, and comparing biochar filtered FWP as a media. The observations and results of these studies indicate that, on their own, these approaches may not be ideal solutions to increase microalgae growth using FWP. Although growth and remediation capacities did not significantly vary between the conditions tested, the biochemical composition of the microalgae possibly changed.

Lipid accumulation in *C. sorokiniana* is too low to be viable as an alternative biofuel feedstock. It is widely known that *C. sorokiniana* accumulates starch in addition to lipids as a method to store excess energy; this fact was observed using transmission electron microscopy (TEM) images to compare *C. sorokiniana* cultivated using phosphorus supplementation.[24] The

starch production pathway can be a significant inhibitory factor in the lipid accumulation by *C. sorokiniana*, so starch inhibition studies should be performed with *C. sorokiniana* to determine the capacity for lipid accumulation when lipid production is the primary energy storage mechanism.

The unexpected, insignificant difference between conditions in the N:P ratio experiments suggests that either phosphorus availability in FWP is not a significant factor in microalgae growth on FWP, or the effects of phosphorus supplementation are not observed due to some outside factor, likely in the chemical composition of the FWP. The high ammonia concentrations were addressed through pretreatment of FWP with NB, but no significant changes in growth were observed upon cultivating microalgae on this treated media. There was, however, an increase in harvested biomass with the increased volume of the cultures. Therefore, it is recommended that future studies should investigate the use of phosphorus supplementation on NB-treated FWP, in combination, with at least 250-mL working volumes. A hypothesis consistent with these observations is that there were no growth differences in phosphorus supplementation or nitrifying bacteria pretreatment experiments because the ammonia concentrations were still at inhibitory levels in the phosphorus experiments, and phosphorus concentrations were limiting growth in the NB experiments. Similarly, there was no difference between the NB-treated and untreated FWP conditions because phosphorus was still limiting. Considering these hypotheses, it would be beneficial for future studies to investigate the combination of these two ideas—phosphorus supplementations and NB-treatments.

There is significant potential for microalgae cultivation on FWP and SFWP if inhibitory factors such as the high ammonia concentrations and low phosphorus concentrations are

studied further and investigated, as necessary. However, it is important to note that these supplementations and pretreatments may affect the economic feasibility of microalgae cultivation using FWP on an industrial scale, so a techno-economic assessment should be performed to evaluate their use in large-scale systems and judge accordingly.

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CHAPTER 4 – Using Nitrogen and Oxidative Stress to Increase Lipid Productivity in *C. sorokiniana* and *C. vulgaris* and Addressing Lipid Quantification Limitation

4.1. INTRODUCTION

Increasing lipid yield in microalgae is an ever-present endeavor in the microalgae cultivation sector. Lipids are valuable and pose to be a sustainable alternative feedstock for biofuels. Besides feedstocks, lipids are prevalent in the nutraceutical market as a source of unsaturated fatty acids.[1] Many methods are applied to increase microalgae lipid productivity, including nutrient depletion and oxidative stress.[2–4] Previous chapters of this dissertation have highlighted the low lipid accumulation by *C. sorokiniana* and *C. vuglaris* when cultivated on 10% FWP and 30% SFWP. The feasibility of microalgae as an alternative biofuel feedstock hinges on its ability to accumulate significantly higher lipid yields, higher than the < 15% w/w observed in my previous studies.[5] So, finding methods to increase lipid production is essential to the applicability of *C. sorokiniana* or *C. vulgaris* as a biofuel feedstock.

Nitrogen limitation is a well-studied method to increase lipid accumulation in microalgae.[2,6–8] Chapter 2 demonstrated that after the cultivation and harvesting of *C. sorokiniana* and *C. vuglaris*, the supernatant media measured high concentrations of remaining nitrogen. Because the microalgae do not experience nitrogen deprivation before they reach stationary phase and are harvested, I hypothesized that induction of lipid accumulation does not occur. In this chapter, I will discuss implementing a two-phase cultivation method to increase lipid productivity in microalgae, specifically *C. sorokiniana* and *C. vuglaris*.

Two-phase cultivation methods utilizing a secondary stress phase have been used in previous studies to increase lipid productivity in microalgae.[9] One method includes utilizing a secondary, nitrogen-deficient phase to induce lipid accumulation. The application of a secondary nitrogen-deficient phase following cultlivation under FWP conditions would address the hypothesis that lipid accumulation in *C. sorokiniana* and *C. vulgaris* is low because it does not experience nitrogen deprivation before reaching stationary phase. In combination with nitrogen deficiency, oxidative stress has been shown to increase lipid production further.[2] Hydrogen peroxide is an inexpensive, readily available chemical that can be added to microalgae cultures in late stationary phase to induce lipid accumulation, especially when added to nitrogen-deficient cultures.[2,10] Applying a dilute H_2O_2 treatment at the end of the final stage of the proposed two-stage method is hypothesized to further increase lipid accumulation by increasing the stress of the surrounding environment.

Upon completion of these cultivation studies, the lipids must be extracted from biomass for quantification. Sonication, bead-beating, and microwave-assisted extraction (MAE) are three effective methodologies for disrupting microalgae membranes.[11] Sonication is the standard method used throughout this dissertation. Alongside sonication, bead-beating and MAE were considered as alternatives for difficult-to-lyse microalgae strains such as the *S. acutus f. alternans* and *S. obliquus* strains discussed in previous chapters.[12] Evaluating the efficiency and efficacy of these lipid extraction methods requires an accurate and precise extraction and quantification method to derive proper conclusions and decisions.

The bleach-assisted Nile Red assay is a standard non-chromatographic quantification method for neutral lipids and is the assay used throughout this dissertation. The accuracy and

precision of this measurement are vital in making decisions on how to move forward with this research and determining the viability/applicability of this research in larger-scale operations. Optimizing this assay for highly pigmented lipid extracts is necessary to mitigate pigments as a source of error in the NRLA. It is hypothesized that graphitized carbon and biochar are possible sorbents for the interfering pigments, allowing for more accurate lipids measurements in the lipid extract.

This chapter covers two main discussion topics: lipid enhancement through two-phase methodology and the extraction and quantification of accumulated lipids in microalgae extracts. It is hypothesized that the two-phase procedure will increase lipid yield in *C. sorokiniana* and *C. vulgaris*. In addition, the supplementation of H_2O_2 in the final stages of the cultivation will further enhance lipid accumulation. Alternative extraction methods to extract neutral lipids are investigated as an alternative to sonication for difficult-to-lyse microalgae species. These alternative extraction methods include bead-beating and microwave-assisted extraction and are hypothesized to be more effective in membrane disruption compared to sonication. This chapter also investigates the reliability of the NRLA and possible methods to control interference to allow for more accurate and precise lipid measurements using this assay.

4.2. MATERIALS AND METHODS

4.2.1. Microalgae Culture Conditions

Axenic cultures of *C. sorokiniana* (UTEX 1230) and *C. vulgaris* (UTEX 2714) were obtained from UTEX (University of Texas, Austin, TX). For experiments, *C. sorokiniana* was inoculated and maintained in BG-11 liquid culture (Sigma-Aldrich, St. Louis, MO), and *C. vulgaris* was inoculated and maintained in Jaworski's Medium (JW) liquid culture (CCAP, Argyll PA37 1QA, Scotland, United Kingdom). All cultures were inoculated in 1-L Corning Pyrex media bottles (Corning 1395-1L) equipped with stir bars and pumped air for mixing and aeration (Petco Air Pump). Incoming air is sterile filtered by Polyvent 4 disposable filters (Whatman, Kent, UK). The culture suspensions were maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All equipment and materials were autoclaved and maintained under sterile conditions. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA).

4.2.2. Anaerobic Digester Wastewater

Ultra-filtered food waste permeates (FWPs) were obtained from the thermophilic Sacramento South Area Transit Station (SATS).[18] An additional ammonia-stripping process using sodium carbonate was performed on a subset of this FWP to remove high ammonia concentrations, yielding a stripped FWP (SFWP).[19] Initial characterization was performed externally by Denele Analytical, Inc (Table 18). Over the course of the experiments, permeate was stored at room temperature in air-tight containers to mimic wastewater storage at larger

scale operations. Containers were minimally disturbed when setting up a new experiment. Compositional analysis performed after the initial characterization by Denele Analytical Inc was determined with in-house analytical methods, including ion chromatography using a Dionex™ Aquion™ Ion Chromatography (IC) System equipped with a Dionex™ IonPac™ IC AS23 4x250 mm capillary column and Dionex™ IonPac™ IC AG23 4x50 guard column (ThermoFisher, San Jose, CA) and Hach test kits.

Table 18. Summary of nutrient concentrations (mg/L) in anaerobic digester permeates before and after the stripping process. FWP = food waste permeate; SFWP = stripped food waste permeate.

Nutrient	Nutrient Concentrations (mg/L)	
	FWP	SFWP
TKN	2710 ± 100	278 ± 10
NH_3-N	2340 ± 100	207 ± 20
P	18 ± 2	47 ± 2
K	1710 ± 30	1155 ± 7
Ca	6.85 ± 0	n/a
Mg	8 ± 4	2.7 ± 0.6
Na	819.50 ± 0.09	3340 ± 40
TOC	20750 ± 6000	21250 ± 6000
pH	8.4	8.6

* Measurements were performed by Denele Analytical, Inc.

4.2.3. Two-Phase Microalgae Cultivation

4.2.3.1. Two-Phase Cultivation of *C. sorokiniana*

For the first cultivation phase, 1.5-L volumes of 10/90 (10% FWP) and 30/70 (30% SFWP) were prepared. Cultures were inoculated into 2-L Corning Pyrex media bottles (Corning 1395-2L) equipped with magnetic stir bars and a 6% CO_2/air mixture for mixing and aeration. A 150-mL aliquot (10% inoculation v/v) of late exponential phase *C. sorokiniana* stock culture was

dispensed into each flask. Ultrapure water was added to reach a final volume of 1.5 L at the final FWP/SFWP concentrations. Cultures were placed on stir plates and maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All culture flasks were autoclaved before preparation to mitigate the possibility of outside contamination. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA). The CO_2 mixture used to aerate the samples was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA).

Upon reaching late exponential phase, 130-mL volumes were aliquoted into 9- 500 mL centrifuge bottles and pelleted by centrifugation. Pellets were resuspended in 60 mL of nitrogen-deplete, ultrapure water (FW), filtered seawater (SW), or original 10% FWP/30% SFWP media and were combined in 500-mL media bottles. A 40-mL aliquot of the appropriate medium was used to rinse the bottles when transferring to avoid biomass loss. When combined, 90-mL of the appropriate media was added to each respective flask for a total of 6-500-mL flasks containing 390 mL of each condition (i.e., FW, SW, or FWP/SFWP). In triplicate, 125 mL aliquots of each condition were aliquoted into sterile 500-mL culture flasks. Phase two cultivation utilized the same cultivation setup as the first, and the cell density was again measured spectrophotometrically at 680 nm. All experimental conditions were performed at N=3. The cultures were harvested upon reaching and maintaining stationary phase for at least seven days (as determined based on optical density at 680 nm). The growth rate and biomass productivity were determined using equations 4.1 and 4.2, respectively.

$$\text{Growth rate } (\mu) = \frac{\ln\left(A_f/A_0\right)}{t_f - t_0} \quad [4.1]$$

where A_f is the absorbance of the culture at 680 nm on the day that the culture entered stationary phase, and A_0 is the absorbance of the culture at 680 nm on the day that the culture entered exponential phase. The t_f is the day that the culture entered stationary phase, and t_0 is the day that the culture entered exponential phase. Biomass productivity was calculated using Equation 2.2.

$$\text{Biomass productivity} = (m)(v)(\mu) \quad [4.2]$$

where m is the mass of harvested algae biomass, v is the volume of culture when harvesting, and μ is the growth rate.

4.2.3.2. Two-Phase Cultivation of *C. vulgaris*

For the first cultivation phase, a 1.5-L volume of 10/90 (10% FWP) was prepared. The culture was inoculated into 2-L Corning Pyrex media bottles (Corning 1395-2L) equipped with a magnetic stir bar and a 6% CO_2/air mixture for mixing and aeration. A 150-mL aliquot (10% inoculation v/v) of late exponential phase *C. vulgaris* stock culture was dispensed into each flask, and sterile-filtered industrial tap water was added to reach a final volume of 1.5 L and final FWP concentration. Cultures were placed on stir plates and maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All culture flasks were autoclaved before preparation to mitigate the possibility of outside contamination. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis

Spectrophotometer (ThermoFisher, San Jose, CA). The CO_2 mixture used to aerate the samples was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA).

Upon reaching late exponential phase, 125-mL volumes were aliquoted into 500 mL centrifuge bottles and pelleted by centrifugation. Pellets were resuspended in 125 mL of nitrogen-deplete, sterile filtered industrial tap water (FW), filtered seawater (SW), or original 10% FWP media using clean, 500-mL culturing bottles. Phase two cultivation utilized the same cultivation setup as the first, and the cell density was again measured spectrophotometrically at 680 nm. All conditions were performed at N=3. The cultures were harvested upon reaching and maintaining stationary phase for at least seven days (as determined based on optical density at 680 nm). The growth rate and biomass productivity were determined using Equations 4.1 and 4.2, respectively.

4.2.3.3. Two-Phase Cultivation of *C. sorokiniana* and *C. vulgaris* with H_2O_2 Stressor

For the first cultivation phase, 250-mL volumes of 10/90 (10% FWP) were prepared. All cultures were inoculated into 500-mL Corning Pyrex media bottles (Corning 1395-500) equipped with magnetic stir bars and a 6% CO_2/air mixture for mixing and aeration. A 25-mL aliquot (10% inoculation v/v) of late exponential phase *C. vulgaris* stock culture was dispensed into each flask. Ultrapure water was added to reach a final volume of 250 mL and final FWP/SFWP concentrations. All experimental conditions were performed in triplicate. Cultures were placed on stir plates and maintained at a constant temperature of 23 ± 2 °C with full-spectrum incident uniform lighting (High-Efficiency T-5 Grow Lights – Gardeners Supply Co, VT) at 16:8 h light/dark cycle (60-120 M photons/m/s). All culture flasks were autoclaved before

preparation to mitigate the possibility of outside contamination. Cell density was measured at 680 nm during culturing using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA). The CO_2 mixture being used to aerate the samples was monitored using an SBA-5 CO_2 gas analyzer (PP Systems, Amesbury, MA).

Upon reaching late exponential phase, the cultures were pelleted by centrifugation and resuspended in 250 mL of nitrogen-deplete, sterile filtered industrial tap water, filtered seawater, or original 10% FWP media using clean, 500-mL culturing bottles. Phase two cultivation utilized the same cultivation setup as the first phase, and the cell density was again measured spectrophotometrically at 680 nm. Upon reaching and maintaining stationary phase for at least three days (as determined based on optical density at 680 nm), samples were treated with either 5 mL of 5 mM H_2O_2 or tap water. Samples were harvested 24 hours after the hydrogen peroxide addition. The growth rate and biomass productivity were determined using Equations 4.1 and 4.2, respectively.

4.2.4. Biomass Determination

Upon reaching and maintaining stationary phase for at least three days (as determined based on optical density at 680 nm), the microalgae cultures (250 mL) were each pelleted by centrifugation and washed with deionized water. The algae pellets were flash-frozen in liquid nitrogen and lyophilized to dryness (Labconco Freezone 6, Kansas City, MO). Lyophilized biomass was weighed and recorded as biomass per liter of culture. Biomass was stored at –20 °C under argon for analysis.

4.2.5. Lipid Extraction

Lipids were extracted from5 mg freeze-dried biomass using a modified Folch method.[14] Microalgae biomass was suspended in chloroform and sonicated for 1 min using a hand sonicator (Fisher Scientific Model 120 Sonic Dismembrator, ThermoFisher, San Jose, CA). After sonication, a 2:1 methanol/chloroform mixture was added to each sample and sonicated for 1 min. Sonication was repeated one more time for a total of 2 x 1 min sonication cycles following the addition of the methanol/chloroform mixture. After the final sonication, 0.1 M PBS was added to each sample, mixed, and centrifuged for layer separation. The bottom chloroform layer was transferred to a glass vial and stored at –20 °C for analysis.

4.2.6. Bleach-Assisted Nile Red Microplate Assay for Neutral Lipid Quantification

4.2.6.1. Graphitized Carbon Pretreatment

Extracts were treated with graphitized carbon to remove interfering pigments using a method derived from Cao et al. [15] In a 2-mL Eppendorf tube, 25 mg of graphitized carbon (Sigma Aldrich, St. Louis, MO) was added to 1 mL of lipid extract and vortexed for 15 sec. The tubes were placed on a benchtop shaker and shaken for 30 min at room temperature. After shaking, the extract was passed through a 0.22 µm syringe filter and stored at –20 °C for analysis. All analyses were done within 24 hours of treatment.

4.2.6.2. Neutral Lipid Assay

Neutral lipids in extracts were quantified using a bleach-enhanced Nile red microplate assay adapted from Higgins et al. (2014).[22] A 1 mg/mL algae oil standard in chloroform was prepared using Thrive® Culinary Algae Oil. The lipid extracts and the 1 mg/mL algae oil standard in chloroform were diluted 2:1 with methanol. Diluted samples and standards were added to a 96-well polypropylene microplate (Greiner, Millipore Sigma, St. Louis, MO) in quadruplicate and triplicate, respectively. Lipid standard was added to the microplate to achieve a range of 0 – 100 µg per well. The plate was heated at 55 °C to evaporate solvent then cooled to room temperature on the benchtop. Lipids were resuspended in 2-propanol followed by adding a Nile Red solution in DMSO (final concentration of Nile Red ~ 160 µg/mL) and incubated in obscurity for 5 min. Next, a 3% aqueous sodium hypochlorite solution was added to each cell and incubated in obscurity for 30 min. After bleaching, fluorescence measurements were taken using a Synergy HI Multi-Mode Plate Reader at excitation wavelengths of 530/40 nm and emission wavelengths of 590/40 nm. The neutral lipid content of the lipid extract was quantified using a standard curve derived from the lipid standards.

