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# Algae: Opportunities for Biomass Feedstock Production, Wastewater Treatment and Educational Outreach

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Algae: Opportunities for Biomass Feedstock Production,

Wastewater Treatment and Educational Outreach

by

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## DEDICATION

I dedicate this body of work to my loving family and friends.

*"When I am operating at my best, my work is my prayer. It comes out of the same place that prayer comes out of – the center, the heart."*

has always supported my academic endeavors. I could never ask for more. My brother's legacy

lives on and inspires me daily.

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**ABSTRACT**

Algae are a diverse group of simple organisms that lack roots, stems or leaves and are able to use sunlight, carbon dioxide, and nutrients to produce complex compounds, such as carbohydrates, proteins and lipids. These compounds, especially lipids, are highly sought-after by agricultural, nutraceutical and energy interests. Although there is great potential for algae derived biofuels, there are technical and economic challenges associated with their cultivation. Relevant to this dissertation, the environmental impacts associated with algae cultivation can be reduced by using municipal and agricultural wastewaters as a water and nutrient source. This research was divided into three sections to address current challenges in the algal industry and science, technology, engineering and math (STEM) education. The sections were: 1) examination of the growth of indigenous algae on wastewater (centrate) produced from dewatering anaerobically digested municipal sludge, 2) examination of the effect of non-axenic conditions on the growth of three different algal cultures using wastewater from a recirculating aquaculture system (RAS), and 3) using wastewater treatment and algae to increase scientific inquiry in authentic science research with high school students. In the first section, indigenous algae were cultivated on centrate under natural light conditions in a semi-continuous photobioreactor. A non-linear bio-optical model was developed considering Michaelis-Menten photosynthesis-irradiance response. The bio-optical model was applied to fit the cumulative biomass data and had an R-squared value of 0.96. The second section examined the growth and accumulation of storage product. Higher calorific values were observed for all algae cultures when grown under non-axenic conditions, most likely due to significantly higher lipid contents. Significantly higher algal lipid

contents under non-axenic conditions may be attributed to the stress of the presence of RAS microorganisms. Finally, having a university-based algal project with involvement of University of South Florida (USF) researchers, teachers and high school (HS) students facilitated increased scientific understanding and skills among HS students. Outcomes included graduate students gaining greater in-depth practical understanding as these students had to learn skills, such as designing a photobioreactor and then immediately had to teach HS students how to construct photobioreactors, design and conduct experiments, and gather scientific data. HS students gained a greater understanding of biological and chemical processes, such as photosynthesis. In addition, they learned important skills, such as calculating means and standard deviations using Excel, orally communicating scientific concepts and preparation of a PowerPoint presentation.

# CHAPTER 1: INTRODUCTION

## 1.1 Background

Algal biofuels can help to meet ever-increasing United States energy demands [2,3]. In 2010, the United States was the largest energy consumer in the world, using approximately 98 quadrillion British Thermal Units (BTU) [4,5]. At present, petroleum accounts for 37% of total energy use. Most (94%) of this petroleum is used for transportation [6]. Renewable forms of energy currently account for only 8% of total energy consumption [6,4]. Biomass only accounts for half of the energy derived in the United States from renewable forms [6]. It is estimated that by 2035, there will only be a 1.7% increase in the use of renewable forms of energy [7]. The two main reasons why renewable forms do not account for a higher percentage in the United States are: (1) they are highly politicized and (2) there needs to be more research and development to make new innovations commercially viable.

The use of algal biofuel production systems is a promising technology for meeting future energy needs [8,9]. Microalgae have the ability to fix carbon dioxide through multifarious photosynthetic activities. Algae are capable of utilizing sunlight, carbon dioxide, nutrients and water from wastewater streams as the building blocks to produce complex compounds, such as carbohydrates and lipids. These valuable compounds, especially lipids, are highly sought-after by large energy and nutraceutical entities [10,2]. Of all the advanced biomass feedstocks, such as switchgrass and organic waste, algae-based biofuel are very promising [10], as algal productivity can be between 20 and 100 times higher than terrestrial energy crops and they can be produced in a manner that does not compete with arable land. However, some researchers do not anticipate

algae biofuel becoming an economically feasible option in the immediate future due to the many technical challenges [11,12].

The use of wastewater as a growth medium can reduce water and fertilizer needs for algae production, making the process more practical and economical [12]. Using wastewater as an algal growth medium may present mutually beneficial effects, especially when considering nutrient removal from the wastewater [13-15]. However, high strength wastewater streams may contain compounds, such as ammonia, that are toxic to algae at high concentrations (i.e. total ammonia nitrogen [TAN] > 100 mg/L as nitrogen). This problem may be overcome by bio-prospecting indigenous algal species that are already adapted to or possess the ability to become adapted to wastewater environments. However, in some cases genetic transformation or bio-engineering may be required to increase productivity of desired end-products, usually lipids, with comparable characteristics to petroleum-derived products.

Prior research on algal biofuels has focused on very unnatural monoculture systems, with significant investments required to keep cultures axenic (free of non-target microbial agents), or at least preventing contamination, particularly predation [16,13,17]. Previous studies suggest that species and niche diversity are crucial in creating resilient (able to produce valuable products despite stressors) natural and engineered systems; however, little is known about how algal-microbial diversity influences energy product outputs and nutrient removal [18,19]. The contribution of each of these sections is summarized below in Figure 1.1. The overall goal of this research was to contribute to greater understanding of how indigenous microbial-algal interactions influence biomass and end-product generation. Algal Wastewater Reactor Sytems (AWRS).
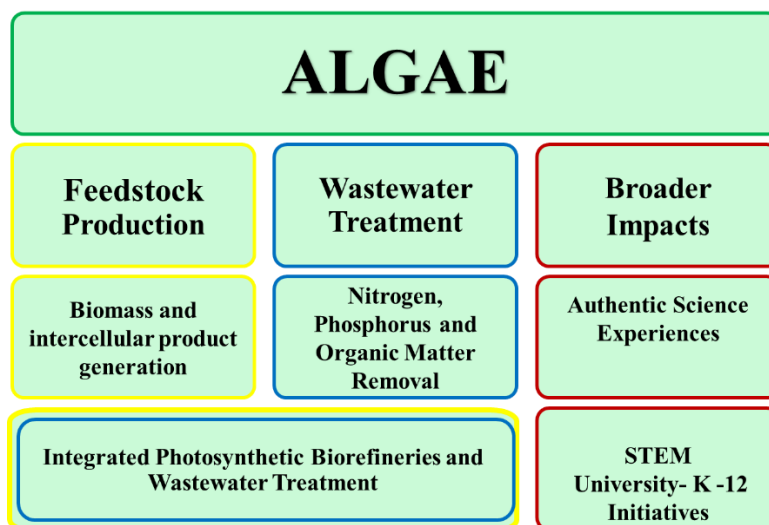
Figure 1.1: Algal biofuel research. This study's algal biofuel research contributed to the areas of: 1) wastewater treatment, 2) feedstock production, and 3) broader impacts. The 2nd tier represents the main accomplishments in these areas, while the 3rd tier highlights the future anticipated outcomes of this research.


## 1.2 Research Goals

The specific goals of this research included:

1. Examine biomass and lipid production of an indigenous algae consortium when municipal centrate and aquaculture wastewaters were used as growth substrates.

2. Determine the effect of natural irradiance variability on biomass production in pilot-scale photobioreactor systems.

3. Investigate the effects of indigenous microbes on algal system performance as defined by: 1) productivity of a desirable end-product (biomass, chlorophyll, starch and lipids), and 2) removal of nutrients and organics from aquaculture wastewater.

4. Facilitate greater understanding of scientific principles and interest in science among high school students through authentic scientific research on algal biofuel production.

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The following research questions and objectives guided this research:

*Research question I: Can high growth and nutrient removal rates can be achieved in wastewater centrate using a consortium of indigenous algae?*

Objectives:

1. Acclimate an algal consortium capable of growing on high ammonia strength wastewater from dewatering anaerobically digested municipal sludge centrate with total nitrogen as ammonia (TAN) greater than 100 mg/L.

2. Design, construct and operate a semi-continuous photobioreactor with indigenous algae using sludge centrate as the growth medium.

3. Determine biomass and lipid production and nutrient removal rates for the indigenous algal consortium in the photobioreactor under natural irradiance.

4. Develop and apply an irradiance-based model to understand the effect of light availability on biomass production.

*Research question II: Does algal species diversity and presence of wastewater microbes increases system performance in AWRS?*

Objectives:

1. Investigate the characteristics of aquaculture wastewater as a growth medium for algae production.

2. Grow an indigenous consortium, *Chlorella* and *Scenedesmus* cultures on aquaculture wastewater.

3. Investigate the effect of indigenous microbes on biomass, chlorophyll, starch and lipid production and nutrient removal efficiencies in aquaculture wastewater.

4. Investigate the effect of algal diversity on biomass and end-product generation and nutrient removal efficiencies in aquaculture wastewater.

*Research question III: Does authentic science research experiences for high school students increase participation, STEM interests, scientific knowledge and skills among HS and graduate students?*

Objectives:

1. Collaborate with a faculty member and graduate student in the USF College of Education to design, implement and evaluate an authentic science research experience for high school students.

