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# Algal Wastewater Treatment and Biofuel Production: An assessment of measurement methods, and impact of nutrient availability and species composition

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Algal Wastewater Treatment and Biofuel Production: An assessment of measurement methods, and impact of nutrient availability and species composition

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**Abstract**

In order to move towards a more sustainable future, human civilization will need to decrease its impacts on air and water quality and minimize consumption of fossil fuels. Using algae for wastewater treatment and biofuel production offers one approach to progress towards these goals simultaneously. Algae are capable of removing nutrients from wastewater and carbon dioxide from the air, and the biomass formed in that process can be used to generate a wide-range of products including fertilizer or fuels that could power combustion engines, power plants, or zero-emission fuel cell vehicles. Using wastewater for biofuel production has the potential to lower biofuel costs and environmental impacts as it provides an existing, free source of nutrients and water. Growing algae during the wastewater treatment process further improves the effluent quality, thus helping prevent eutrophication and costly dead zones in downstream ecosystems.

Maximizing the benefits of algal wastewater treatment and biofuel production will require optimizing total nutrient removal, biomass production, and lipid content of the biomass; a complicated problem given that many algal species known for high nutrient removal and lipid production are easily suspended single-celled microalgae that are difficult to harvest efficiently by gravity. In wastewater treatment, growing pure cultures of single-species algae is not practical, so positive and negative impacts of growing algae in the presence of endogenous microbial communities should be evaluated.

This dissertation seeks to move algal wastewater treatment forward by improving our understanding of the roles of species composition and nutrient availability on nutrient removal, lipid and biomass production, and settling; and by assessing alternative methods used to quantify algal productivity in order to improve future research. In comparison with traditional

quantification methods, alternative methods investigated provided reliable results for a range of algal species and concentrations, with significantly less sample volume, reagents and processing time. For the nutrient and species experiments, *Chlorella vulgaris* was grown in real and synthetic wastewater and in combination with other algal species and endogenous wastewater microbial communities. Multiple synergistic effects were observed from mixing species with implications that could improve both secondary and tertiary treatment of wastewater while providing biofuel feedstocks.

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**List of Abbreviations**

AI – Autotrophic Index

APHA - American Public Health Association

BOD - Biochemical Oxygen Demand

COD – Chemical Oxygen Demand

*C. vulgaris – Chlorella vulgaris*

DBP – Disinfection Byproduct

DDI - Distilled De-Ionized

DO – Dissolved Oxygen

EPS - Extracellular Polymeric Substances

IC – Ion Chromatography

LCA – Life-cycle Analysis

N – Nitrogen

N:P – Nitrogen to Phosphorus Ratio

P - Phosphorus

*S. dimorphus – Scenedesmus dimorphus*

*S. epidermidis – Staphylococcus epidermidis*

SS – Suspended Solids

TN – Total Nitrogen

TP – Total Phosphorus

WWTP – Wastewater Treatment Plant

**List of Papers**

**Chapter #1**

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**Chapter #2**

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## Introduction

The term "algae" refers to a diverse group of (mostly) autotrophic, aquatic organisms that are found throughout the world from sea ice in the Arctic to microbiotic crusts in deserts (Larkum et al. 2012, Vymazal 1995). Given their diversity, it is perhaps not surprising that algae present many challenges and potential benefits to human society. Excess growth of algae can both poison our drinking water and clean our wastewater. It can provide dissolved oxygen (DO) to other aquatic species or reduce DO so much that massive fish die-offs occur. People and other organisms eat many species of algae and it forms an important part of the global food web.

## Algae and Biofuel Production

Algae can be used to produce a wide range of biofuels including hydrogen, methane, bioethanol, biodiesel, and simple dried biomass (Pittman et al. 2011, Singh and Olsen 2011). Algal biofuels offer many benefits compared to more traditional biofuel crops since algae grow exponentially, have higher biomass productivity per unit area, and do not directly compete with production of food crops (Pittman et al. 2011). Microalgae are capable of producing many times more oil per acre (30-200X more according to some estimates) than traditional oil-producing terrestrial crops, and can be grown in locations where they do not compete for land with food crops (Smith et al. 2010, Singh et al. 2011, Singh and Olsen 2011). In fact, algae can be grown in buildings and in arid or desert areas using seawater, meaning algal biofuels need not compete with food production for land or water (Singh and Olsen 2011). In terms of $CO_2$ emissions and climate change, current biofuel crops perform poorly and in cases where they replace natural ecosystems or even food crops, they cause the emission of greater amounts of $CO_2$ than they prevent; whereas algae's greater land use and growth efficiencies allow algal biofuels to be

closer to carbon-neutral (Smith et al. 2012) and the carbon intensity of this fuel source will improve as more of the energy that goes into growing, harvesting, and processing algae comes from more sustainable sources.

The processes for generating the various types of algal biofuels range from quite simple to very complex and energy intensive. Combustion of dried biomass simply requires harvesting and drying of algae using heat or the sun, but this form of energy is limited in terms of usefulness. Though optimization of inputs can add complexity, anaerobic digestion is also a fairly simple technology and, in addition to methane, can produce useful co-products that can be utilized for fertilizer or compost, thus recycling key nutrients and offering greater benefits in terms of sustainability (Park and Li 2012, Singh and Olsen 2011). Methane output, both total and relative to $CO_2$ production, can be maximized by careful balancing of lipid, carbohydrate, and protein composition of the material being digested, either by supplementing algal biomass with other feedstocks and lipid sources or by using algae with high lipid content (Park and Li 2012). Biogas production can also be improved using a range of chemical and physical treatment techniques (Singh and Olsen 2011).

Algae offer many benefits over more traditional feedstocks for bioethanol production. They can require less pretreatment than popular plant inputs and some species directly produce ethanol (Singh and Olsen 2011, John et al. 2011). Algal species with high starch and cellulose contents are particularly ideal for ethanol production (Hirano et al. 1997). Algae can also be used to generate hydrogen gas which can be used in fuel cells or combustion engines whose only emission product is water. Growing certain species, like *Chlamydomonas reinhardtii,* and then depriving them of sulfur and $O_2$ can lead to the production of harvestable hydrogen (Melis and

Happe 2001, Singh and Olsen 2011). Gasification of algae involves heating it to high temperature (800-1000 °C) to produce a burnable gas. Due to the high heat requirements, the process typically has an energy balance near 1, but as with anaerobic digestion, the process generates products that can be reused to grow more algae (Singh and Olsen 2011).

The species *Chlorella vulgaris* and *Scenedesmus dimorphus*, which were used in this research, are popular in biofuel production due to their relatively high lipid content compared to other algal species. Under certain conditions *C. vulgaris'* lipid content can account for 70-80% of dry biomass weight (Liang et al. 2009, Held and Raymond 2011). The species is also capable of heterotrophic growth when supplied with a carbon source such as glucose, acetate, or glycerol, allowing it to reach greater cell concentrations and grow in shaded conditions or in the dark (Liang et al. 2009).

**Algae and Wastewater Treatment**

With life-cycle assessments (LCAs) for many algal biofuel processes showing them to have net energy ratios around 1 and perhaps only nearing carbon neutrality (Singh and Olsen 2011), other environmental and economic benefits such as improving WWTP effluent quality, improved downstream fisheries health, and fertilizer production boost the case for algal biofuels being a more sustainable technology than existing popular fuel sources.

Algae have historically been seen as a problem for wastewater treatment. Unicellular microalgae can be difficult and costly to remove and failing to adequately do so can cause problems downstream or lead to the creation of dangerous disinfection byproducts (DBPs) (Coral et al. 2013, Nguyen et al. 2005, Vuuren and Duuren 1965). While algae can have negative impacts on water treatment processes, it has the potential to be used to treat wastewater and

improve WWTP effluent while generating biomass that could be used to produce biofuels (Schumacher et al. 2003, Wang et al. 2010, Sturm et al. 2012, Arbib et al. 2014, Park et al. 2011, Pittman et al. 2011). As far back as the 1970's, researchers were exploring the use of algae to remove nutrients from wastewater. Garrett and Allen found that *C. vulgaris* was capable of removing high percentages (95+%) of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Suspended Solids (SS), Total Phosphorus (TP), and Total Nitrogen (TN) from diluted hog waste slurry (1976).

In wastewater applications, algae could be incorporated into the secondary treatment process or added on as a tertiary, polishing stage. In secondary treatment, oxygen-producing algae could reduce the need for aeration, though optimal solar irradiation may be difficult to achieve under the turbid conditions common at this stage (Humenik & Hanna 1971, Humenik & Hanna 1970). Adding a tertiary treatment process would increase operating costs, though these may be offset by the generation of biofuels or other useful products. Using algae for tertiary treatment allows it to have better access to light, likely improving its ability to remove nutrients remaining following secondary treatment. Algae used in secondary treatment would be settled out with the other biosolids, while algae produced in a tertiary treatment step would require an additional settling/harvesting step (Van Den Hende et al. 2011). In order for this technology to enter mainstream use, issues with harvesting efficiency and maintenance of target algal species in the presence of numerous other microbial species and under fluctuating wastewater conditions will have to be resolved, while ideal species are identified to maximize nutrient removal and generation of useful products.

## Algae Quantification

Whether algae are being studied in the context of drinking water safety, biofuel production, or wastewater treatment, the same general set of methods targeting biomass or chlorophyll are used to quantify algal concentration, growth, and composition. One popular way to quantify algae is by measuring chlorophyll. Traditional methods involve a lengthy, and lethal, extraction process while some alternative methods are capable of quickly measuring chlorophyll inside living cells. Due to its structure and properties, chlorophyll can be measured using autofluorescence or absorbance methods on a variety of instruments (APHA 2005, Held 2011).

Cell counts and concentrations can be determined via microscopy or by using more automated particle counter methods (Krediet et al. 2015). Microscopic counts are time-consuming and require an even distribution of cells across a filter to achieve accurate results. Particle counters can rapidly count many more cells, but unlike microscope counts, the user is not able to directly observe cells to verify counts.

Algal biomass production is of interest for the production of certain biofuels and is commonly quantified using total dried biomass, which can be difficult to accurately assess from small sample volumes. Due to the time required for drying this method cannot be used to provide real time measurements. As mentioned previously, the lipid content of algal biomass is of interest in the production of multiple types of biofuel. Neutral lipids are of particular interest in the generation of biodiesel and their proportion of total lipids present can vary greatly depending on species and growth conditions (Higgins et al. 2014, Hu et al. 2008) providing additional value to the ability to measure neutral lipids specifically and rapidly. Traditional lipid methods involve a time-consuming extraction and drying process. More rapid fluorescence methods utilizing dyes

that target neutral lipids specifically are becoming popular (Mujtaba et al. 2012, Held and Raymond 2011, Chen 2009).

**Research Objectives**

Interest in using algae for biofuel production and wastewater treatment has been growing recently. Given their ability to grow exponentially, algae have the potential to generate more biomass or biofuel per unit area than other popular biofuel crops (Demirbas and Demirbas 2011, Sturm and Lamer 2011). Algae have also shown the ability to remove nitrogen and phosphorus even at relatively low concentrations found in wastewater treatment plant (WWTP) effluent. Given the difficulty treatment plant managers can have in meeting stringent effluent requirements, algae have the potential of providing beneficial tertiary treatment. Recycling nutrients and water from wastewater effluent to grow algae for biofuel production has the potential to boost the environmental and economic sustainability of our treatment plants and energy systems. Chapter 2 explores this concept of using algae for wastewater treatment and assesses what impacts nutrient limitation and nutrient ratios have on overall nutrient removal, algal growth, and lipid production.

These experiments, and the preliminary tests that helped design them, utilized a wide variety of methods to measure algal productivity and growth. Research on the use of algae for water quality enhancement and biofuel production also use a diverse set of methods to measure lipids, biomass, and algal concentration which can make direct comparison of results difficult. For example, lipids can be measured via chloroform/methanol extraction and also via Nile Red staining and fluorescence. Extraction methods alone vary greatly (Li et al. 2011, Mujtaba et al. 2012, Liang et al. 2009, Woertz et al. 2009) and it is difficult to guarantee only lipids have been

extracted, while Nile Red fluorescence focuses in on neutral lipids only and is also performed differently between publications (Held and Raymond 2011, Chen 2009, Higgins et al. 2014). Chapter 1 compares alternative and traditional methods for measuring cell concentration, lipid content, and chlorophyll concentration in algal samples based on precision, comparability, time requirements, sample volumes, and chemical consumption.

While algae can assist in nutrient removal at treatment plants, they can also be difficult to efficiently remove from treated water. Effective, energy efficient removal of algae is critical for protecting downstream water quality and making the concept of algae for wastewater treatment and biofuel production economically viable and less energy intensive. Chapter 3 expands on the work done in Chapter 2 to look at settling, nutrient removal, and biomass and lipid production by single and mixed species of algae and bacteria and it explores the possibility of using these species earlier in the treatment process when nutrient concentrations are much higher. The species used were *Chlorella vulgaris*, *Staphylococcus epidermidis*, a *Lyngbya* sp. dominated consortium isolated from a hog waste lagoon (Savoy, AR), and activated sludge from a local WWTP (West Side Wastewater Treatment Facility, Fayetteville, AR). *C. vulgaris* was chosen for its well-studied high lipid content and use in biofuel production (Arbib et al. 2014, Liang et al. 2009, Liu et al. 2008). *S. epidermidis* was used due to its ability to produce extracellular polymeric substances (EPS) and aid in formation of biofilms and flocs and due to its presence on humans and in wastewater (Bitton 2005, Evans et al. 1994). The *Lyngbya* sp. dominated consortium was chosen due to its filamentous nature, its presence in a high-nutrient concentration hog waste lagoon, and for its ability to form attached growths and large flocs.

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## Chapter 1 - Assessment of Alternative Quantification Methods for Biomass, Chlorophyll, and Lipids in Microalgae


### Abstract

Algal research in the field of biofuel production and wastewater treatment is growing in popularity. Traditional quantification methods such as cell count, chlorophyll *a* measurement and lipid extraction are widely used, however, most requires long processing time and the accuracy is highly dependent on the algae concentration measured. This study seeks to assess alternative methods including Coulter counter and fluorescence measurement for chlorophyll and neutral lipids, by directly comparing with the traditional methods in terms of accuracy, time and sample size required, and the use of hazardous materials. Microalgae of different morphologies were used, and correlations between the alternative and traditional methods were provided. The results showed the alternative methods tested can produce reliable results over a wide range of algae concentration, with faster speed and smaller sample volume. The adoption of these methods will benefit algal research in multiple applications.



**Keywords:**

Microalgae, Chlorophyll, Lipids, Cell Count, Quantification Methods


### 1. Introduction

Microalgae have been extensively studied for biofuel production, wastewater treatment, drinking water source quality/taste and odor issues, and geoengineering (Arbib 2014, Lam & Lee 2013). Despite the specific application, biomass and chlorophyll concentrations are important parameters indicating algal growth, monitored in almost all algal systems. In addition, lipid content and biomass production are important characteristics to measure when microalgae are

cultivated for biofuel conversion. For example, numerous studies have identified species of green algae that are capable of developing lipids exceeding 40% of their total mass for biodiesel extraction (Mata et al. 2010); algal biomass is also explored to produce biogas via methanogenic bacteria (Beer et al. 2009). In other cases where algae removal is required (such as in drinking water treatment), microscopy examination is often performed to confirm the presence of algae (Lam et al. 1995). Due to the prevalence of applications involving microalgae, quantification methods for algal biomass, chlorophyll, and lipid content is of great importance. The current practice for these quantifications include total dried biomass, chlorophyll *a* extraction and measurement, and lipid extraction, respectively.

Chlorophyll *a* extraction and measurement is the most widely used quantification method for algae quantification. But the standard extraction process takes multiple steps and requires the use of hazardous chemicals like acetone and hydrochloric acid (APHA 2005). Total dried biomass is easy to perform, but the accuracy suffers when the sample is low in volume or concentration. In addition, real time measurement is impossible to achieve due to the physical process such as filtration and drying. Lipid extraction is a multi-step procedure including separation, extraction, drying, and weighing. It is time-consuming, requires large sample volumes, and involves the use of chloroform (Mujtaba et al. 2012). Other quantification method such as light microscopy requires sample fixation and tedious counting, which is also time-consuming. Due to these limitations, researchers have started to seek alternative methods that are rapid and reliable, such as fluorescence measurement as surrogates for chlorophyll and lipid concentration (Held 2011, Held & Raymond 2011), and automated particle counts (Krediet et al. 2015) for algal cells.

Fluorescence can be utilized as a promising quantification method for microalgae. Autofluorescence of chlorophyll has long been established, and used to analyze phytoplankton communities (Booth 1987). However, this technique was not frequently used to quantify chlorophyll in microalgae, mostly due to the difficulty in fluorescence quantification. Lipid content can also be assessed using fluorescence. Nile red fluorescence of neutral lipids has been used sparingly in algal research, and is more established on certain species such as *Chlorella vulgaris* (Huang et al. 2009, Higgins et al. 2014). However, this method was rarely tested on other species, and often not used independently. Automated particle counters such as Coulter counter were used to quantify algal biomass in the 1970s, but the method was never widely adopted due to the inability to differentiate biological and inorganic particles. Recent development on instruments such as microplate reader and new models of Coulter counter enabled the re-emergence and improvements of these methods. The advantages of these alternative methods include easy to perform, small sample size, and rapid process. However, the accuracy and reliability is largely unknown on difference species of microalgae, which hinders the wide adoption of these quantification methods.

This study seeks to assess these emerging methods by compare with existing methods for quantifications of biomass, chlorophyll, and lipid content of microalgae in terms of accuracy, repeatability, ease of operation, and time and materials required. The alternative methods include automated particle count for algal cells, and fluorescence measurement for chlorophyll and lipid content. These methods will be assessed using three algal species representing different morphology, including *Chlorella vulgaris*, *Scenedesmus dimorphus*, and a consortium of species isolated from a hog waste lagoon dominated by *Lyngbya sp*. *C. vulgaris* is a round, single-celled green alga; *S. dimorphus* is a unicellular alga that has an elongated shape and tends to aggregate

together; *Lyngbya* consortium are comprised of filamentous cyanobacteria *Lyngbya sp.* and *Leptolyngbya sp*. Both *C. vulgaris* and *S. dimorphus* have been studied for their lipid production capabilities (Arbib et al. 2014, Shen et al. 2009, Liang et al. 2009, Liu et al. 2008), and *Lyngbya sp.* is proven to successfully sustain growth within high strength wastewater (Rana et al. 2014, Sood et al. 2015). Therefore, the results from this study can directly benefit algal research and industries with multiple applications.