4.2.7. Evaluating Pigment Interference in the Bleach-Assisted Nile Red Assay

A modified Folch extraction was used to extract lipids from a 10 mg sample of harvested *C. sorokiniana* biomass using 1.5x the volume of reagents described in the extraction procedure above.[14] Extractions were performed in triplicate. A 1-mL volume of each replicate was aliquoted into 2-mL Centrifuge tubes, and 100 µL of a 1 mg/mL lipid standard in chloroform was added for the “Extract + standard” conditions, and 100 µL of $CHCl_3$ was added for the “Extract +

$CHCl_3$” conditions. An unpigmented control was prepared by combining 100 µL of 1 mg/mL standard and 300 µL $CHCl_3$. Samples were vortexed and placed on ice while performing the bleach-assisted Nile red assay. All conditions were performed in triplicate.

4.2.8. Comparing the Sulfo-Phospho-Vanillin Assay Versus the Bleach-Assisted Nile Red Assay for Lipid Quantification of Highly Pigmented Extracts

4.2.8.1. Bleach-Assisted Nile Red Assay of *C. sorokiniana* Lipid Extracts

A modified Folch extraction was used to extract lipids from a 10 mg sample of harvested *C. sorokiniana* biomass using 1.5x the volume of reagents described in the extraction procedure above.[14] A 1-mL volume of each replicate was aliquoted into 2-mL Centrifuge tubes, and 150 µL of a 1 mg/mL lipid standard in chloroform was added for the "Extract + standard" conditions, and 100 µL of $CHCl_3$ was added for the “Extract + $CHCl_3$" conditions. An unpigmented control was prepared by combining 150 µL of 1 mg/mL standard and 300 µl $CHCl_3$. Samples were vortexed and placed on ice when performing the bleach-assisted Nile red assay. All conditions were performed in triplicate.

4.2.8.2. Sulfo-Phospho-Vanillin Assay on *C. sorokiniana* Biomass

The sulfo-phospho-vanillin (SPV) reagent was prepared by combining 42 mL ultrapure water, 8 mL concentrated H_3PO_4, and 8 mg vanillin. Fresh SPV reagent was performed before each assay. A 1 mg sample of *C. sorokiniana* biomass was added to 50-mL glass centrifuge tubes along with 100 µL of ultrapure water and 2 mL of 85% H_2SO_4. Stoppers were used to seal each tube, and the tubes were placed in a 100 °C water bath for 10 min and then placed on ice for 5

min. A 5-mL aliquot of SPV reagent was added to each tube along with a magnetic stir bar. Tubes were placed in a 37 °C water bath for 15 min with constant mixing. After heating, a 1-mL aliquot of each sample was transferred to individual cuvettes, and the samples were measured spectrophotometrically at 530 nm using a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA).

4.2.9. Using Biochar Versus Graphitized Carbon for Pigment Removal

A modified Folch extraction was used to extract lipids from a 10 mg sample of harvested *C. sorokiniana* biomass using 2x the volume of reagents described in the extraction procedure above.[14] Microalgae samples were first treated with 4 mL of chloroform and sonicated (Fisher Scientific Model 120 Sonic Dismembrator, ThermoFisher, San Jose) for 1 min. Next, a 3.34 mL of a 2:1 mixture of methanol/chloroform was added to each sample and sonicated for 1 min. Then, a 6-mL aliquot of 0.1 M PBS was added to samples, mixed vigorously, and centrifuged to separate layers. The lipid-containing chloroform layer was removed and stored at –20 °C for further analysis. Extractions were performed in triplicate and combined to create a pooled extract.

A 1-mL volume of each replicate was aliquoted into 2-mL Eppendorf tubes, and 25 mg of either biochar or graphitized carbon (GC) was added. Samples were vortexed for 1 min to ensure complete suspension of biochar/GC in the extract. Tubes were placed on an orbital shaker at 250 rpm for 1 hour. The extract was filtered through a 0.22 µm syringe filter and stored at –20 °C for analysis. Conditions were performed at N=2.

4.2.10. Graphitized Carbon Pretreatment of Lipid Extracts

4.2.10.1. Evaluating the Precision of Bleach-Assisted Nile Red Assay on Microalgae Lipid Extracts Treated with Graphitized Carbon

A modified Folch extraction was used to extract lipids from a 10 mg sample of harvested *C. sorokiniana* biomass.[14] Microalgae samples were first treated with 3 mL of chloroform and sonicated (Fisher Scientific Model 120 Sonic Dismembrator, ThermoFisher, San Jose) for 1 min. Next, 2.51 mL of a 2:1 mixture of methanol/chloroform was added to each sample and sonicated for 1 min. Then, a 4.5-mL aliquot of 0.1 M PBS was added to samples, mixed vigorously, and centrifuged to separate layers. The lower organic (chloroform) layer containing lipids was removed and stored at –20 °C for GC treatment. Extractions were performed in triplicate.

A 0.75-mL volume of each replicate was aliquoted into 2-mL Eppendorf tubes and graphitized carbon (GC) was added to each tube at concentrations of 0 mg/mL GC, 5 mg/mL GC, 10 mg/mL GC, and 25 mg/mL GC. Samples were vortexed for 1 min to ensure complete suspension of GC in the extract. Samples were placed on an orbital shaker for 60 min at 250 rpm. After treatment, all samples were filtered through a 0.22 µm syringe filter to remove GC and stored at –20 °C for lipid analysis. All conditions were performed in triplicate. Statistical analyses were performed to determine precision between different experiments.

4.2.10.2. Optimizing the Duration of Graphitized Carbon Pretreatment

Extractions were performed on 15 mg *C. sorokiniana* biomass obtained from 10% FWP and BG-11 cultivation conditions. All extractions were performed in triplicate. The triplicate extracts were combined in a scintillation vial to create a "pooled extract," allowing all samples to be treated and analyzed simultaneously to lessen systematic error. The averaged extracts were treated with 25 mg graphitized carbon (GC) for 0 min, 15 min, 30 min, or 60 min. For treatment, 25 mg GC was added to each tube and vortexed to ensure complete suspension of the GC. Samples were then placed on a shaker plate at 250 rpm for their designated amount of time. Then, samples were removed, and the extracts were filtered through a 0.22 µm syringe filter into 2-mL Eppendorf tubes. All treated extracts were stored at –20 °C for analysis.

4.2.10.3. Evaluating Different Graphitized Carbon Treatment Methods

A 0.75-mL aliquot of lipid extract was added to 1.5-mL centrifuge tubes. Sample extracts were either extracted with $CHCl_3$ or 2:1 $CHCl_3$/MeOH. The samples extracted with $CHCl_3$ were treated and evaluated with a single replicate due to low extract volumes. Samples extracted with 2:1 $CHCl_3$/MeOH were treated and evaluated at N=2. Graphitized carbon (GC) was added to each tube according to the condition being evaluated: 37.5 mg GC (50 mg GC treatment), 18.75 mg GC (25 mg GC (x2) treatment), and 18.75 mg GC (25 mg GC treatment). The samples were vortexed and placed on a shaker plate and shaken at 250 rpm for 30 min. Extracts were filtered through a 0.22 µm syringe filter into 2-mL Eppendorf tubes. Another 18.75 mg GC was added to the 25 mg GC (x2) samples, vortexed, and shaken for another 30 min. The extracts were filtered through a 0.22 µm syringe and measured spectrophotometrically at 680 nm using

a Thermo Scientific Genesys 10S Vis Spectrophotometer (ThermoFisher, San Jose, CA) to evaluate pigment presence. All samples were stored at –20 °C for analysis.

A 0.5-mL aliquot of lipid extract was added to 1.5 mL centrifuge tubes. These extracts were extracted with ultrapure water, and each condition was performed in triplicate. Graphitized carbon (GC) was added to each tube according to the condition being evaluated: 25 mg GC (50 mg GC treatment) and 12.5 mg GC (25 mg GC treatment). The samples were vortexed and placed on a shaker plate at 250 rpm for 30 min. The extracts were filtered through a 0.22 µm syringe and stored at –20 °C for analysis.

4.2.11. Bead Beating and Sonication Extraction Methods

4.2.11.1. Comparison Between Sonication and Two Bead-Beating Methods to Evaluate Lipid Extraction Efficiency and Recovery

For the bead-beating samples, 1 mL of 0.6 mg/mL lipid standard in $CHCl_3$ was added to 2-mL screw-cap tubes along with 500 µL MeOH. Silica beads (100 µm) were added to each tube according to the appropriate condition: 250 mg beads or 0.75 mL beads. For the sonication samples, 1 mL of 0.6 mg/mL lipid standard in $CHCl_3$ was added to 50-mL glass centrifuge tubes with rubber stoppers along with 1 mL of $CHCl_3$. A 1-mL aliquot of 0.6 mg/mL lipid standard was set aside to act as a control. All conditions were performed in triplicate.

The bead beating samples were shaken at high speed for six 20 sec beating periods with 30-sec intervals on ice water. Then, the samples were transferred to 50 mL glass centrifuge tubes by filtering through a 5-mL syringe containing a stainless-steel wire mesh disk (#40 mesh). The trapped beads were washed three times with 1.5 mL Folch solvent (2:1 $CHCl_3$/MeOH), and

the rinsate was dispensed into the glass tube. A 3-mL aliquot of 0.1 M PBS was added to each glass tube and shaken to mix. The tubes were centrifuged to separate layers, and the lipid-containing $CHCl_3$ layer was transferred to a scintillation vial. All samples were stored at –20 °C for further analysis.

The sonication samples were sonicated using a hand sonicator (Fisher Scientific Model 120 Sonic Dismembrator, ThermoFisher, San Jose) for 1 min. Next, a 1.67 mL of a 2:1 MeOH/$CHCl_3$ was added to each sample and sonicated for 1 min. Then, a 3-mL aliquot of 0.1 M PBS was added to samples, mixed vigorously, and centrifuged to separate layers. Lastly, the lipid-containing $CHCl_3$ layer was transferred to a scintillation vial and stored at –20 °C for further analysis.

4.2.11.2. Comparison Between Bead-Beating and Two Sonication Methods to Evaluate Lipid Extraction Efficiency and Recovery

For the bead-beating samples, 1 mL of 0.6 mg/mL lipid standard in $CHCl_3$ was added to 2-mL screw-cap tubes along with 500 µL MeOH. Silica beads (filled to the 0.75-mL mark) were added to each tube. For the sonication samples, 1 mL of 0.6 mg/mL lipid standard in $CHCl_3$ was added to 50-mL glass centrifuge tubes with rubber stoppers along with 1 mL of $CHCl_3$. A 1-mL aliquot of 0.6 mg/mL lipid standard was set aside to act as a control. All conditions were performed in triplicate.

The sonication samples were sonicated using a hand sonicator (Fisher Scientific Model 120 Sonic Dismembrator, ThermoFisher, San Jose) for 1 min. Next, a 1.67 mL of a 2:1 MeOH/$CHCl_3$ was added to each sample and sonicated two times in 1 min intervals. For the 3x

sonication samples, the samples were sonicated for another 1 min. Following sonication, 3-mL of 0.1 M PBS was added to samples, mixed vigorously, and centrifuged to separate layers. The lipid-containing $CHCl_3$ layer was transferred to a scintillation vial and stored at –20 °C for further analysis.

The bead beating samples were shaken at high speed for six 20 sec beating periods with 30-sec intervals on ice water. Then, the samples were transferred to 50 mL glass centrifuge tubes by filtering through a 5-mL syringe containing a stainless-steel wire mesh disk (#40 mesh). The trapped beads were washed three times with 1.5 mL Folch solvent (2:1 $CHCl_3$/MeOH), and the rinsate was dispensed into the glass tube. A 3-mL aliquot of 0.1 M PBS was added to each glass tube and shaken to mix. The tubes were centrifuged to separate layers, and the lipid-containing $CHCl_3$ layer was transferred to a scintillation vial. All samples were stored at –20 °C for further analysis.

4.2.11.3. Sonication versus Bead-Beating of Microalgae Extracts and Lipid Standards to Evaluate Lipid Recovery

A 20 mg sample of *C. sorokiniana* biomass was added to 2-mL screw-cap tubes. Lipid standard (1 mg/mL) and $CHCl_3$ were added to each tube to achieve final lipid concentrations of 100 µL/mL, 200 µL/mL, or 400 µL/mL. Next, a 500 µL aliquot of MeOH was added to each tube and mixed. For those conditions without lipid standard, 1 mL of $CHCl_3$ and 500 µL of MeOH were added. An additional set of samples was prepared without algae by combining lipid standard (1 mg/mL) and $CHCl_3$ to each tube to achieve final lipid concentrations of 100 µL/mL, 200 µL/mL, or 400 µL/mL followed by 500 µL of MeOH. For the control, samples were prepared

by combining 100 µL, 200 µL, or 400 µL of lipid standard (1mg/mL) and 3.9 mL, 3.8 mL, or 3.6 mL $CHCl_3$, respectively, into 50-mL glass centrifuge tubes. All conditions were performed in triplicate.

The bead beating samples were shaken at high speed for six 20 sec beating periods with 30-sec intervals on ice water. Then, the samples were transferred to 50 mL glass centrifuge tubes by filtering through a 5-mL syringe containing a stainless-steel wire mesh disk (#40 mesh). The trapped beads were washed three times with 1.5 mL Folch solvent (2:1 $CHCl_3$/MeOH), and the rinsate was dispensed into the glass tube. A 3-mL aliquot of 0.1 M PBS was added to each glass tube and shaken to mix. The tubes were centrifuged to separate layers, and the lipid-containing $CHCl_3$ layer was transferred to a scintillation vial. All samples were stored at –20 °C for further analysis.

4.2.12. Evaluating Different Solvent Systems for Microwave-Assisted Extractions of Lipids

4.2.12.1. Microwave-Assisted Extraction Method with $CHCl_3$ Solvent

Magnetic stir bars were added to microwave vials (0.5 – 2 mL; Neta Scientific, Hainesport, NJ) and the vials were flame dried. After drying, 40 mg of *C. sorokiniana* biomass was added to each tube along with either 2 mL $CHCl_3$ or 100 µL 1 mg/mL lipid standard and 1.9 mL $CHCl_3$. Each condition was performed in duplicate. Samples were microwaved at 62 °C for 5 min with a 15 sec stir time. After microwaving, the sample was transferred to a 50-mL glass centrifuge tube with a rubber stopper. The microwave vials were rinsed with two 1-mL aliquots of $CHCl_3$, and the rinsate was added to the centrifuge tube. Methanol was used to rinse the microwave tube, and the methanol rinsate was added to the glass tube. Samples were placed

on a shaker for one hour at 250 rpm. A 4.5-mL aliquot of 0.1 M PBS was added to each vial, gently shaken to mix, and centrifuged for 20 min. The $CHCl_3$ layer containing the lipids was transferred to a glass vial and stored at –20 °C for graphitized carbon (GC) treatment.

4.2.12.2. Microwave-Assisted Extraction with 2:1 $CHCl_3$/MeOH Solvent

Magnetic stir bars were added to microwave vials (0.5 – 2 mL; Neta Scientific, Hainesport, NJ) and the vials were flame dried. After drying, 40 mg of *C. sorokiniana* biomass was added to each tube in along with either 2 mL 2:1 $CHCl_3$/MeOH or 100 µL 1 mg/mL lipid standard and 1.9 mL 2:1 CHCl3/MeOH. Samples were microwaved at 100 °C for 5 min with a 15 sec stir time. After microwaving, the sample was transferred to a 50-mL glass centrifuge tube with a rubber stopper. The microwave vials were rinsed with three 2-mL aliquots of 2:1 $CHCl_3$/MeOH, and the rinsate was added to the centrifuge tube. Samples were placed on a shaker for one hour at 250 rpm. A 4.5-mL aliquot of 0.1 M PBS was added to each vial, gently shaken to mix, and centrifuged for 20 min. The $CHCl_3$ layer containing the lipids was transferred to a glass vial and stored at –20 °C for graphitized carbon (GC) treatment.

4.2.12.3. Graphitized Carbon Treatment of Microwave Extracts

A 1-mL aliquot of each lipid extract was added to 2-mL Eppendorf tubes. Graphitized carbon (25 mg) was added to each tube, vortexed, and the extracts were placed on a shaker plate for 30 min at 250 rpm. The extracts were filtered through a 0.22 µm syringe and stored at –20 °C for analysis.

4.2.13. Evaluating Lipid Recovery After Microwave-Assisted Extraction from Microalgae using a 2:1 $CHCl_3$/MeOH Solvent System

Magnetic stir bars were added to microwave vials (0.5 – 2 mL; Neta Scientific, Hainesport, NJ) and the vials were flame dried. After drying, a 20 mg sample of *C. sorokiniana* biomass was added to the dried microwave vials along with a 200 µL aliquot of either 1 mg/mL lipid standard or $CHCl_3$. Vials were sealed, purged with argon, and 1 mL of 2:1 $CHCl_3$/MeOH was added to each vial through the cap membrane using a syringe. Both conditions were prepared in triplicate. Samples were microwaved for 10 min at 100 °C with a 15 sec stir time. After microwaving, the sample was transferred to a 50-mL glass centrifuge tube with a rubber stopper. The microwave vial was rinsed with four 1-mL aliquots of 2:1 $CHCl_3$/MeOH, and the rinsate was added to the centrifuge tube. Samples were placed on a shaker for one hour. A 4-mL aliquot of 0.1 M PBS was added to each vial, gently shaken to mix, and centrifuged for 20 min. The $CHCl_3$ layer containing the lipids was transferred to a glass vial and stored at –20 °C for graphitized carbon (GC) treatment.

4.2.13.1. Treatment of Lipid Extracts with Two-Time Graphitized Carbon Treatment Method

A 1-mL aliquot of each sample was transferred to 2-mL centrifuge tubes, and 25 mg of GC was added to each tube, vortexed, and placed on a shaker at 250 rpm for 30 min. After 30 min, the extracts were filtered through a 0.22 µm syringe filter into new 2-mL centrifuge tubes, and another 25 mg of GC was added to the samples. The samples were vortexed and shaken for an additional 30 min. After the second 30 min, the treated extracts were filtered through a 0.22 µm syringe filter and stored at –20 °C for analysis.

4.2.13.2. Treatment of Lipid Extracts with Different Graphitized Carbon Masses

A 500-mL aliquot of each sample was transferred to 2-mL centrifuge tubes, and GC was added at a concentration of either 25 mg/mL extract and 50 mg/mL extract. All samples were vortexed and placed on a shaker at 250 rpm for 30 min. Then, the treated extracts were filtered through a 0.22 µm syringe filter and stored at –20 °C for analysis.