2. Construct a photobioreactor using easily assessable equipment.

3. Work with local high school teachers and students to investigate algal growth in photobioreactors under varying conditions.

4. Assess the attitudes and perceptions of HS and graduate students of this authentic science experience.

5. Determine the contribution of the high school students in generating useful data for this project.

## 1.3 Dissertation Organization

A significant amount of the initial research was based on foundational work and data collected during experiments conducted with the indigenous algal consortium. Subsequent steps were taken to determine how indigenous microbes and algal diversity influence performance (biomass and valuable end-product production). Figure 1.2 shows the interconnectedness of the different phases. The dissertation chapters address the following topics:

1. Chapter 1: Introduction, research objectives, hypotheses and organization

2. Chapter 2: A literature review discussing algae use and wastewater treatment

3. Chapter 3: Algal biomass production using municipal sludge centrate as a growth medium and development of an irradiance-based model

4. Chapter 4: Production of algal biomass, chlorophyll, starch and lipids using aquaculture wastewater under axenic (algal monocultures without other microorganisms) and non-axenic conditions

5. Chapter 5: Authentic science research among high school students

6. Chapter 6: Conclusions and recommendations

**Phase I: Pilot scale experiments**

- Pilot scale experments using an indigenous algal consortium.
- Algal biomass production and nutrient removal determined.
- Irradiance based model developed

**PhaseII: High school student experiments**

- High school students were taught key coneplts.
- Effect of wastewater strength and nitrogen form on biomass production was determined

**Phase III: Bench scale experiments**

- Experiments determined if there are significant differences in biomass and end-prodect development between axenic and non-axenic conditions.

**Overall scientific contribution and broader impact**

- Quantify effects of algal cultivation on:1)feedstock production and 2) wastewater treatment.
- Assess the effect of authentic science experiences.

Figure 1.2: Interconnected phases of this study in chronological order. The overall scientific and community contributions are dependent on the synergy between the phases

## CHAPTER 2: ALGAE AND WASTEWATER TREATMENT

### 2.1 Introduction to Algae

Algae are a large and diverse group of simple organisms that lack roots, stems or leaves. Most algae are eukaryotic and are able to utilize inorganic carbon sources to support their photosynthetic metabolism. There are both unicellular and multicellular forms of algae. The largest and most complex forms are marine seaweeds; some kelp species are able to grow to a total length of 65 meters [20]. They are ubiquitous and have many varying forms and functions that allow them to adapt to different environments, such as freshwater, saltwater, soil, streams, slow pools and lakes. Some algae can also thrive in extreme environments, such as hot springs and brine lakes.

Algae can be harmful in the environment, as algal blooms in marine and freshwater ecosystems occur in response to nitrogen and phosphorus inputs. A summary of common algae at different levels of nutrient enrichment is shown in Table 2.1. An algae bloom in Lake Erie that was approximately 1,920 square miles and crippled fishing and tourism industries in 2011 is shown in Figure 2.1. In these eutrophic environments, algae blanket the water; light penetration becomes very limited and submerged plants' photosynthesis and subsequent oxygen production becomes severely constrained. In addition, when nutrients are depleted in eutrophic systems and the algal population dies-off, opportunistic aerobic bacterial communities utilize the organic matter. When dissolved oxygen levels reach critically low levels (<4mg/L), many aquatic organisms, such as fish, will die. In addition, algae can cause taste and odor problems in drinking water and can produce toxins, which cause gastroenteritis outbreaks [21].

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Table 2.1: Common algae and their relation to nutrient levels

| Lake Trophy | Nutrient characteristics | Common species |
|---|---|---|
| Oligotrophic | Low | *Straurastrum*, Cryptophytes, many oligotrophic diatoms, Melosira, Dinobryon |
| Mesotrophic | Intermittent periods of high nutrients | *Dinoflagellates, Ceratium spp., Glenodinium* |
| Eutrophic | High | *Rhodomonas minuta*, major contributor to blue-green algae blooms |

Adapted from Crittenden et al [21], pp 206



Figure 2.1: Satellite photo of Lake Erie on October 5th, 2011. Photo source: National Aeronautics and Space Administration (NASA)

Although algae cause the human and environmental problems described above, algae also have many beneficial uses including treating wastewater, and providing food products for both animal and human consumption. Treatment lagoons, which are also called stabilization ponds or oxidation ponds, have been used to treat domestic and animal wastewater [22]. The algae in treatment lagoons provide oxygen for the biodegradation of organic matter [23], uptake nutrients

[24-27], and remove other pollutants, such as heavy metals [28-31] and endocrine disrupting compounds [32-34]. The more recent research, including this study, focuses on optimizing algae production and treatment of various wastewater feeds (Section 2.3) and the production of high value intracellular products within the biomass feedstock (Chapter 4). Section 2.3.1 and 2.3.2 examine municipal centrate and aquaculture wastewater sources in greater detail as these two waste streams were considered as culture media in experiments.

**2.2 Requirements for Algal Growth**

A number of factors affect growth rates of algae, including light irradiance, carbon source (inorganic carbon for photoautotrophs, organic carbon for heterotrophs and mixotrophs), inorganic macronutrients (nitrogen and phosphorus), and trace nutrients, such as vitamins and metals (Table 2.2). Irradiance is one of the necessary ingredients in supporting the metabolism of photoautotrophs: algae and plants. Most (45%) of the visible light spectrum between 400-700nm is available for algal growth [35-37]. Approximately 8.5 MJ are required to produce one mole of glucose [14]. Chapter 3 further examines the effect of fluctuating solar insolation on algal biomass generation.

Carbon metabolism is dependent on the species and the strain of algae. Some species demonstrate autotrophic metabolism, and only utilize inorganic carbon compounds [38]. *Chlorella* is a mixotroph algal species, which is capable of utilizing inorganic carbon for its metabolism [39,40]. Although *Chlorella* grows well under autotrophic conditions, lipid productivity tends to be highest under mixotrophic conditions [39,41]. Lipid yields per dry weight of algae as high as 48.7% can be achieved under these conditions [13]. Chemical oxygen demand (COD) levels of at least 3.75 mg/L have been shown to be required to support

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mixotrophic algae species, such as *Chlorella* [42]. Optimal cell growth and lipid productivity were attained using glucose at 1% (w/v), whereas higher concentrations were inhibitory [39,41].

Nitrogen and phosphorus are the macronutrients required in the largest amount to support algal growth (Table 2.2). The ratio and quantities of nitrogen and phosphorus in individual waste streams vary widely within any given wastewater treatment plant [43]. Equations 2.1 and 2.2 show that a theoretical mass ratio of 7.2 grams of nitrogen per gram of phosphorus is required for algae production via biosysnthesis [38]. The actual optimal growth N/P mass ratio has been shown to vary between 6.8 and 10. Algae prefer to utilize nitrogen species in the following order: $NH_4^+ > NO_3^-$ > simple organic-N compounds such as urea and simple amino acids [44]. However, $NH_4^+$ and high pH pose problems as the unionized form of ammonia ($NH_3$) is more toxic than the ionized form ($NH_4^+$). Section 2.3 discusses the problems associated with ammonia in high strength wastewaters, such as centrate, in greater detail.

$$16NH_4^+ + 92CO_2 + 92H_2O\ +\ 14HCO_3^- + HPO_4^{2-} \xrightarrow{h\upsilon} C_{106}H_{263}O_{110}N_{16}P + 106O_2 \text{ (Eq. 2.1)}$$

$$16NO_3^- + 124CO_2 + 140H_2O\ +\ HPO_4^{2-} \xrightarrow{h\upsilon} C_{106}H_{263}O_{110}N_{16}P + 138O_2 + 18HCO_3^- \text{ (Eq. 2.2)}$$
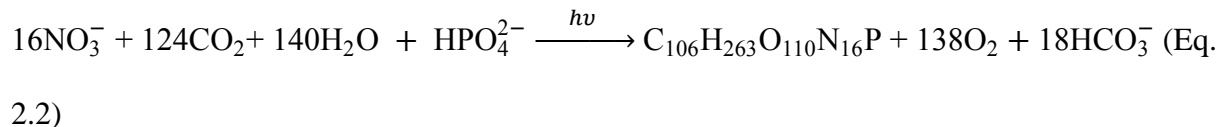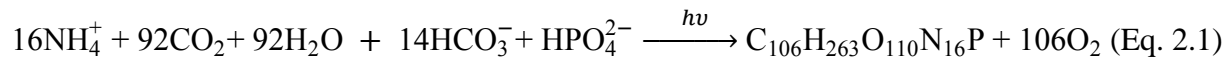
## 2.3 Wastewater as a Growth Substrate

Synthetic media tend to be more expensive and less sustainable than using wastewater as a growth media to support algal production [45]. Use of wastewater offers the additional benefit of nutrient removal, prior to effluent discharge. A number of different wastewater types can be used as a substrate to support algal growth (Table 2.3). Most of these wastewater streams have high concentrations of ammonia, as most organic nitrogen, including urea decomposes to form ammonia. High ammonia strength wastewaters that have been used as a nutrient source for

indigenous algae, including livestock wastewater [46,47], synthetic anaerobic digestate [48], dairy wastewater [49,50] and centrate from dewatering municipal wastewater sludges [51-53]. This study focuses on two wastewater streams: 1) centrate from dewatering sludges and aquaculture wastewater.