## 2. Materials and Methods

### *2.1 Algal Species*

*C. vulgaris* and *S. dimorphus* were purchased on agar slants from UTEX algae center (UTEX number: 2714 and 1237, respectively) at the University of Texas at Austin (Austin, Texas) and maintained in Bristol media ($NaNO_3$ (25 mg $L^{-1}$), $CaCl_2.H_2O$ (2.5 mg $L^{-1}$), $MgSO_4.7H_2O$ (7.5 mg $L^{-1}$), $K_2HPO_4$ (7.5 mg $L^{-1}$), $KH_2PO_4$ (17.5 mg $L^{-1}$), NaCl (2.5 mg $L^{-1}$), Peptone (1 g $L^{-1}$)) at 26°C. Liquid cultures of both species were periodically isolated on agar plates prepared in the same media to ensure the purity of the strain. The *Lyngbya* dominated mix was isolated from a hog waste lagoon (Savoy, Arkansas) and identified by the UTEX algae center. The *Lyngbya sp.* were maintained in BG-11 media ($NaNO_3$ (1.5 g $L^{-1}$), $K_2HPO_4$ (0.04 g $L^{-1}$), $MgSO_4 \cdot 7H_2O$ (75 mg $L^{-1}$), $CaCl_2 \cdot 2H_2O$ (36 mg $L^{-1}$), Citric acid (6 mg $L^{-1}$), Ferric ammonium citrate (6 mg $L^{-1}$), EDTA (disodium salt) 1 mg $L^{-1}$), $Na_2CO_3$ (0.02 g $L^{-1}$), $H_3BO_3$ (2.86 mg $L^{-1}$), $MnCl_2 \cdot 4H_2O$ (1.81mg $L^{-1}$), $ZnSO_4 \cdot 7H_2O$ (0.222 mg $L^{-1}$), $NaMoO_4 \cdot 2H_2O$ (0.39mg $L^{-1}$), $CuSO_4 \cdot 5H_2O$ (79 µg $L^{-1}$), $Co(NO_3)2 \cdot 6H_2O$ (49.4 µg $L^{-1}$)) also at 26°C. The pH of growth media used was adjusted to an initial pH of 7.0 using hydrochloric acid or sodium hydroxide. The liquid

algae cultures were maintained in 1 L glass bottles on stir plates with continuous illumination at 150 µmol $s^{-1}$ $m^{-2}$.

*2.2 Sample Preparation*

Liquid cultures of all three species were allowed to grow for one week to reach the exponential growth phase. *C. vulgaris* and *S. dimorphus* reached the optical density (OD) of 0.28-0.30 at 680 nm on a spectrophotometer (Beckman Coulter DU720 UV/VIS Spectrophotometer, Beckman Coulter Inc., Brea, CA) after one week. The *Lyngbya* consortium was not monitored for OD due to their filamentous nature. The biomass concentration of *C. vulgaris*, *S. dimorphus*, and *Lyngbya sp.* reached 89±1.2, 151±7.6, and 88±15 mg $L^{-1}$ respectively at the end of one week. Dilutions were created with blank growth media for each species to acquire six known algal concentrations, referred to as "relative concentration" herein (1.0, 0.5, 0.2, 0.1, 0.02 and 0.01). The conversion between relative concentrations and actual biomass concentrations is shown in Table 1. These dilutions are used in the following analyses.

***Table 1:*** *Conversion between biomass concentrations and relative concentrations for the three algal species.*

| **Species** | **Biomass (mg $L^{-1}$) in Dilutions (Relative Concentration)** | | | | | |
|---|---|---|---|---|---|---|
| | **1.0** | **0.5** | **0.2** | **0.1** | **0.02** | **0.01** |
| *C. vulgaris* | 89±1 | 45±1 | 17±3 | 11±1 | 5±3 | 2±3 |
| *S. dimorphus* | 151±8 | 79±5 | 31±4 | 14±3 | 1±4 | 3±2 |
| *Lyngbya sp.* | 88±15 | 43±3 | 18±2 | 9±7 | -1±3 | 2±2 |

*2.3 Sample Analysis*

Algae dilutions were measured for cell count, chlorophyll and lipid concentration. A summary of methods compared is listed in Table 2. For each method tested, average values were obtained from triplicate samples at each relative concentration. Paired T-tests were performed on results

from methods compared, and standard deviations were calculated using Microsoft Excel (Microsoft, Redmond, WA). Linear regression is fitted on all data and presented with the coefficient of determination $R^2$.

***Table 2:*** *A summary of quantification methods compared in this study.*

| Parameter | Method | Instrument | Reference |
|---|---|---|---|
| Cell Count | Particle count | Beckman Multisizer 4 | Zhang et al. 2014 |
| | Standard Method 10200-F | Nikon Eclipse Ni-E upright microscope | APHA 2005 |
| Chlorophyll* | Standard Method 10200-H | Beckman Coulter UV/Vis spectrophotometer | APHA 2005 |
| | Standard Method 10200-H | Turner Designs fluorometer | APHA 2005 |
| | Autofluorescence | Biotek H1 microplate reader | Held 2011 |
| Lipids | Chloroform extraction | Centrifuge/sonicator/oven | Mujtaba et al. 2012 |
| | Nile Red fluorescence | Biotek H1 microplate reader | Held & Raymond 2011 |

*2.3.1 Cell Counts*

Cell counts were performed on *C. vulgaris* and *S. dimorphus*, but not on the *Lyngbya* consortium due to their filamentous morphology. Cell counts were performed using both Coulter counter and light microscopy, and the procedures are briefly described below.

For Coulter counter measurement, a 100 µm aperture tube was used on a Multisizer 4 Coulter Counter (Beckman Coulter, Brea, CA) to capture particles with a diameter between 2 and 60 µm. One hundred µL samples were pipetted into accuvettes (Beckman Coulter, Brea, CA) and diluted with Isotone II solution to a final volume of 10 mL. An analytical volume of 75 µL was drawn for measurement, and a particle distribution was calculated automatically by the

instrument software. Total numbers per mL were calculated by summing the particle counts ranging from 2.5 to 15µm. These size limits were chosen to include single algal cells and possible aggregates.

For microscopy counts, samples ranging from 1-3 mL (depending on relative concentration) were filtered through Whatman 0.2 µm nucleopore membrane filters (GE Healthcare Life Sciences, Buckinghamshire, U.K.), allowed to vacuum dry, and then mounted to slides for counting. Standard Method 10200-F was followed for this procedure and calculation (APHA 2005). The average of cells counted from 5 areas was recorded for each sample and used to calculate total cells/mL.

*2.3.2 Chlorophyll measurements*

Chlorophyll was measured on all three algal species using the three methods indicated in Table 2. The chlorophyll *a* extraction and measurements were performed according to Standard Method 10200-H (APHA 2005). Following filtering and acetone extraction, samples were analyzed via both fluorometer (acid-corrected) and spectrophotometer (uncorrected) for chlorophyll *a* concentrations.

For chlorophyll autofluorescence measurement, 100 µL sample aliquots were pipetted into black-sided clear bottomed 96-well plates (Corning 3603, Corning, Tewksbury, MA) and then measured on a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc., Winooski, VT). Chlorophyll autofluorescence was measured using excitation at 440 nm and emission at 685 nm for chlorophyll *a* concentration (Held 2011).

*2.3.3 Lipid Measurements*

The lipid content of all three species was measured using the two methods indicated in Table 2. Lipid extraction and measurement was performed using a modified Bligh and Dyer method described in Mujtaba et al. (2012). The procedures include: 50 mL samples were centrifuged at 1,600g for 15 minutes and supernatant removed. The algal pellet was resuspended in 1 mL deionized distilled water, and 4 mL chloroform/methanol/water mixture (1/2/0.8, v/v/v) was added before a series of sonication and vortexing. The extracted portion was dried in an oven overnight prior to being weighed.

For fluorescence measurement, neutral lipids were measured on the microplate reader following methods described in Held & Raymond (2011). After the sample was measured for chlorophyll autofluorescence, 100 µL of 2× working solution (1 µg/mL) of Nile Red (Enzo, Ultra Pure, Farmingdale, NY) was added to each well and allowed to incubate in the dark for 10 minutes before measuring the fluorescence at 530 nm/570 nm (excitation/emission wavelength); the amount of fluorescence collected corresponds to the amount of neutral lipids inside the algal cells.

## 3. Results and Discussion

### *3.1 Cell counts*

Counting cell numbers in liquid algae culture is a direct method for algae quantification. In this study, particle count using a Coulter counter was compared with traditional cell count using light microscopy. According to the light microscopy analysis, cell size for *C. vulgaris* ranges from 2.5 – 8 µm, and *S. dimorphus* (which is more ellipsoidal) ranges from 2.5 – 13 µm. The length of filamentous *Lyngbya sp.* can extend up to hundreds of µm, thus this species is not suitable for cell count using either method. As a result, the inclusion size range for Coulter

counter measurement was determined to be 2.5-10 µm for *C. vulgaris*, and 2.5-15 µm for *S. dimorphus*.

Figure 1 shows a comparison of cell counts from the Coulter counter and light microscopy. While these two methods resulted in cell numbers within an order of magnitude of each other, they yielded significantly different results for *S. dimorphus* ($p = 0.04$), but not for *C. vulgaris* ($p = 0.47$). The lower count from Coulter counter for *S. dimorphus* indicates a potential strength of the microscopy method. This species (and others like *Pediastrum*) often forms aggregates/colonies of two or more cells (Graham and Wilcox 2000). These aggregates were counted as a single (larger) particle on the Coulter counter, but as multiple cells via microscopy. In addition, the standard deviation for microscopy counts was consistently larger than those from the Coulter counter in both species, indicating a more stable and consistent measurement using the Coulter method. As a result, Coulter counter is concluded to work best for counting non-colony forming unicellular algae. These findings match those of Maloney et al. who concluded that Coulter counters were "well suited for the counting of spherical unicellular algae in pure culture" and "much less tedious than the conventional microscopic methods" (1962). The lower count on *S. dimorphus* is consistent with a much more recent paper by Krediet et al., which also noted the potential issue of undercounting due to clumping (2015). However, this problem can be overcome by the use of detergent and homogenization prior to Coulter measurement (Krediet et al. 2015).

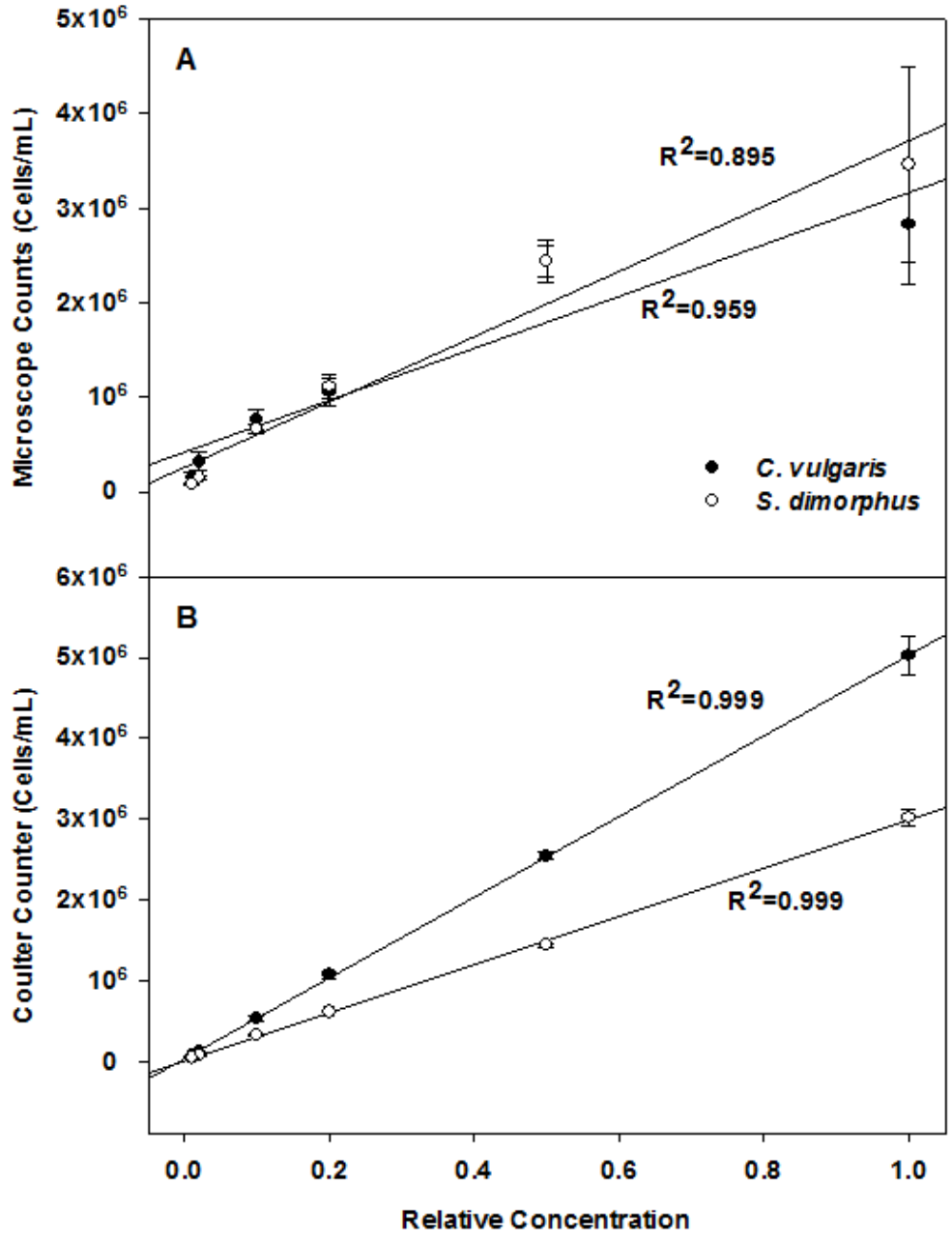


***Figure 1:*** *Cell count results via (A) light microscopy and (B) Coulter counter. Relative concentration represent dilutions of known biomass concentration, and conversion can be found in Table 1.*

*3.2 Chlorophyll measurements*

Chlorophyll is a widely used indirect measurement for algae concentration. There are many types of chlorophyll, related accessory pigments and bacteriochlorophylls. For example, chlorophyll *a* is the most widespread in photoautotrophs; chlorophyll *b* is found mostly in plants; chlorophyll *c1* and *c2* are found in algae, and chlorophyll *d* and *f* are found in cyanobacteria. All forms of chlorophyll feature chlorin or protoporphyrin rings with magnesium ions in the center, but they vary in structure which produces differences in absorbance and fluorescence spectra (Douglas et al. 2003).

Chlorophyll *a* extractions were performed on both a spectrophotometer (uncorrected) and fluorometer (acid correction). Acid correction is used to detect and subtract interference from pheophytin, a natural degradation product of chlorophyll. While both produced comparable results (p=0.23, 0.57, 0.11 for *C. vulgaris*, *S. dimorphus*, and *Lyngbya* sp. respectively), the fluorometer, which factored in acid correction and automatically computes concentrations, occasionally produced negative chlorophyll *a* results due to perceived high pheophytin values. For the discussion below, the results from the fluorometer (acid-corrected) are used.

Chlorophyll *a* concentrations obtained from the Standard Method and the autofluorescence measurement for all three species are shown in Figure 2. Linear relationships were established for all three species for both methods ($R^2 > 0.94$). However, the standard deviations for the Standard Method were consistently larger than those for the autofluorescence method. The level of autofluorescence varied with species. Chlorophyll *a* was produced at $(1.23\pm0.13) \times 10^5$ RFU per mg of dried biomass in *C. vulgaris*, $(7.48\pm0.60) \times 10^4$ RFU per mg of dried biomass in *S. dimorphus*, and $(4.16\pm0.92) \times 10^3$ RFU per mg of dried biomass in *Lyngbya*

*sp*. Among these algal species with different morphology, chlorophyll concentration from the *Lyngbya* consortium measured the lowest using both methods. This may be due to the fact that *Lyngbya* is a cyanobacteria, which have a different balance of photosynthetic pigments than Chlorophyll *a* in green algae (Douglas et al. 2013). A later wavelength scan revealed the optimal excitation/emission wavelength for chlorophyll autofluorescence of this particular species was 430/692 nm. Though slightly varies from the original wavelength for chlorophyll a (440/685 nm), the fluorescence signal collected yielded $(6.51 \pm 0.13) \times 10^4$ RFU per mg of dried biomass. This shows the flexibility of the autofluorescence method in assessing chlorophyll in algae, as adjustment in wavelength pairs can be made quickly to prevent underestimation. Nonetheless, the results indicate autofluorescence measurement for chlorophyll was a good substitute for the traditional chlorophyll *a* extraction method. Given the time required to perform the Standard Method, chlorophyll autofluorescence can be a fast and nondestructive alternative for algae quantification. However, the Standard Method is still valuable for relating relative fluorescence signal to actual concentration.

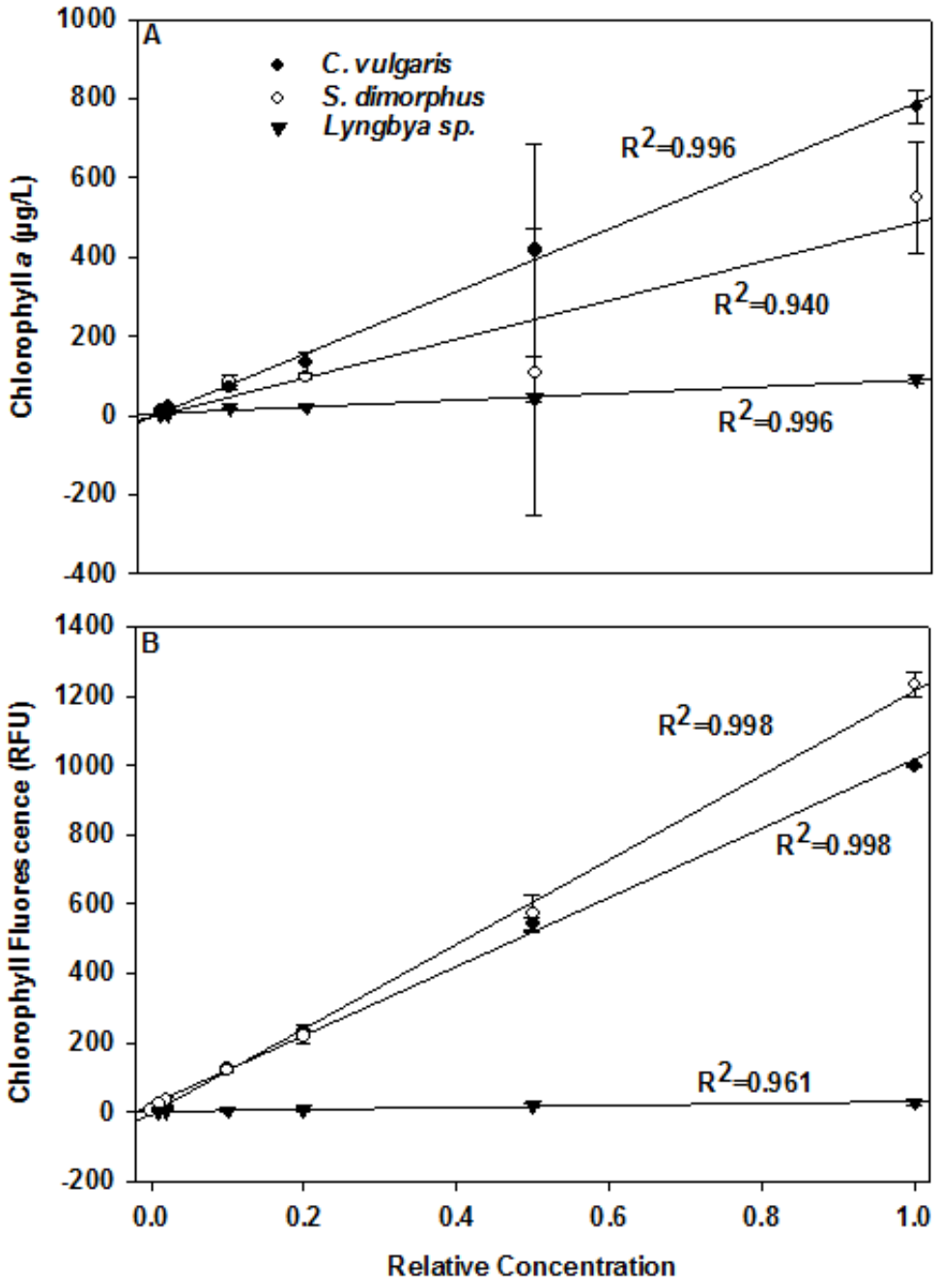


***Figure 2:*** *Chlorophyll a measurement by (A) Standard Method (acid-corrected) and (B) autofluorescence. Relative concentration represent dilutions of known biomass concentration, and conversion can be found in Table 1.*

*3.3 Lipids measurements*

Microalgae can be processed in many ways to produce different types of biofuels. Algal lipid extraction is often used to generate biodiesel, while biomass is commonly used to produce methane gas. Neutral lipids like triacylglycerol esters, which make up a highly variable fraction of the total lipids in algae depending on environmental conditions, are particularly useful in biodiesel production. This session compared the two methods for measuring lipids in algae: (1) traditional chloroform/methanol extraction, and (2) a fluorescence method using the Nile Red stain that binds to neutral lipids. The results of this comparison are shown in Figure 3.