4.2.14. Statistical Analysis

All results are reported as mean values ± standard deviation. Student's t-test, one-way ANOVA, and post hoc Tukey's HSD tests were used to determine significant differences between microalgae strains and between experimental conditions of the growth data at a 5% significance level.

4.3. RESULTS AND DISCUSSION

4.3.1. Two-Phase Cultivation of *C. sorokiniana*

Lipid accumulation by *C. sorokiniana* increased with resuspension in nutrient-deficient conditions after an initial growth phase in 30% SFWP (Table 19). After cultivating *C. sorokiniana* on 30% SFWP, resuspension in filtered seawater (SW) resulted in a 133 ± 20% increase in the percentage of neutral lipids. A 102 ± 17% increase in lipid accumulation was observed when *C. sorokiniana* was resuspended in freshwater (FW) after an initial growth phase of 30% SFWP. With the observed increase in lipid accumulation, there was a decrease in biomass growth as determined by cell density (Figure 25a). The decline in biomass productivity was expected, considering nutrient-limited environments are less conducive for microalgae growth.[16] The

costs and benefits of neutral lipid accumulation versus biomass accumulation must be evaluated to determine if this two-phase methodology is beneficial in the biofuel industry.

After initial cultivation on 10% FWP, the *C. sorokiniana*'s growth varied between resuspension media (Figure 25). The lipid accumulation increased when resuspended in SW and decreased when resuspended in FW compared to resuspension in 10% FWP; however, these changes were not significantly significant (Table 19). Concomitantly, *C. sorokiniana*'s biomass productivity increased by 37 ± 7% and 27 ± 4% when resuspended in FW and SW, respectively. This productivity trend is the opposite of the trend observed in the 30% SFWP two-phase experiment, where there was an overall decrease in biomass productivity with resuspension in FW or SW (Table 19). These results indicate that resuspension in nutrient-deficient conditions after initial cultivation on 10% FWP is not ideal for increasing lipid accumulation in *C. sorokiniana*; however, this method may be beneficial for increasing biomass production.

The neutral lipid content was determined to be low in all conditions reaching up to only 12% neutral lipid by weight (Table 19). These values are much lower than previously reported lipid values, and this disparity highlights a gap in the literature that should be investigated further.[17,18] That said, it is important to note that the lipid values for *C. sorokiniana* in the 30% SFWP two-phase experiment were determined before a procedural change addressing pigment interference using the bleach-assisted Nile Red assay (NRLA) on highly pigmented extracts. However, the lipid content in *C. sorokiniana* measured in subsequent studies in 2019 (Tables 19) using the modified NRLA remains low. Thus, the occurrence of low lipid measurements in subsequent studies supports the hypothesis that there will still be a significant disparity

between the lipid measurements in this study and literature values even after using the modified NRLA.

Upon repeating the 10% FWP two-phase cultivation, *C. sorokiniana* accumulated more biomass, increased biomass productivity, and increased lipid accumulation (Table 19). Biomass accumulation and biomass productivity increased dramatically in the 2019 experiment compared to the 2018 experiment (Table 19). An increase in harvested biomass could be due to the optimization of the CO_2 manifold and the CO_2 sensor. By 2019, the issues with the system had been addressed, improving the reliability and accuracy of CO_2 supplementation. This optimization could have also played a role in increasing lipid accumulation.[19,20]

In the 2019 experiment, *C. sorokiniana* accumulated 47 ± 10% more lipids when resuspended in FW and 17 ± 10% more lipids when resuspended in SW (Table 19). The increased lipid accumulation could be attributed to the longer duration of time in stationary phase before harvesting (Figure 25). Longer durations in stationary phase would provide prolonged periods of exposure to nutrient-deficient conditions. As a result of the extended time in nutrient-deficient conditions, the microalgae can accumulate more lipid bodies. After cultivation and resuspension in 10% FWP, *C. sorokiniana*'s lipid yields were at 10% in both the 2018 and 2019 experiments. Therefore, the consistent lipid accumulation between experiments could be attributed to a nutrient-replete environment of 10% FWP compared to the nutrient-depleted environment after resuspension in FW and SW.

Further studies should investigate this hypothesis that the nutrient-dense conditions of 10% FWP prevent lipid accumulation. These studies could further support the hypothesis that *C. sorokiniana* has low lipid yields due to the premature induction of stationary phase,

preventing the 10% FWP from reaching the nitrogen deplete conditions to increase lipid production. I hypothesize that early stationary phase is induced by phosphorus depletion, so studies should investigate if phosphorus is the factor initiating stationary phase or if another unknown factor is controlling it.

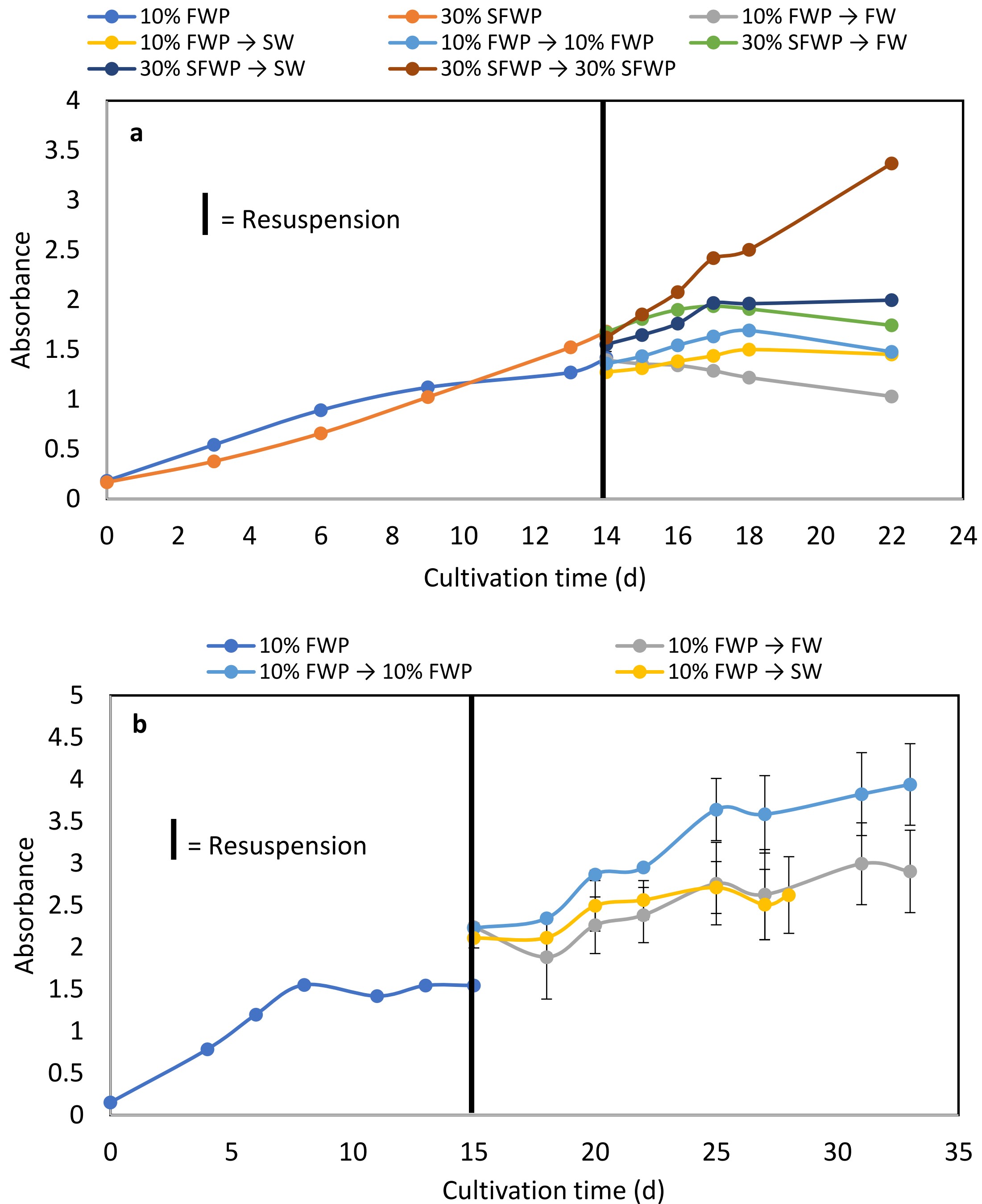

Figure 25. Growth curves for *C. sorokiniana* cultivated in 1.5 L of 10% FWP and 30% SFWP followed by a 125-mL scale second phase of either freshwater (FW), filtered seawater (SW), or the original media performed in (a) 2018 and (b) 2019. The first phase was performed at N=1, and the second phase conditions were performed at N=3. FWP = food waste permeate; SFWP = stripped food waste permeate.

Table 17. Growth characteristics for *C. sorokiniana* tested at 125-mL scale using a two-phase nutrient depletion method.[a] FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater.

Experiment	Condition	Harvested Biomass[b] (mg)	Biomass productivity ($mgL^{-1}d^{-1}$)	Percent neutral lipid content (w/w)
May 2018	10% FWP → FW	47.8 ± 0.6	84 ± 1	9 ± 1
	10% FWP → SW	63 ± 2	64 ± 2	12 ± 2
	10% FWP → 10% FWP	54 ± 4	58 ± 5	10 ± 1
	30% SFWP → FW	78 ± 2	85 ± 2	2.8 ± 0.2
	30% SFWP → SW	98 ± 5	101 ± 5	4.5 ± 0.4
	30% SFWP → 30% SFWP	113 ± 2	113 ± 2	0.9 ± 0.2
April 2019	10% FWP → FW	144 ± 11	133 ± 20	14.5 ± 0.6
	10% FWP → SW	120 ± 20	134 ± 28	14.4 ± 0.9
	10% FWP → 10% FWP	156 ± 18	159 ± 23	10.5 ± 0.9

[a] Values presented as mean ± SD of triplicates

[b] Biomass based on harvesting at stationary phase; strains reached stationary phase at different days throughout the experiments and were therefore harvested on different days

Table 18. Nutrient composition of initial FWP/SFWP and subsequent final resuspension media (125 mL) for *C. sorokiniana* cultivated using a two-phase method.[a] FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater

Condition	Total Nitrogen (mg/L)			Ammonia Nitrogen (mg/L)		
	Initial	Final	% Change	Initial	Final	% Change
10% FWP → FW	298 ± 7	8.3 ± 0.6	na	275 ± 4	0.3 ± 0.6	na
10% FWP → SW	298 ± 7	5 ± 5	na	275 ± 4	0.13 ± 0.6	na
10% FWP → 10% FWP	298 ± 7	212 ± 33	29 ± 30	275 ± 4	145 ± 15	47 ± 6
30% SFWP → FW	108 ± 2	8 ± 4	na	73 ± 3	0.1 ± 0.3	na
30% SFWP → SW	108 ± 2	0 ± 3	na	73 ± 3	0 ± 0.1	na
30% SFWP → 30% SFWP	108 ± 2	42 ± 4	61 ± 7	73 ± 3	8 ± 2	89 ± 22

na = not applicable

[a] Values presented as mean ± SD of triplicates

The act of resuspension into the original media did not significantly affect remediation efficiency by *C. sorokiniana* (Table 20). Remediation capacity was higher when cultivated and resuspended in 30% SFWP with a 61 ± 7% removal of total nitrogen and 89 ± 22% removal of ammonia. The remediation of total nitrogen from 10% FWP reached 29 ± 30%, and ammonia concentrations decreased by 47 ± 6%. The increase in remediation efficiency when cultivated on 30% SFWP is consistent with the remediation results described in Chapter 2 of this dissertation (Table 8); further discussion about this disparity can be found in that chapter.

4.3.1.1. Two-Phase Cultivation of *C. sorokiniana* with Oxidative Stress

C. sorokiniana cultures treated with hydrogen peroxide showed increased lipid accumulation after 24 hs of treatment for all conditions. (Table 21). These increases in lipid accumulation after H_2O_2 treatment were not statistically significant ($\alpha = 0.05$) but demonstrate the potential of H_2O_2 treatment to increase lipid yields in *C. sorokiniana (*Figure 27*)*. Further work needs to be performed to optimize this treatment strategy and achieve statistically significant increases in lipid accumulation.

The first phase of this experiment was allowed to continue until early stationary phase before resuspension in FW, SW, or 10% FWP (Figure 26). The first phase of the initial two-phase studies discussed previously was set at 14 days and utilized smaller culture volumes (125 mL) (Figure 25). Resuspending at early stationary phase rather than at 14 days allows the microalgae to accumulate the maximum amount of biomass before being exposed to nitrogen-deficient conditions. This procedural change could account for the differences in the biomass

productivity values observed in the first two-phase experiment (Table 19) versus this second two-phase experiment with an H_2O_2 stressor (Table 21).

The optical density of the *C. sorokiniana* cultures decreased when resuspended in SW (Figure 26). A subsequent decrease in optical density of the SW cultures was observed with the addition of H_2O_2. This decrease in optical density was also observed after the addition of the H_2O_2 treatment (Figure 26). The high stress of resuspension and nitrogen-deficient media followed by oxidative stress are likely the main contributors to this observed cell death.[2,10]

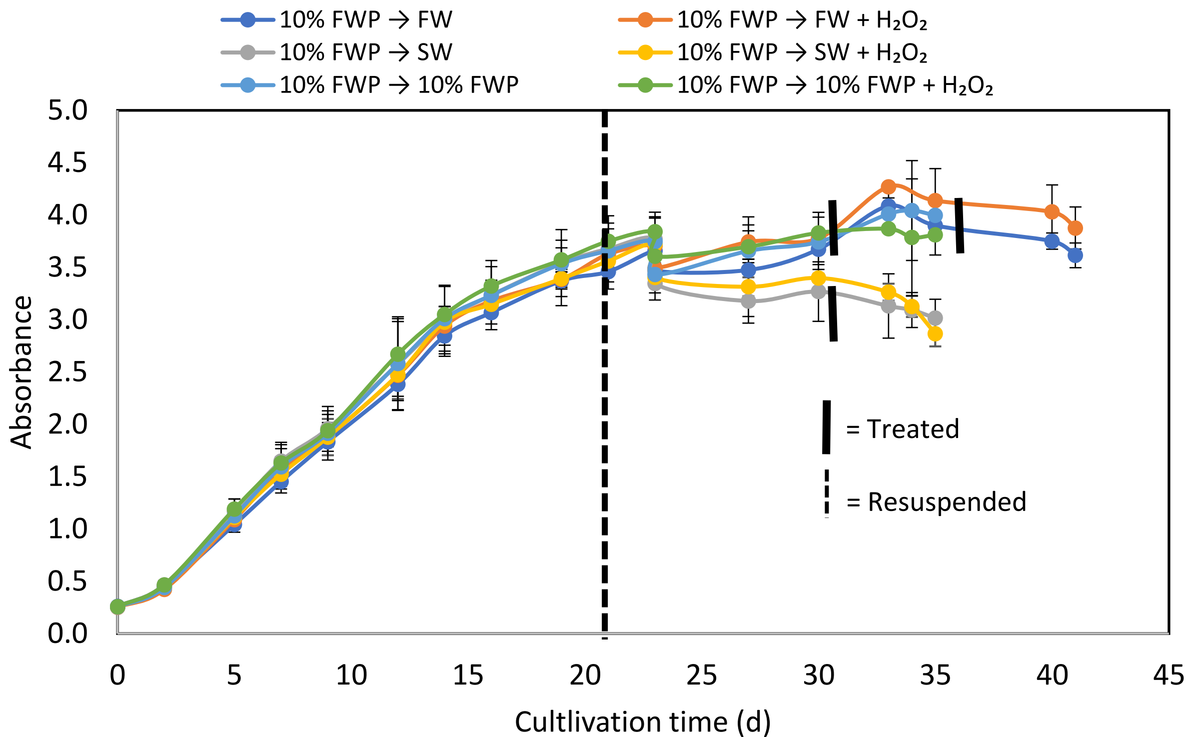

Figure 26. Growth curves for *C. sorokiniana* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide. All conditions were performed in triplicate. FWP = food waste permeate; SFWP = stripped food waste permeate

Table 19. Growth characteristics for *C. sorokiniana* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide.[a] FWP = food waste permeate; SFWP = stripped food waste permeate

Condition	Harvest day	Harvested biomass (mg)[b]	Biomass productivity ($mgL^{-1}d^{-1}$)	Percent neutral lipid content (w/w)
10% FWP → FW	41	261 ± 9	123 ± 7	11.6 ± 0.9
10% FWP → FW + H_2O_2	41	266 ± 6	131 ± 6	12.4 ± 0.9
10% FWP → SW	35	193 ± 15	92 ± 7	8.2 ± 0.6
10% FWP → SW + H_2O_2	35	168 ± 5	80 ± 4	9.6 ± 0.5
10% FWP → 10% FWP	35	265 ± 4	129 ± 6	9 ± 2
10% FWP → 10% FWP + H_2O_2	35	247 ± 20	120 ± 10	11 ± 1

[a] Values are presented as mean ± SD of triplicates
[b] Biomass based on harvesting at stationary phase; conditions reached stationary phase at different days throughout the experiments and were therefore harvested on different days