Table 2.2: Summary of growth requirements for green microalgae (adapted from Zeng et al., 2011).

| Nutrient / growth requirement | Main forms | Function | Appropriate range |
|---|---|---|---|
| Carbon | $CO_2$, $HCO_3^-$, $CO_3^{2-}$ | Backbone for most cellular structures | 1-10g/L |
| Nitrogen | $NO_3^-$, $NH_4^+$ | Required for amino acid production | 10-2000mg/L |
| Phosphorus | Hydrophosphate, phosphate | Needed for photosynthetic processes | 1-200mg/L |
| Inorganic salts | K, Ca, Na, Mg, etc. | Increases photosynthetic activities | 0.1-100mg/L |
| Sulfur | Sulfate | Needed for amino acids and enzymes production | 0.1-100mg/L |
| Trace elements | Fe, Zn, Mn, Cd | Needed for co-enzyme production | 0.01-10mg/L |
| Vitamins | $V_B$, $V_C$, $V_E$, etc. | Aids cell division | 0.01-1000µg/L |

Some algal species have a reasonable toxicity tolerance for ammonia and tolerate ammonia concentrations up to 34 mg/L. Prior exposure to high concentrations of ammonia, allowed for greater tolerance and acclimation [44]. Algae were able to grow in wastewater lagoon oxidation ponds, municipal wastewater and oxidation ponds, where high ammonia concentrations are typical. *Scenedesmus*, a dominant species in most oxidation ponds, was inhibited by ammonia concentrations greater than 34 mg/L and a pH greater than 8.0 [61]. Indigenous benthic algae, with *Microspora willeana spp.* being dominant, grew well on anaerobically digested dairy wastewater [50]. The mean growth rate over a nine-week period varied between 5.3 and 5.5

g/m$^2$/day. A high productivity was achieved with indigenous algae (0.5g/L/day) on municipal

wastewater centrate [52].

Table 2.3**:** Studies that utilized algae to treat different industrial wastestreams.

| Industrial wastestream | BOD concentration (mg/L) | Major contributions or comments | References |
|---|---|---|---|
| Meat and poultry | NP | 200 hen operation. Pond system. | [54] |
| Pulp, paper, starch | >10,000 | *Microcystis* sp. removed 70% of color. Adsorption is the main removal mechanism. | [54,55] |
| Aquaculture | NP | 50-60% TN removal efficiencies when *Scenedesmus* is used. | [56] |
| | NP | The maximum values removal rates for nitrogen was 10.5mg N/L/ day when *Chlorella* was used. | [57] |
| Municipal | 7620 | Aeration used. Pond system. | [54,58] |
| Metal finishing | NP | *Scendesmus* absorbed >90% $Cu^{2+}$within 1 min of exposure. Metals removed by absorption or adsorption. | [59] |
| Pharmaceutical | 2000-5000 | No aeration required. Pond system. | [54] |
| Food and dairy | 2000-5000 | 61% reduction in COD. Optimal strength was75%. Mean nitrogen removal was 70%. | [54,60] |

NP- Not provided

High $NH_4^+$ and high pH poses a toxicity concern, as free (unionized) ammonia ($NH_3$) dissipates

transmembrane proton gradients in algae [61,46,62]. The equilibrium shift between these two

forms (Equation 2.3) is highly influenced by pH. Concentrations of free ammonia increase with

increasing pH (pH > 9.25). Strategies that have been used to overcome this problem include: 1)

using indigenous algal species that can utilize wastewaters with high ammonia concentrations, 2)

or operating algae culturing systems in continuous or semi-continuous mode, so that ammonia

concentrations in the reactors are maintained at a relatively low level through dilution.

$$NH_3 + H_2O \leftrightharpoons NH_{4^+} + OH^- \qquad \text{(Eq. 2.3)}$$
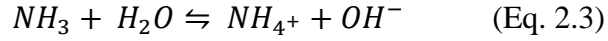
An additional concern with using wastewater as a growth substrate for algae production is the presence of other toxicants that might inhibit algae growth or bioaccumulate in algal products (e.g. neutraceuticals). In particular, algae of have been shown to bioacumulate metals, as shown in Table 2.4. The main mechanism is adsoption and is attributed to the carboxyl groups. The aquatic chemistry, temperature and metabolic stage all influence the soption process. Although this topic is outside the scope of this research, Chapter 4 investigates the presence of metals in aquaculture wastewater.

Table 2.4: Summary of studies of effects of metals on algae growth

| Metals investigated | Species | Key findings | Reference |
|---|---|---|---|
| Cr, Pb, Cu Cd, Zn and Al | *Laminaria japonica* | 94.1, 348, 100, 136, 56.9, and 75.3 mg/g was the soption capacity at pH 4.5. | Lee [63] |
| Ag, Cu, Cd, Zn | *Chlorella vulgaris, Scenedesmus quadricauda* | General binding efficiencies decrease in the order: Ag > Cu > Cd > Zn. Soption rates were rapid. 90% Cu sorbed in less than 15 mins. >92.6% Cu and Ag removal were achieved for both species. | Harris and Ramelow [64] |
| Zn, Cd, Pb, Cu, Hg, Ag and Au. | *Chlamydomonas* | Heavy metals: Pb, Cd, Hg and Cu, bind to metallothionein | Rajamani et al. [65] |
| Pb (II) | *rhizoclonium* | The adsorption process was spontaneous, endothermic and favored at higher temperature. | Velan and Kayalvizhi [66] |

*2.3.1 Centrate as a Nutrient Feed*

A key challenge with using raw or treated municipal wastewater for algae cultivation is that wastewater nutrient concentrations are relatively low (total nitrogen [TN] concentrations < 40 mg/ L, total phosphorous [TP] concentrations < 10 mg/L). The low nutrient concentrations support low algal biomass densities, resulting in high downstream costs for thickening and dewatering [67,68]. Using centrate, or the liquid waste derived from sludge dewatering, to support algal growth has been proposed to overcome this challenge [69]. The TN and TP concentrations present in centrate are the highest found in wastewater treatment plants [70,52]. Centrate is normally recycled to the head of the wastewater treatment plant, resulting in high irregular nutrient loads that can upset mainstream treatment processes, increase energy and chemical costs, and reduce efficiency by retreating pollutants. Therefore, the treatment of centrate using algae is particularly advantageous. Although using centrate for algae cultivation offers high growth potentials compared to other wastewater streams, approximately 60% of the TN in centrate is present as ammonia, with the other major fraction being organic nitrogen [71]. This introduces the problem of ammonia toxicity described above.

*2.3.2 Aquaculture Wastewater a Nutrient Feed*

The aquaculture industry has grown to meet increasing worldwide fish and protein demands [72]. The aquaculture industry in Florida alone has more than 900 aquaculturists, and annual sales in excess of $80 million [73]. As the scale and intensity of production increase, the volume and concentration of wastewater from aquaculture systems also increases [74]. Lekang [1] classified the main compounds in aquaculture wastewater: phosphorus, nitrogen, biochemical oxygen demand (BOD), suspended solids, pathogen, and chemicals, such as hormones and stabilizers. Although, aquaculture RAS wastewater tends to generally have lower concentrations of nutrients

but higher water flow rates than industrial and municipal wastestreams.[74], overall nutrient loadings may be high due to higher mass flow rates and larger scale.

Nutrient enrichment is the most notable environmental problem associated with aquaculture [75]. The primary contributor of most of these nutrients stems from feed application. Most of the nutrients are not fully assimilated by the fish [76]. It is estimated that only 30% of total nitrogen and phosphorus from feed inputs are assimilated. Estimates suggests that one metric ton of fish produces approximately 0.8 kg nitrogen/day and 0.1 kg phosphorus/day [77,78]. Nitrogenous compounds (ammonia, nitrite, and nitrate) are considered major contaminants in aquaculture wastewater. Although, ammonia is the principal nitrogenous waste produced by aquatic animals, nitrate is the main form when a recirculating system is utilized [77,76], as organic and ammonia are converted to nitrate through ammonification and nitrification processes [77].

There is an increasing emphasis on the need for aquaculture facilities to meet effluent standards for wastewater contaminants such as solids, nutrients (nitrogen and phosphorus) and organics. Aquaculture wastewater treatment systems can be classified into physical, chemical and biological, as shown in Table 2.5. Most of these wastewater treatment processes have high capital, energy and chemical costs and do not recover nutrients to produce useful or commercially viable end-products. Using an integrated, biological approach that facilitates energy and cost savings and produces useful end-products, such as algal biomass, should be favored [79,80].

Aquaculture wastewater has been used previously to support symbiotic photoautotrophic growth for using various co-cultivation approaches, such as aquaponics [81,82,80,83]. Algal co-cultivation may be more advantageous than aquaponics because it provides the potential to

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improve water quality and increase dissolved oxygen concentrations, which improves the target

species' health, while producing a feedstock for onsite energy production and/or feed

supplementation [80,82,81,84,85]. Drapcho and Brune [81] used algae in a partitioned

aquaculture system to reduce ammonia concentrations and increase dissolved oxygen

concentrations required for fish health. Haglund and Pedersén [84] used macrospecies algae,

*Gracilaria tenuistipitata*, for wastewater treatment and epiphyte control in a rainbow trout

system.