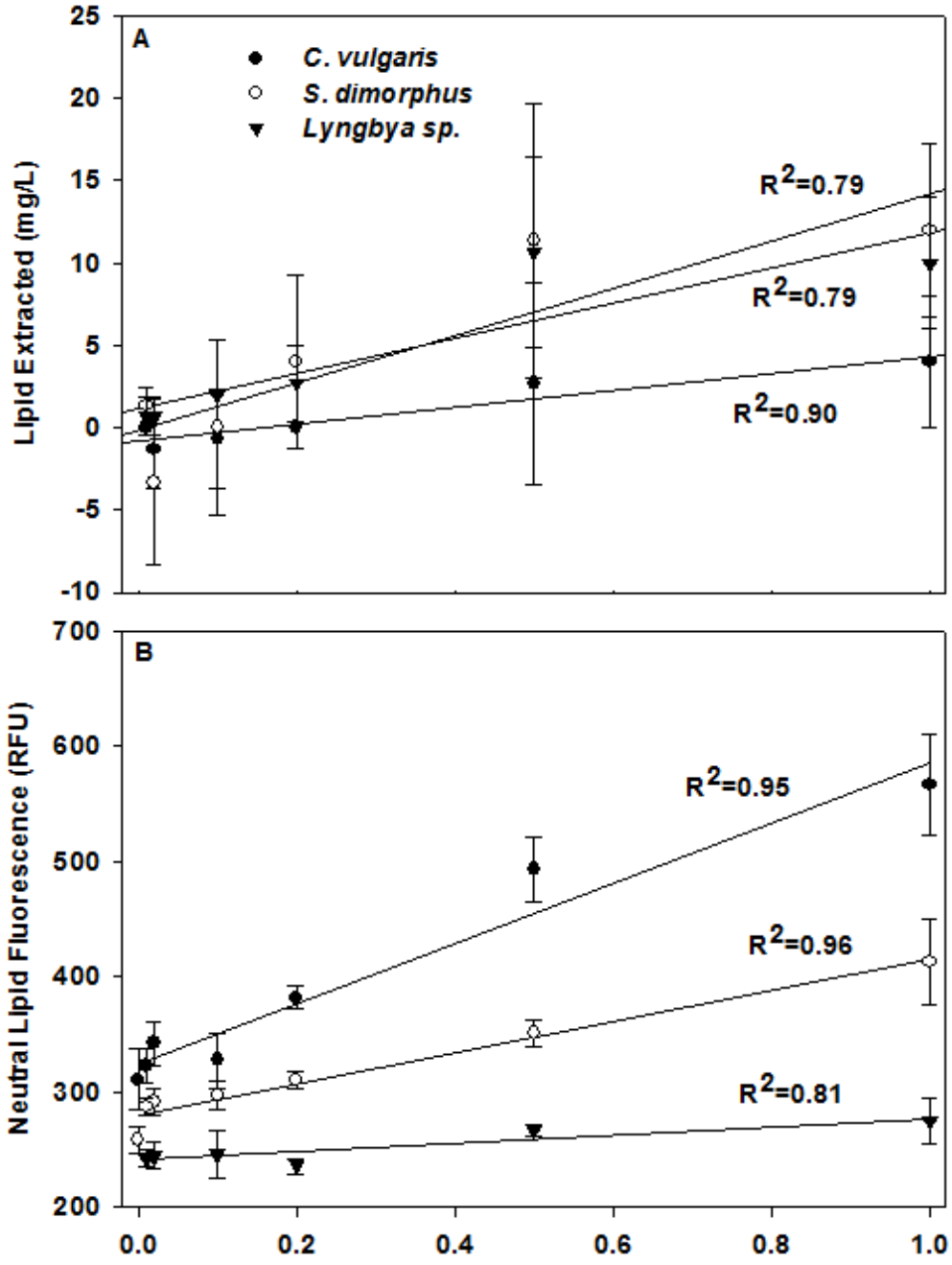


***Figure 3***: *Lipids measurement using (A) modified Bligh and Dyer method and (B) neutral lipid fluorescence. Relative concentration represent dilutions of known biomass concentration, and conversion can be found in Table 1.*

The fluorescence method produced results with smaller standard deviations and better $R^2$ values than the lipid extraction method for both *C. vulgaris* and *S. dimorphus*. However, the fluorescence method produced very low fluorescence signal for Lyngbya sp. and resulted in a weakly fitted linear trend line ($R^2 = 0.81$). Two reasons were suspected for this result; (1) The filamentous nature led to the heterogeneous distribution of the algal cells, which affected the dye-cell interaction and measurement of the fluorescent signal; (2) the Nile Red stain could not penetrate the rigid cell wall, as reported in Chen et al. (2009). They also investigated the effect from other parameters, such as temperature, dye concentration, and staining time, and have identified the optimal procedure for high throughout lipid measurement.

The results also showed the fluorescence method was particularly helpful when assessing microalgae with low lipid concentrations. For example, based on the traditional solvent extraction method, results revealed the three species under the experimental condition only yielded (5.12±1.01)%, (11.73±3.37)%, and (16.99±6.98)% of lipid (mass based) in *C. vulgaris*, *S. dimorphus*, and *Lyngbya sp.*, respectively. In the lipid extraction procedure, centrifuging 50 mL of lipid culture produced very small masses of lipids (less than 1 mg), leading to comparatively large standard deviations. Based on observations from these results and other researchers, tens of milligrams, or even grams of biomass is required to get consistent results with improved accuracy. For example, published studies using this method presented biomass concentrations 10-20 times higher than this study (Mujtaba et al. 2012, Liang et al. 2009). On the other hand, the fluorescence method produced reliable result with only 100 µL of liquid culture despite the low lipid concentration.

Other disadvantages of the traditional extraction method include the difficulty to exclusively extract lipids from the biomass (Archanaa et al. 2012). Alternatively, the

fluorescence method offers a direct measurement of neutral lipids, which can vary greatly within total lipids depending on growth conditions, providing a more useful and accurate indicator for biodiesel generation (Higgins et al. 2014).

*3.4 Additional Comparisons*

In addition to differences discussed above, the methods compared for each measurement varied in sample size and time required (Table 3). Alternative methods tested in this study significantly reduced the required sample size and processing time. Meanwhile, these new methods produced consistent and stable results over a wide range of algae concentrations, thus minimum effort is needed for adjusting the ideal sample volumes. In comparison, sample size required by the traditional methods is highly dependent on the algae concentration, consequently diluting or concentrating the samples is commonly performed prior to measurement, such as in microscopy counts and chlorophyll *a* extraction. As a result, the alternative methods assessed here will be especially suitable for samples with unknown algae concentration.

***Table 3:*** *Sample volume and time requirements for methods compared in this study.*

| **Parameter** | **Method** | **Sample Volume Required (mL)** | **Average Time Required** |
|---|---|---|---|
| Cell Count | Standard Method 10200-F | 1-3 | 1 hour |
| | Particle count | 0.1 | 5 minutes |
| Chlorophyll | Standard Method 10200-H | 50** | 1 day |
| | Autofluorescence | 0.1* | 1 minute |
| Lipids | Chloroform extraction | 50 | 1 day |
| | Nile Red fluorescence | 0.1* | 15 minutes |
| * Performed on the same samples in microplate reader. | | | |
| ** Same extraction used for fluorometer and spectrophotometer | | | |

The alternative methods assessed also have the advantage of little to no usage of hazardous materials compared to the traditional methods. For example, chlorophyll *a* extraction requires the use of acetone and/or hydrochloric acid, while chlorophyll autofluorescence uses no hazardous chemicals; lipid extraction uses methanol and chloroform, while neutral lipid fluorescence only requires the Nile Red stain. In addition, chlorophyll autofluorescence and neutral lipid fluorescence can be measured using the same sample, and the operation of microplate readers allows for high throughput measurement, further reducing the sample size and time required.

However, the traditional methods for algae quantification still have some unique benefits over the alternative methods tested. For instance, microscopy counts provide more accurate results for microalgae species that tend to form colonies (such as *S. dimorphus*); the excitation/emission wavelength for chlorophyll autofluorescence will require adjustment to capture different type of chlorophyll in cyanobacteria. Furthermore, fluorescence can only be measured in relative fluorescence unit, where the correlation to actual biomass concentrations can only be achieved through the traditional measurements.

## 4. Conclusions

The alternative methods assessed here consistently provide rapid and stable measurements over a wide range of algae concentrations. Compared to microscope counts, the Coulter counter offered faster processing and small sample requirements, but likely undercounted colony forming *S. dimorphus*. The chlorophyll autofluorescence method worked well for *C. vulgaris* and *S. dimorphus* by offering advantages in sample size, time required and chemical use; however, the excitation/emission wavelength needs adjustment for the *Lyngbya* consortium. The neutral lipid

fluorescence method offered a wide range of benefits over the lipid extraction method including smaller sample size, fewer hazardous chemicals, less time requirements, and more accurate targeting of the lipid content most useful for biodiesel generation. However, traditional methods are still valuable for correlating relative fluorescence units to actual biomass concentrations, and for counting species that tend to form colonies or flocs.

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## Chapter 2 - Nutrient Removal and Neutral Lipid Production from Wastewater by Chlorella vulgaris under Nutrient Limiting Conditions


### Abstract

A series of experiments was conducted to examine *Chlorella vulgaris'* ability to remove nutrients and produce lipids from domestic wastewater under nutrient limiting conditions. *C. vulgaris* was able to remove both nitrogen and phosphorus under these unfavorable conditions, and continue to consume either nutrient when the other was exhausted. Neutral lipid production was highest when the algae completely consumed nitrogen from wastewater, but decreased significantly following the addition of supplemental nitrate. Synergistic effect was observed between *C. vulgaris* and indigenous microorganisms in nutrient removal from real wastewater effluent, but competition from other species affected the maximization of lipid production by *C. vulgaris*.



**Keywords:**

*Chlorella vulgaris;* wastewater treatment; nutrient removal; lipid production; indigenous microorganisms.

## 1. Introduction

Algae have traditionally been viewed negatively with respect to water quality and water treatment. They can be indicators of poor water quality, and algal blooms can lead to low-dissolved oxygen, dead zones, and other conditions that are harmful to wildlife and humans (Anderson et al. 2002). Some species produce toxins (such as cyanotoxins) when they grow or decay (Chorus, Bartram 1999). Microalgae, especially unicellular species, can be difficult to remove in sedimentation basins during water and wastewater treatment, and the oxidation of algae by common disinfectants in water treatment is known to create harmful disinfection byproducts (DBPs) (Mash et al., 2014, Coral et al. 2013, Nguyen et al. 2005, Vuuren and Duuren 1965).

While algae can cause a variety of issues within water treatment processes, its potential for polishing wastewater treatment plant effluent has been recognized for years and has been the subject of much recent research (Schumacher et al. 2003, Wang et al. 2010, Sturm et al. 2012, Arbib et al. 2014, Park et al. 2011, Pittman et al. 2011). Algae can also be used to generate a wide range of biofuels including ethanol, biodiesel, methane, and hydrogen; and require less land and other inputs compared to other popular biofuel sources such as corn, soybeans, an oil palm (Singh et al. 2011). Since algae only require water and certain nutrients to grow, they rarely compete with food production the same ways as the other popular biofuel sources grown on agricultural land. Nutrients from wastewater can be recovered by algae and no additional costly inputs in the form of fertilizer will be needed for algal growth; in addition, the added benefits include improving wastewater effluent quality, freeing up agricultural land, and sequestering carbon dioxide from the atmosphere.

Many factors such as carbon source, pH, light wavelength and intensity, and availability of key metals and nutrients can impact the growth of algae (Schindler 1977, Park et al. 2011, Cade-

Menun and Paytan 2010). Scientists have known for years that availability of nutrients plays an important role in the species composition in natural water bodies (Schindler 1977), and researchers have attempted to identify the ideal nitrogen to phosphorus (N:P) mass ratios for algae based on their elemental compositions or nutrient removal abilities (Mandalam and Palsson 1998, Wang et al. 2010, Rhee 1978). Wang et al. (2010) concluded that while the optimal N:P mass ratio for freshwater algae species is thought to be 6.8-10, high nutrient removal rates from wastewater have been seen at a much wider range of ratios (Wang et al. 2010).

*Chlorella vulgaris* has been studied extensively in wastewater treatment and biofuel production. This freshwater species grows at a range of pH and in media varying from swine slurry to secondary clarifier effluent. It can utilize multiple carbon sources in place of carbon dioxide and grow in the absence of light (Liang et al. 2009). It is also known for its high lipid production and can reach 80% lipid by weight (Held and Raymond 2011, Illman et al. 2000, Khan et al. 2009, Mujtaba et al. 2012, Deng et al. 2011). However, wastewater quality fluctuates widely and continuously, which can have detrimental impacts on the initiation and maintenance of algal growth and their ability to remove nutrients. For example, the pH of secondary effluent can range from 6-9 and the concentration of key nutrients such as nitrate and phosphate can vary by more than an order of magnitude. Conditions in raw wastewater can be even more extreme in terms of light transmission, competition from other microorganisms, and concentrations of potentially inhibitory constituents. The impact of these challenging conditions was rarely studied for algal wastewater treatment and biofuel production. Due to these uncertainties, although algae have been shown to successfully remove nutrients and produce lipids/biomass from municipal wastewater (Park et al. 2011), implementation of algal treatment in wastewater treatment plants is still rare, nor is there large scale biofuel production using wastewater currently.

This study seeks to explore the capability of *C. vulgaris* to remove nutrients and produce lipids from domestic wastewater under nutrient limiting conditions. *C. vulgaris* was grown on synthetic wastewater with variable levels of nitrogen and phosphorus as well as real wastewater effluent to study nutrient removal and lipid production simultaneously. The impact of indigenous microorganisms from wastewater effluent was also investigated on *C. vulgaris*. It is expected the result of this study will shed light on a variety of environmental conditions in wastewater that could benefit from *C. vulgaris* for effective algal nutrient removal and biofuel production.

## 2. Materials and Methods

### *2.1 Algal Growth*

*C. vulgaris* was purchased on agar slants from UTEX algae center at the University of Texas at Austin (Austin, Texas) and maintained in Bristol media ($NaNO_3$ (25 mg/L), $CaCl_2.H_2O$ (2.5 mg/L), $MgSO_4.7H_2O$ (7.5 mg/L), $K_2HPO_4$ (7.5 mg/L), $KH_2PO_4$ (17.5 mg/L), NaCl (2.5 mg/L), Peptone (1 g/L)) at 26°C for inoculation. Liquid cultures of *C. vulgaris* were periodically isolated on agar plates prepared in the same media to ensure the purity of the strain.

Several growth parameters were tested prior to the nutrient ratio experiments to avoid interference from other environmental factors, including pH, light condition, and air exchange method. To test pH, jars containing Bristol media at a range of pH values from 6-10 were inoculated with *C. vulgaris*. Overhead fluorescent lighting and T5 high-output aquarium lights (Coralife, Franklin, WI) were compared for algae growth. Active aeration using aquarium pumps, tubing, and air filters was compared to more passive aeration using magnetic stir bars. For the following experiments, the algae were maintained in a consistent manner: jars placed on a stir plate in a 26-27 °C warm room lit continuously by fluorescent ceiling lights and three T5 high-output aquarium lights.

*2.2 Media Preparation and Inoculation*

All glassware, stir bars, and tongs used in this experiment were washed in phosphorous-free laboratory detergent, rinsed with tap water three times, and with distilled deionized (DDI) water three times. To minimize risk of contamination, stir bars were soaked in dilute bleach solution overnight, rinsed with sterile DDI, and added to autoclaved media while it was still hot using autoclaved tweezers.

For the duration of the experiments, three types of media were used: secondary clarifier effluent, Bristol (described above) and synthetic wastewater with varied levels of nitrate and phosphate concentrations. Secondary clarifier effluent was collected from two local wastewater treatment plants (Westside and Noland Wastewater Treatment Plants, Fayetteville, AR) and inoculated on the same day. The synthetic wastewater media was composed of $NaNO_3$ (varied), $CaCl_2 \cdot 2H_2O$ (2.5 mg/L), $MgSO_4 \cdot 7H_2O$ (7.5 mg/L), $K_2HPO_4$ (varied), $KH_2PO_4$ (varied), NaCl (2.5 mg/L), $FeSO_4 \cdot 7H_2O$ (3 mg/L), $Na_2CO_3$ (19 mg/L), $C_6H_8O_7$ (6 mg/L), KCl (16 mg/L), $H_3BO_3$ (2.86 mg/L), $MnCl_2 \cdot 4H_2O$ (1.81 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.22 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (0.39 mg/L), $CuSO_4 \cdot 5H_2O$ (79 µg/L), $Co(NO_3)_2 \cdot 6H_2O$ (49.4 µg/L). A preliminary screening experiment was conducted to determine the nitrogen and phosphorus limiting conditions from a range of nitrogen and phosphorus gradients (Supplemental Table 1). For inoculation, five mL of suspended algae were added to 1L clear glass jars filled with secondary clarifier effluent or synthetic wastewater. Prior to inoculation, synthetic wastewater was adjusted to pH of 8.0 using hydrochloric acid or sodium hydroxide. Filtration using 0.45 µm filters on secondary effluent was performed to remove indigenous microorganisms, and algal performance was compared with and without indigenous microorganisms. The effluent was measured for initial pH and nutrient concentrations prior to the inoculation.

*2.3 Sample Analyses*

Ten ml of samples were extracted from the jars and used for a variety of analyses including growth monitoring, nutrient analysis and lipid quantification via ion chromatography (IC), spectrophotometer, and microplate reader. For the synthetic wastewater experiment, triplicate averages were taken for the nutrient, chlorophyll and lipid measurements; for the real wastewater experiment, the experiment was repeated three times at different seasons, with varying nitrate and phosphate levels in the secondary clarifier effluent. Statistical significance calculations were applied to triplicate datasets to different treatments (paired student's t-test), along with standard deviations calculated using Microsoft Excel (Microsoft, Redmond, WA).