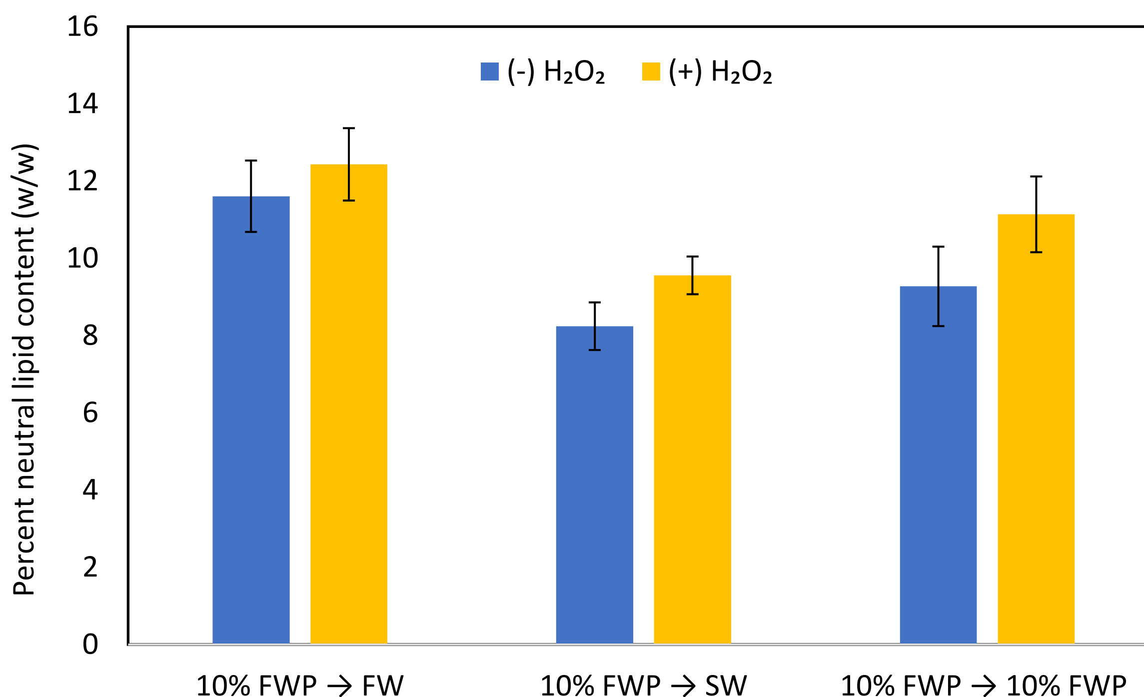

Figure 27. Comparison of neutral lipid content in *C. sorokiniana* when cultivated using two-phase methodology followed by treatment with or without hydrogen peroxide as a metabolic stressor. FW = tap water; SW = filtered seawater; FWP = food waste permeate

The variation in lipid accumulation is an indication that this two-phase methodology, both with and without H_2O_2 stress, is a possible strategy to increase lipid yields in *C. sorokiniana*; however, adjusting the method to optimize lipid yield is essential to afford its application at a large production scale. Further studies should evaluate different oleaginous microalgae strains and different H_2O_2 concentrations to optimize neutral lipid production by microalgae. This optimization is necessary to achieve the biofuel feedstock targets described in Chapter 1 of this dissertation. Future research projects could also align with the discussion in Chapter 3 regarding *C. sorokiniana*'s tendency to accumulate large starch stores rather than lipid stores (Figure 15). So, if the starch production pathway is inhibited somehow (e.g., chemical, biological), a significant increase in lipid yields could be observed.[21]

4.3.2. Two-Phase Cultivation of *C. vulgaris*

Lipid accumulation by *C. vulgaris* significantly increased when resuspended in nutrient-deficient conditions after an initial growth phase in 10% FWP. After cultivation of *C. vulgaris* on 10% FWP, resuspension in FW resulted in a 72 ± 6% increase in lipid yield compared to cultures resuspended in 10% FWP (Table 22). When resuspended in SW, *C. vulgaris* increased its lipid yield by 89 ± 5% compared to cultures resuspended in 10% FWP. With the observed increase in lipid accumulation, there was also an increase in biomass accumulation and biomass productivity (Table 22 and Figure 28). The most significant biomass increase was observed after resuspension in FW (Table 22). The harvested biomass and biomass productivity of *C. vulgaris* increased by 55 ± 5% and 50.8 ± 0.9%, respectively (Table 22). An increase in biomass productivity is unexpected, and the possible causes for this increased growth in the FW/SW

resuspensions should be investigated further. Nonetheless, these results indicate that *C. vulgaris* may be a better cultivar option compared to *C. sorokiniana* when implementing a two-phase system to increase lipids.

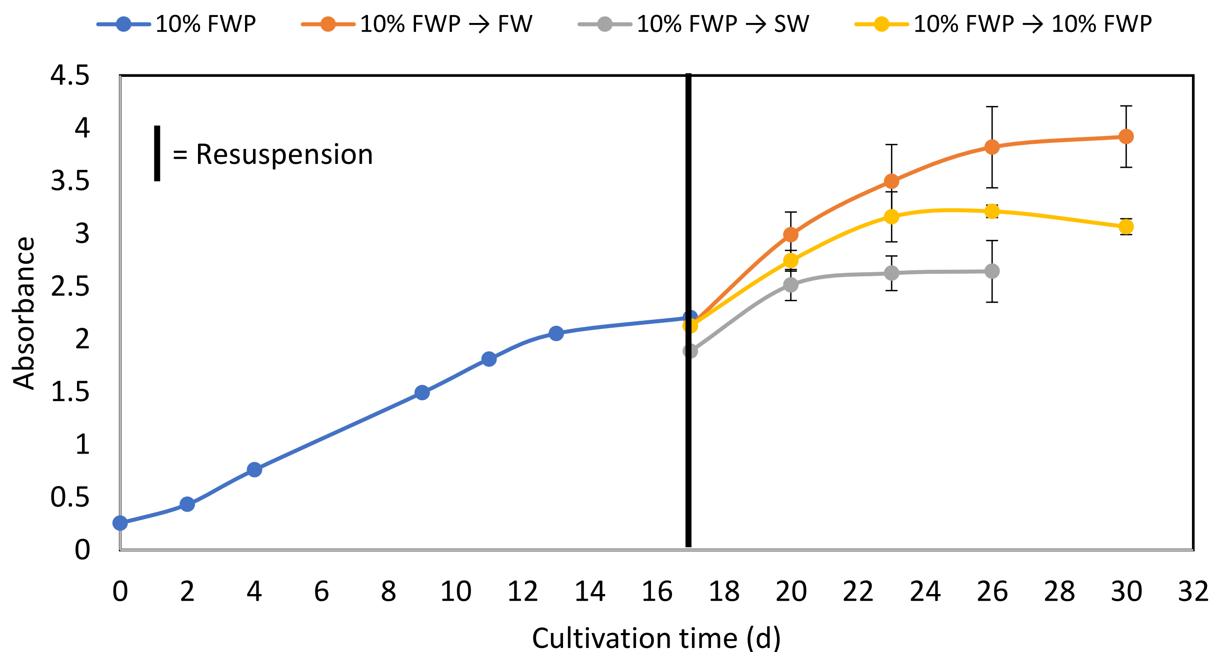

Figure 28. Growth curves for *C. vulgaris* cultivated in 1.5 L of 10% FWP and 30% SFWP followed by a 125-mL scale second phase of either freshwater (FW), filtered seawater (SW), or the original media. The first phase was performed at N=1, and the second phase conditions were performed at N=3. FWP = food waste permeate; SFWP = stripped food waste permeate

Table 20. Growth characteristics for *C. vulgaris* tested at 125-mL scale using a two-phase nutrient depletion method.[a] FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater.

Condition	Harvested Biomass (mg)	Biomass productivity ($mgL^{-1}d^{-1}$)	Percent neutral lipid content (w/w)
10% FWP → FW	174 ± 6	126.2 ± 0.8	25 ± 1
10% FWP → SW	120 ± 15	94 ± 1	30.9 ± 0.9
10% FWP → 10% FWP	99 ± 2	75.1 ± 0.4	11.8 ± 0.3

[a] Values are presented as mean ± SD of triplicates

The remediation capacity of *C. vulgaris* (Table 23) is consistent with the remediation capacity of *C. vulgaris* discussed in Chapter 2, Table 8. The remediation of ammonia increased with this two-phase method. This increase is likely due to increased volatilization of NH_3 during aeration, possibly due to low CO_2 concentrations in the aeration manifold (see Chapter 2), but the increase should be investigated further. The remediation of total nitrogen from 10% FWP reached 38 ± 2%, and ammonia concentrations decreased 47 ± 6%. Due to time constraints and the overall goal to determine if nutrient deficiency will increase lipid accumulation, nutrients were not monitored extensively. These methods may benefit from more extensive nutrient monitoring to find possible opportunities to optimize the current two-phase method.

Table 21. Nutrient composition of initial FWP/SFWP and subsequent final resuspension media (125 mL) for *C. vulgaris* cultivated using a two-phase method.[a] FWP = food waste permeate; SFWP = stripped food waste permeate; FW = freshwater; SW = filtered seawater

Condition	Total Nitrogen (mg/L)			Ammonia Nitrogen (mg/L)		
	Initial	Final	% Change	Initial	Final	% Change
10% FWP → FW	225	nd	na	275 ± 4	nd	na
10% FWP → SW	225	nd	na	275 ± 4	nd	na
10% FWP → 10% FWP	225	140 ± 5	38 ± 2	275 ± 4	145 ± 15	47 ± 6

na = not applicable; nd = not detected

[a] Values are presented as mean ± SD of triplicates

4.3.2.1. Two-Phase Cultivation of *C. vulgaris* with Oxidative Stress

C. vulgaris cultures resuspended in all resuspension media followed by H_2O_2 treatment accumulated fewer lipids than cultures without H_2O_2 treatment (Table 24 and Figure 30). The lipid yields in the initial two-phase experiment (Table 22) are consistent with the lipid yields

observed in this study except in cultures resuspended in SW (Table 24). In the cultures resuspended in SW, *C. vulgaris* had an 85 ± 6% lower lipid yield compared to the lipid yield in the initial *C. vulgaris* two-phase study. This significant decrease in lipid accumulation could be attributed to the larger culture volume used in this study, providing a less constrained space and, therefore, a less stressful environment. The first phase of this experiment was allowed to continue until early stationary phase before the culture was resuspended in FW, SW, or 10% FWP (Figure 29). The significant dip in optical density observed on day 21 is when the samples were reconstituted with tap water to maintain the correct culture volume. In the initial *C. vulgaris* two-phase study, the samples were resuspended on day 17 (Figure 29) and utilized smaller culture volumes (125 mL). The longer exponential phase allows the microalgae to accumulate a more significant amount of biomass before being exposed to nitrogen-deficient conditions. This procedural change could account for the differences in harvested biomass between the first two-phase experiment and this study (Tables 22 and 24).

The optical density of the *C. vulgaris* cultures remained constant when resuspended in all conditions (Figure 29). Additionally, there was no decrease in optical density with the addition of H_2O_2 and 24 h treatment. A stable optical density in these stressful conditions indicates that *C. vulgaris* may be more tolerant than *C. sorokiniana* to outside stressors such as nutrient deficiency and oxidative stress.

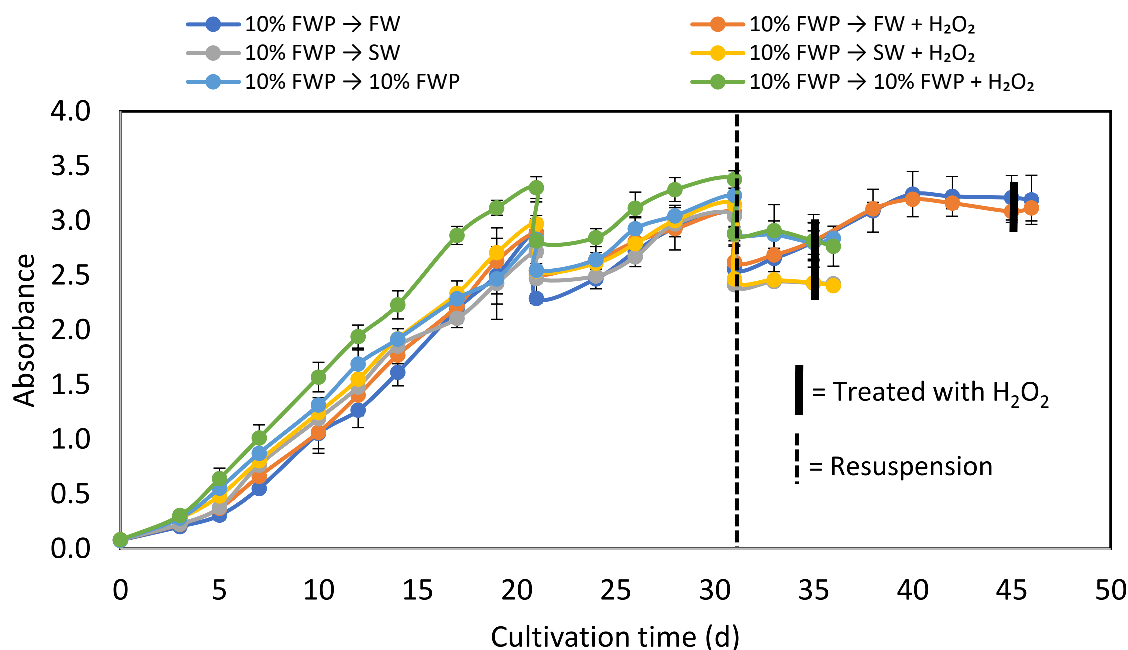

Figure 29. Growth curves for *C. vulgaris* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide. All conditions were performed in triplicate. FWP = food waste permeate; SFWP = stripped food waste permeate

Table 22. Growth characteristics for *C. vulgaris* cultivated in 250-mL volumes of 10% FWP followed by resuspension in either freshwater (FW), filtered seawater (SW), or the original media and treated with hydrogen peroxide.[a] FWP = food waste permeate; SFWP = stripped food waste permeate

Condition	Harvest Day	Harvested Biomass (mg)[c]	Biomass productivity ($mgL^{-1}d^{-1}$)	Percent neutral lipid content (w/w)[b]
10% FWP → FW	46	278 ± 4	75.3 ± 0.2	20 ± 1
10% FWP → FW + H_2O_2	46	278 ± 8	63.7 ± 0.3	19 ± 1
10% FWP → SW	36	185 ± 15	98 ± 8	12.5 ± 0.9
10% FWP → SW + H_2O_2	36	193 ± 6	120 ± 20	11 ± 1
10% FWP → 10% FWP	36	197 ± 6	100 ± 40	10 ± 1
10% FWP → 10% FWP + H_2O_2	36	192 ± 18	120 ± 6	7.7 ± 0.7

[a] Values are presented as mean ± SD of triplicates
[b] There may be pigment interference making these values lower than the actual neutral lipid content
[c] Biomass based on harvesting at stationary phase; conditions reached stationary phase at different days throughout the experiments and were therefore harvested on different days

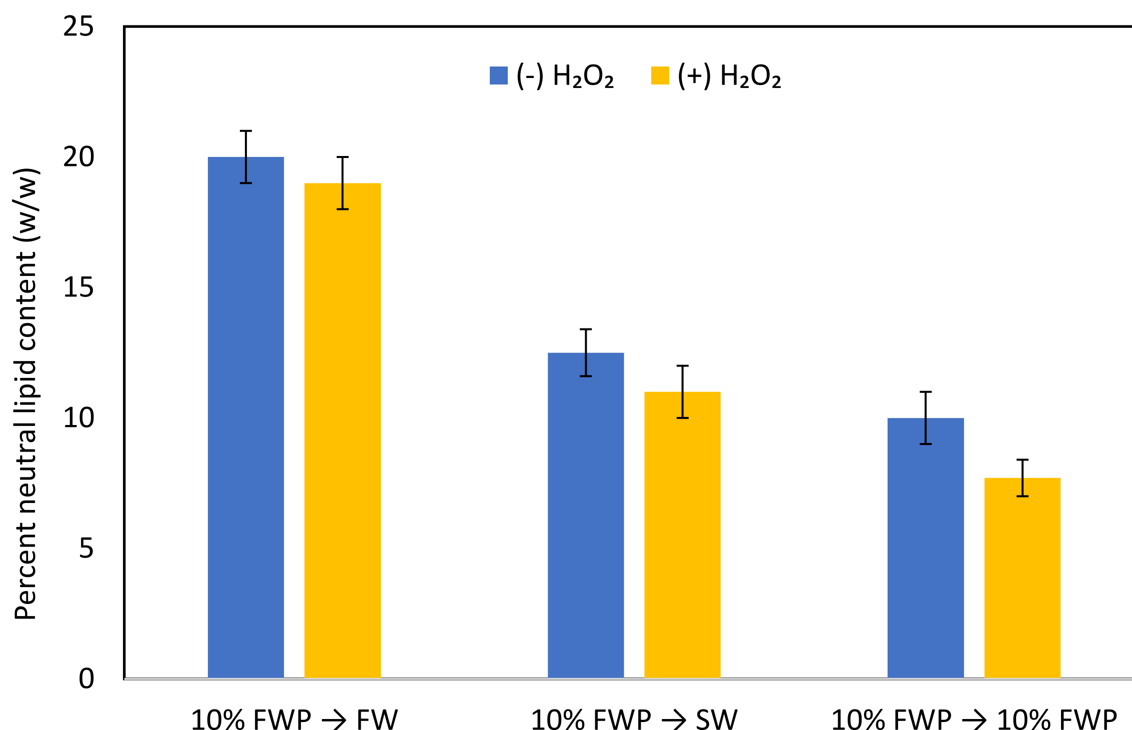

Figure 30. Comparison of neutral lipid content in *C. vulgaris* when cultivated using two-phase methodology followed by treatment with or without hydrogen peroxide as a metabolic stressor. FW = tap water; SW = filtered seawater; FWP = food waste permeate

4.3.3. Pigment interference in the Bleach-Assisted Nile Red Assay

4.3.3.1. Preliminary Pigment Interference Studies in the Bleach-Assisted Nile Red Assay

A significant reduction in fluorescence signal was observed when quantifying neutral lipids in highly pigmented *C. sorokiniana* extracts using the bleach-assisted Nile Red assay (NRLA) (Figure 31). The combined mass of neutral lipids in the two extract conditions should be greater than that of the standard control, and the difference between the two extract conditions was expected to be equal to the control (100 µg). Instead, the difference between the two algae extract conditions was 4 ± 5 µg, which is 104 ± 5% lower than the expected mass

of 100 µg (Table 25). These results demonstrate that some factor in the highly pigmented extracts is inhibiting accurate quantification of neutral lipids using the NRLA. It is important to note that the control also varied from the expected 100 µg lipid spike. The mass of neutral lipids in the control sample was determined to be 64 ± 4 µg which is 36 ± 4% lower than that of the expected 100 µg (Table 25). This indicates that there is likely a systematic error in the assay that should be investigated further. While this systematic error could contribute to the large error observed in the extract's lipid measurements, it is hypothesized that the pigments are interfering somehow, preventing accurate fluorescence readings from being measured.

Although the bleaching in the assay is added to destroy the pigments, the pigment concentrations in these algae extracts are concentrated to the point where the current bleaching is not sufficient to mitigate the interference.[22] This hypothesis is further supported by experiments that evaluate the bleach concentrations used during this bleach-assisted Nile Red assay. Matt Paddock tested higher bleach concentrations in the VanderGheynst lab at UC Davis. He found that lipids will degrade even slightly higher bleach concentrations, so it was decided that increasing the bleaching will not solve the pigment interference issues. Future studies should optimize the NRLA for highly pigmented extracts or develop a new rapid quantification assay for these pigmented samples that better addresses pigment interference.