Several prior studies produced algae for use as an onsite feed supplement and found that algae

grown on aquaculture wastewater had higher growth rates and protein contents and were more

nutritious (containing a more complete amino acid profile) than non-leguminous plants such as

oat, barley and rye [86,87,85,80]. Bio-flocs technology (BFT) is an example of co-cultivation

that takes advantage of the synergy between aquaculture, algae and microorganisms [83].

Bioflocs formed are an aggregate combination of heterotrophic bacteria, algae, colloidal particles

and polymeric substances that can be used to supplement fish feed.  In addition, this process also

facilitates nitrogen immobilization and recovery [88]. Chapter 4 further examines algal biomass

and intercellular product production using aquaculture wastewater.

Table 2.5**:** Summary of physical, chemical and biological methods to treat aquaculture
wastewater (Adapted from information derived from Lekang [1])

| Physical and chemical | Biological |
|---|---|
| Reverse osmosis | Trickling filters |
| Ion exchange | Fluidized bed reactors |
| Carbon adsorption | Rotating biological reactors |
| Electrodialysis | Bioflocs |
| | Wetland retention systems |
| | Electrochemical |
| | Algae reactors |

*2.3.3 Algal Wastewater Reactor (AWRS) Interactions*

Wastewater is nutrient rich; however, it facilitates the growth of both the target algal species and other microorganisms and non-target algae. These organisms may influence production of the target algal species, as well as intracellular product generation, positively or negatively, as shown in Table 2.6 [17,89].  Beneficial relationships exist when the presence of one species facilitates greater health of another.  One species may provide nutrients or other resources for another. Typically when the relationship is competitive in nature, the species occupy similar ecological niches and strive to maintain dominance using the same resources, such as nutrients.[90]. Contamination with native, invasive microbial species is one of many major challenges in ensuring algal biofuel commercial viability [13]. Chapter 4 discusses this issue in greater detail in the context of algal biomass and intercellular product generation using aquaculture wastewater as a feed source.

Table 2.6: Summary table of interaction mechanisms between algae and bacteria

| Mechanism | Nature of Relationship | Description | Ref. |
|---|---|---|---|
| Phtyohormone production | Positive to algae. No effect on bacteria | Indole- 3- Acetic Acid (IAA) and cytokinins promote cell division in Chlorella. | [91-93] |
| Morphogenesis of algae associated with bacterial products | Positive results most times but the change could be negative. Bacteria are not affected. | Morphogenesis refers to the structural and functional changes. Changes could occur at an enzymatic level of effect the immunochemistry. There may be differences in the spatial orientation of enzymes in the cell wall. | [94] |
| Provision of primary metabolites | Positive for bacteria. Effect on algae is dependent on whether the bacteria are acting as host or scavengers. | Bacteria benefits from production of primary metabolites such as carbohydrates, amino acids, peptides and proteins. Microorganisms entering the algal membrane may be detrimental if they penetrate the tissue. | [95] |
| Microniche and habitat provision | Positive for bacteria. Mostly negative for algae | Algae surfaces present a favorable microniche for opportunistic bacteria, as there is large surface area and a lot of food resources, reproduction and subsequent reproduction. In addition, algal cell walls contain polysaccharides, complex and inviting for a number of bacteria. May be negative for algae as it may cause floc formations and reduction in photosynthetic surface area. | [95] |
| Mineralization and provision of growth factors | Positive for algae. No effect on bacteria. | Bacterial respiration provides carbon dioxide and other metabolites, such as vitamins, chelators and other growth factors, which support algal growth. | [95] |
| Production of bioactive metabolites from bacteria | Positive for bacteria. Negative for algae if no defense response is elicited. | Bacteria produce secondary metabolites, which are bioactive. These are produced in an effort of gain a competitive advantage. Algae produce antimicrobial secondary metabolites in an effort to reduce microbial attack. | [95] |
| Lysis | Positive for bacteria but negative for algae. | Gram-negative myxobacteria attack and cause lysis of algal cells. | [20] |

**CHAPTER 3: AN IRRADIANCE-BASED MODEL FOR PREDICTING ALGAL BIOMASS PRODUCTION USING MUNICIPAL SLUDGE CENTRATE AS A GROWTH MEDIUM[1]**

## 3.1 Background

Algal biofuel production is recognized as a promising future source of renewable energy [9,12]. Although the potential for algae derived biofuels is high, there are many technical and economic challenges associated with algal biomass production, harvesting and processing that must still be overcome [9]. In particular, a number of recent life cycle assessment (LCA) studies have shown that a large portion of the energy and environmental impacts associated with algal biofuel production are due to the provision of water, nutrients and carbon dioxide needed for algae growth [12]. These impacts can be greatly reduced by using wastewater as the water and nutrient source for algae cultivation [53]. A major advantage of this approach is that the eutrophication potential of wastewater is reduced, as the macro-nutrients (nitrogen [N] and phosphorous [P]) present in wastewater support the growth of algae within the confines of a photobioreactor. In addition, organic matter present in wastewater favors mixotrophic metabolism (*i.e.* utilization of sunlight as an energy source and organic carbon for biosynthesis), which has been shown to increase biomass and lipid productivity [41]. Wastewater also contains micro-nutrients that support algal growth [43].

A key challenge with using raw or treated municipal wastewater for algae cultivation is that wastewater nutrient concentrations are relatively low (total nitrogen [TN] concentrations < 0.04

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[1] Material in this chapter has been submitted to Bioenergy Research. Reference: Halfhide et al [96].

g L$^{-1}$, total phosphorous [TP] concentrations $< 0.01$g L$^{-1}$). The low nutrient concentrations support low algal biomass densities, resulting in high downstream costs for thickening and dewatering [67,68]. The use of centrate (a waste stream with a high ammonia concentration produced from dewatering wastewater sludge) to support algal growth has been proposed to overcome this challenge [69]. The TN and TP concentrations present in centrate are the highest found in wastewater treatment plants [53,70,52]. Centrate is normally recycled to the head of the wastewater treatment plant, resulting in high irregular nutrient loads that can upset mainstream treatment processes, increase energy and chemical costs, and reduce efficiency by retreating pollutants. Therefore, the treatment of centrate using algae is particularly advantageous.

Although using centrate for algae cultivation offers high growth potentials compared to other wastewater streams, approximately 60% of the TN in centrate is present as ammonia (NH$_4^+$), with the other major fraction being organic nitrogen [71]. The high NH$_4^+$concentration is a toxicity concern, as free (unionized) ammonia (NH$_3$) dissipates transmembrane proton gradients in algae [61,62]. Prior studies have addressed this problem by using different measures, which are discussed later [47,14,97].

In this paper, the cultivation of an indigenous algal consortium using centrate derived from anaerobically digested municipal sludge was demonstrated in semi-continuous column photobioreactors under natural sunlight conditions. Biomass production was modeled using a simplified irradiance-based model developed according to Michaelis-Menten photosynthesis-irradiance kinetics. Treatment of the centrate was evaluated by measuring influent and effluent concentrations of nutrients and organics.

## 3.2 Materials and Methods

### 3.2.1 Indigenous Algae Collection and Reactor Start-up

A filamentous, indigenous algal mat was harvested from a secondary clarifier at the Howard F. Curren Advanced Wastewater Treatment Facility (HFCAWTF) in Tampa, Florida. The algal mat was gently swirled in filtered centrate (described below) to suspend the microalgae. The mixture was allowed to grow in 0.4 L of 0.2 µm-filtered centrate in a 1-L flask. A 2% $CO_2$ - air mixture was bubbled through the flask at a flow rate of 0.5 L min$^{-1}$. The flask was maintained at room temperature (~22 °C) with a 16-hr light/dark cycle under artificial light conditions of 20.1 mol m$^{-2}$day$^{-1}$. A 10 day growth period was initially allowed before transferring the suspended microalgae into a 1-L bottle containing 600 mL of filtered centrate. Serial transfers were carried out by incubating the suspension until the total suspended solids (TSS) concentration reached 2.0 g-DW L$^{-1}$ and then transferring 0.05 L of the suspended indigenous algal consortium into 0.6 L of fresh filtered centrate. The resulting algal culture was used to inoculate the pilot-scale photobioreactors.

### 3.2.2 Scale-up, Photobioreactor Setup and Maintenance

Vertically hanging tubular plastic bag photobioreactors were obtained from the Faculty of Plant and Environmental Sciences at the Norwegian Life Sciences University (UMB), Ås, Norway. Each photobioreactor column had a height of 2.73 m, a diameter of 0.12 m and a total volume of 10 L. Centrate was added until a total operating volume of 7.0 L was achieved. The algal culture described above was added to achieve an initial TSS concentration of 0.6 g-DW L$^{-1}$. The photobioreactor was operated as a batch system for two weeks to increase the initial biomass density. Subsequently, the system was operated as a semi-continuous batch photobioreactor at a

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mean cell residence time of 7-days by removing 14% (1 L) of the contents of each cell on a daily basis and replacing it with new centrate (Table 3.1).