*2.3.1 Ion Chromatography*

Nine mL of each sample was filtered by VWR Sterile 0.45µm Cellulose Acetate Membrane Syringe Filters (VWR, Radnor, PA) and diluted to concentrations within the detection range of the IC method. A range of anions, including nitrite, nitrate, phosphate, and sulfate were analyzed on IC (Metrohm 850 Professional IC, 887 Professional UV/VIS Detector, 858 Professional Sample Processor). The IC method used a Metrosep A Supp 7 250/4.0mm column, a run-time of 40 minutes at 0.7ml/min flow, and 3.6 mM sodium carbonate eluent. Standards used for standard curves and check standards were made using Metrohm Custom Anion Mix 3 (Metrohm USA, Riverview, FL). A relative standard deviation <5% and correlation coefficient >0.999 was achieved on standard curves before running samples, and frequent check standards and blanks were employed to ensure precision.

In addition to the analyses described above, Hach Kits (PhosVer 3 and NitraVer 5, Hach, Loveland, CO) were occasionally used to quickly analyze phosphate and nitrate concentrations during experiments.

*2.3.2 Spectrophotometer Method*

One mL of each sample was transferred into disposable cuvettes (VWR PS Semi-micro, VWR, Radnor, PA) and analyzed for OD680 on a spectrophotometer to monitor *C. vulgaris* growth (Beckman Coulter DU720 UV/VIS Spectrophotometer, Beckman Coulter Inc., Brea, CA).

*2.3.3 Microplate Methods*

A hundred µL sample aliquots were pipetted into black-sided clear bottomed 96-well plates (Corning 3603, Corning, Tewksbury, MA) for analysis on a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc., Winooski, VT). Samples were first tested for fluorescence using excitation at 440 nm and emission at 685 nm, a measure of chlorophyll concentration (Held 2011). Following these measurements, neutral lipids were measured following methods described in Held and Raymond (2011): 100 µL of 2X working solution (1 µg/mL) of Nile Red (Enzo, Ultra Pure, Farmingdale, NY) was added to each well and allowed to incubate in the dark for 10 minutes before being tested for fluorescence using 530nm excitation and 570nm emission wavelengths; the fluorescence measurement corresponds to the amount of neutral lipids inside the algal cells.

*2.3.4 Biomass Determination*

Following the Standard Method for TSS (USEPA 160.2, SM 2540-D), 100 mL of liquid from each jar at the end of each experiment was filtered using a vacuum flask onto pre-rinsed 47mm glass microfiber filters. Filters were then dried in an oven at 105°C, allowed to cool in a desiccator, and then weighed (APHA 2005, USEPA 1999).

## 3. Results and Discussion

*3.1 Growth Condition Determination*

Though other studies concluded *C. vulgaris* growing in a wider pH range (Powell 2009,

Lam and Lee 2013), in this study the species only grew within the pH range of 6.3 to 10 and the fastest at pH 8.0, but not at all below pH of 6 or above 10 (data not shown), which agrees with findings discussed in Park et al. (2011). While overhead fluorescent light was sufficient to maintain algal cultures, *C. vulgaris* grew faster and to greater final concentrations with high output aquarium lights placed at close proximity. The light was kept on at all times to promote maximum growth of algae. The use of brighter, fuller spectrum bulbs is common in algal research, though excessive light can impede growth (Sorokin and Kraus 1958, Ogbonna et al. 2000). Active aeration using filtered ambient air was compared to stirring using magnetic bars, and *C. vulgaris* grew faster and to a higher final concentration using stir bars with less incidence of contamination. As a result, these optimal growth conditions were employed in the following experiments to ensure these environmental parameters such as pH, light and aeration were not the limiting factors for *C. vulgaris* growth.

*3.2 Algal Biomass and Neutral Lipid Production under Nutrient Limiting Conditions*

The predicted N:P consumption value based on elemental ratios found in *C. vulgaris* is about 5:1 (Mandalam and Palsson 1998), while other sources place the ideal N:P ratio for freshwater algae at 6.8-10:1 (Wang et al. 2010). Based on these ratios and nutrient levels in real secondary wastewater effluent, a wide range of N:P mass ratios (Supplemental Table S1) were screened to identify nutrient limiting conditions for *C. vulgaris* growth. The results of these preliminary experiments are provided in the Supplementary Figures section (Sup. Figs. 1 & 2) and they showed: (1) nitrogen or phosphorus continued to be consumed when one of the nutrients ran out; (2) supplemental nitrate boosted phosphate removal in nitrogen-free starting condition; and (3) *C. vulgaris* produced, and often later consumed low levels of nitrite. This experiment showed

nitrogen was consumed completely within 2 days with an N:P mass ratio of 0.5:1, and phosphorus was exhausted within 3 days with an N:P ratio of 1:0.06. To reveal how *C. vulgaris* performs with low to exhausted nutrient conditions in a short time frame, these two ratios were chosen to represent N- and P-limiting conditions in the following experiments.

Current algal biofuel generation processes depend either on sheer biomass production (anaerobic digesters) or the lipid content of the algae (biodiesel). For this reason, both algal biomass and lipid production were investigated in this study. Lipid production varies greatly by species and growth conditions. A major reason *C. vulgaris* has been studied so extensively is its relatively high lipid content compared to other species of algae. While algae form both polar and neutral lipids, stored neutral lipids (i.e., triacylglycerol) are of greater interest when it comes to biofuel production (Held and Raymond 2011). Depending on environmental conditions, lipids account for anywhere from 20 - 80% of the dry mass of *C. vulgaris* (Khan et al. 2009), with greater lipid accumulation taking place under unfavorable growth conditions such as nitrogen stress (Illman et al. 2000; Mujtaba et al. 2012; Deng et al. 2011). However, sudden changes from nutrient rich to nutrient depleted conditions were usually created in all these studies by human intervention to study these unfavorable conditions. For this reason, synthetic wastewater with limiting nutrients was used to achieve the shift from nutrient rich to depleted conditions naturally, and nutrient removal as well as algal biomass and lipid production were studied during this transition. Figure 1 shows the phosphate (1a) and nitrate (1b) removal, and chlorophyll (1c) and neutral lipids (1d) production over time by *C. vulgaris* under N- and P-limiting conditions. Four types of starting conditions were included: N:P ratio of 1:0.06 without inoculation (control), ideal growth condition with no limiting nutrients (Bristol media), N:P ratio of 0.5:1 (N-limited), and N:P ratio of 1:0.06 (P-limited).

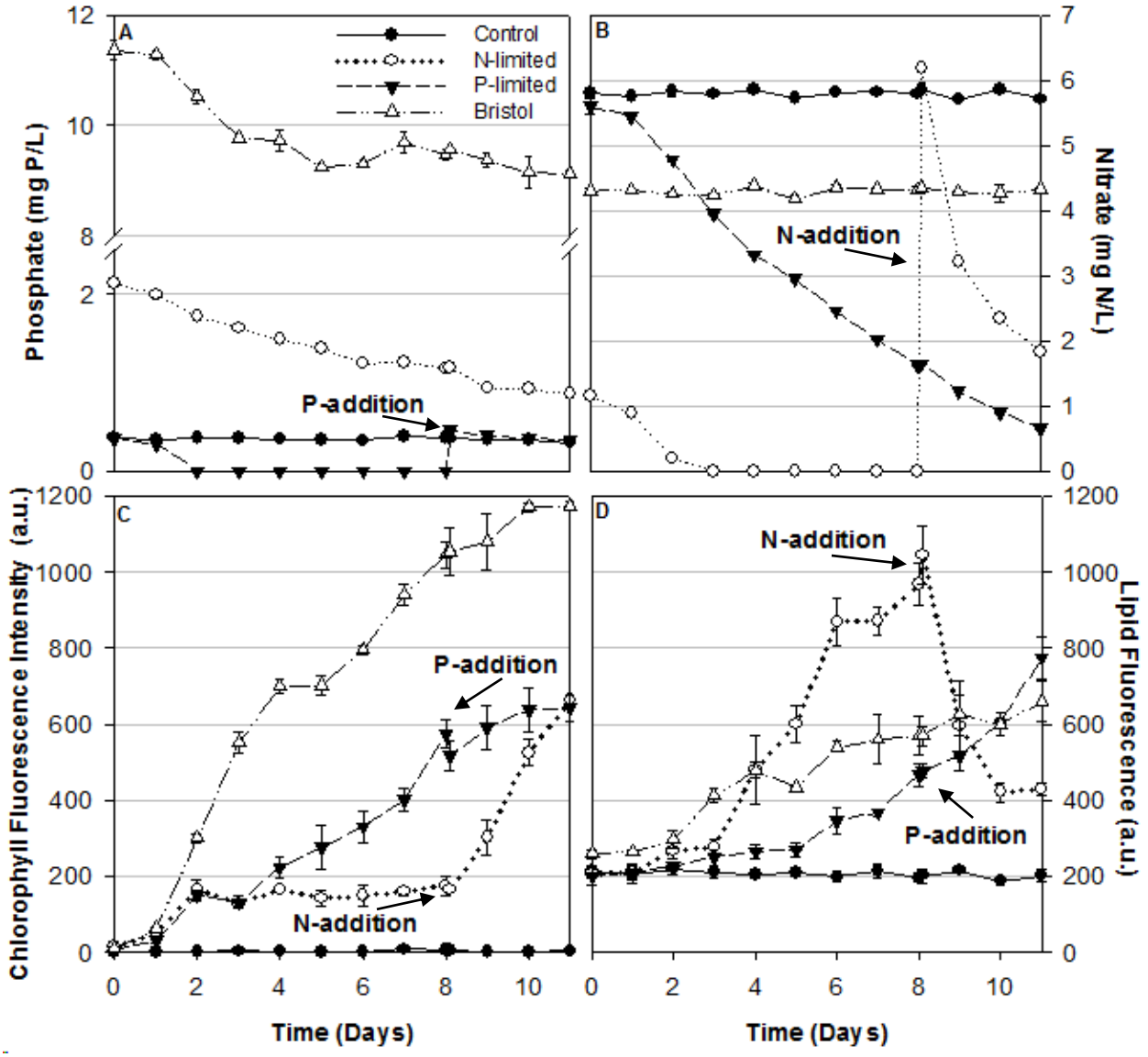


***Figure 3***. *The productivity and nutrient reduction from C. vulgaris in synthetic wastewater under nutrient limiting conditions and Bristol media over an 11-day period. Exhausted nutrients were added on Day 8 to N- and P-limited jars. The figures include: (A) phosphate removal, (B) nitrate removal, (C) relative chlorophyll production, and (D) relative neutral lipid production.*

The result showed *C. vulgaris* can continue to consume nitrate or phosphate when the other nutrient becomes exhausted, and the availability of both nutrients did not alter the consumption rate of the other nutrient (Figure 1a and 1b). However, the added nitrate on Day 8 was quickly consumed (13 mg/L removed in one day), while no such trend was observed in added phosphate consumption. Nitrite concentration was also monitored throughout the experiment (Supplemental

Figure 3). Nitrite was produced at low levels (< 0.4 mg/L) when nitrate was available, and then exhausted when nitrate became scarce. The ability of *C. vulgaris* to both produce and uptake nitrite was also reported by Wang et al. (2010) who theorized that nitrite was released during the reduction of nitrate in the cells' chloroplasts.

Figure 1c shows the chlorophyll concentrations of *C. vulgaris* under different nutrient conditions. While chlorophyll *a* is often measured using ESS 150.1/Standard Method 10200H to represent the quantity of algae, here chlorophyll levels (including chlorophyll *a* and several other forms of chlorophyll) were monitored using fluorescence, with the benefits of smaller sample size and shorter processing time. A high fluorescence value represents high chlorophyll levels. The chlorophyll concentration increased the most in the ideal growth medium, Bristol, indicating the most algal growth; its production was not affected by the phosphorus limitation and addition (P-limited jar), but was affected by the nitrogen limitation and boosted by the nitrogen supplement (N-limited jar). This can be explained by the elemental composition of chlorophyll ($C_{55}H_{72}O_5N_4Mg$). At the end of this experiment, chlorophyll reached comparable levels in both N- and P-limited jars ($p$=0.44) (Fig. 1c). The biomass quantification revealed at 100.7 mg/L, the P-limited jar yielded 12% greater final biomass than the N-limited jar which yielded 90 mg biomass/L ($p$<0.0001). The rich Bristol medium yielded the highest biomass of 216 mg/L ($p$<0.0001), which agreed with the chlorophyll measurement.

Figure 1d shows the neutral lipid production of *C. vulgaris* throughout the experiment. A high fluorescence value represents high neutral lipid production by the algae. Under N-limited conditions, the algae produced much higher levels of neutral lipids. While algal growth in the N-limited jar plateaued, neutral lipids increased rapidly from Day 3 to Day 8, with levels three times

higher than those found in the P-limited jar ($p$=0.0002). However, the lipid concentration in the N-limited jar returned to a much lower level following the addition of supplemental nitrogen, confirming that lipid production is maximized under nitrogen limiting conditions, even at gradual exhaustion of the nitrogen source. At the end of the experiment, even though the algae in the Bristol medium produced the highest level of chlorophyll and biomass, the neutral lipid production was not significantly different than the P-limited jar ($p$=0.06) (Fig 1d). This finding has important implications for maximizing algal biofuel production using waste sources. In fact, this shows nutrient limitation and exhaustion in wastewater can favor the lipid production in algae, which adds to the benefit of algal treatment of wastewater. Together with previous studies such as Mujtaba et al. (2012), which found that lipid production was maximized by growing *C. vulgaris* in nutrient rich conditions followed by 24 hours in nitrogen-deficient media, it is confirmed that both sudden and gradual nitrogen deficiency can boost lipid production by *C. vulgaris*. Neutral lipids were not found to increase dramatically under phosphorus-limiting conditions. The continuing increase of neutral lipids in the P-limited jar at the end of the experiment coincided with the near complete removal of nitrate and nitrite, agreeing with the results shown in the N-limited jar.

*3.3 Nutrient Removal and Lipid Production in Real Wastewater Effluent*

Real wastewater effluent after secondary treatment collected from two local wastewater treatment plants was inoculated with *C. vulgaris* in the same fashion as the previous experiments with synthetic wastewater. In all three rounds of testing, effluent from both treatment plants had low phosphate concentrations (≤ 0.2 mg/L). The low starting phosphate concentration enabled the growth of *C. vulgaris*, but the nutrient quickly became undetectable, leaving the effluent P-limited. In all rounds of testing, the addition of *C. vulgaris* improved nitrate removal leading to lower final

concentrations compared to un-inoculated treatment ($p$<0.0001), with starting nitrate concentrations ranging from 11 to 56 mg/L. Figure 2 shows the nitrate (2a), nitrite (2b), neutral lipid (2c) and chlorophyll (2d) change over the course of 6 days during the third round of experiments. In addition to nutrient removal and neutral lipid production, the impact of existing microbial cultures within the secondary effluent was also studied by comparing filtered and unfiltered effluent. The indigenous species played an important role in nitrate removal, as unfiltered treatment resulted in a lower final concentration in both the Noland plant ($p$=0.02) and the Westside plant ($p$=0.01). The Noland plant is the older plant of the two and had higher effluent nutrient concentrations and much more visible indigenous algae in the clarifier and its secondary effluent. These differences are likely responsible for the large difference between the filtered and unfiltered treatment in Noland effluent (Fig. 2a). The unfiltered, inoculated effluent from Noland saw the greatest total removal of nitrate at 32 mg/L, compared to 15 and 20 mg/L for the existing microbial community and just *C. vulgaris* respectively, showing that mixed microbial species could potentially have a synergistic effect on nutrient removal. While nutrient removal in the Westside effluents was dominated by *C. vulgaris*, the unfiltered inoculated jar had a slightly higher nitrate removal percentage than the filtered one (81% vs. 77%) and both were higher than the unfiltered, non-inoculated control (37%). This synergistic effect of *C. vulgaris* and indigenous microbial cultures in nutrient removal can be attributed to other species directly through removing additional nutrients (see controls in Figure 2) or indirectly by stabilizing pH or dissolved $CO_2$ levels. When grown in isolation, *C. vulgaris* consumes $CO_2$ and other carbon sources, increasing the pH of the growth media, which could negatively impact its growth. This result adds to the benefit of algal treatment of wastewater, as the presence of other species could improve nutrient removal rather than being a source of concern (such as contamination in photobioreactors).

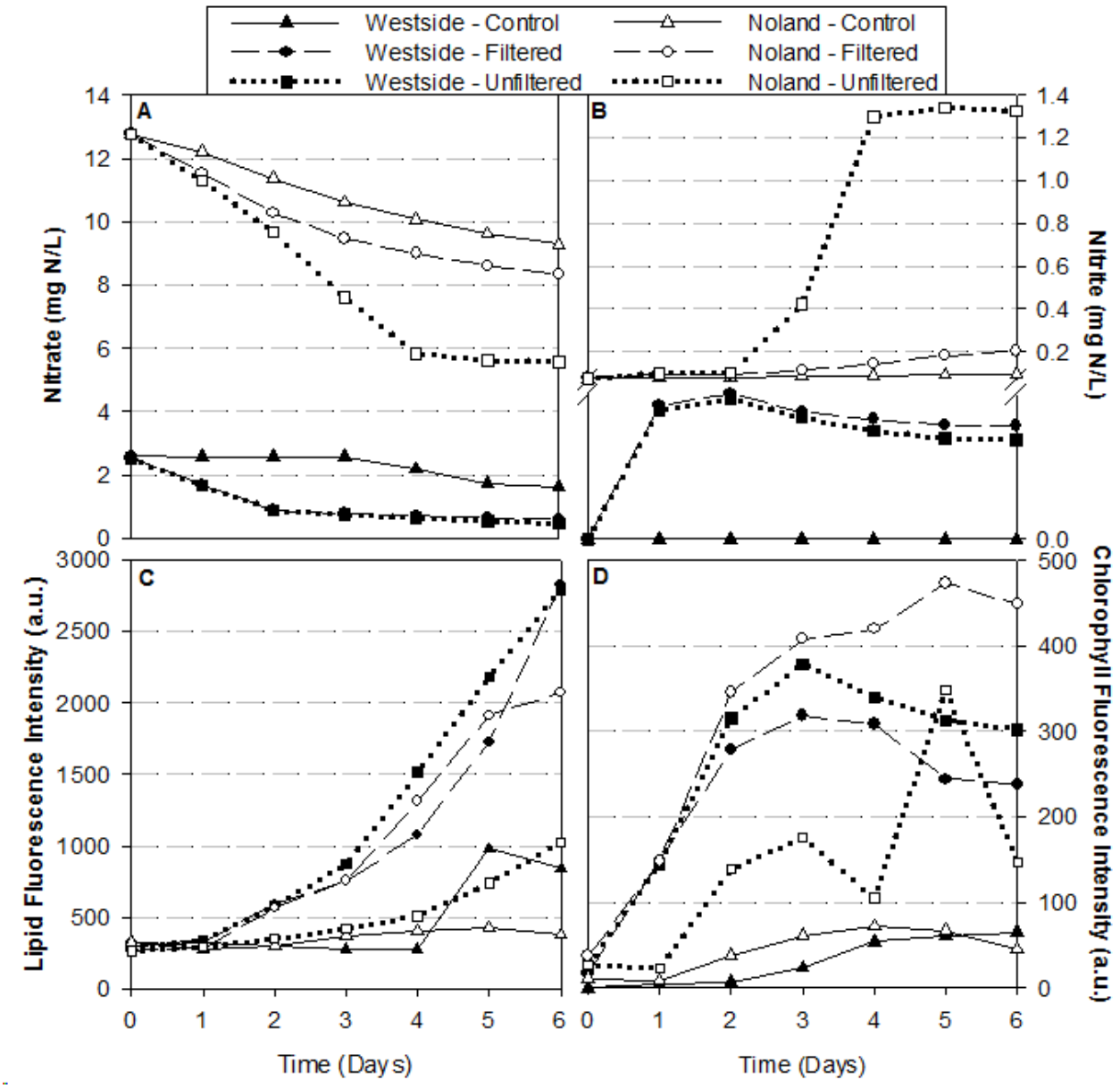


***Figure 4***. *Nutrient reduction and lipid production in the secondary clarifier effluent from two wastewater treatment plants: (A) nitrate removal, (B) nitrite concentration, (C) relative neutral lipid concentration, and (D) relative chlorophyll concentration over time. Variables tested include filtration and inoculation with C. vulgaris. Controls were not inoculated with C. vulgaris and not filtered.*

The presence of other species may also help with separating the introduced algae from treated water. As single-celled algae, *C. vulgaris* are easily suspended and therefore difficult to settle in the sedimentation basin. At the end of this study, unfiltered inoculated jars settled much

faster than filtered jars. In the presence of filamentous algae or biofilm producing bacteria, *C. vulgaris* may form larger aggregates or attach to existing biofilms. While a potential benefit for treatment processes, this increased aggregation or attached growth likely interfered with accurate fluorescence measurements of growth. Figure 2d shows chlorophyll measurements over the course of this experiment. Chlorophyll levels were highest for the four inoculated jars, but the measured values for the Noland unfiltered jar fluctuated greatly. This is likely due to the heterogeneous algal growth which resulted in biomass aggregation.