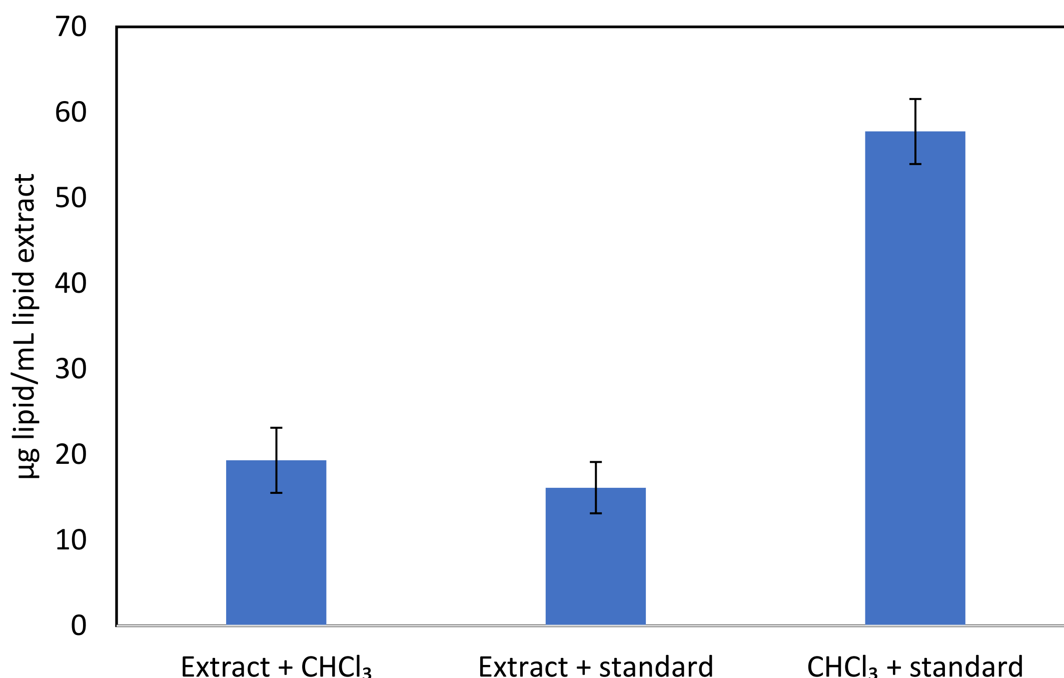

Figure 31. Graph demonstrating the neutral lipid content in 1 mL of *C. sorokiniana* lipid extracts quantified by bleach-assisted Nile Red assay. The "standard" was a 100 µL aliquot of 1 mg/mL lipid standard.

Table 23. Neutral lipid content in 1.1 mL of *C. sorokiniana* lipid extracts as quantified by bleach-assisted Nile Red assay. The analysis was performed between relative conditions and their comparison to the theoretical value.[a]

<table>
<tr><th>Condition</th><th>Mass of neutral lipids (µg)</th><th>Mass difference between experimental conditions (µg)</th><th>% Difference from theoretical (100 µg)</th></tr>
<tr><td>Extract + $CHCl_3$</td><td>19 ± 3</td><td rowspan="2">-4 ± 5</td><td rowspan="2">-104 ± 5</td></tr>
<tr><td>Extract + standard</td><td>18 ± 3</td></tr>
<tr><td>$CHCl_3$ + standard</td><td>64 ± 4</td><td>na</td><td>-36 ± 4</td></tr>
</table>

[a] Values presented as mean ± SD of triplicates

4.3.3.2. Comparing the Sulfo-Phospho-Vanillin Assay Versus the Bleach-Assisted Nile Red Assay for Lipid Quantification of Highly Pigmented Extracts

The sulfo-phospho-vanillin assay resulted in significantly more accurate neutral lipid measurements than the bleach-assisted Nile Red assay (NRLA). The interference in this study is similar to that observed in the preliminary interference studies (Figure 32 and Table 26). In this study, the difference between the extract conditions should be 150 µg, the mass of the standard spike in the extract. However, the mass difference between the extract conditions was only 4.9 ± 0.3 µg, which is 145.1 ± 0.3% lower than the expected 150 µg. The mass of lipids in the standard control was 133 ± 25 µg, which is 11 ± 17% lower than the expected 150 µg (Table 26). As stated previously, the systematic error (as demonstrated by the control) in these measurements could contribute to the large percent difference observed between extract conditions; however, most of the error is hypothesized to be due to pigments.

The error with the SPV assay was significantly smaller than the error seen in the NRLA (Table 26 and Figure 32). The calculated mass difference between the two extract conditions should be equal to the standard added to the "Extract + standard" sample, 150 µg. The calculated difference between the extract conditions was 113 ± 31, which is 25 ± 21% lower than the expected value (150 µg). This difference is significantly lower than the 104 ± 5% difference observed in the NRLA (Table 25). The lipid standard control was measured at 172 ± 13 µg, which is a 15 ± 9% difference from the expected. This preliminary study demonstrates that SPV is a possible alternative method to quantify lipids in highly pigmented samples. The SPV assay should be further optimized to mitigate the error observed in this study.

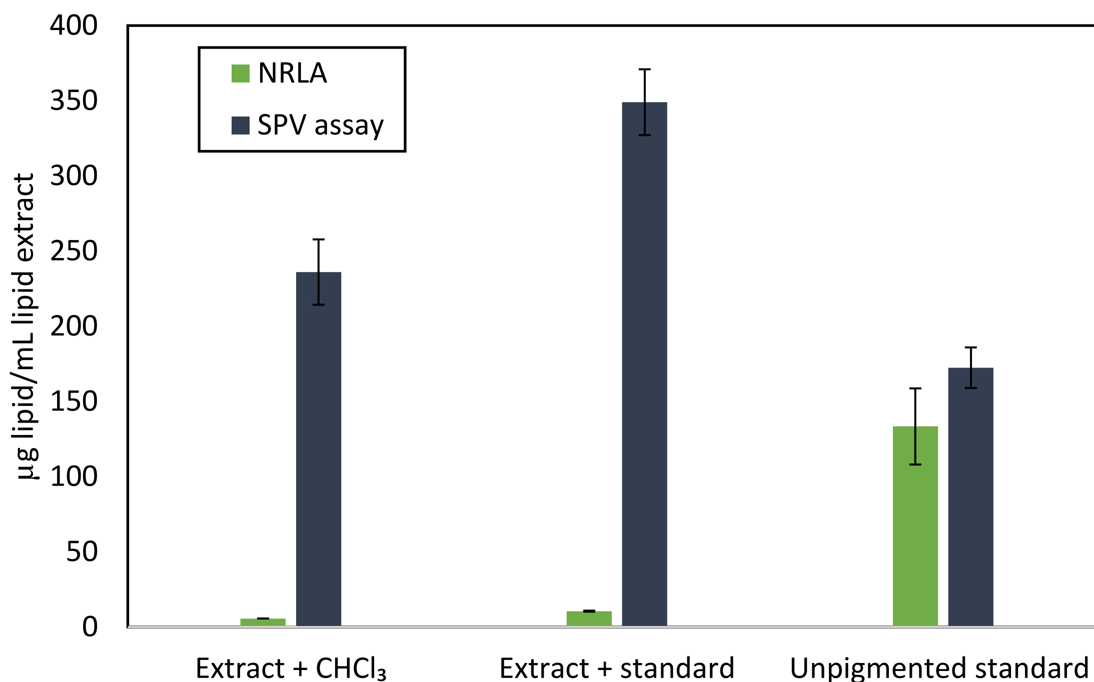

Figure 32. The concentration of lipids in *C. sorokiniana* lipid extracts as determined by a bleach-assisted Nile red assay (NRLA) and sulfo-phospho-vanillin assay (SPV) demonstrating pigment inference in the NRLA. All conditions were performed in triplicate.

Table 24. The concentration of lipids in *C. sorokiniana* lipid extracts as determined by a bleach-assisted Nile red assay (NRLA) and sulfo-phospho-vanillin assay (SPV).[a]

Analysis Method	Condition	μg lipid/mL of sample	Mass difference between experimental conditions (μg)	% Difference from theoretical (150 μg)
NRLA	Extract + $CHCl_3$	5.68 ± 0.03	4.9 ± 0.3	-145.1 ± 0.2
	Extract + standard	10.6 ± 0.3		
	$CHCl_3$ + standard	133 ± 25	na	-11 ± 17
SPV Assay	Extract + $CHCl_3$	236 ± 22	113 ± 31	-25 ± 21
	Extract + standard	349 ± 22		
	$CHCl_3$ + standard	172 ± 14	na	15 ± 9

[a] Values presented as mean ± SD of quadruplicates

While the SPV assay is more accurate at quantifying lipids in highly pigmented extracts, it is not a rapid screening method; only a limited number of samples can be processed at a time. This will introduce error when analyzing related samples that need to be compared to each other. The SPV assay also utilizes substantially more reagents and materials, including concentrated sulfuric acid and concentrated phosphoric acid. The low throughput and the hazardous, large volumes of chemicals used in the SPV assay reduce its workability as an alternative to the NRLA in quantifying neutral lipids. Therefore, I developed a method that addresses pigment interference as a pretreatment before performing the NRLA.

4.3.4. Optimizing Graphitized Carbon Treatments of Lipid Extracts for lipid quantification

4.3.4.1. Evaluating Pigment Reduction with Graphitized Carbon Pretreatments and Its Correlation with Neutral Lipid Quantification

Graphitized carbon (GC) as a pretreatment removed pigment from algae extracts and resolved the pigment interference issues. As the absorbance of the pigments goes down with increasing amounts of GC, the neutral lipid measurement increases (Figure 33). This correlation supports the hypothesis that the removal of pigments from lipid extracts will increase measured lipid yields. There was an observable variation in color between the different treatments evaluated in this study (Figure 34).

A 25 mg aliquot of GC to a 1 mL extract sample effectively reduces pigment concentrations to a level conducive to performing a NRLA. The 25 mg GC and 50 mg GC conditions used to treat extracts from *C. sorokiniana* cultivated on 10% FWP were not significantly different (Figure 33). The difference between 25 mg GC and 50 mg GC is more

apparent in the extracts of *C. sorokiniana* cultivated on synthetic media than the extracts of *C. sorokiniana* cultivated on 10% FWP. However, this difference still is not significant enough to warrant the use of 50 mg GC. Considering these insignificant differences, I concluded that 25 mg GC per mL of the extract is adequate in reducing pigment concentrations to a level conducive to performing accurate NRLAs. This decision was applied in all consequent lipid analyses of highly pigmented lipid extracts.

There is a noticeable increase in the error bars with larger masses of GC (Figure 33). This error could be due to inadequate suspension of the GC in the extracted sample being treated. Excess clumping of the GC in solution would limit available surface area and reduce the effectiveness of pigment removal. Further studies should be performed to determine the cause of this increased error and identify solutions to mitigate this issue.

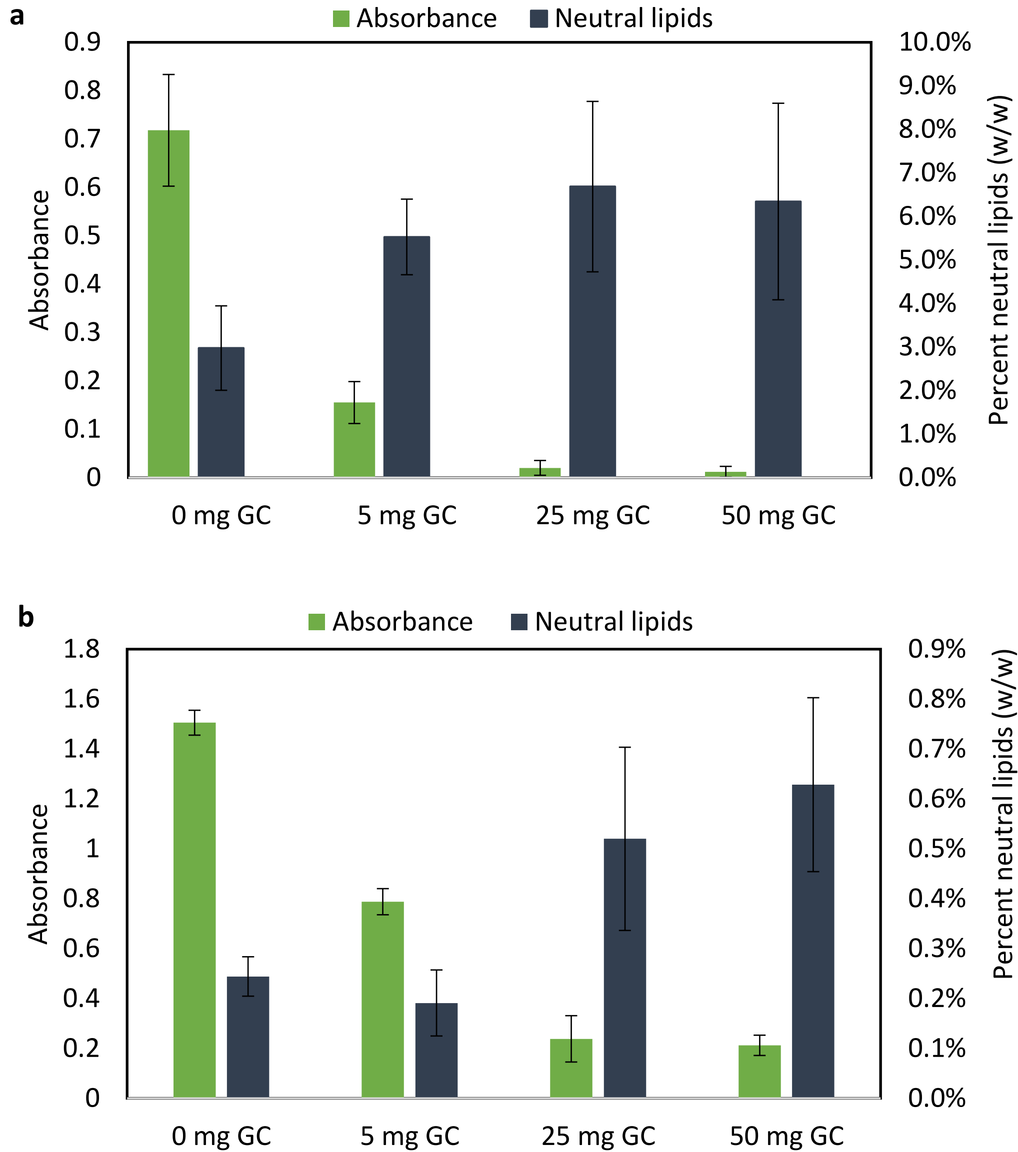

Figure 33. Comparison between chlorophyll pigments measured at 680 nm and neutral lipid content as measured by bleach-assisted Nile Red assay. The conditions evaluated the mass of graphitized carbon (GC) used to reduce pigment concentrations in lipid extracts of *C. sorokiniana* cultivated on (a) 10% FWP and (b) BG-11 synthetic media.

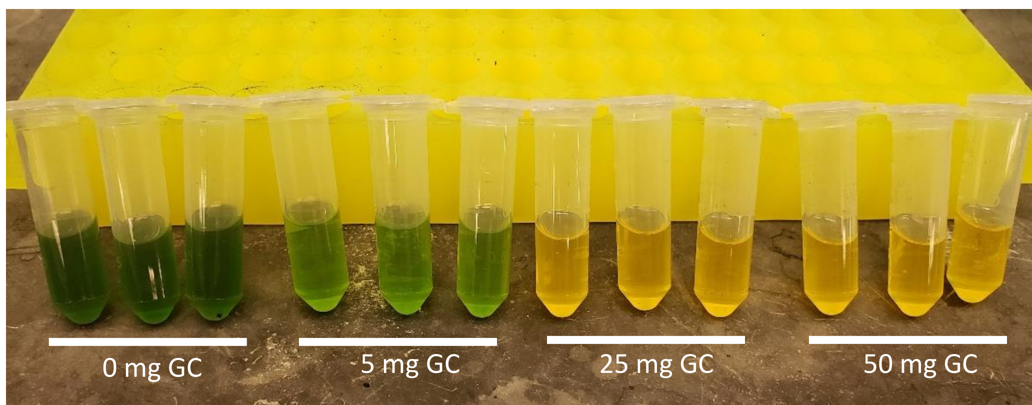

Figure 34. Comparison of the color changes observed of *C. sorokiniana* lipid extracts treated with varying amounts of graphitized carbon (GC).

4.3.4.2. Optimizing the Duration of Graphitized Carbon Pretreatment of *C. sorokiniana* Lipid Extracts

Treatment of lipid extracts with 25 mg of graphitized carbon (GC) for 30 min effectively reduces pigment concentrations to levels conducive to performing NRLAs reliably (Figure 35). After 15 minutes of treatment to the extracts from *C. sorokiniana* cultivated on 10% FWP, they did not vary widely over the next 45 min. There was a more apparent increase between 15 min and 30 min of treatment to the extracts cultivated on BG-11. The insignificant difference observed between 30 min and 60 min in both types of extract support the decision to establish that 30 min on a shaker at 250 rpm is an adequate amount of time to reach the maximum reduction potential of the 25 mg GC. Similar to the experiments evaluating GC masses (Figure 33), the error bars are larger the longer the extracts are treated (Figure 35). This increase should be investigated further and addressed to reduce error in the assay.