Table 3.1: Mean nutrient values for influent and effluent

| Parameter | Influent | Effluent |
|---|---|---|
| Mean TN concentration (mg/L) | 220.0 | 76.2 |
| Mean $NH_4^+$- N concentration (mg/L) | 218.8 | 50.0 |
| Mean TP concentration (mg/L) | 34.7 | 12.5 |
| Mean COD concentration (mg/L) | 130.5 | 119.0 |

Algal growth experiments were conducted under natural illumination (discussed in detail in the results section) in a temperature controlled (25-32°C) greenhouse at the University of South Florida Botanical Gardens in Tampa, Florida between November 1st and December 19th 2011. A 2% $CO_2$/air mixture was bubbled into the culture from the bottom of each photobioreactor column using compressed gas sources. The gas flow rate was maintained at 0.5 L min$^{-1}$ in each column using rotameters supplied with needle valves (Cole Parmer Inc., Vernon Hills, IL) and coarse bubble diffusers.

HFCAWTF digests a mixture of primary and waste activated sludge (WAS) in a mesophilic (35°C) single-stage anaerobic digester with a 21-day SRT. Biosolids are dewatered using a gravity belt thickener, with polymer addition. The belts are periodically washed with treated wastewater effluent that may significantly dilute the centrate. Centrate was collected weekly from HFCAWTF and filtered using a filter cloth to remove large biosolids, increase light transmission and reduce solids degradation in the feed. Total nitrogen (TN) and total phosphorous (TP) concentrations in the centrate were measured on the day of collection and

adjusted to between 0.20-0.25 g L$^{-1}$ TN and $2.5\times10^{-2}$ and $7.63\times10^{-2}$ g L$^{-1}$ TP, by dilution with local groundwater or addition of $(NH_4)_2SO_4$ and/or $KH_2PO_4$ .

*3.2.3 Sampling and Analytical Methods*

Photobioreactor samples were analyzed daily for TSS, dissolved oxygen (DO), dissolved $CO_2$, optical density at 670 nm and pH. Influent and effluent concentrations of TN, TP, chemical oxygen demand (COD), nitrate ($NO_3^-$-N) and $NH_4^+$-N were measured weekly. Changes in TSS were used as an indication of areal biomass productivity, which is reported here as g dry weight (DW) m$^{-2}$ day$^{-1}$. An Onset® HOBO U12 data-logger was used to record irradiance, ambient temperature, culture temperature and relative humidity every 15 minutes.  The logged data was in units of lux (1 lux = $1.85\times10^{-2}$ µmol-photon m$^{-2}$ sec$^{-1}$).

Analyses were conducted according to *Standard Methods* for TS (2540G), TSS (2540B), DO (4500-O C), $NO_3^-$-N (4500-$NO_3$ B), TN (4500-N), TP (4500-P C), COD (5220 D) [20].  $NH_4^+$- N concentration was determined by the salicylate method using Hach test vials (Loveland, CO). The estimated method detection limit (MDL) for TN, TP and $NH_4^+$-N were (g L$^{-1}$): $7.0\times10^{-3}$, $0.06\times10^{-3}$ and $0.6\times10^{-3}$, respectively. Culture pH was measured using a calibrated pH meter and probe (Metrohm, Riverview FL or Teledyne Isco, Lincoln, NE). Lipid content was determined gravimetrically at the end of the experiment (day 47) using the method of Bligh and Dyer [98]. Chlorophyll content for the consortium was determined using a methanol extraction method described by Franco et al.[99]. Total chlorophyll was calculated using Liechtenthaler equations [100]. For more detailed method protocols adopted for chlorophyll and lipids, refer to Appendix A.1.

*3.2.4 Algal Species Identification and Enumeration*

Samples were collected at the end of the experiment (day 47) and shipped to the Environmental

Biotechnology Laboratory in the Department of Soil & Water Science at the University of

Florida for species identification and enumeration. Algae were microscopically observed using a

Nikon Labophot (Nikon Corporation Tokyo, Japan) after brief (10 sec) centrifugation at 15,000

rpm (Eppendorf 5414, Hamburg, Germany).  Each resultant cell paste was observed and keyed to

genus level following Wehr and Sheath [101]. Algal cells were counted on a Bright-Line

hemacytometer with improved Neubauer ruling (American Optical Co., Buffalo, New York).

Triplicate counts were made from two grab samples and the average counts were taken. Cell

numbers per mL were calculated [102]. Genera were counted separately and compiled for a total

cell count and relative species composition. Taxonomic composition was recorded as percent

relative abundance of the total population.

*3.2.5 Algal Growth Modeling[2]*

 It was assumed that the photobioreactor system is a completely mixed semi-batch reactor. An

overall mass balance for the photobioreactor system yields the following:

$$\frac{dB}{dt} = r - \frac{Q}{V} B \qquad \text{(Eq 3.1)}$$

where $B$  is the biomass concentration (g-DW m$^{-3}$) , $V$ is the working volume of the

photobioreactor (m$^3$) and $Q$ is the flow rate (m$^3$ s$^{-1}$). The average mean cell residence time can be

calculated as V/Q, which was maintained at 7 days.

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[2] Modeling work shown in this section was mainly carried out by Omayoto K. Dalrymple and Qiong Zhang and is included here for completeness.

The modeled biomass prior to the time of harvest ($B_{tp}$) was calculated from:

$$B_{tp} = B_{t-\Delta t} + r(\Delta t) \qquad \text{(Eq. 3.2)}$$

where r is the growth rate (g-DW m$^{-3}$ s$^{-1}$) and $\Delta t$ is the elapsed time since the last harvest (s). The

biomass concentration after harvest ($B_{ta}$) was calculated as:

$$B_{ta} = B_{tp}\left(1 - \frac{V_H}{V}\right) \qquad \text{(Eq. 3.3)}$$

where $V_H$ was the harvest volume (m$^3$), or the volume of the reactor contents removed each day.

The model was programmed to match the semi-continuous operation of the photobioreactor,

such that the predicted biomass concentration at 15:00 hours (once a day) was adjusted to match

the feed and harvest flow, and algal growth rate. The algal growth rate depends on both nutrient

availability and irradiance. However, in this study, irradiance was considered the limiting factor

for microalgae growth as nutrients were assumed to be in excess (Table 3.1). Since growth rate is

directly related to carbon fixation rate, a simple irradiance-based model was applied in this work

according to the Michaelis-Menten formulation [103], which relates light to carbon fixation:

$$P(z) = P_m \frac{I(z)}{E_k + I(z)} \qquad \text{(Eq. 3.4)}$$

where P(z) is the gross carbon photosynthetic rate (μmol-C m$^{-2}$ s$^{-1}$), $P_m$ is the maximum

photosynthetic rate (μmol-C m$^{-2}$ s$^{-1}$), $I(z)$ is the irradiance (μmol-photon m$^{-2}$ s$^{-1}$) at depth $z$ (m)

and $E_k$ is the light half saturation constant (μmol-photon m$^{-2}$ s$^{-1}$), that is, the irradiance value at

which the photosynthetic rate is half of the maximum value. The propagation of light through the

culture can be defined according to a modified Beer-Lambert relationship as [103]:

$$I(z) = I_0 exp\left(-\frac{aBz}{b + B}\right) \qquad \text{(Eq. 3.5)}$$

where $I_0$ is incident irradiance (μmol-photon m$^{-2}$ s$^{-1}$), $a$ (m$^{-1}$) and $b$ (g m$^{-3}$) are attenuation

constants and z (m) is the cross-sectional light path [103]. In this study, values for $a$ and $b$ were

obtained from Yun and Park [103], and are shown in Table 3.2.  By integrating through the

effective light path, $d_{eff}$, (m), the net photosynthetic rate per unit surface area, $P_{net}$ (μmol-C m$^{-2}$ s$^{-1}$), is given by:

$$P_{net} = P_m \left(\frac{b+B}{aB}\right) \ln\left(\frac{I_0 + E_k}{E_k + I_0\exp\left(-\frac{aBd_{eff}}{b+B}\right)}\right) - R_B \qquad \text{(Eq. 3.6)}$$

where $R_B$ is the biomass dependent respiration rate (μmol-C m$^{-2}$ s$^{-1}$) and was obtained by:

$$R_B = \frac{R_0 BV}{A} \qquad \text{(Eq. 3.7)}$$

where $A$ is the illuminated surface area (m$^2$) and the specific biomass respiration rate, $R_0$ (μmol-

C g-DW$^{-1}$ s$^{-1}$), was obtained by fitting the data.

The algae growth rate, r, needed for Equation 3.1 was calculated from $P_{net}$ (Eq. 3.6) from:

$$r = \frac{24(10)^{-6}}{d_{eff}} P_{net} \qquad \text{(Eq. 3.8)}$$

The effective path length of the photobioreactor ($d_{eff}$) was calculated as the working volume

divided by the illuminated surface area (d$_{eff}$ = V/A).  In Equation 3.8, the numerator was

obtained by assuming that the dry weight of algae consists of 50% carbon (numerator = 12 g-C

mol$^{-1}$ x 2 g-DW biomass g C$^{-1}$ x 10$^{-6}$ μmol mol$^{-1}$).