A correlation between chlorophyll, lipids and biomass were observed in jars where *C. vulgaris* was the dominant algae species. The Westside filtered, Westside unfiltered and Noland filtered jars all had high lipid concentrations at the end of the experiment with high chlorophyll and biomass values (Fig. 2c & d). However, no such correlation was observed in jars where indigenous microorganisms played a major role, such as in un-inoculated and unfiltered Noland jars. This shows competition from indigenous microorganisms might hinder this ability and lower the overall lipid production from *C. vulgaris*. While algal treatment can benefit nutrient removal from wastewater even at nutrient limiting conditions, lipid production for biofuel extraction may not achieve the maximum level when *C. vulgaris* suffers from competition with other species.

*3.4 Nutrient Removal from Waste Sources*

Multiple studies have focused on algal nutrient removal from waste sources using *C. vulgaris* (Figure 3).

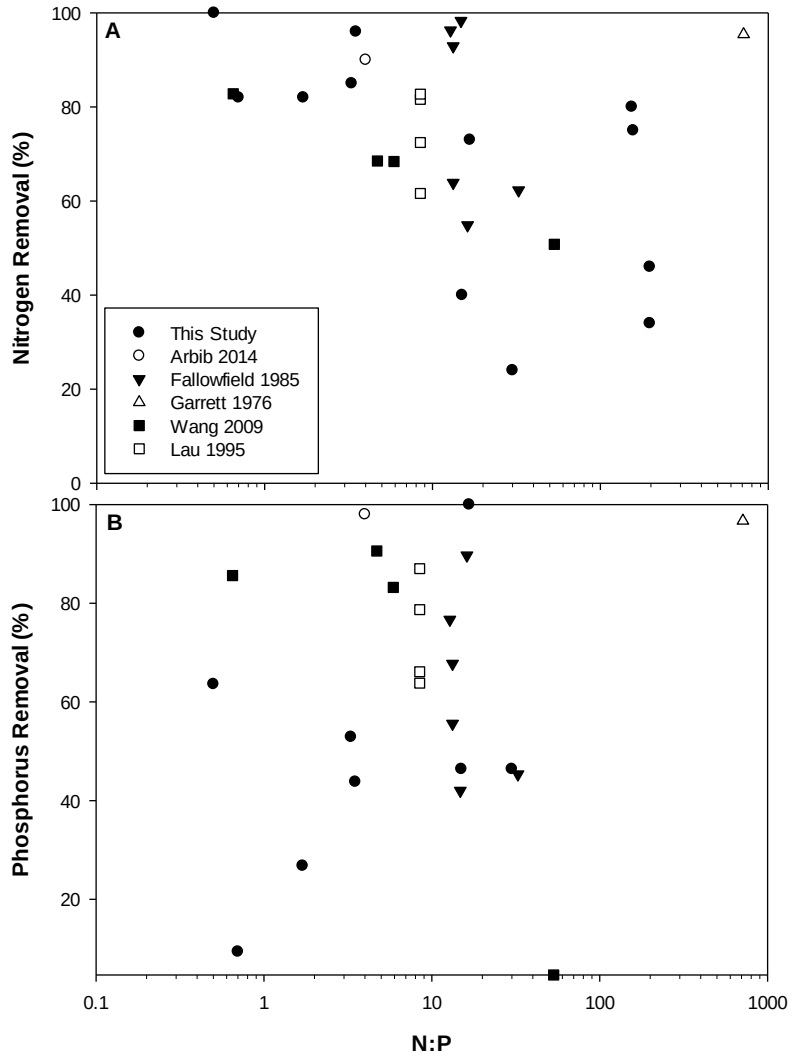


***Figure 3.*** *Comparing nutrient removal by C. vulgaris at a range of N:P ratios from multiple studies: (A) nitrogen removal, (B) phosphorus removal.*

As shown in the above figure, the nitrogen to phosphorus ratio in most studies fall within the range of 3.5 to 16, where neither nutrient is limiting. Nitrogen and phosphorous removal percentages varied greatly over these experiments and even within individual experiments at the same N:P ratio. The study by Fallowfield and Garret (1985) was conducted in an outdoor raceway in the United Kingdom with data collected at different times of the year, so temperature and light

intensity impacted algal productivity and species composition; Lau et al. (1995) varied initial *C. vulgaris* inoculum sizes in primary settled sewage and found a range of nutrient removal rates; Wang et al. (2010) used water from various stages of the wastewater treatment process. It is likely that other factors such as the presence of indigenous microorganisms, light intensity/penetration, residence time, temperature, and mixing/aeration method strongly influenced the nutrient removal. This study filled the knowledge gap by investigating the growth of *C. vulgaris* with limited nitrogen and phosphorus in wastewater (at both ends of the N:P ratios). It is also unique by monitoring lipid production and nutrient removal simultaneously. The role of nitrogen deficiency in lipid production from *C. vulgaris* was confirmed in this study; and the role of indigenous microorganisms from domestic wastewater effluent was shown in nutrient removal and lipid production. While the synergy from other species improved *C. vulgaris*' ability to remove nutrients from wastewater, the level of lipid production was affected by the competition from other species.

## 4. Conclusions

*C. vulgaris* was grown in synthetic wastewater under various nutrient limiting conditions and in secondary effluent from two wastewater treatment plants. This species has shown to continue consuming nitrogen or phosphorus after the other nutrient became exhausted. Neutral lipid production was highest when nitrogen became exhausted in wastewater. Synergistic effects were observed for nutrient removal when *C. vulgaris* co-existed with indigenous microorganisms, but not for lipid production.

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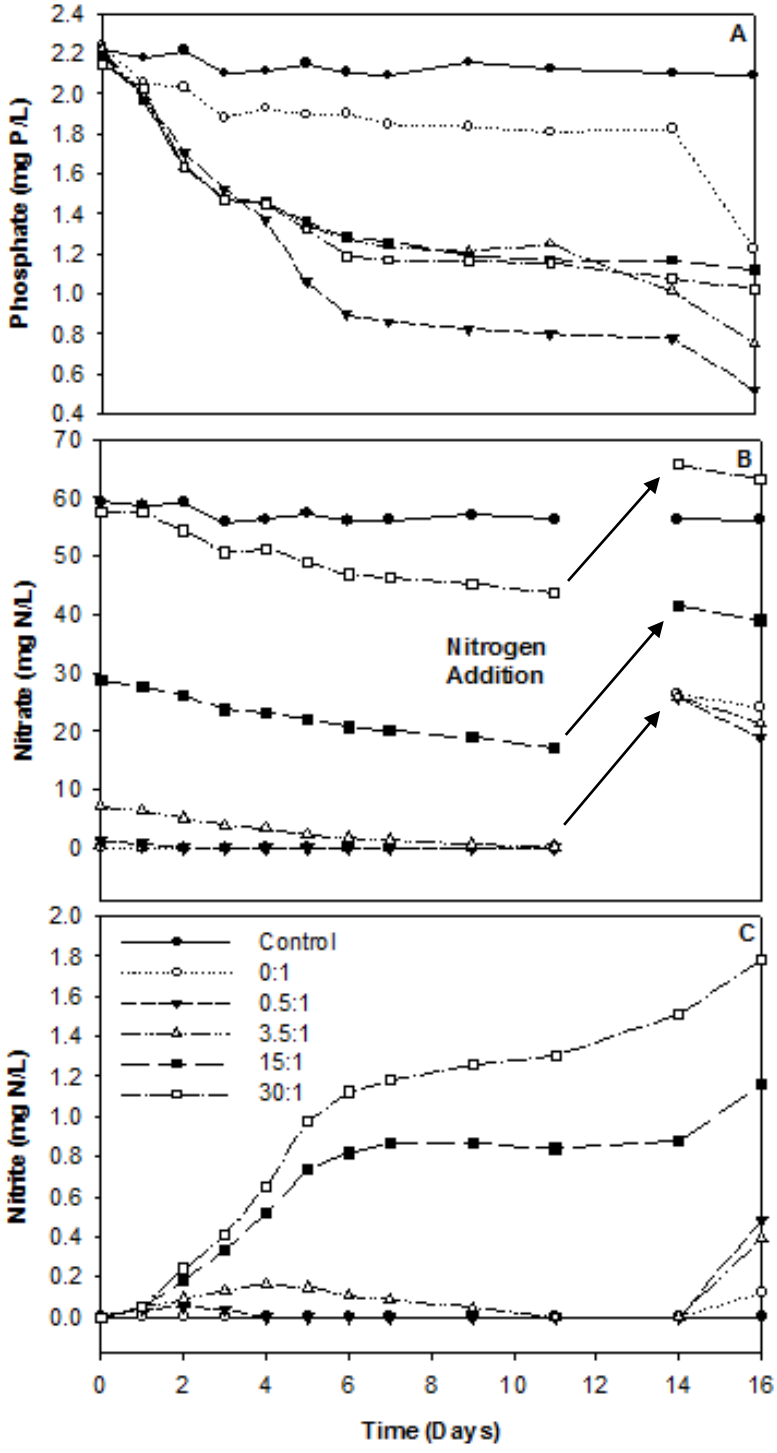
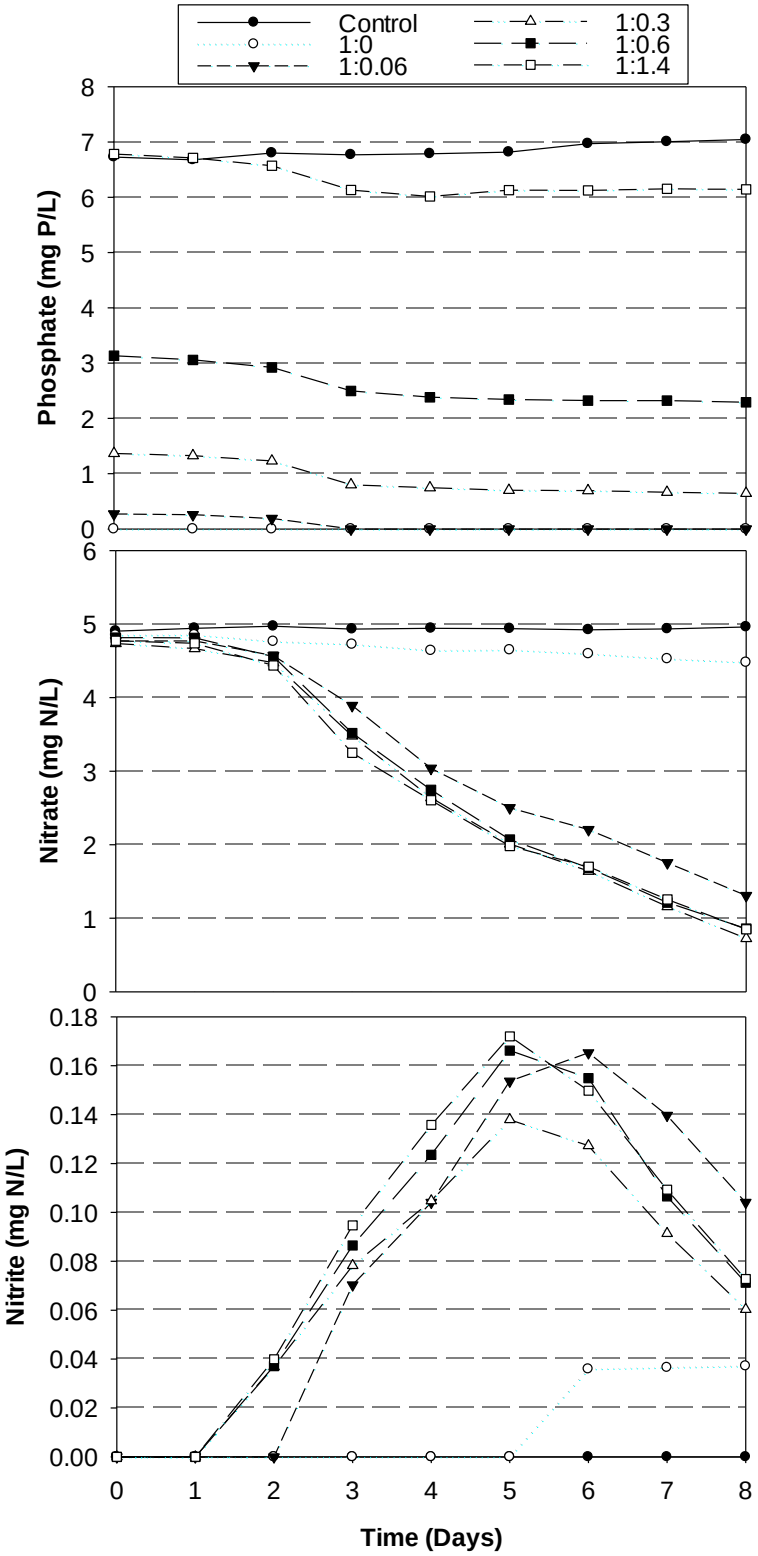
***Supplemental Table 1***. *N:P Ratios in synthetic wastewater used for phosphorus and nitrogen gradient experiments.*

| | **Phosphorus Gradient** | | | | | **Nitrogen Gradient** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Designed N (mg/L)** | 5 | 5 | 5 | 5 | 5 | 0 | 1 | 7 | 30 | 60 |
| **Designed P (mg/L)** | 0 | 0.3 | 1.5 | 3 | 7 | 2 | 2 | 2 | 2 | 2 |
| **Measured $NO_3^-$ (mg/L)** | 21.5 | 21.1 | 21.0 | 21.3 | 21.1 | 0 | 5.4 | 31.4 | 127 | 255 |
| **Measured $PO_4^{3-}$ (mg/L)** | 0 | 0.8 | 4.2 | 9.6 | 20.8 | 6.9 | 6.7 | 6.8 | 6.7 | 6.6 |
| | | | | | | | | | | |
| **Designed N:P** | 1:0 | 1:0.06 | 1:0.3 | 1:0.6 | 1:1.4 | 0:1 | 0.5:1 | 3.5:1 | 15:1 | 30:1 |

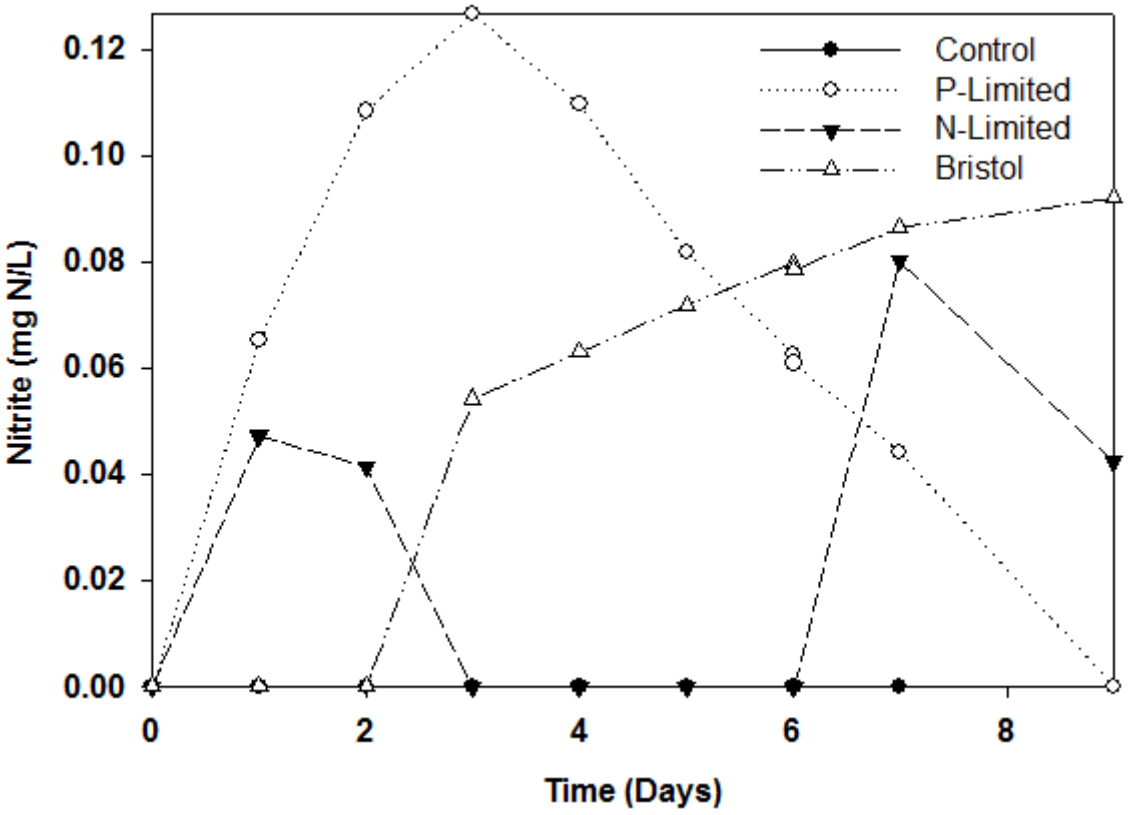
**Supplemental Figure S1-** Nutrient reduction from *C. vulgaris* in synthetic wastewater with the nitrogen gradient: (1a) phosphate removal, (1b) nitrate removal, and (1c) nitrite concentration change over time. Five N:P ratios are shown with the non-inoculated control of 30:1.