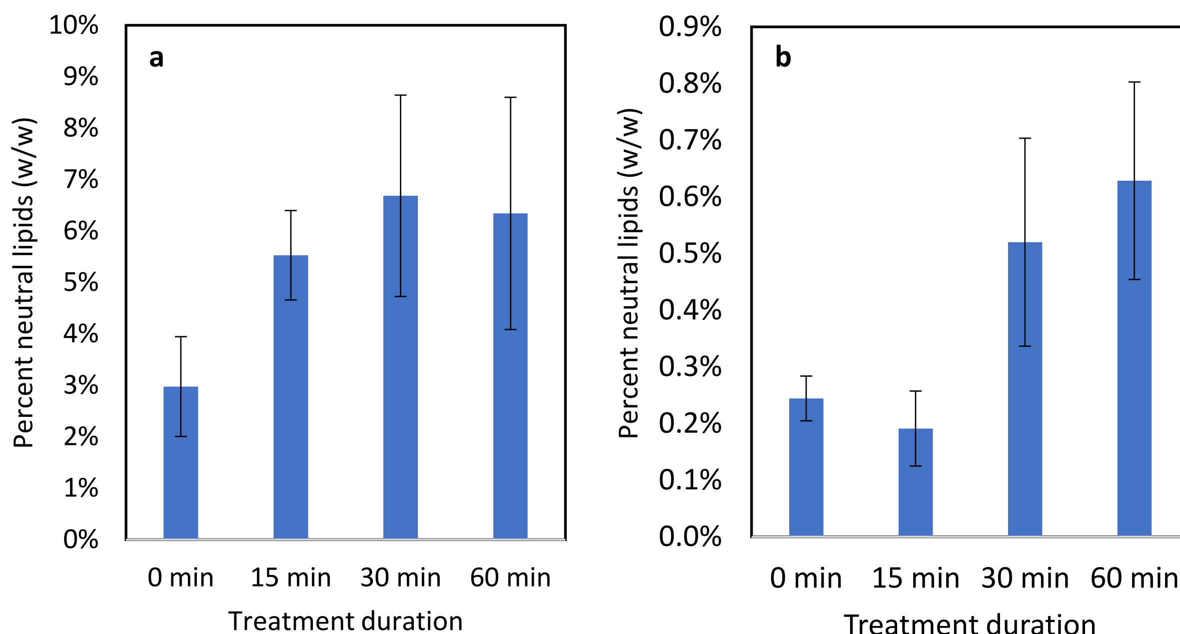

Figure 35. Comparison between graphitized carbon (GC) treatment durations using 25 mg GC to treat lipid extracts from *C. sorokiniana* cultivated on (a) 10% FWP and (b) BG-11 synthetic medium. FWP = food waste permeate.

4.3.5. Using Biochar versus Graphitized Carbon to Remove Pigments from Lipid Extracts

Biochar and graphitized carbon black were both effective in removing pigment from algae extracts. The assay was repeated two times using the same algae extract to validate initial results and determine the repeatability of each method (Figure 36). The biochar pretreatment method provided different lipid yields between the two NRLAs. The second NRLA measured 35 ± 6% more neutral lipids by weight compared to the first NRLA. The graphitized carbon pretreatment method provided similar lipid yields between the two NRLA with only a 5 ± 5% difference between the two assays. That said, preliminary results indicate that both biochar and graphitized carbon are effective pretreatments for the NRLA. However, biochar may be more economically feasible and provide a more sustainable option compared to graphitized carbon

black. Other types of biochar should be evaluated to determine the optimally efficient biochar source. On the other hand, graphitized carbon may be more reliable, and the pretreatments will be more repeatable than biochar treatments. Other forms of carbon, such as multiwalled carbon nanotubes or activated carbon, should be considered as a treatment material as well.[15] Overall, further studies should be conducted before making overarching conclusions regarding the use of biochar versus graphitized carbon to remove pigments from algae extracts.

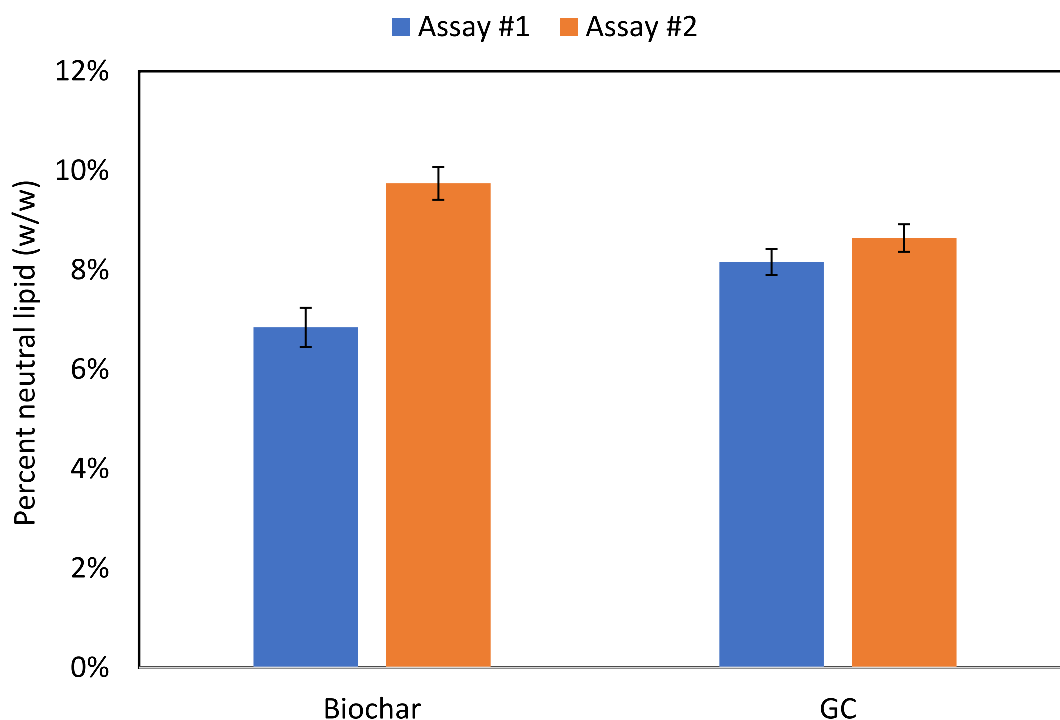

Figure 36. Comparison between biochar versus graphitized carbon as a pretreatment for highly pigmented *C. sorokiniana* lipid extracts being analyzed by a bleach-assisted Nile Red assay. The assay was performed two times with a triplicate design.

4.3.6. Evaluating Bleach-Assisted Nile Red Lipid Assay Precision Between Both Individuals and Replicate Assays

Significant variations were observed in the measurements of neutral lipids in *C. sorokiniana* extracts taken by different individuals using the bleach-assisted Nile Red assay (NRLA) (Table 27). However, less variation was observed between assays being performed by the same individual over the course of 2 days. The only significant ($p < 0.05$) differences observed between assays performed by the same individual were the “No GC + $CHCl_3$” and the “Control + std” with p-values of 0.0498 and 0.0138, respectively. All other differences were insignificant at an α of 0.05.

A significant difference was observed when comparing NRLA measurements between different individuals (Figure 37). The only conditions with insignificant differences between individuals were the two control conditions (Table 27). The difference is increased in those extracts treated with GC. The large observable error in lipid measurements after GC treatment has been discussed in previous sections. The reasoning behind this error could also contribute to the larger differences between individuals seen here.

These variable results question the reliability of this assay to provide accurate and precise measurement of neutral lipids in algae extracts. There is a lot of room for error in this assay, such as pipetting error and error in the resuspension of the lipid extract after drying. The latter is likely the primary source of error within this assay. Investigations should be performed to identify the source of this error within assays being conducted by the same individual and between assays performed by different individuals.

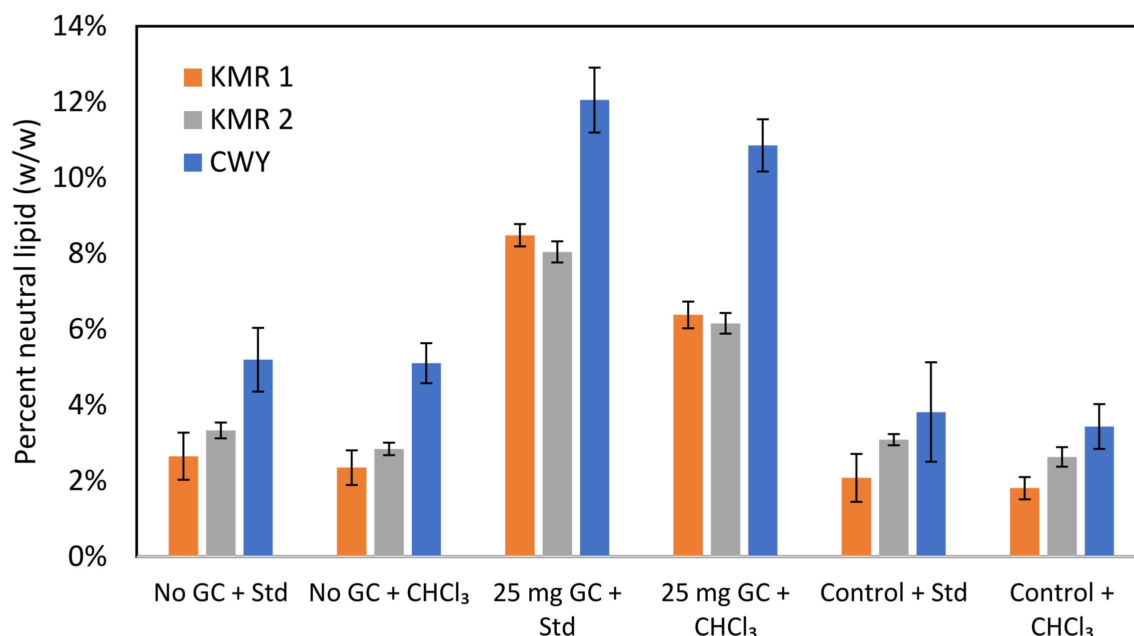

Figure 37. Neutral lipids by weight as determined by bleach-assisted Nile Red assay. Individual assays were performed by: CWY = Cody Yothers, KMR 1 & 2 = Kayla Rude. Samples were of the same algae extract with and without graphitized carbon (GC) treatment. Standard was 100 µL of a 1 mg/mL lipid standard in $CHCl_3$.

Table 25. Percent neutral lipids by weight as determined by bleach-assisted Nile Red assay. Individual assays were performed by: CWY = Cody Yothers, KMR 1 & 2 = Kayla Rude. Samples were of the same algae extract with and without graphitized carbon (GC) treatment.[a] Standard was 100 µL of a 1 mg/mL lipid standard in $CHCl_3$.

Condition	Percent neutral lipids (w/w)			p-value (α = 0.05)[b]	
	Trial 1 KMR 1	Trial 2 KMR 2	Trial 3 CWY	KMR 1 versus KMR 2	KMR 1 & 2 versus CWY
No GC + Std	2.7 ± 0.6	3.3 ± 0.2	5.2 ± 0.8	0.0511	0.000871
No GC + $CHCl_3$	2.3 ± 0.5	2.8 ± 0.2	5.1 ± 0.5	0.0498	0.0000175
25 mg GC + Std	8.5 ± 0.3	8.0 ± 0.3	12.1 ± 0.9	0.491	0.00000924
25 mg GC + $CHCl_3$	6.4 ± 0.4	6.2 ± 0.3	10.9 ± 0.7	1	0.000000431
Control + Std	2.1 ± 0.6	3.1 ± 0.1	4 ± 1	0.0138	0.0514
Control + $CHCl_3$	1.8 ± 0.3	2.6 ± 0.3	3.4 ± 0.6	0.943	0.527

[a] Values are presented as mean ± SD of quadruplicates
[b] Significant difference at p-value < 0.05

The lipid recoveries observed in these assays were low across those conditions with *C. sorokiniana* extract and the control, unpigmented condition (Table 28). Samples without algae had consistently variable recovery rates that ranged from a 17.7 ± 0.8% loss to a 139.6 ± 0.8% loss and from a 46 ± 1 % increase in measured lipids to a 122 ± 2% increase in lipid content. While there were still variable recovery rates in the conditions with algae extract, the variation between individuals within each condition was relatively the same; the only exception being the condition in which the algae extracts were pretreated with GC. The variation observed in the GC treated samples could relate to the increase in standard deviation between replicates observed in the GC optimization assays (Figures 33 and 35).

Table 26. Comparisons between the spiked and unspiked conditions to determine the accuracy and evaluate the precision of the bleach-assisted Nile Red assay. Individual assays were performed by: CWY = Cody Yothers, KMR 1 & 2 = Kayla Rude. Samples were of the same *C. sorokiniana* extract with and without graphitized carbon (GC) treatment. Standard was 100 µL of a 1 mg/mL lipid standard in $CHCl_3$.[a]

Condition		µg lipid in sample aliquot (1.1 mL)			Mass difference between spiked and unspiked conditions (µg)			% difference from theoretical (Theoretical = 100 µg)		
		KMR1	KMR2	CWY	KMR1	KMR2	CWY	KMR1	KMR2	CWY
(+) algae	No GC + Std	84 ± 20	106 ± 7	165 ± 27	10 ± 25	16 ± 8	3 ± 32	-90.3 ± 0.2	-84.47 ± 0.08	-97.1 ± 0.3
	No GC + $CHCl_3$	74 ± 15	90 ± 5	162 ± 17						
	25 mg GC + Std	270 ± 9	256 ± 9	383 ± 27	67 ± 15	60 ± 12	38 ± 35	-33.0 ± 0.1	-40.0 ± 0.1	-61.9 ± 0.4
	25 mg GC + $CHCl_3$	203 ± 11	196 ± 9	345 ± 22						
	Control + Std	66 ± 20	98 ± 5	121 ± 42	8 ± 22	14 ± 9	12 ± 46	-91.3 ± 0.2	-85.51 ± 0.09	-87.8 ± 0.5
	Control + $CHCl_3$	57 ± 9	83 ± 8	109 ± 19						
(-) algae	No GC + Std	857 ± 152	647 ± 89	546 ± 73	79 ± 160	46 ± 96	29 ± 81	-20 ± 160	-54 ± 1	-70.7 ± 0.8
	No GC + $CHCl_3$	777 ± 48	601 ± 36	517 ± 35						
	25 mg GC + Std	903 ± 78	682 ± 51	518 ± 58	146 ± 131	82 ± 76	-39 ± 77	46 ± 1	-17.7 ± 0.8	-139.6 ± 0.8
	25 mg GC + $CHCl_3$	756 ± 105	600 ± 56	558 ± 51						
	Control + Std	1255 ± 90	1117 ± 117	934 ± 49	221 ± 197	189 ± 176	221 ± 230	122 ± 2	89 ± 2	121 ± 2
	Control + $CHCl_3$	1033 ± 175	927 ± 132	713 ± 224						

[a] Values presented as mean ± SD of quadruplicates

4.3.7. Evaluating Bead-Beating Versus Sonication Methods for Neutral Lipid Extraction from Microalgae Biomass

4.3.7.1. Comparison Between Sonication and Two Bead-Beating Methods to Evaluate Lipid Extraction Efficiency and Recovery

After sonication and bead beating as measured by the bleach-assisted Nile Red assay (NRLA), the percent recovery of neutral lipids was significantly decreased compared to the untreated control (Table 29 and Figure 38). Sonication resulted in a higher lipid recovery compared to the two bead beating methods. After sonication, the lipid recovery was calculated to be 58 ± 1% compared to the 39 ± 2% recovery observed with the bead beating methods. There was no significant difference between the two bead beating methods indicating that lipid recovery is unaffected by using 250 mg beads versus 0.75 mL of beads.

The lipid recovery of the untreated control was 109 ± 12%, indicating that the NRLA is not likely the source of low lipid measurements in the sonication and bead-beating methods. Instead, the low lipid recoveries observed in this experiment are likely due to transfer loss during the extraction procedures. Transfer loss would also explain why sonication resulted in a higher lipid recovery. There are fewer procedural steps and transfers in sonication in which material could be lost compared to the bead-beating methods. Further studies should be done to identify the specific steps at which significant losses in lipids are observed. Once this is understood, procedural changes should be considered to mitigate this loss.

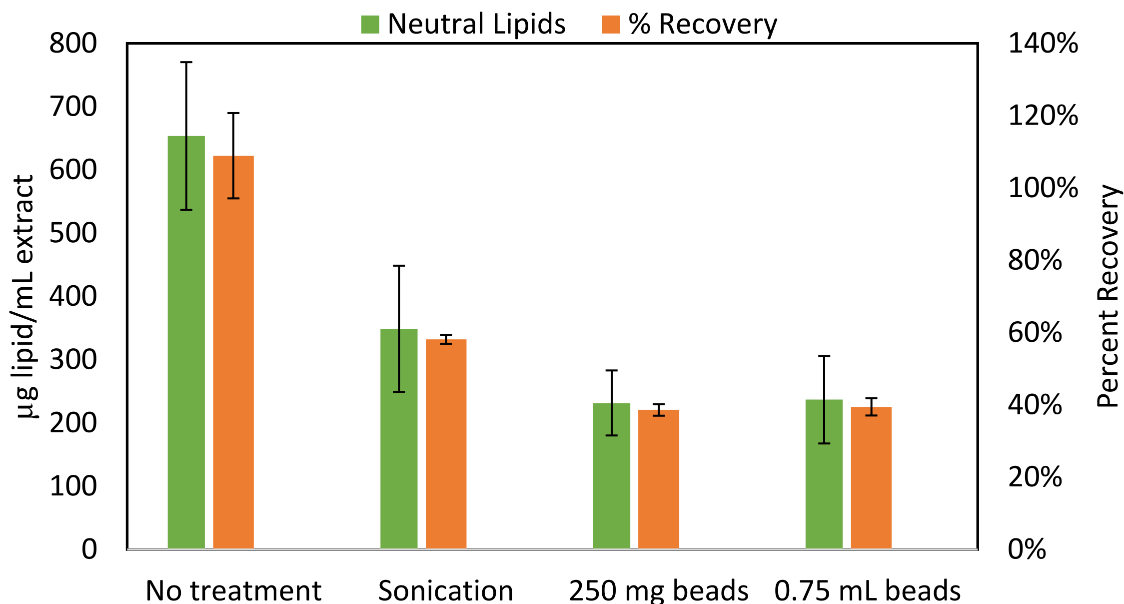

Figure 38. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by sonication, bead-beating using 250 mg beads, or filling the extraction tube with beads up to the 0.75 mL mark (0.75 mL beads).