## 3.3 Results and Discussion

### 3.3.1 Microscopic Identification and Enumeration of Algae

Identifying and enumerating the indigenous species in the algal consortium is important to determine their relative contribution to biomass and lipid content and provide greater understanding of ecological relationships. The primary genera identified within the photobioreactor samples were *Chlorella, Chlamydomonas,* and *Stichococcus*, which comprised 95.2, 3.1, and 1.1% of the total cell population respectively (Figure 3.1). Several other species of algae were rarely observed and included: *Scenedesmus*, *Trachelomonas* and unidentified diatoms. These genera, along with unidentified algae, comprised ~0.6% of the total algae population. Rotifers were also observed, but were not identified or counted. An image taken of a view under the light microscope of the algal community is shown in Figure 3.2. Most of the cells were spherical, which is typical for *Chlorella*.
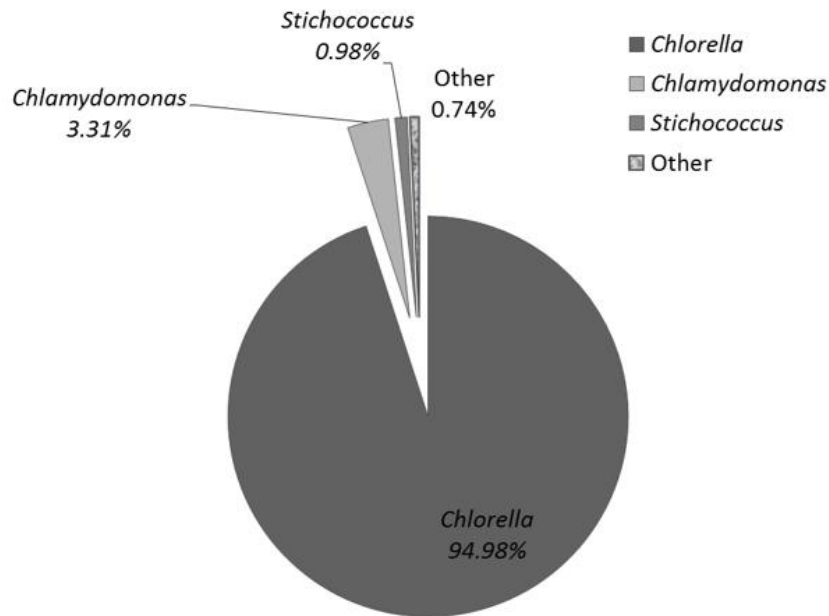


Figure 3.1**:** Composition of indigenous algal consortium

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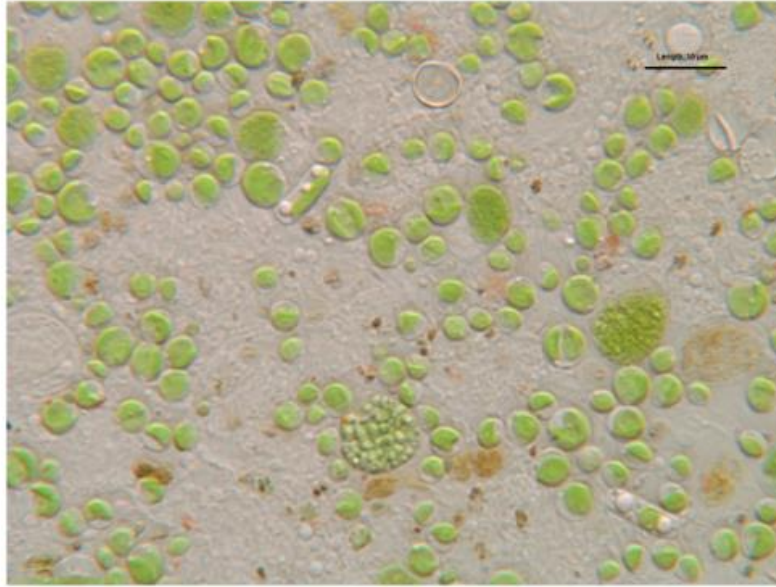
Figure 3.2: Light microscope image under X1250 magnification

*3.3 2 Lighting Conditions*

Light is one of the necessary ingredients supporting the metabolism of photoautotrophs. Most (45%)

of the visible light spectrum between 400 and 700 nm is available for algal growth [35].

Approximately 8.5 MJ are required to produce one mole of glucose [14]. The amount of

instantaneous photosythetically active radiance (PAR) and total daily insolation varied over the

cultivation period from November through December 2011. Incident irradiance was on average

low given the time of the year. The maximum instantaneous PAR was 566 $\mu$mol-photon m$^{-2}$ sec$^{-1}$

(Figure 3.3). The mean insolation over the period was $6.1 \pm 1.5$ mol-photons m$^{-2}$ day$^{-1}$. The

maximum and minimum insolation was 9.4 and 2.3 mol-photons m$^{-2}$ day$^{-1}$, respectively (Figure

3.4). Cultivation in the greenhouse reduced outdoor PAR by 60-70%. However, since the

photosynthetic rate saturates at high irradiance, significant biomass productivity was still

observed (Figures 3.5 and 3.6). It appears that through semi-continuous dilution a continuous

production process can be achieved that effectively utilizes the available PAR.

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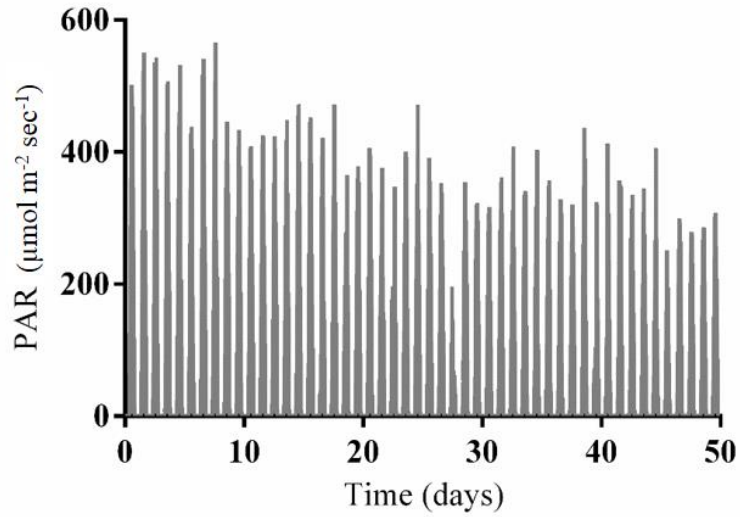
Figure 3.3: Instantaneous PAR ($\mu$mol m$^{-2}$ sec$^{-1}$) over the experiment
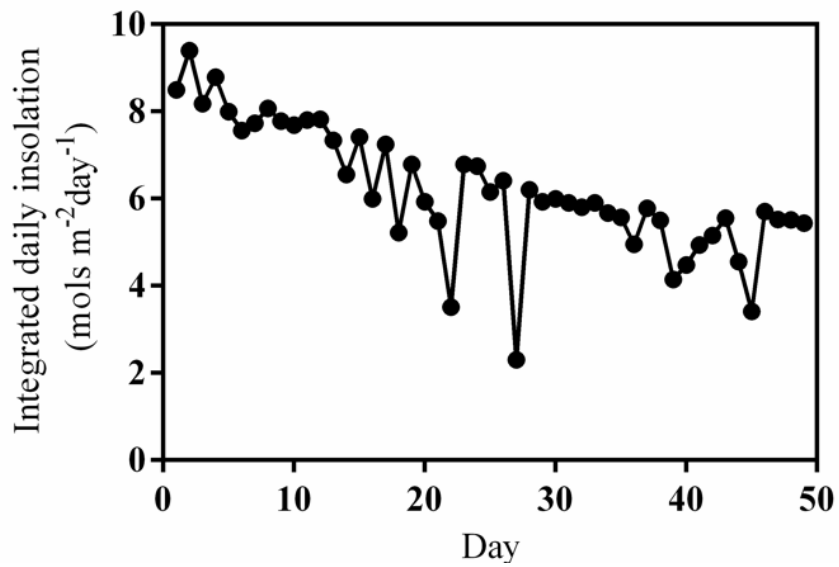


Figure 3.4: Integrated daily insolation (mols m$^{-2}$ day$^{-1}$)

*3.3.3 Algal Biomass Growth*

The indigenous algal consortium was able to grow and survive on the wastewater centrate under semi-continuous photobioreactor conditions. The standing biomass (g-DW m$^{-2}$) refers to the total mass of algae in the photobioreactor normalized by the illuminated surface area. Harvested

biomass (g- DW m$^{-2}$) refers to the normalized biomass collected daily from the photobioreactor. The sum of the standing and harvested biomass was used to calculate the cumulative or total biomass over time (g-DW m$^{-2}$). The maximum standing biomass achieved was 84 g-DW m$^{-2}$ (Figure 3.5). Final cumulative biomass at the end of the growth period was 299 g-DW m$^{-2}$ (Figure 3.6). Although there was significant variability in the observed standing biomass, a pseudo-steady state was observed, where the measured standing biomass ranged between 30 and 90 g-DW m$^{-2}$. It is suspected that the variability could be attributed to periodic settling of biomass as a result of cell flocculation. Flocculation could be associated with growth of bacteria in the system and daily variations in medium pH [104,105].