**Supplemental Figure S2** – Nutrient reduction from *C. vulgaris* in synthetic wastewater with the phosphorus gradient: (2a) phosphate removal, (2b) nitrate removal, and (2c) nitrite concentration change over time. Five N:P ratios are shown with the non-inoculated control of 1:1.4.



**Supplemental Figure S3**- Nitrite concentration over time in various media inoculated with *C. vulgaris*.



## Chapter 3 - Performance of Algal-Bacterial Flocs in Wastewater Treatment and Lipid Production


### Abstract

The concurrent use of algae for wastewater treatment and biofuel production offers a sustainable approach towards the seemingly unrelated goals of meeting higher treatment plant effluent quality requirements and efficiently generating less carbon-intensive fuels. Species of algae, such as *Chlorella vulgaris*, known for their biofuel production capabilities, can be difficult to efficiently harvest. Using synthetic wastewater of both high and low nutrient levels, this study evaluated combinations of *C. vulgaris*, a *Lyngbya* dominated consortium isolated from a hog waste lagoon, EPS-forming *Staphylococcus epidermidis*, and activated sludge for nutrient removal, floc formation, settle-ability, neutral lipid production, and biomass formation. Two combinations, *C. vulgaris* with *Lyngbya*, and *C. vulgaris* with activated sludge demonstrated improved settling and neutral lipid production compared to *C. vulgaris* alone, while maintaining similar levels of nutrient removal and biomass formation. *C. vulgaris* with activated sludge performed better than activated sludge alone in terms of settling, neutral lipid production, nutrient removal, and biomass production, demonstrating potential benefits of incorporating algae into secondary wastewater treatment.



**Keywords:**

Algae, Biofuels, Lipids, Biomass, Methods


### 1. Introduction

Due to stricter effluent regulations and the need for more sustainable energy sources, interest in the use of algae for wastewater treatment and biofuel production is growing. Algal species, such as *Chlorella vulgaris* and *Scenedesmus dimorphus*, that are popular in biofuel

research due to their high lipid content, present difficulties in wastewater treatment applications due to slow settling rates (de-Bashan and Bashan, 2010, Pittman et al. 2011). Research on growing these species in the presence of other microbial species has shown both advantages and disadvantages in terms of nutrient removal and ease of harvesting during treatment and biofuel production potential (Olguín 2012, Pittman et al. 2011, Gonçalves et al. 2016). In mixed settings, it can be difficult to prevent the growth or dominance of less desirable species (Christenson and Sims 2011). On the other hand, algae and bacteria can bio-flocculate and improve settling, while growth of both can be improved due to algae providing bacteria with oxygen and bacteria providing $CO_2$ (Gutzeit et al 2005). In fact, the use of mixed algal-bacterial flocs may enable the removal of nutrients under aerobic conditions without supplementary aeration, while aiding the removal of typically easily suspended single-celled algae like *Chlorella*, which become "integrally enmeshed" in the flocs and settle rapidly (Humenik & Hanna 1971, Humenik & Hanna 1970). While using mixed flocs to treat sewage supplemented with flue gas, researchers found the flocs maintained their structure, contained high concentrations of microalgae, and were higher in neutral lipids than typical activated sludge (Van Den Hende et al. 2011). These mixed flocs hold together under stress commonly found in mixing of aerated activated sludge and when used in aquaculture, they can remove nutrients from fish waste and provide fish with a nutrient-rich food source (De Schryver et al. 2008). Nonflocculating microalgae, like *C. vulgaris,* can also be bio-flocculated using fungi or naturally flocculating microalgal species rather than bacteria (Wrede 2014, Salim 2011).

The goal of this experiment was to compare the performance of multiple algal and bacterial consortia in wastewater treatment, with the goal of improving nutrient removal and subsequent separation from treated wastewater, and the potential of generating biomass/lipids for

biofuel production. The role of extracellular polymeric substances (EPS) was also assessed using the bacteria *Staphylococcus epidermidis* (which is commonly found in wastewater and on humans and is known to produce EPS) in the *C. vulgaris*-*S. epidermidis* consortium, and the performance of algae-activated sludge consortiums were studied in synthetic wastewater. This experiment is unique in that it measured settling, biomass production, neutral lipid content, and nutrient removal for a variety of algal and bacterial combinations in synthetic wastewater.

## 2. Materials and Methods

### *2.1 Algal & Microbial Growth*

*C. vulgaris* was purchased on agar slants from UTEX algae center at the University of Texas at Austin (Austin, Texas), and maintained in Bristol medium at 26°C prior to its use in the following experiments. The *Lyngbya* dominated mix was isolated from a hog waste lagoon (Savoy, Arkansas) and maintained in BG-11 medium. *S. epidermidis* was purchased from American Type Culture Collection (ATCC, Manassas, VA) and inoculated in nutrient broth 24 hours prior to use. Activated sludge samples was taken from a nearby wastewater treatment plant (West Side Wastewater Treatment Facility, Fayetteville AR) and used for inoculation on the same day.

For this experiment, growth conditions were maintained in a consistent manner: inoculated jars were plugged with autoclaved cotton balls, covered with autoclaved aluminum foil, and placed on a stir plate in a 26-27°C warm room lit continuously by fluorescent ceiling lights and three T5 high-output aquarium lights with continuous illumination at 150 µmol $s^{-1}$ $m^{-2}$.

*2.2 Preparation of Algae-Bacteria Consortia*

To prepare algae-bacteria consortia, different combinations of algae and bacteria were centrifuged, rinsed in experimental media, and resuspended before being added to the synthetic wastewater. The media were adjusted to pH of 7.0 using hydrochloric acid or sodium hydroxide and then autoclaved in 1L clear glass jars before use. The experimental matrix shown in Table 1 demonstrates the combinations of organisms and wastewater media used. With the exception of the 10:1 *C. vulgaris/S. epidermidis* combinations, inoculation was performed using equal volumes of centrifuged biomass. Species in their original media were centrifuged and washed twice, by replacing the supernatant with the experimental media and centrifuged again before pipetting biomass into jars. Synthetic wastewater media approximating both raw wastewater (Yoo et al. 1999) and secondary effluent (Chapter 2) were tested to investigate the performance of algal-bacterial flocs in wastewaters with both high and low nutrient levels.

*Table 1. Experimental matrix for combinations of species and media used.*

| | **Species** | | | | **Media** | |
|---|---|---|---|---|---|---|
| **Experiment** | *C. vulgaris* | *Lyngbya* Mix | *S. epidermidis* | Activated Sludge | Synthetic Raw Wastewater | Synthetic Effluent |
| **1** | X | | | | | X |
| | X (10:1) | | X | | | X |
| | X (1:1) | | X | | | X |
| **2** | X | | | | X | |
| | X (10:1) | | X | | X | |
| | X (1:1) | | X | | X | |
| **3** | X | | | | X | |
| | X | X | | | X | |
| | X | | | X | X | |
| | | X | | | X | |
| | | X | | X | X | |
| | | | | X | X | |

*2.3 Sample Analysis*

Experiments 1 and 2 from Table 1 were designed to access the role of bacterial EPS in floc formation. In these experiments, chlorophyll *a* extraction, biomass, and settling were performed at the end of the 4-day growth period. Activated sludge was added into the experimental matrix in Experiment 3, where daily samples of 10.5 mL were taken and used for nutrient analysis via ion chromatography (IC), and algal growth and lipid production analyses via microplate reader. IC samples were filtered through sterile 0.45 µm cellulose acetate filters (VWR, Radnor, PA) and diluted with DDI. For the microplate reader analysis, 9 mL samples were homogenized (Polytron 1300 D, Kinematica Inc., Bohemia, NY) at 30,000 rpm prior to analyses on the microplate reader. Chlorophyll *a* extraction, biomass, settling and pH were all measured at the end of the experiment. Triplicate samples were taken from each consortium and analyzed in all experiments. Where applicable, ANOVA and the Tukey-Kramer analysis were performed to determine the statistical difference between different consortia combinations using alpha values of 0.05 for both.

*2.3.1 Microplate Methods*

One hundred µL sample aliquots were pipetted into black-sided clear bottomed 96-well plates (Corning 3603, Corning, Tewksbury, MA) for analysis on a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc., Winooski, VT). Samples were first measured for autofluorescence using excitation at 440 nm and emission at 685 nm for chlorophyll concentration (Held 2011). Following these measurements, neutral lipids were measured following methods described in Held & Raymond (2011): 100 µL of 2X working solution (1 µg/mL) of Nile Red (Enzo, Ultra Pure, Farmingdale, NY) was added to each well and

allowed to incubate in the dark for 10 minutes before measuring for fluorescence using 530nm excitation and 570nm emission wavelengths; the fluorescence measurement corresponds to the amount of neutral lipids inside the algal cells.

### *2.3.2 Chlorophyll a*

For chlorophyll *a* measurement, 25-50 mL samples were vacuum filtered and analyzed following SM 10200-H (APHA 2005).

### *2.3.3 Nutrient Analysis*

Phosphate, ammonium, nitrate, and nitrite were measured daily via IC (Metrohm USA, Riverview, FL). A relative standard deviation <5% and correlation coefficient >0.999 was achieved on 5-6 point standard curves before running samples and frequent check standards and blanks were employed to ensure precision. Total nitrogen was measured using Hach Kits (Hach Company, Loveland, CO).

### *2.3.4 Microscopy, Biomass, and Settling*

Fifteen µL samples from each consortium were viewed at 200X and 1000X magnification (Nikon Eclipse Ni-E upright microscope, Melville, NY), to analyze floc morphology and the presence of algal species. At the end of the experiment, biomass was determined by filtering 25 mL samples on pre-rinsed filters following SM 2540-D (APHA 2005). Settling was analyzed by pipetting 1 mL samples into cuvettes and analyzing them on a spectrophotometer for OD750 over the course of an hour following the method used in Salim 2011.

## 3. Results and Discussion

### *3.1 The role of EPS in algae-bacteria consortium*

*C. vulgaris* is a single-celled green alga that grows in suspension, which has been proven difficult to remove in gravitational sedimentation (Salim et al 2011). In order to take advantage of *C. vulgaris*' capability in lipid production and nutrient removal, algal-bacterial floc could potentially improve the biomass settling and ease the application in wastewater treatment. To encourage the formation of algal-bacterial flocs, EPS forming bacteria *S. epidermidis* was introduced and allowed to grow simultaneously with *C. vulgaris*. The consortia were cultivated in both low and high nutrient conditions mimicking secondary effluent and raw wastewater for 4 days. Under low nutrient conditions, the species combinations showed differences in settling (Figure 1), biomass, and autotrophic index (AI) (Table 2), while chlorophyll values were similar. Autotrophic index is the ratio of biomass to chlorophyll *a*, which is an indicator of the portion of the biomass composed of algae. A high AI means algae make up less of the total biomass. The differences observed among the species combinations largely disappeared under high nutrient conditions, but chlorophyll *a* was 40% lower than the other two combinations for *C. vulgaris*/*S. epidermidis* 1:1, a significant difference by ANOVA and Tukey Test standards. While the 1:1 biomass was higher than the other two combinations at low nutrient conditions; at high nutrient levels, biomass differences between jars were not statistically significant. It is clear from the AI data (Table 2) that bacteria grew, but the number and size of visible flocs was much more limited under high nutrient conditions which likely resulted in the lack of difference in settling. Of most interest here, is that at low nutrient levels similar to those found in wastewater effluent, *C. vulgaris* showed improved floc formation, settling and biomass production when grown in the presence of *S. epidermidis*, while differences in lipid production were not significant

(Supplemental Figure 2). This result is similar to the wastewater experiment in Chapter 2, where *C. vulgaris* was grown with endogenous secondary clarifier microorganisms and demonstrated no difference in lipid production in effluent from one wastewater treatment plant. The formation of algal-bacterial flocs confirmed the role of EPS, which is likely to contribute to the "enmeshment" effects and incorporated the *C. vulgaris* cells into the floc (Humenik & Hanna 1971).

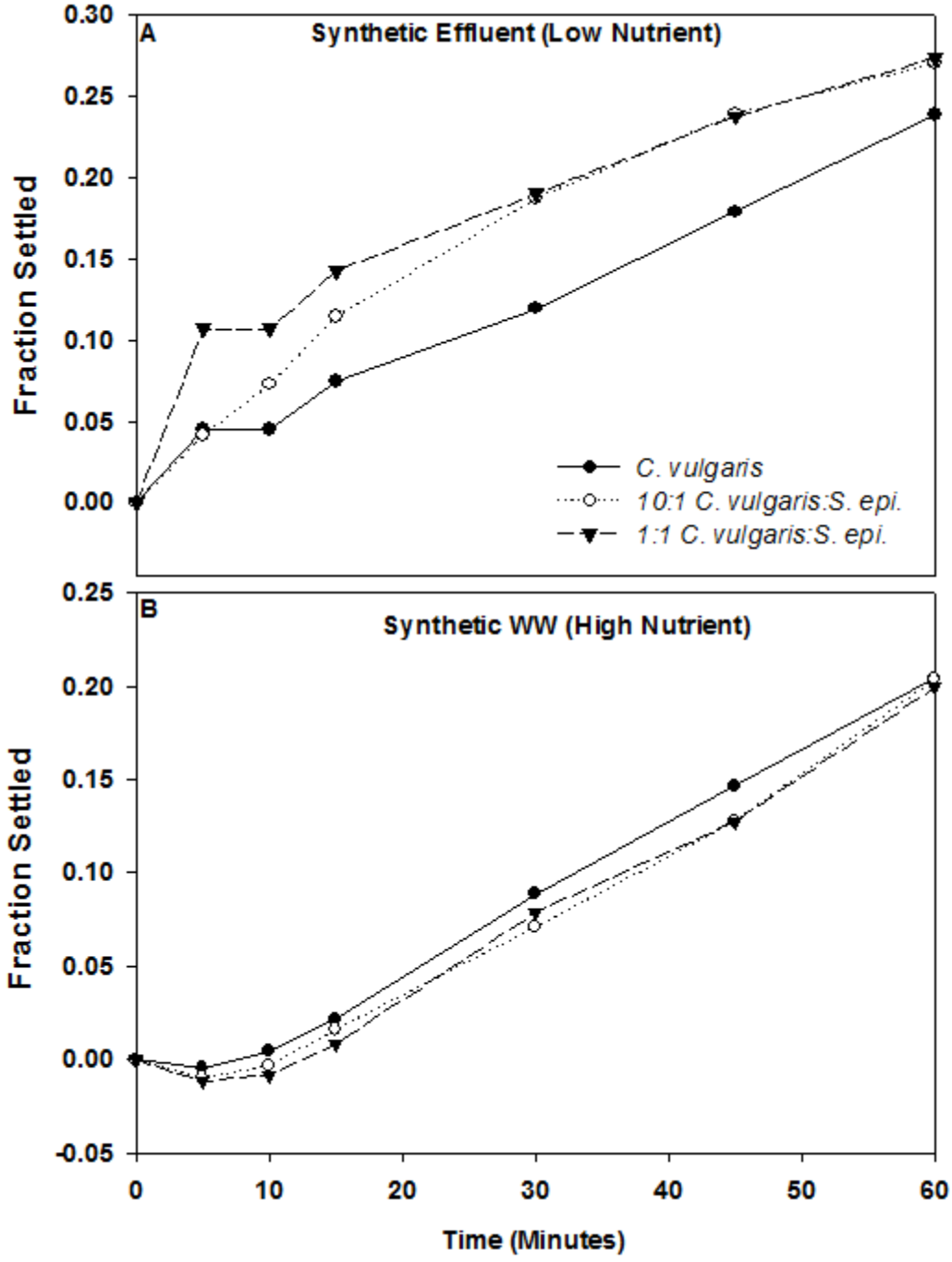



***Figure 1.*** *Algal and bacterial settling in A) Low Nutrient/Synthetic Effluent media and B) High Nutrient/Raw Wastewater media*

*Table 2.* Biomass, Chlorophyll *a* and Autotrophic Index values for *C. vulgaris* and *S. epidermidis* experiments.

| | **Species** | **Biomass (mg/L)** | **Chlorophyll *a* (µg/L)** | **Autotrophic Index** |
|---|---|---|---|---|
| **Experiment #1 Low Nutrient** | *C. vulgaris* | 16±2 | 325±20 | 49 |
| | 10:1 *C. vulgaris*:*S. epi.* | 17±2.3 | 333±18 | 52 |
| | 1:1 *C. vulgaris*:*S. epi.* | 24±3.5 | 312±36 | 77 |
| **Experiment #2 High Nutrient** | *C. vulgaris* | 121±5 | 2098±111 | 58 |
| | 10:1 *C. vulgaris*:*S. epi.* | 116±4 | 2095±44 | 55 |
| | 1:1 *C. vulgaris*:*S. epi.* | 113±2 | 1256±166 | 90 |

*3.2 Settling of Algal-Activated Sludge Flocs*

With the confirmation of floc formation from the initial *C. vulgaris-S. epidermidis* experiment, activated sludge was introduced to multiple species of algae to assess the performance of algae-activated sludge consortia in wastewater treatment. Since activated sludge is the main step in biological nutrient removal, only high strength synthetic wastewater representative of wastewater after primary sedimentation was used in this experiment. Equal amounts of settled biomass (algal or activated sludge) were used to inoculate synthetic wastewater after initial tests showed seed cultures with a range of *C. vulgaris*:activated sludge ratios yielded similar floc formation.

Compared to the *C. vulgaris* and *S. epidermidis* test, even more distinct differences in settling were observed at the end of the six-day algal-activated sludge experiment (Figure 2). The three *Lyngbya*-containing combinations settled the most rapidly, with the *Lyngbya* alone and *Lyngbya*/activated sludge combinations being mostly settled in 5-10 minutes (above 40% of the total biomass). Of particular interest, given its use in biofuel production, combining *C. vulgaris*

with *Lyngbya* or activated sludge improved settling compared to both *C. vulgaris* alone or in combination with *S. epidermidis*. *C. vulgaris* combined with activated sludge settled better than either species did alone.