Table 27. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by sonication, bead-beating using 250 mg beads, or filling the extraction tube with beads up to the 0.75 mL mark (0.75 mL beads).[a]

Treatment Condition	Lipid Concentration (µg/mL)	Theoretical Lipid Concentration (µg/mL)	Lipid Recovery (%)
No treatment	654 ± 117	600	109 ± 12
Sonication	349 ± 100	600	58 ± 1
250 mg beads	232 ± 51	600	39 ± 2
Beads to 0.75 mL mark	237 ± 69	600	39 ± 2

[a] Values presented as mean ± SD of triplicates

4.3.7.2. Comparison Between Bead-Beating and Two Sonication Methods to Evaluate Lipid Extraction Efficiency and Recovery

After sonication and bead beating as measured by the bleach-assisted Nile Red assay (NRLA), the percent recovery of neutral lipids was significantly decreased compared to the untreated control (Figure 39). Both sonication methods resulted in higher lipid recoveries compared to the bead-beating method. After bead- beating the percent recovery of neutral lipids was determined to be 38 ± 4%. This recovery is consistent with the two bead-beating experiments performed in the previous study, where the lipid recovery with bead-beating was determined to be 39 ± 2% (Table 30). The percent recovery for the two sonication methods was 65 ± 9% when using the 2x sonication method and 74 ± 5% when using the 3x sonication method. These results indicated that more rounds of sonication are possible if extra sonication is necessary to lyse algal cells effectively.

The lipid recovery of the untreated control was 143 ± 12%, indicating that there could be some error in the NRLA in this study. The difference between this study's NRLA measurements and those of the previous sonication/bead-beating study is not unexpected, considering the variability of the assay as discussed previously in this chapter. Similar to the previous sonication/bead-beating experiment, the low lipid recoveries observed in this experiment are likely due to transfer loss during the extraction procedures. Therefore, I concluded that the bead-beating method used in this study is not ideal for extracting neutral lipids. Further studies could investigate other bead-beating methods and materials, but currently, sonication is a better alternative for neutral lipid extractions.

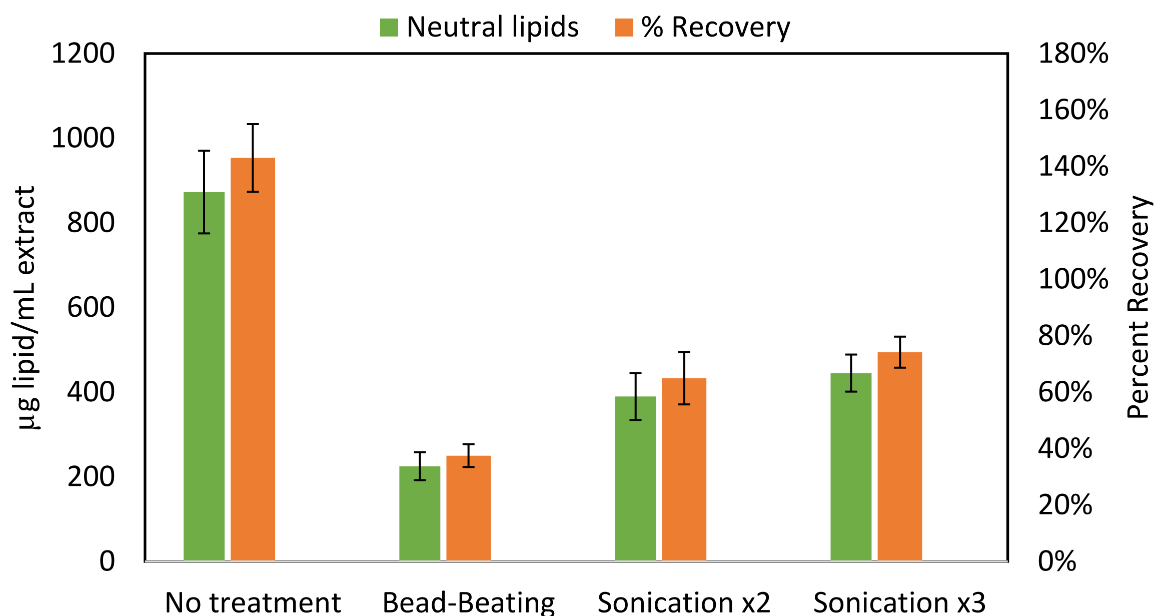

Figure 39. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by bead-beating, two rounds of sonication (sonication x2), or three rounds of sonication (sonication x3).

Table 28. Comparison between lipid concentrations and recoveries when a lipid standard is put through extraction by bead-beating, two rounds of sonication (sonication x2), or three rounds of sonication (sonication x3).[a]

Treatment Condition	Lipid Concentration (µg/mL)	Theoretical Lipid Concentration (µg/mL)	Lipid Recovery (%)
No treatment	873 ± 97	600	143 ± 12
Bead-Beating	225 ± 33	600	38 ± 4
Sonication x2	390 ± 55	600	65 ± 9
Sonication x3	445 ± 44	600	74 ± 5

[a] Values are presented as mean ± SD of quadruplicate measurements

4.3.7.3. Sonication versus Bead-Beating of Microalgae Extracts and Lipid Standards to Evaluate Lipid Recovery

This study supports the conclusion that sonication is a better lysing method than bead beating for lipid extraction from microalgae extracts (Tables 31 and 32). Sonication afforded higher lipid recoveries ranging from 65 ± 8% to 80 ± 80%. Both bead beating experiments afforded substantially lower lipid recoveries ranging from 8 ± 15% to 45 ± 69%. The lipid standard samples demonstrated a wide variation in lipid recoveries. The percent recoveries were abnormal for the lipid standard samples put through the extraction processes (Table 32). However, this trend was not seen in the lipid standard samples not put through the extraction methods (the standard control). The lipid recoveries in the standard control for the sonication experiment were consistent with those lipid recoveries in the sonication extraction experiment. This consistency in lipid recoveries demonstrates that the composition of the algae extract does not interfere with or affect the sonication extraction process or the ability of the NRLA to quantify lipids. The lipid recoveries observed in the bead-beating experiments were significantly higher than 100%, which was not a trend observed in the algae extract experiment. Lower percent recoveries were observed in those samples with larger spike volumes (400 µL), but this could be misleading considering all values in the extracted lipid standards were around 400 µg. Although the lipid recoveries were high in the bead-beating experiment, the results between the two bead-beating experiments are relatively consistent (Tables 31 and 32) with differences of 10 ± 16, 5 ± 18%, and 12 ± 16% for extracted lipid standard conditions and 9 ± 20%, 0%, and 3 ± 10% for the lipid control conditions. Nonetheless, the wide variations and excess recoveries should be investigated further to identify sources of error.

Due to experimental size limitations, each condition was performed with a single replicate, and the reported standard deviations are N=4 technical replicates measured with the NRLA. This lower replicate count could explain the large standard deviations observed in this study. This experiment should be repeated with N=3 biological replicates o better establish both lipid recovery rates and the reliability of sonication over bead-beating. Using N=3 biological replicates would afford 12 total replicates between the biological and technical replicates. The increased number of replicates should lower the standard deviation and provide a more accurate and precise lipid recovery determination.

Table 29. Summary of sonication and bead-beating extractions of microalgae extracts to evaluate lipid detection and recovery.[a]

Sample	µg lipid added (expected)	Sonication			Bead-beating – 2/18/20			Bead-beating – 3/2/20		
		µg in $CHCl_3$ extract	Difference between sample and control	% recovery	µg in $CHCl_3$ extract	Difference between sample and control	% recovery	µg in $CHCl_3$ extract	Difference between sample and control	% recovery
Algae Extract + Std	+100	655 ± 76	80 ± 80	80 ± 80	563 ± 40	15 ± 42	15 ± 42	486 ± 65	45 ± 69	45 ± 69
	+200	719 ± 35	144 ± 42	72 ± 21	563 ± 27	15 ± 30	8 ± 15	483 ± 50	41 ± 55	21 ± 28
	+400	871 ± 22	396 ± 32	65 ± 8	641 ± 32	93 ± 34	23 ± 9	531 ± 54	89 ± 59	23 ± 15
Algae Extract (no std)		575 ± 23			548 ± 12			442 ± 23		

[a] Values are presented as mean ± SD of quadruplicate measurements

Table 30. Summary of sonication and bead-beating extractions of a lipid standard without algal biomass to evaluate lipid detection and recovery.[a]

Sample	µg lipid added (expected)	Sonication		Bead-beating – 2/18/20		Bead-beating – 3/2/20	
		µg in 4 mL $CHCl_3$ extract	% recovery	µg in 4 mL $CHCl_3$ extract	% recovery	µg in 4 mL $CHCl_3$ extract	% recovery
Lipid Standard + treatment	+100	401 ± 32	401 ± 32	346 ± 20	346 ± 20	312 ± 48	312 ± 48
	+200	490 ± 7	245 ± 4	367 ± 33	184 ± 17	349 ± 53	175 ± 27
	+400	732 ± 99	183 ± 25	393 ± 39	98 ± 10	346 ± 44	87 ± 11
Lipid Standard (no treatment)	+100	81 ± 18	81 ± 18	144 ± 13	144 ± 13	132 ± 24	132 ± 24
	+200	120 ± 7	60 ± 4	253 ± 19	127 ± 10	253 ± 20	127 ± 10
	+400	266 ± 21	67 ± 5	487 ± 29	122 ± 7	471 ± 41	118 ± 10

[a] Values are presented as mean ± SD of quadruplicate measurements

4.3.8. Microwave-Assisted Extraction (MAE) of Neutral Lipids from Microalgae Biomass

4.3.8.1. Evaluating the Use of Different Extraction Solvents for MAE of Lipids from Microalgae Biomass

The utilization of a 2:1 $CHCl_3$/MeOH solvent system for lipid extraction using microwaves resulted in significantly higher lipid yields than the $CHCl_3$ extraction solvent (Figure 40). A 4.007 ± 0.004% lipid yield was observed for those samples extracted using 2:1 $CHCl_3$/MeOH, which is 83 ± 16% higher than the 1.653 ± 0.001% lipid yield observed with the $CHCl_3$ extraction (Table 33). However, the lipid recoveries were 60 ± 160% higher for those samples extracted with $CHCl_3$ compared to 2:1 $CHCl_3$/MeOH (Table 33). With these results, the 2:1 $CHCl_3$/MeOH solvent was determined to be the best solvent to move forward with, considering its higher lipid yield by weight. Although the lipid recovery was lower with the 2:1 $CHCl_3$ solvent system, it has the potential to extract more lipids than the $CHCl_3$ solvent upon further optimization of the extraction method.

It is important to note that this experiment was performed with a single biological replicate for each condition to allow them to be analyzed in a single NRLA. Running a single NRLA avoided possible errors associated with running two separate assays. Because I used single replicate conditions, the standard deviations for these measurements were large. After optimizing the NRLA to quantify lipids in *C. sorokiniana* lipid extracts, this experiment should be repeated with more biological replicates to establish more precise percent recoveries of each solvent system. Studies should also investigate at which steps lipids are being lost in this extraction and analysis process.

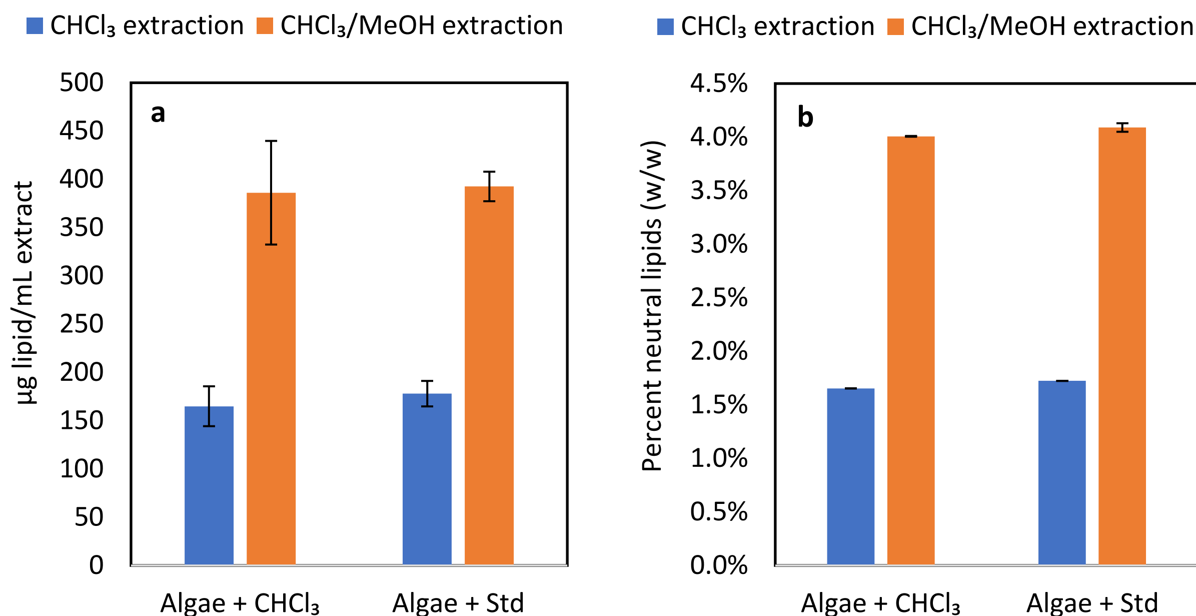

Figure 40. Summary of both (a) lipid concentrations and (b) neutral lipid content extracted from microalgae using microwave-assisted extraction (MAE) with either $CHCl_3$ or 2:1 $CHCl_3$/MeOH solvent to evaluate lipid content. A standard was added to evaluate lipid recovery using MAE.

Table 31. Summary of neutral lipid content extracted from microalgae using microwave and either $CHCl_3$ or 2:1 $CHCl_3$/MeOH solvent to evaluate lipid content. A standard was added to evaluate lipid recovery using MAE.[a] Extract = 4 mL $CHCl_3$

Solvent	Condition	µg in $CHCl_3$ extract	Difference between conditions	% recovery	Percent neutral lipids (w/w)
$CHCl_3$	Algae + $CHCl_3$ Algae + Std	660 ± 21 712 ± 16	52 ± 23	52 ± 25	1.653 ± 0.001 1.7237 ± 0.0008
2:1 $CHCl_3$/MeOH	Algae + $CHCl_3$ Algae + Std	1544 ± 54 1570 ± 15	26 ± 56	28 ± 56	4.007 ± 0.004 4.09 ± 0.04

[a] Values are presented as mean ± SD of quadruplicate measurements

4.3.8.2. Evaluating Treatment Variations of MAE extracts on Pigment Removal and Neutral Lipid Quantification

Extraction with 2:1 $CHCl_3$/MeOH yielded the highest percentage of lipids compared to the $CHCl_3$ solvent system (Table 34). MAE extracts are very highly pigmented (Figure 42), so the efficacy of the treatment of 25 mg GC for 30 min in reducing pigment concentration enough for an accurate NRLA was investigated. There was slight variation between the different GC treatments except in the treatment of the $CHCl_3$ extract with 25 mg GC (Figure 41). After treatment with 25 mg GC, the extracted lipids were measured at 2.0 ± 0.2%, which is relatively low compared to the other two GC conditions. The 2:1 $CHCl_3$/MeOH solvent system samples all measured on average 4.6 ± 0.2% neutral lipids by weight, but when evaluating pigment reduction, the 2x 25 mg GC method was the most efficacious in lessening pigment absorbance. Because it is hypothesized that pigments are a significant source of error in the NRLA, the 2x 25 mg GC treatment was chosen for consequent studies.

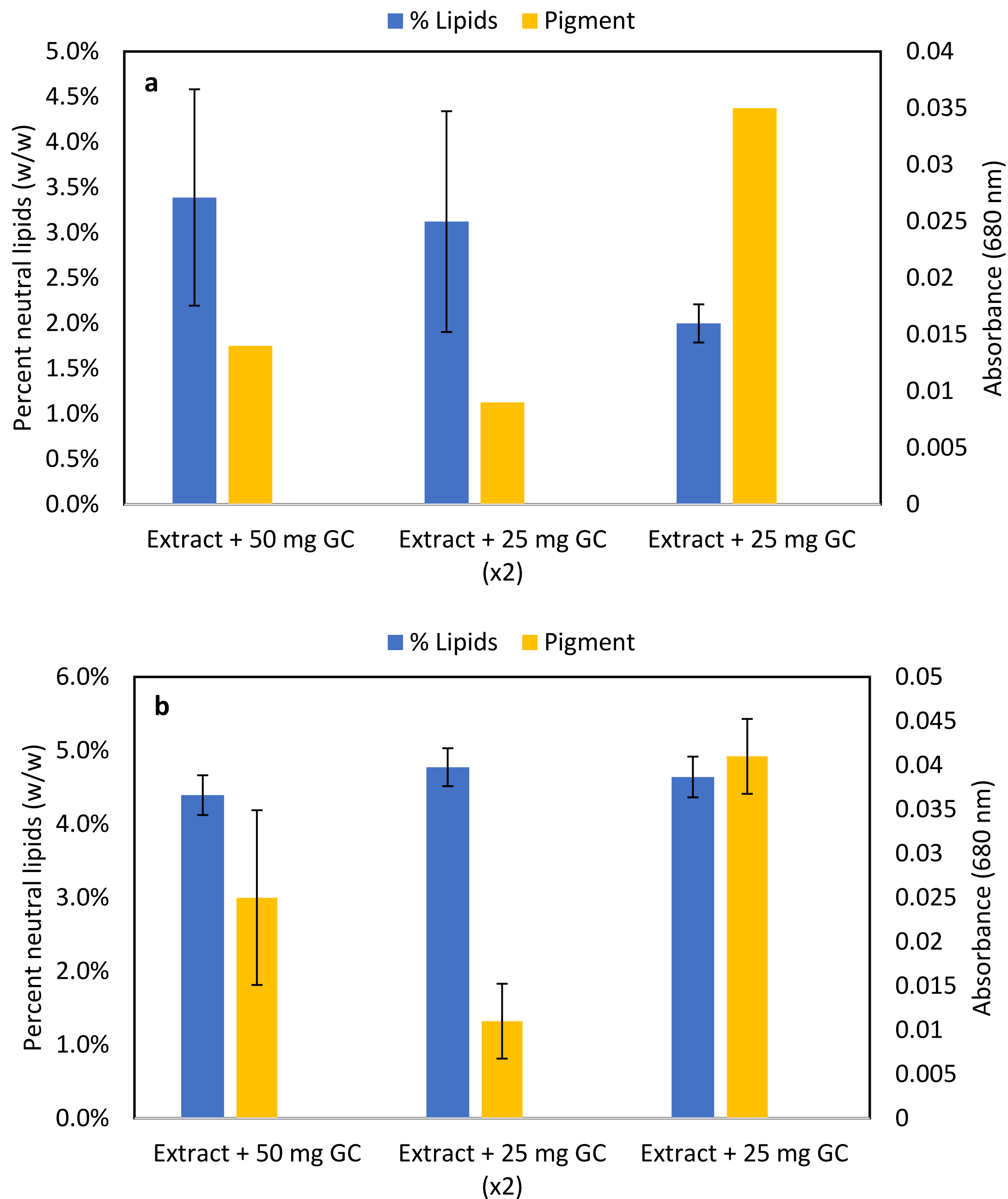

Figure 41. Comparison between neutral lipid quantification and pigment removal in *C. sorokiniana* lipid extracts after being treated with 50 mg graphitized carbon (GC), 25 mg GC, or 25 mg GC twice, consecutively. This comparison was performed on samples extracted with either (a) $CHCl_3$ or (b) 2:1 $CHCl_3$/MeOH.