*3.3.4 Biomass Production Modeling*

Comparisons of the measured and predicted standing and cumulative biomass are shown in Figures 3.5 and 3.6, respectively. The model captures the increase in standing biomass over the first two weeks of cultivation (Figure 3.5). Thereafter, the model predicts a pseudo-steady state in the standing biomass. However, as previously discussed, the measurement of biomass varies significantly between 30-90 g-DW m$^{-2}$, likely due to periodic settling and re-suspension of cells. An excellent fit of the model to the cumulative biomass data was achieved ($R^2 = 0.96$).

Values of $E_k$ and $P_m$ were obtained using a non-linear least square fitting procedure and are shown in Table 3.2. The observed $E_k$ and $P_m$ values are similar to those reported by other authors for *Chlorella* [103]. The results demonstrate that the simple irradiance-based model applied here was sufficient to describe the photobioreactor system, indicating that biomass productivity was mainly light limited. The simplicity of the approach lends itself to ease of application for industrial prediction of biomass under similar conditions or a determination of how irradiance will influence biomass productivity.

Table 3.2. Model parameters.

| $a$ | 1,041 m$^{-1}$ | Yun and Park [91] |
|---|---|---|
| $b$ | 1.03 g-DW m$^{-3}$ | Yun and Park [91] |
| $d_{eff}$ | 0.12 m | measured |
| $E_k$ | 73.1 μmol photon m$^{-2}$ s$^{-1}$ | calibrated |
| $P_m$ | 5.53 μmol-C m$^{-2}$ s$^{-1}$ | calibrated |
| $R_o$ | 0.15 μmol-C g DW$^{-1}$ s$^{-1}$ | calibrated |

*3.3.5 Lipid Production*

Lipid analyses conducted at the end of the experiment showed that lipids accounted for 10% of

the total dry biomass. The lipid productivity may have increased if the mean cell residence time

was increased, which would result in decreased photobioreactor nutrient concentrations [47].

Prior studies have shown an inverse relationship between lipid production and TN concentration

[41]. Therefore, it is not surprising that lipid content was low for algae grown on high TN

strength wastewater. Lipid content greater than 30% is generally required for biodiesel

production to be economically viable [12]. However, alternative forms of fuel production can

include methane production via anaerobic digestion [69] or hydrothermal liquefaction of algal

biomass for fuel production [106].

*3.3.6 Nutrient and COD Removal*

Mean removal efficiencies for $NH_4^+$, TN and TP were 74.2, 65.0, and 72.6%, respectively, as

shown in Figure 3.7.  The TN removal efficiency (91.4%) and maximum TN removal rate (0.03

g L$^{-1}$day$^{-1}$) were high, especially considering that the mean cell residence time was half that of

similar studies (Table 3.2).  The main nitrogen removal mechanism was most likely cell

synthesis, as very little nitrogen removal could be attributed to $NH_3$ stripping or denitrification.

The maximum photobioreactor pH was 7.32, and free $NH_3$ would have accounted for only 1% of

the total ammonia nitrogen at this pH [107]. Denitrification was an unlikely mechanism since the system was fully aerobic.

Nitrogen and phosphorous are the macronutrients required in the largest amount to support algal growth. The ratio, quantities and forms of N and P vary widely in different types of waste streams and at different points within wastewater treatment plants [43]. The N/P ratio required for optimal algal growth is between 6.8 and 10 g/g [70]. Although an N/P ratio of 7.2 g/g can be calculated from an assumed algal biomass molecular formula of $C_{106}H_{263}O_{110}N_{16}P$, the actual N/P ratio required is dependent on the form of the nutrients supplied (e.g. $NH_4^+$, $NO_3^-$, organic N) and their bioavailability[38]. In this study, the average N/P ratio in the municipal centrate was maintained at 6.3, which is slightly below the optimal N/P ratio, indicating that nitrogen limited growth.
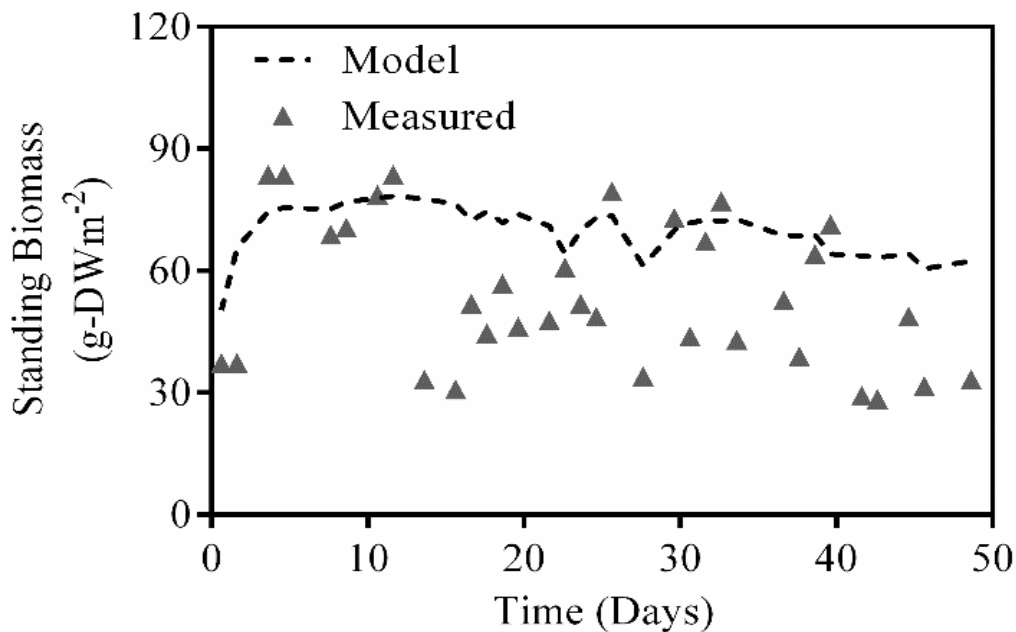


Figure 3.5: Standing biomass over the duration of the experiment

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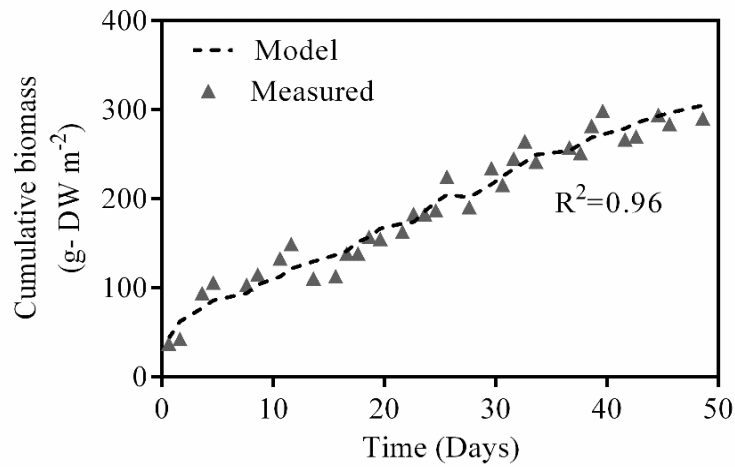
Figure 3.6: Cumulative biomass over the duration of the experiment

The COD removal efficiency observed in this study was relatively low (8%). *Chlorella* sp. are capable of mixotrophic metabolism; however, in this study they mainly utilized inorganic carbon from the carbon dioxide provided. This was most likely due to the low bioavailability of organic carbon in centrate from anaerobic digesters, as most of the easily degradable organics are converted to biogas (a mixture of methane and carbon dioxide) during the anaerobic digestion process [69].
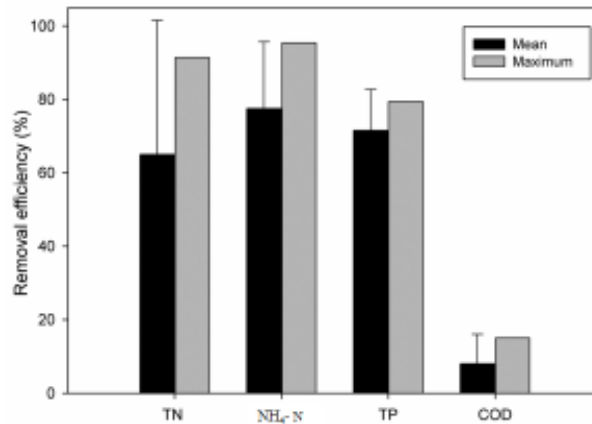


Figure 3.7: Removal efficiency of nutrients and COD

*3.3.7 Comparison with Other Studies*

A summary of recent studies that investigated the growth of algae on centrate is shown in Table 3.3. The mean algal productivity achieved in this study (5.2 g DW m$^{-2}$ day$^{-1}$) was higher than many of these studies. As discussed previously, the high concentrations of $NH_4^+$ typical of anaerobically digested sludge centrate poses a potential toxicity problem for algae cultivation, as concentrations greater than 0.2 g $NH_4^+$- N L$^{-1}$ have been shown to significantly inhibit algal productivity [46]. Operational measures that can be used to reduce ammonia inhibition include: 1) combining different waste streams to reduce ammonia concentrations, 2) using indigenous algae species and/or 3) using a semi-continuous or continuous mode to dilute ammonia concentrations. Cabanelas et al. [70] and Travieso et al. [47] combined waste streams. Cabanelas et al. [70] compared algal growth on 13 different waste streams, including centrates with 5 different N/P ratios (0.7- 15.0) and determined that algal productivity was higher with centrate with a N/P ratio of 2.0, than with all other waste stream sources [70]. Travieso et al. [47] used *Chlorlla vulgaris* to treat a combination of settled swine waste (with $NH_4^+$- N concentrations of 0.34 g L$^{-1}$) and raw municipal wastewater in a 1:60 volume ratio.