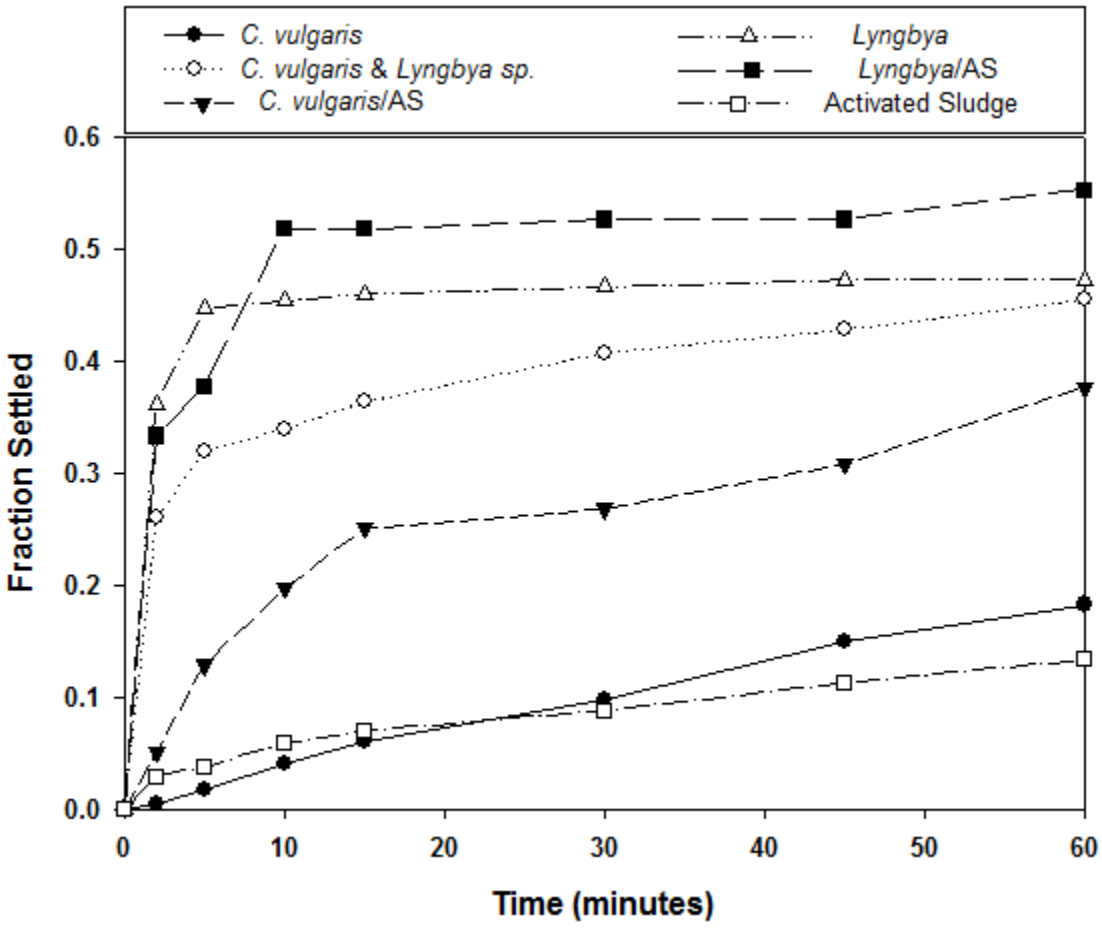



***Figure 2.*** *Settling in algal activated-sludge tests using synthetic raw wastewater.*

The images in Figure 3 provide evidence as to why this difference in settling occurred. *C. vulgaris* alone is a single-celled alga that settles slowly, however it formed flocs that were visible both under a microscope and with the naked eye when grown in combination with other species. *Lyngyba* mixes formed the largest flocs, some with diameters over an inch. Inspection of flocs in

the *Lyngyba*/*C. vulgaris* jars showed large aggregations of *C. vulgaris*, implying the majority of *C. vulgaris* was bound up in these flocs. Other researchers have reported similar results for *C. vulgaris* being bound to flocs with other species of algae or bacteria and not floating free in large numbers (Salim et al. 2011, Van Den Hende et al. 2011).

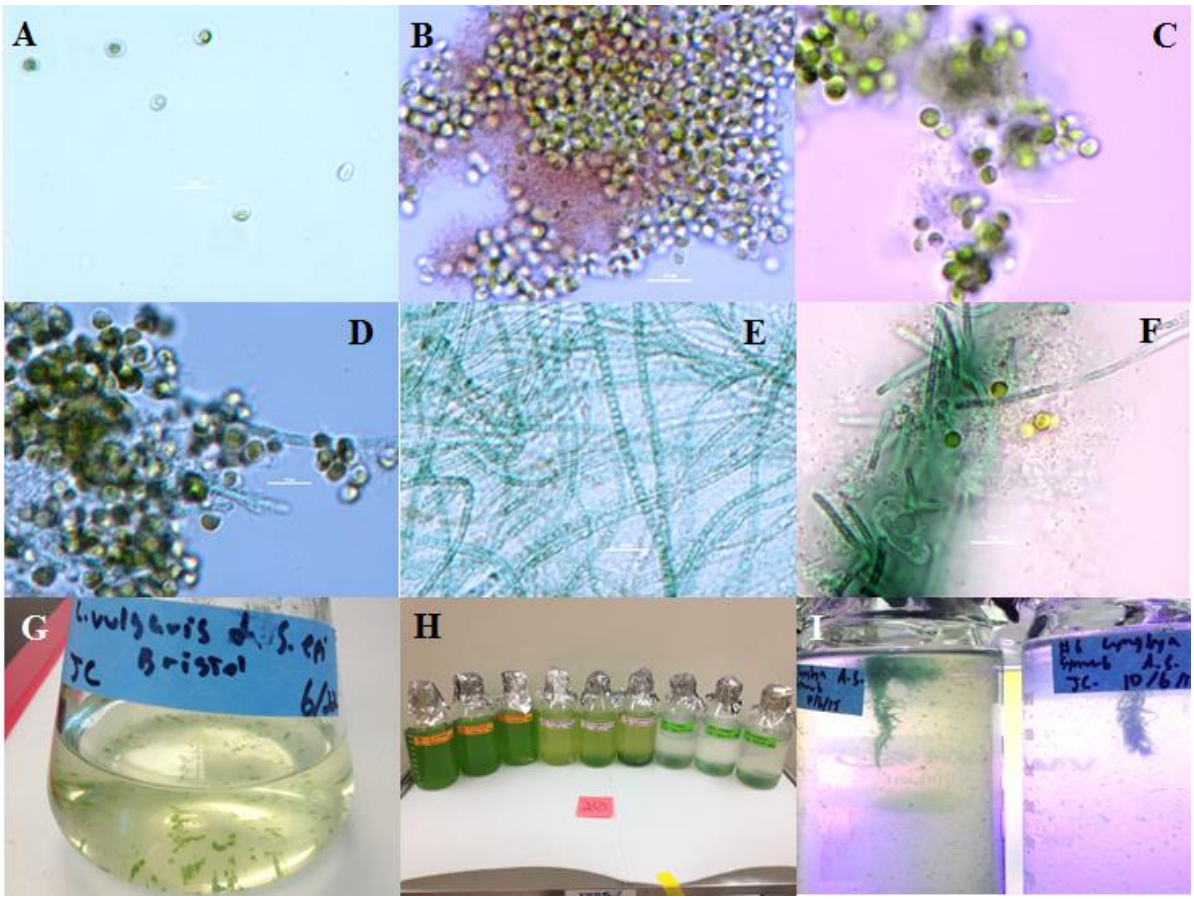



***Figure 3.*** *Images of different species combinations. A-F taken at 1000X magnification A) C. vulgaris alone. B) C. vulgaris & S. epidermidis C) C. vulgaris & Activated Sludge D) C. vulgaris & Lyngbya mix E) Lyngbya mix F) Lyngbya mix & Activated Sludge. Scale bars are 10µm*
*G) C. vulgaris/S. epidermidis flocs H) From Left to Right, Settling differences between C. vulgaris, C. vulgaris/Lyngbya mix, and Lyngbya mix after 20 minutes I) Example of flocs in Lyngbya/Activated Sludge Jars*

*3.3 Nutrient Removal from Algae-Activated Sludge Flocs*

Given the current practice of biological nutrient removal, activated sludge is commonly used in wastewater treatment plants to remove organic carbon, nitrogen and phosphorus. Though effective, anoxic/anaerobic zones are required in addition to aerobic zones to achieve removal of nitrate and phosphate. Using mixed algal bacterial flocs for treatment could save energy on aeration while producing more useful biomass in terms of lipid, fuel, or fertilizer production (Humenik & Hanna 1971, Humenik & Hanna 1970, Pittman et al. 2011, Van Den Hende et al. 2011). During the six-day experiment, phosphate and ammonium concentrations, the major sources of phosphorus and nitrogen in the media, were monitored and are provided in Figure 4. Total Nitrogen (TN) was also measured on three days (Day 0, 3, and 6) and showed a similar trend to ammonium (Supplemental Figure 1). Nitrate and nitrite were monitored but the concentrations were stable and low (~0.8 mg/L) for the duration of the experiment for all consortia, indicating ammonium was consumed by algae directly rather than ammonia oxidizing bacteria in activated sludge. The *C. vulgaris* combinations showed the greatest overall nutrient removal. For phosphate, *C. vulgaris* containing combinations formed a statistically distinct group separate from non-*C. vulgaris* containing combinations, with no significant differences within those groups according to Tukey-Kramer analysis. Though the three *C. vulgaris* combinations recorded the lowest ammonium and TN concentrations on the final day, these differences were not significant by ANOVA/Tukey-Kramer analyses (with the exception of *C. vulgaris* compared to *Lyngbya* for TN) (Table 4). For ammonium, the other activated sludge combinations ended with concentrations similar to the *C. vulgaris* groups, showing more rapid declines on the last two days which corresponded with the visible increase in single-celled algae in those jars that was likely present in the activated sludge inoculum but at low initial concentrations. Figure 5

shows evidence of this and shows chlorophyll fluorescence over time for all species combinations.

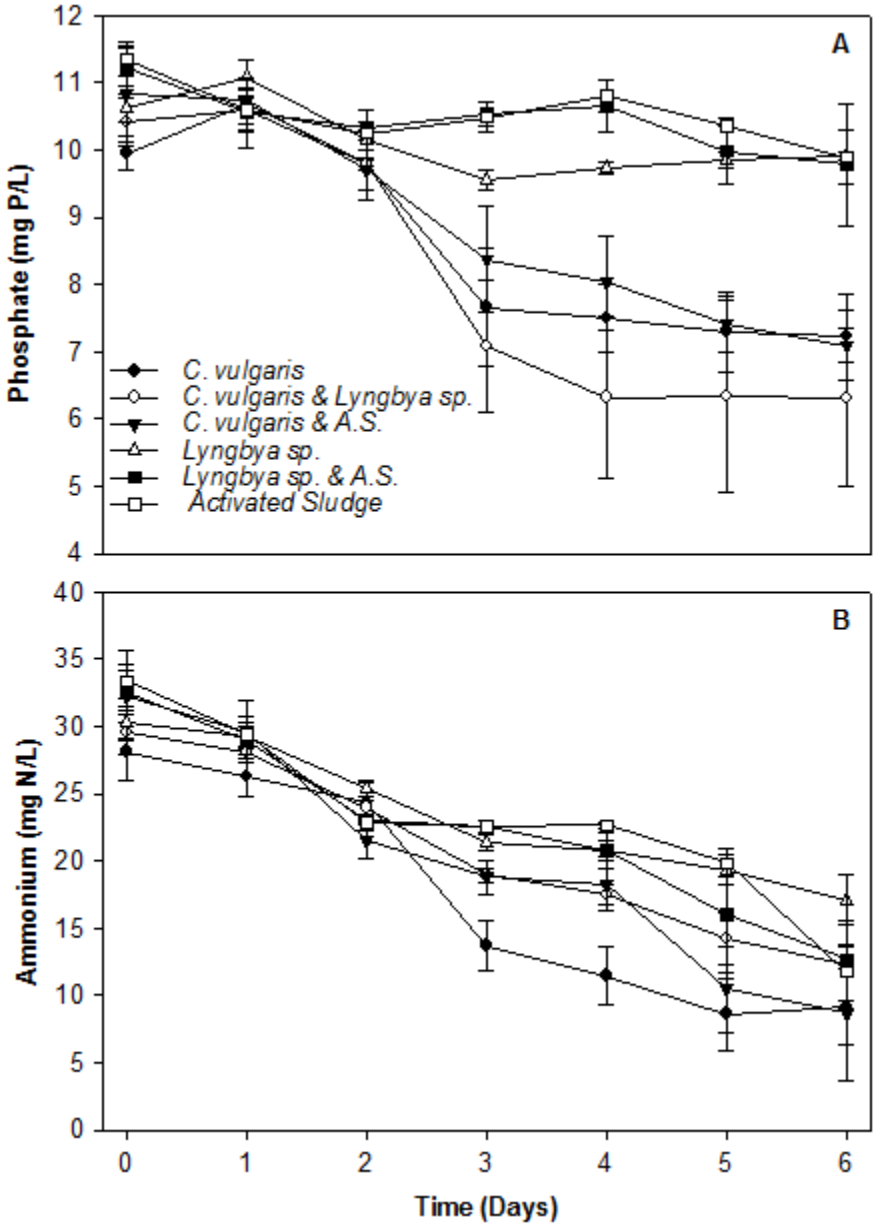



***Figure 4.*** *Nutrient Concentrations Over Time.*

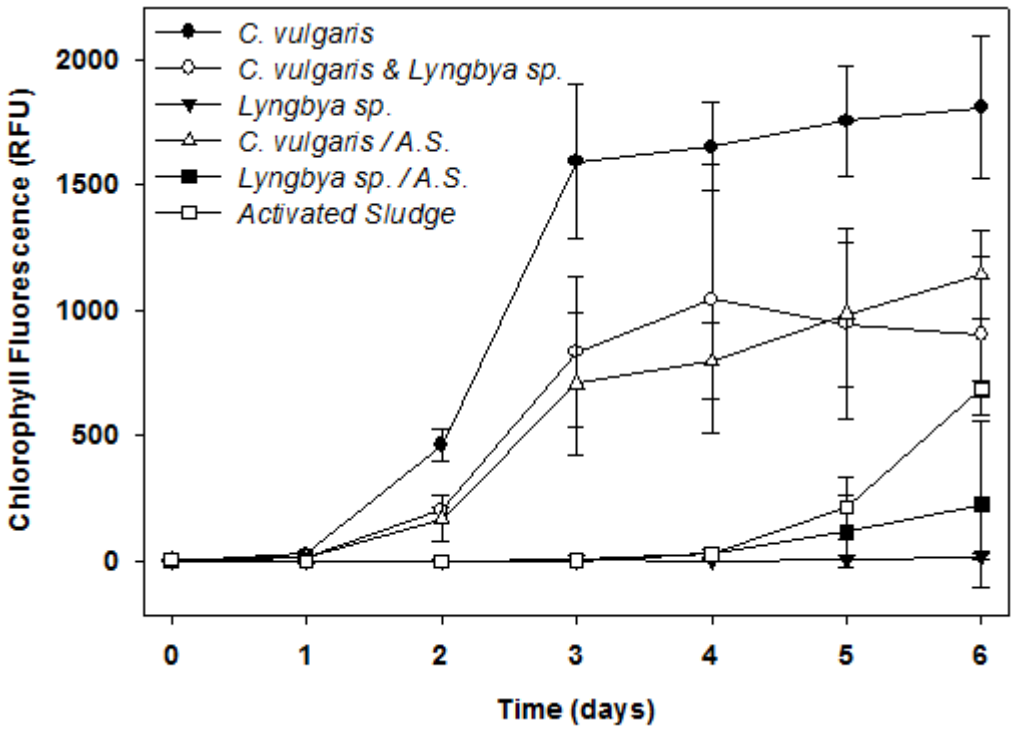



***Figure 5.*** Chlorophyll Fluorescence Over Time.

Along with having generally higher nutrient removal, the three *C. vulgaris* mixes showed the highest measured chlorophyll fluorescence and final biomass, though by Tukey-Kramer analysis, only the two highest biomasses, *C. vulgaris* and *C. vulgaris*/activated sludge, were significantly greater than the two lowest biomasses (*Lyngbya* alone and activated sludge) (Table 4). Since a previous study (Chapter 1) showed a different relationship between values for traditional chlorophyll *a* extraction and chlorophyll fluorescence for *Lyngbya sp.* (a type of cyanobacteria) than for two species of green algae, we also conducted chlorophyll *a* extraction at the end of the experiment. Figure 6 shows the percent phosphorus and nitrogen removed standardized for both chlorophyll *a* and biomass; which could highlight any differences in the relative contributions to nutrient removal by bacteria and algae in the consortia. For example, compared to *C. vulgaris* alone, *C. vulgaris* with activated sludge showed greater N and P removal per unit of biomass and

less per unit of Chlorophyll *a*, showing that bacteria played a larger role in nutrient removal in the combination with activated sludge.

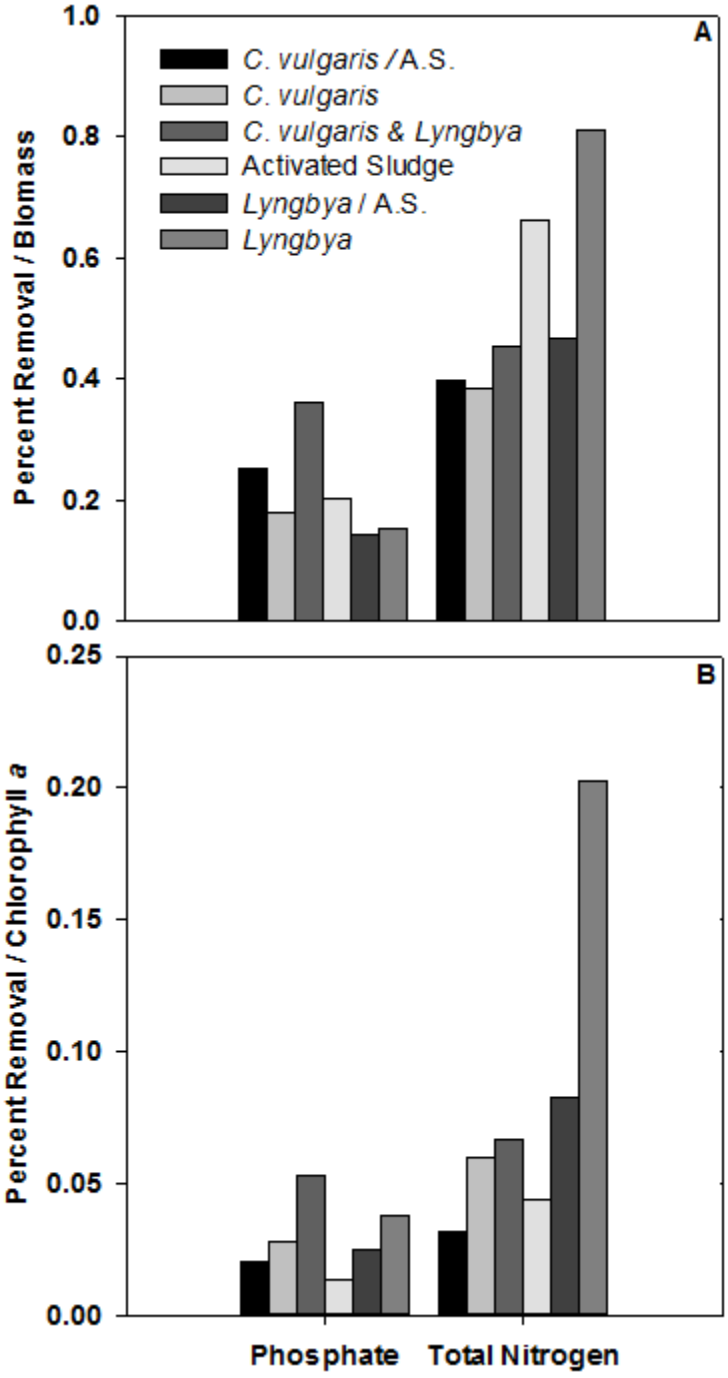



***Figure 6.*** Nutrient Removal Per Unit of A) Biomass & B) Chlorophyll *a*

Compared to activated sludge alone, *Lyngbya* and activated sludge had lower N & P removal per unit of biomass and higher removal per unit of Chlorophyll *a*, showing that bacteria played a larger role in nutrient removal in the jar with just activated sludge, which is consistent with the nature of activated sludge (mostly bacteria). While *Lyngbya* and activated sludge had weaker total nutrient removals, they had the highest nitrogen removal per unit of biomass and *Lyngbya* had much higher TN removal per unit of chlorophyll *a*. The *Lyngbya* / *C. vulgaris* combination had the greatest phosphate removal overall, per unit of biomass, and per unit of chlorophyll *a*.