Table 32. Comparison between neutral lipid content in *C. sorokiniana* lipid extracts extracted with either $CHCl_3$ or 2:1 $CHCl_3$/MeOH solvent and treated with 50 mg graphitized carbon (GC), 25 mg GC, or 25 mg GC twice, consecutively.[a]

Extraction solvent	Treatment Condition	Percent neutral lipids (w/w)
$CHCl_3$[b]	Extract + 50 mg GC	3 ± 1
	Extract + 25 mg GC (x2)	3 ± 1
	Extract + 25 mg GC	2.0 ± 0.2
2:1 $CHCl_3$/MeOH	Extract + 50 mg GC	4.6 ± 0.3
	Extract + 25 mg GC (x2)	4.5 ± 0.3
	Extract + 25 mg GC	4.8 ± 0.3

[a] Values are presented as mean ± SD of multiple measurements of duplicates.
[b] Small volume of extract available, so values are presented as mean ± SD of quadruplicate measurements of single replicates.

The lipid recovery for neutral lipids in *C. sorokiniana* lipid extracts reached a maximum of 56 ± 18% when the extract was treated with 25 mg GC for 30 min (Table 35). The lowest lipid recovery was 32 ± 24%, which was observed for those extracts treated with two rounds of 25 mg GC for 30 min.

With a percent lipid recovery of between 50% and 60%, the actual percent lipid by weight is likely higher, possibly even double that observed in Tables 34 and 35. These studies support the conclusion that 25 mg GC is the best for the pretreatment of MAE extracts. However, the extracts after treatment are still highly pigmented (Figure 42d), and therefore interference could still play a role in the NRLA measurements. Further investigations should be performed to mitigate pigments further or identify other possible sources of error in the procedure/measurements.

Table 33. Comparison of *C. sorokiniana* MAE extracts pretreated with graphitized carbon (GC) the associated percent recoveries and percent neutral lipids by weight.[a]

Assay	Condition	GC used for treatment (mg)	µg lipid in $CHCl_3$ extract	Difference between conditions	% recovery	Percent neutral lipid content (w/w)
5/29/20	Algae + $CHCl_3$	25 (x2)	667 ± 15	66 ± 47	32 ± 24	3.292 ± 0.007
	Algae + Std	25 (x2)	733 ± 45			3.64 ± 0.02
6/2/20	Algae + $CHCl_3$	25	677 ± 31	113 ± 36	56 ± 18	3.39 ± 0.02
	Algae + Std	25	790 ± 18			3.95 ± 0.01
	Algae + $CHCl_3$	50	681 ± 51	100 ± 53	50 ± 27	3.4 ± 0.8
	Algae + Std	50	781 ± 17			3.9 ± 0.3

[a] Values are presented as mean ± SD of triplicates

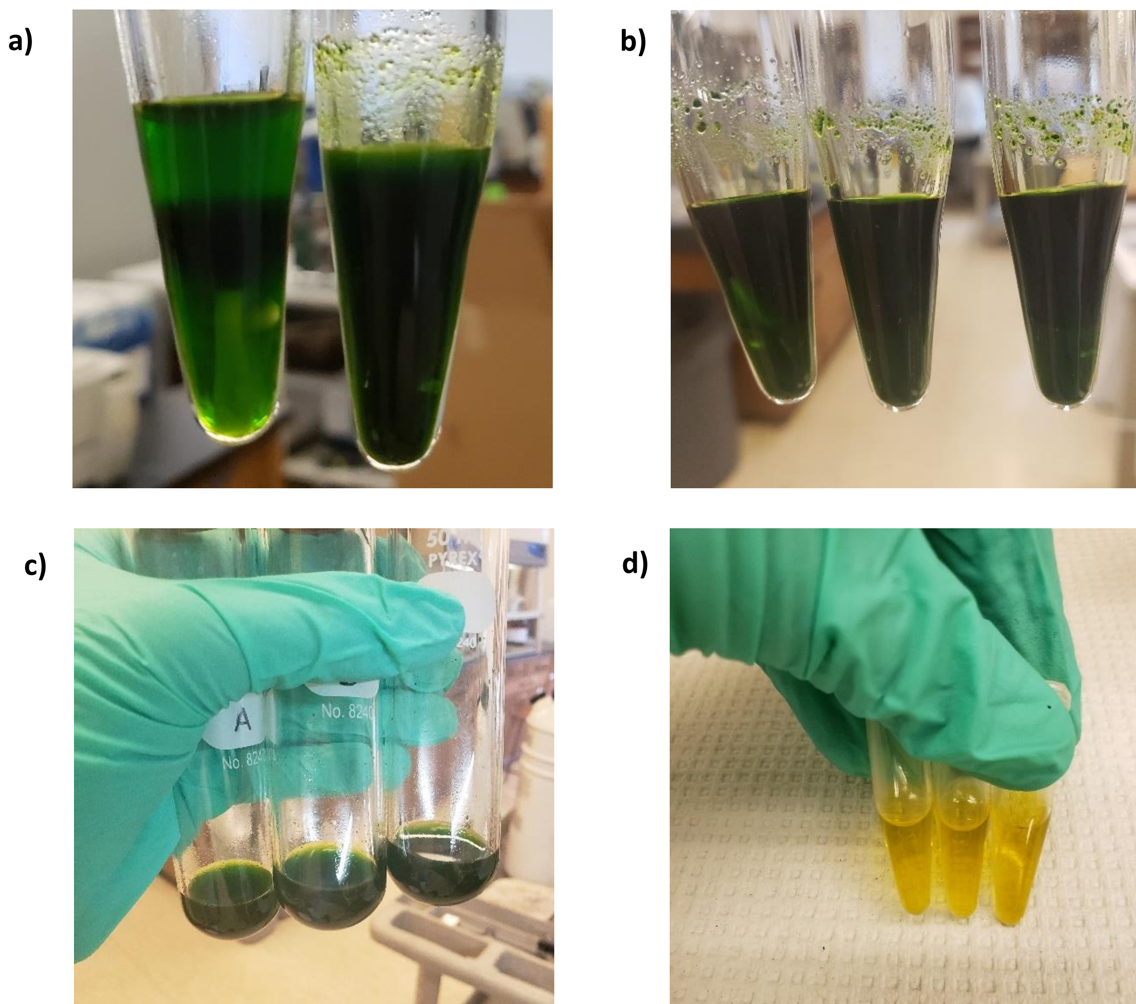

Figure 42. extracts from *C. sorokiniana* biomass extracted using microwave-assisted extraction with a 2:1 $CHCl_3$/MeOH solvent system. The images are in procedural order: (a) extract before MAE (left) and after MAE (right), (b) extracts at N=3 after MAE, (c) extracts after shaking, and (d) extracts after graphitized carbon treatment.

4.4. CONCLUSIONS

These studies demonstrate the positive potential of using a two-phase methodology to increase lipid production in *C. sorokiniana*. Although an increase in *C. sorokiniana* lipid production was observed, the overall lipid yield was low at $< 15\%$. Higher lipids yields were observed overall when cultivating *C. vulgaris* using the two-phase cultivation method.

Resuspension on filtered seawater (SW) appears to be the most efficacious resuspension media for both *C. vuglaris* and *C. sorokiniana*. Future studies should investigate approaches that will further increase lipid production, including utilizing a different resuspension media or media supplementations in the second phase. Hydrogen peroxide addition in the second phase was used to investigate oxidative stress on lipid yield. A minor increase in lipid production was observed in *C. sorokiniana,* but hydrogen peroxide negatively impacted resuspended *C. vulgaris* cultures. Oxidative stress is a common method to increase lipid yields, so this method should be investigated further, optimizing it for individual species.

The bleach-assisted Nile Red assay (NRLA) used to quantify neutral lipids in these microalgae extracts was inaccurate and unreliable. Pigment interference was demonstrated to be a significant contributing factor to the inaccuracy of the NRLA. After reducing pigment concentrations in the extracts using graphitized carbon (GC), the lipid measurements became much more accurate and reliable. A treatment duration of 30 min with 25 mg GC/mL extract was determined to be optimal for *C. sorokiniana* extracts. This pretreatment was used in subsequent studies to reduce pigment interference. Further optimization should be performed to decrease the increased error observed between technical replicates in the NRLA after treatment.

Sonication is a standard lysing method used to extract neutral lipids from microalgae.[23] However, this method was ineffective in lysing *Scenedesmus acutus f. alternans* or *S. obliquus*, prompting studies into different extraction methods. Bead beating was tested alongside sonication but afforded significantly lower lipid recoveries than sonication, so it was determined to be an ineffective alternative extraction method. Microwave-assisted extractions

were evaluated but also yielded low lipid recoveries. These MAE extracts were highly pigmented, so I hypothesize that these pigments may be a recurrent interfering factor. Different GC treatments were utilized, but the extracts were still pigmented after all treatments. Different carbon allotropes such as nanotubes should be investigated to determine their pigment reduction potential. MAE is the most promising extraction method alternative to sonication and warrants further optimization studies.

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APPENDIX

Table A1. Days at which each microalgae strain and condition reached the specified stage of cultivation (exponential phase, stationary phase, harvested). The growth rate was calculated between the designated beginning of exponential phase and the designated beginning of stationary phase.

Strain	Condition	Exponential phase (d)	Stationary phase (d)	Harvested (d)
Chlorella sorokiniana	10% FWP	0	15	25
	30% SFWP	4	15	25
	BG-11	4	15	25
Chlorella vulgaris	10% FWP	3	13	22
	30% SFWP	3	38	48
	JW	0	6	17
Scenedesmus acutus f. alternans	10% FWP	0	17	25
	30% SFWP	4	25	28
	BG-11	0	8	17
Scenedesmus obliquus	10% FWP	0	19	29
	30% SFWP	0	31	41
	BG-11	0	8	18

Table A2. Summary of nutrient concentration and nutrient remediation for permeate used for each strain and condition.[b] nd = below detection limit

			Inorganic nutrients (mg/L)				
			Nitrates	TN	NH_3-N	Phosphates	Sulfates
C. sorokiniana	10% FWP	Initial	2 ± 3	228 ± 16	186 ± 13	11 ± 4	7 ± 1
		Final	5.1 ± 0.6	82 ± 48	44 ± 46	nd	0.4 ± 0.7
		% Remediation	-209 ± 36	64 ± 21	76 ± 25	> 95	99.7 ± 0.4
	30% SFWP	Initial	9 ± 10	108 ± 3	62 ± 17	57 ± 1	11.9 ± 0.2
		Final	7.6 ± 0.6	18 ± 3	0.5 ± 0.3	nd	nd
		% Remediation	16 ± 7	83 ± 3	99.1 ± 0.5	> 95	> 95
	Syn media	Initial	1273 ± 165	268 ± 79	n/a	61 ± 4	15 ± 3
		Final	nd	152 ± 8	n/a	nd	14.4 ± 0.6
		% Remediation	> 95	43 ± 3	n/a	> 95	7 ± 4
C. vulgaris	10% FWP	Initial	4 ± 4	201 ± 4	172 ± 6	13 ± 8	1.7 ± 0.6
		Final	5.1 ± 0.6	150 ± 44	130 ± 6	nd	0.4 ± 0.7
		% Remediation	-26 ± 15	25 ± 21	24 ± 3	> 95	99.3 ± 0.6
	30% SFWP	Initial	15 ± 6	113 ± 29	28 ± 4	26 ± 14	5 ± 2
		Final	7.6 ± 0.6	nd	6 ± 4	nd	nd
		% Remediation	48 ± 4	>95	78 ± 15	> 95	> 95
	Syn media	Initial	62 ± 4	15.7 ± 0.6	n/a	17 ± 1	14 ± 3
		Final	nd	1 ± 2	n/a	nd	14.4 ± 0.6
		% Remediation	> 95	89 ± 10	n/a	> 95	-0.08 ± 4
S. acutus f. alternans	10% FWP	Initial	9.0 ± 0.2	263 ± 34	237 ± 33	8 ± 1	1 ± 1
		Final	111 ± 5	252 ± 11	197 ± 2	nd	nd
		% Remediation	-1138 ± 59	4 ± 4	17.0 ± 0.8	> 95	> 95
	30% SFWP	Initial	13 ± 9	76 ± 28	81 ± 7	40 ± 28	2 ± 1
		Final	115 ± 4	69 ± 6	15 ± 1	3 ± 3	n/a
		% Remediation	-809 ± 30	9 ± 8	82 ± 1	93 ± 7	> 95
	Syn media[a]	Initial	n/a	n/a	n/a	n/a	n/a
		Final	17.5 ± 0.8	180 ± 12	n/a	5 ± 1	0.1 ± 0.2
		% Remediation	n/a	n/a	n/a	n/a	n/a
S. obliquus	10% FWP	Initial	n/a	220 ± 53	161 ± 10	8.8 ± 0.2	7 ± 3
		Final	109.3 ± 0.3	153 ± 6	88 ± 3	nd	nd
		% Remediation	n/a	31 ± 3	45 ± 2	> 95	> 95
	30% SFWP	Initial	14 ± 7	135 ± 7	82 ± 3	51 ± 8	11.6
		Final	128 ± 7	52 ± 7	10.1 ± 0.4	20 ± 2	0.1 ± 0.2
		% Remediation	-817 ± 50	61 ± 5	92 ± 7	61 ± 5%	99 ± 1
	Syn media	Initial	1317 ± 147	270 ± 10	n/a	53 ± 36	28.2
		Final	840 ± 9	178 ± 0	n/a	13.9 ± 0.9	13.9 ± 0.9
		% Remediation	36.2 ± 0.7	34 ± 1	n/a	74 ± 2	51 ± 3

[a] Supernatant was lost when harvesting, so analyses could not be performed; therefore, the data is not shown.

[b] Values are presented as a mean ± SD of triplicates.

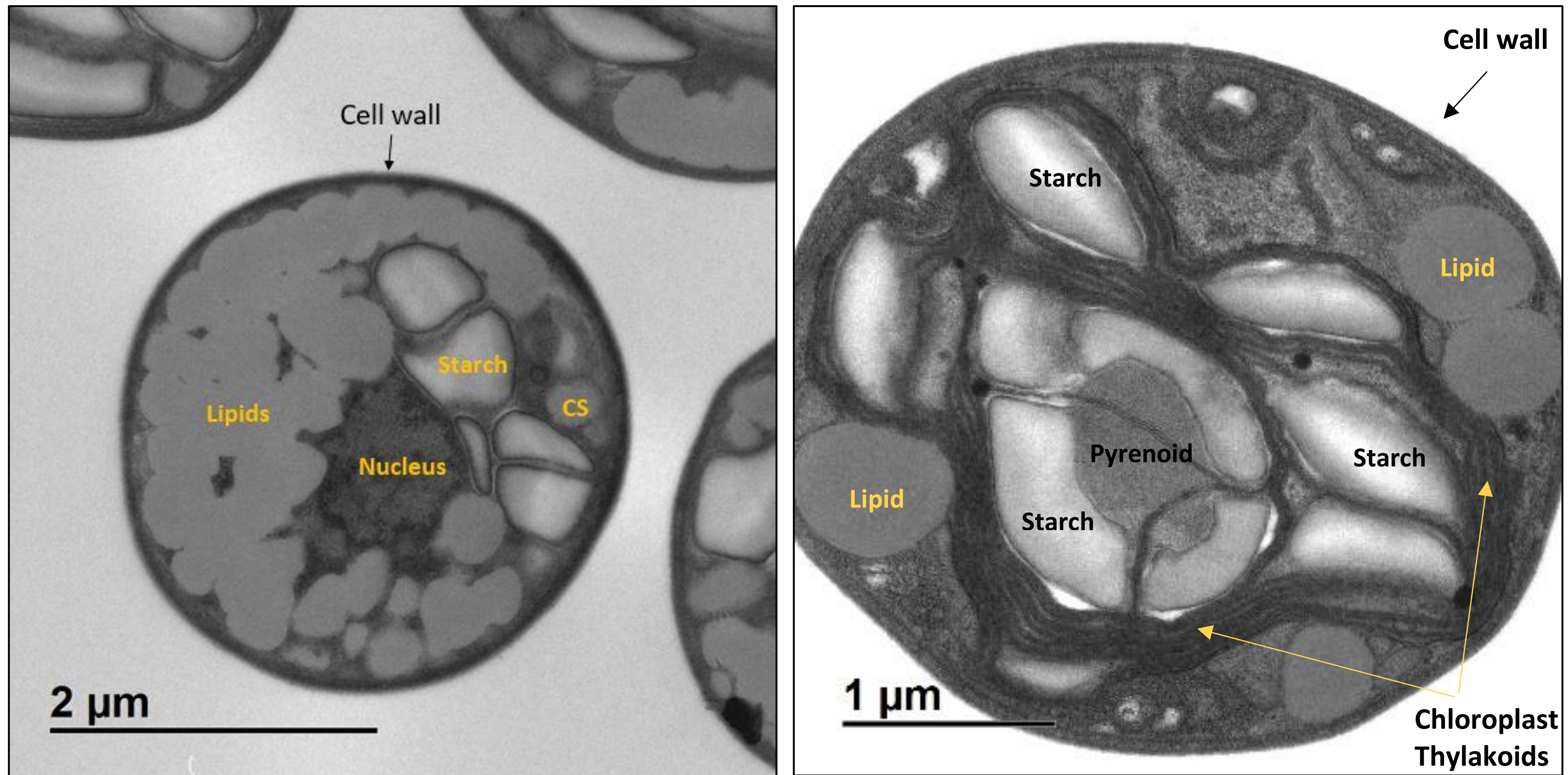

Figure A1. Ultrastructure identification in two *C. sorokiniana* cells imaged using transmission electron microscopy (TEM). CS = cytoplasmic storage material