*3.4 Lipid Production*

Lipid production is shown in Figure 7. Neutral lipids spiked early in the *Lyngbya* jars and this corresponded to rapid growth in the bacteria that are a part of the consortium. As mentioned previously, the balance in growth between the *Lyngbya* and bacteria is determined by the media (lower nutrients favors algae, higher favors bacteria, especially when yeast extract or peptone are used). Neutral lipids gradually increased in the *C. vulgaris* jars in line with the growth (see Chlorophyll fluorescence Figure 5). Chapter 2 and studies by other authors have shown lipid content of this species to increase under nitrogen-limited conditions (Mujtaba et al. 2012). Those conditions were not reached in this experiment (see Figure 4). For final day lipid values, the highest lipid values were recorded for *C. vulgaris*/activated sludge and *C. vulgaris/Lyngbya*, though only the differences between *C. vulgaris*/activated sludge and the two lowest, *Lyngbya*/activated sludge and *C. vulgaris* were determined to be significant via the Tukey-Kramer procedure (Table 4). While Van Den Hende et al. (2011) reported flocs with higher lipid

content than activated sludge, none of the consortiums we looked at had significant differences in neutral lipid concentrations compared to the activated sludge.

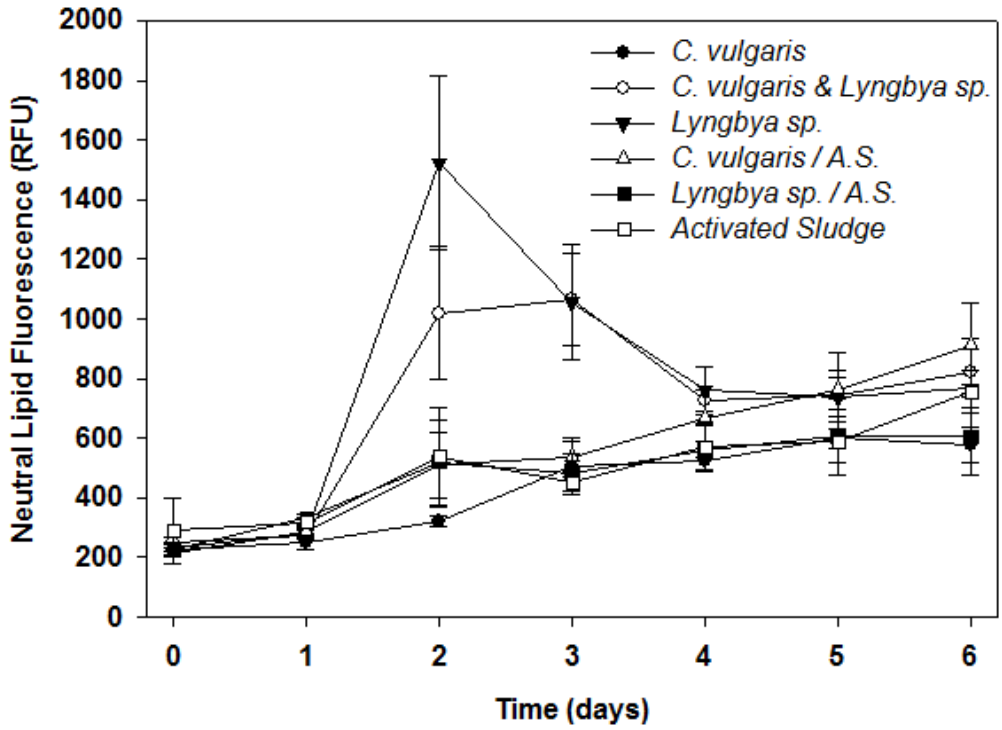



***Figure 7.*** Neutral Lipid Fluorescence Over Time.

*3.5 Overall Comparisons*

As discussed above, the consortia studied in these three experiments often varied in terms of biomass production, floc formation and settling, chlorophyll *a* concentration, nutrient removal, and neutral lipid production. Optimizing the species combinations for simultaneous wastewater treatment and biofuel production requires balancing all these variables at the same time. Table 3 summarizes biomass and chlorophyll *a* production over the three experiments and includes the AI, a measure of the relative contribution of algae to the total biomass (with lower values meaning more algae). Table 4 shows statistically significant differences between the

consortia tested in Experiment 3 in terms of nutrient removal, and production of neutral lipids and biomass.

***Table 3.*** *Biomass, Chlorophyll a, and Autotrophic Index for all conditions tested.*

| | **Species** | **Biomass (mg/L)** | **Chlorophyll *a* (µg/L)** | **Autotrophic Index** |
|---|---|---|---|---|
| **Experiment #1 Low Nutrient** | *C. vulgaris* | 16±2 | 325±20 | 49 |
| | 10:1 *C. vulgaris*:*S. epi.* | 17±2.3 | 333±18 | 52 |
| | 1:1 *C. vulgaris*:*S. epi.* | 24±3.5 | 312±36 | 77 |
| **Experiment #2 High Nutrient** | *C. vulgaris* | 121±5 | 2098±111 | 58 |
| | 10:1 *C. vulgaris*:*S. epi.* | 116±4 | 2095±44 | 55 |
| | 1:1 *C. vulgaris*:*S. epi.* | 113±2 | 1256±166 | 90 |
| **Experiment #3 Mixed Species** | *C. vulgaris* | 152±11 | 970±360 | 157* |
| | *C. vulgaris & Lyngbya* | 109±10 | 740±410 | 148 |
| | *Lyngbya sp.* | 44±14 | 176±44 | 250 |
| | *C. vulgaris*/A.S. | 137±31 | 1701±286 | 80 |
| | *Lyngbya*/A.S. | 90±35 | 508±211 | 177 |
| | Activated Sludge | 63±32 | 954±428 | 66 |

*Value higher than expected.

By comparing the initial species ratio, AI, and settling data for Experiment 1, it is clear that when present, even at low fractions of the total biomass, EPS-producing bacteria can improve settling; though Experiment 2 shows this is not always the case. In Experiment 3, the AI values are more difficult to interpret and it is not clear why such a high value was recorded for *C. vulgaris* alone. Bacterial contamination would be an explanation, but was not visibly observed. High values for *Lyngbya*-containing consortia could be due to bacterial growth and/or low chlorophyll *a* readings

since *Lyngbya* are cyanobacteria, which can have a different set, or ratio, of chlorophyll-like pigments compared to other algae.

The previously discussed groupings for phosphate removal can be seen in Table 4, with *C. vulgaris*-containing consortia removing more than the other groups. While differences in mean values for ammonium existed at the end of the experiment, none of these were significant by the standards employed here, though the difference between the top and bottom performers in TN removal was significant, with *C. vulgaris* removing the most. *C. vulgaris* with activated sludge demonstrated the greatest average neutral lipid production, which was significantly higher than the two lowest consortia. This combination along with *C. vulgaris* alone, had the highest mean biomass production which was significantly higher than the two lowest producers, activated sludge and *Lyngbya*. Activated sludge alone, performed poorly or in the middle of the pack in terms of biomass production, nutrient removal, settling, and neutral lipid production, while *C. vulgaris* with activated sludge did much better. This demonstrates the potential advantages of using algae or mixed-algal bacterial consortiums in wastewater treatment and biofuel production.

***Table 4.*** *Statistically significant differences in nutrient removal, neutral lipid content, and biomass production between consortia.*

| Consortia Compared | Phosphate | Ammonium | Total Nitrogen | Lipid | Biomass |
|---|---|---|---|---|---|
| 1 to 2 | | | | | |
| 1 to 3 | X | | X | | X |
| 1 to 4 | | | | X | |
| 1 to 5 | X | | | | |
| 1 to 6 | X | | | | X |
| 2 to 3 | X | | | | |
| 2 to 4 | | | | | |
| 2 to 5 | X | | | | |
| 2 to 6 | X | | | | |
| 3 to 4 | X | | | | X |
| 3 to 5 | | | | | |
| 3 to 6 | | | | | |
| 4 to 5 | X | | | X | |
| 4 to 6 | X | | | | X |
| 5 to 6 | | | | | |

| Consortia | Species |
|---|---|
| 1 | *C. vulgaris* |
| 2 | *C. vulgaris & Lyngbya* |
| 3 | *Lyngbya* |
| 4 | *C. vulgaris* / A.S. |
| 5 | *Lyngbya sp.* / A.S. |
| 6 | Activated Sludge |

"X" Indicates significant difference between compared means via Tukey Test using $\alpha$=0.05 for ANOVA and Tukey threshold

## 4. Conclusion

This study showed a benefit to combining species with different known strengths when seeking to optimize nutrient removal, settling, and biomass and neutral lipid production during wastewater treatment and biofuel production. The two combinations, *C. vulgaris* and *Lyngbya*, and *C. vulgaris* and activated sludge overall showed the greatest combined nutrient removal, biomass formation, and neutral lipid production, and they settled better than *C. vulgaris* alone, which could improve harvesting in both wastewater treatment and biofuel production. *C. vulgaris* with activated sludge performed better than activated sludge in terms of settling, neutral lipid production, nutrient removal, and biomass production showing a clear benefit to incorporating algae into secondary wastewater treatment.

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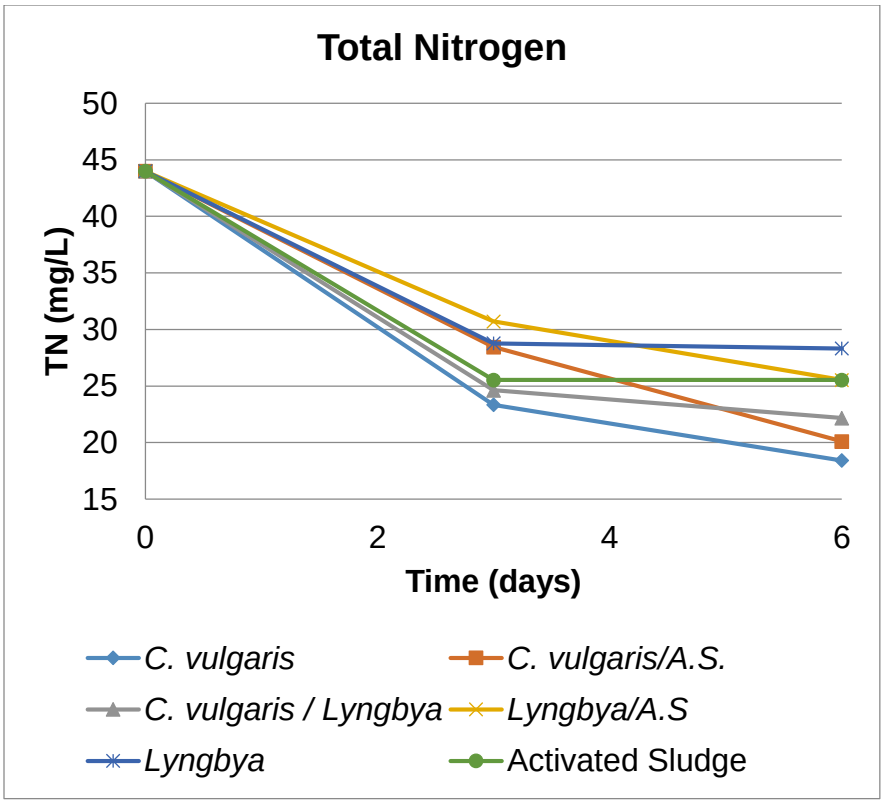
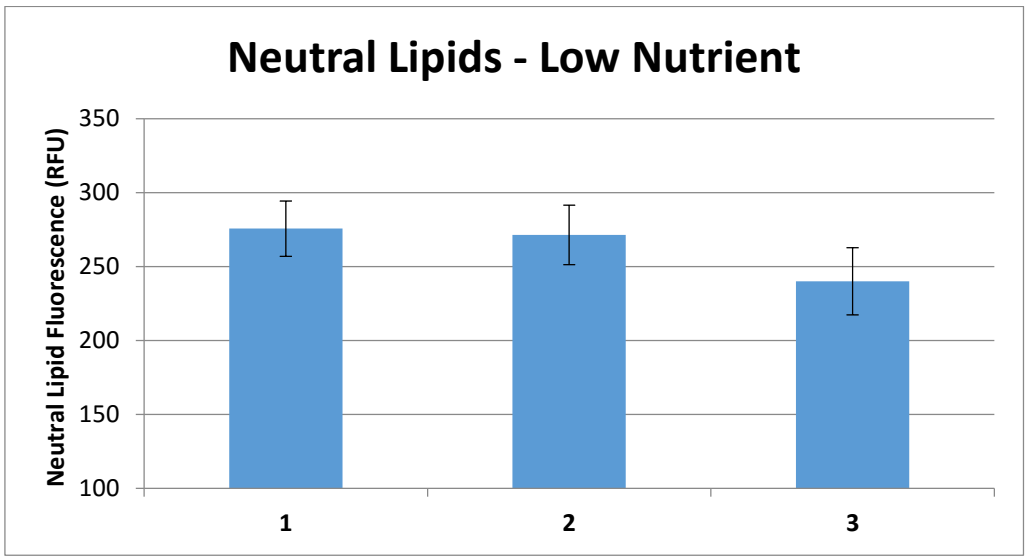
**Supplemental Materials**

***Supplemental Figure S1***- *Total Nitrogen Over Time.*




***Supplemental Figure S2*** *– Experiment 1 Neutral Lipid Fluorescence*



***Supplemental Table T1*** *– Carbon and Nitrogen Percentages for Experiments 1-3*

| | Species | Carbon (%) | Nitrogen (%) |
|---|---|---|---|
| **Low Nutrient** | *C. vulgaris* | 58 | 11 |
| | 10:1 *C. vulgaris* :*S. epi.* | 53 | 10 |
| | 1:1 *C. vulgaris* :*S. epi.* | 33 | 6 |
| **High Nutrient** | *C. vulgaris* | 47 | 8 |
| | 10:1 *C. vulgaris* :*S. epi.* | 49 | 8 |
| | 1:1 *C. vulgaris* :*S. epi.* | 48 | 8 |
| **Mixed Species** | *C. vulgaris* | 47 | 8 |
| | *C. vulgaris & Lyngbya* | 41 | 7 |
| | *Lyngbya sp.* | 35 | 7 |
| | *C. vulgaris* /A.S. | 51 | 9 |
| | *Lyngbya* /A.S. | 51 | 9 |
| | Activated Sludge | 67 | 12 |

***Supplemental Table T2*** *– Example ANOVA & Tukey-Kramer Analysis*

| ANOVA α=0.05 | | | | | | |
|---|---|---|---|---|---|---|
| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between Groups | 240098 | 5 | 48019.6 | 4.858235 | 0.011639 | 3.105875 |
| Within Groups | 118610 | 12 | 9884.1667 | | | |
| | | | | | | |
| Total | 358708 | 17 | | | | |
| | | | | | | |
| | | Critical Value α=0.05 | | 4.75 | | |
| | | | | | | |
| Tukey-Kramer | | Num df | 6 | Den df | 12 | |
| Comparison | Absolute Difference | Critical Range | Results | | | |
| 1 to 2 | 239.7 | 272.6 | Means not sig dif | | | |
| 1 to 3 | 187.7 | 272.6 | Means not sig dif | | | |
| 1 to 4 | 332.0 | 272.6 | Means Sig. Dif. | | | |
| 1 to 5 | 27.7 | 272.6 | Means not sig dif | | | |
| 1 to 6 | 175.0 | 272.6 | Means not sig dif | | | |
| 2 to 3 | 52.0 | 272.6 | Means not sig dif | | | |
| 2 to 4 | 92.3 | 272.6 | Means not sig dif | | | |
| 2 to 5 | 212.0 | 272.6 | Means not sig dif | | | |
| 2 to 6 | 64.7 | 272.6 | Means not sig dif | | | |
| 3 to 4 | 144.3 | 272.6 | Means not sig dif | | | |
| 3 to 5 | 160.0 | 272.6 | Means not sig dif | | | |
| 3 to 6 | 12.7 | 272.6 | Means not sig dif | | | |
| 4 to 5 | 304.3 | 272.6 | Means Sig. Dif. | | | |
| 4 to 6 | 157.0 | 272.6 | Means not sig dif | | | |
| 5 to 6 | 147.3 | 272.6 | Means not sig dif | | | |

## Conclusion

The goal of this dissertation was to increase our understanding of the use of algae in wastewater treatment and biofuel production. Chapter 1 compared alternative and more traditional methods used to study algal productivity in the areas of precision, sensitivity, and time and sample size requirements using algal species of different morphologies to inform the selection of methods used during future algal research. The alternative methods assessed here, using Coulter counter and microplate methods, frequently provided greater precision and accuracy at the concentrations explored in this study while providing more rapid analysis. Traditional methods proved useful for correlating relative units to known concentration units and for dealing with species that present problems for newer methods such as colony or floc formation. The neutral lipid fluorescence microplate method offered a wide range of benefits over the more traditional lipid extraction method including smaller sample and time requirements and more specific targeting of a lipid class that is most useful for biodiesel generation.

Chapter 2 focused on nutrient removal by *C. vulgaris* under a range of nutrient ratios and nutrient limited conditions; in both real and synthetic secondary clarifier effluent. It also examined the impact of endogenous microbial communities on nutrient removal and lipid production by *C. vulgaris*. This experiment demonstrated *C. vulgaris* has the ability to further reduce nutrient levels in WWTP effluent, even after nitrogen or phosphorus becomes limited. Using *C. vulgaris* to treat effluent lead to higher neutral lipid contents in produced biomass and neutral lipid production was highest when nitrogen became exhausted. When *C. vulgaris* co-existed with indigenous microorganisms, synergistic effects were observed for nutrient removal but not for lipid production.

Chapter 3 expanded on this work by looking at multiple combinations of algal and bacterial species to see how settle-ability, nutrient removal, and biomass and neutral lipid production were impacted under nutrient conditions found in effluent and raw wastewater. Combining *C. vulgaris* with other species of bacteria or filamentous algae often improved settling. Compared to *C. vulgaris* alone, two combinations, *C. vulgaris* with *Lyngbya*, and *C. vulgaris* with activated sludge demonstrated improved settling and neutral lipid production, while maintaining similar levels of nutrient removal and biomass formation. *C. vulgaris* combined with activated sludge performed better than activated sludge alone in terms of settling, neutral lipid production, nutrient removal, and biomass production; demonstrating potential benefits of incorporating algae into secondary wastewater treatment.

Overall, this research provides additional evidence that algae has the potential to enhance the secondary or tertiary treatment of wastewater while generating easily harvested biofuel feedstocks. The methods findings have the potential to improve the efficiency, precision, and comparability of future research on the use of algae for wastewater treatment and biofuel production. If utilized, these findings could improve the sustainability of both algal research and the use of algae for wastewater treatment and biofuel production; leading to improvements in water quality, ecological health, fisheries production, and emissions from energy production.

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