Using adapted indigenous algae may be particularly advantageous to overcome the ammonia toxicity problem, while achieving a high level of wastewater treatment for nutrients and organics. High algal growth and nutrient removal rates have been achieved with indigenous algae acclimated to high $NH_4^+$ concentrations, such as landfill leachate [108], livestock waste [47,46], dairy waste [43,109] and centrate from municipal wastewater [52,53]. Growth rates of fourteen strains of indigenous microalgae on centrate were examined by Li et al. [53]. *Chlorella kessleri* and *Chlorella protothecoides*, which were capable of mixotrophic metabolism, had the highest net growth rates.

The photobioreactor system used in this study was operated in semi-continuous mode by removing 14% of the total reactor volume each day and replacing it with fresh centrate. This allowed $NH_4^+$-N concentrations in the photobioreactors to be maintained at a relatively low level through dilution, while providing enough residence time in the photobioreactor for algal growth and nutrient metabolism. This dilution approach has been used in prior studies to reduce the exposure of algae to toxic levels of $NH_4^+$-N found in sludge centrate [47,110,97,109].

## 3.4 Conclusions

A photobioreactor operated under semi-continuous conditions with an indigenous algae consortium was successful at production of algal biomass, while reducing high nutrient levels in wastewater centrate. The consortium, which was harvested from the wastewater clarifier, consisted of more than 95% *Chlorella sp.* The application of a simple irradiance-based model was sufficient to describe biomass development in the photobioreactor, including cumulative and standing biomass. While maximum TN removal rates were high compared with prior studies, low COD utilization may have been due to the low bioavailability of COD in the centrate. The consortium had low lipid content, indicating that it should be used as feedstock for anaerobic digestion.

Table 3.3: Summary comparing results obtained in this and previous studies

| Feed used | Reactor operating conditions | Algae species used | Mean TN feed concentration $(g\ L^{-1})$ | Light period (hr) & Insolation $(mol\ m^{-2}\ day^{-1})$ | Max. productivity $(g\ m^{-2}day^{-1})$ | Max. TN Removal (%) | Lipid content (%) | Reference |
|---|---|---|---|---|---|---|---|---|
| Centrate from the activated sludge process | Batch for 7 days, then continuous for 7 days (Total=14 days) | *Chlorella* | 0.15 | 14/10 13.0 | I* | 89.1 | NP | Cabanelas et al. [10] |
| Raw and autoclaved centrate from the activated sludge process | Batch, 14 days | *Chlorella* | 0.12-0.13 | 24/0 4.3 | 13.0 | 89.0 | 11.0*** | Li et al. [11] |
| Anaerobically digested municipal centrate | Batch, 12 days | *Chlorella* | 0.2- 0.4 | 12/12 5.2 | 6.8 | 91.0 | NP | Yuan et al. [12] |
| Mixture of settled swine waste and sewage | Continuous with 4- 14 day HRT | *Chlorella vulgaris* | 0.02 | Natural lighting 46.8-61.6 | 38.2 | 26.1 | NP | Travieso et al. [15] |
| Anaerobically digested swine centrate | Semi-continuous | *Scenedesmus* | 1.22 | 12/12 8.6 | I* | 89.0 | NP | Park et al. [16] |
| Anaerobically digested municipal centrate | Semi-continuous, 12 days | *Chlorella* | 0.62 | Natural lighting, NP | 13.0 | 98.9 | NP | Rusten and Sahu [17] |
| Anaerobically digested dairy centrate | Semi-continuous, 7 day HRT | *Microspora willeana* | 0.33 | 16/8 3.5 – 12.1 | 5.5 | 39 | NP | Wilkie and Mulbry [31] |
| Centrate from the activated sludge process | Batch, 12 days | *Auxenochlorella protothecoides* | 0.17±0.038 | 24/0 5.2 | I* | 73.6 | 20.8 | Hu et al. [32] |
| Anaerobically digested municipal centrate | Semi-continuous, 7 day HRT | Mixed consortia (*Chlorella* is dominant) | 0.20- 0.25 | Natural lighting 2.3- 9.4 | 5.2** | 91.4 | 10.0 | This study |

NP- Not Provided, *I=insufficient information provided to calculate aerial productivity, **- Mean, *** FAME lipid %. This may be slightly less than total lipid content.

# CHAPTER 4: PRODUCTION OF ALGAL BIOMASS, STARCH AND LIPIDS USING AQUACULTURE WASTEWATER UNDER AXENIC AND NON-AXENIC CONDITIONS[3]

## 4.1 Background

The aquaculture industry has grown to meet increasing worldwide fish and protein demands [72]. As the scale and intensity of production increase, the volume and concentration of pollutants in the wastewater from aquaculture systems also increase. In addition, there is increasing emphasis on the need for aquaculture facilities to meet effluent standards for wastewater contaminants, such as solids organics, nitrogen (N) and phosphorus (P). However, wastewater treatment processes have high capital, energy and chemicals costs and do not recover nutrients to produce useful or commercially viable end-products. Therefore using an integrated, biological approach that facilitates energy and cost savings and produces useful end-products, such as algal biomass, and intracellular products should be favored [79,80].

Aquaculture wastewater has been used previously to support symbiotic photoautotrophic growth using various co-cultivation approaches, such as aquaponics [81,82,80,83]. A potential alternative for integration of algae cultivation with aquaculture is shown in Figure 4.1.  Algal co-cultivation may be more advantageous than aquaponics because it has the potential to improve water quality, and increase dissolved oxygen concentrations, which improves the target species' health, while producing a feedstock for onsite energy production and/or feed supplementation [80,82,81,84,85]. Drapcho and Brune [81] used algae in a partitioned aquaculture system to

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[3] Material in this chapter has been submitted to Algal Research. Reference: Halfhide et al. [111]

reduce ammonia concentrations and increase dissolved oxygen concentrations required for fish health. Haglund and Pedersén [84] used macrospecies algae, *Gracilaria tenuistipitata*, for wastewater treatment and epiphyte control in a rainbow trout system. Several prior studies produced algae for use as an onsite aquaculture feed supplement and found that algae grown on aquaculture wastewater had higher growth rates and protein contents and were more nutritious (containing a more complete amino acid profile) than non-leguminous plants such as oats, barley and rye [86,87,85,80]. Bio-flocs technology (BFT) is an example of co-cultivation that takes advantage of the synergy between aquaculture, algae and microorganisms [83]. Bioflocs formed are an aggregate combination of heterotrophic bacteria, algae, colloidal particles and polymeric substances that can be used to supplement fish feed. The process also facilitates N immobilization and recovery [88].

The use of aquaculture wastewater as a nutrient feed for algae production increases the chances of contamination by microorganisms and non-target algal species. Many prior studies of algae photobioreactor systems have used axenic conditions (i.e. algal monocultures without other microorganisms) [112-115]. However, it would not be practical or economically viable to maintain axenic conditions in large-scale open pond systems [113,114]. Non-target algae, bacteria or protozoans may compete with the target algal species for nutrients and light or may be toxic or predatory in nature [116,115,113,117]. However, some prior studies have shown that the presence of bacteria can improve algae production by making the system more resilient [7,17,18] (i.e. able to maintain its function in the face of external stress and disturbances [118]). This increased resilience may be due to the ability of indigenous microorganisms to: 1) mineralize organic substrates to inorganic forms that are more bioavailable to algae [119,120], 2) produce growth factors and micronutrients that support algal growth and/or 3) convert toxic

ammonia to nitrite and nitrate through nitrification [104,121,122]. In addition, the use of algae-bacteria consortia has the potential to reduce downstream processing costs. When cultures contain a mixture of algae and bacteria, algal cells have been shown to produce a matrix of carrageenan or alginate, which facilitates autoflocculation [67].

This study examined the effect of non-axenic conditions on algal biomass development using aquaculture wastewater as a growth medium. Three algal cultures were studied: a mixed indigenous consortium and pure cultures of *Chlorella* and *Scenedesmus*. The effects of axenic and non-axenic conditions on the ability of the system to maintain function and resilience was also assessed. Two success criteria were used to examine system resilience: productivity of a desirable end-products (biomass, chlorophyll, starch and lipids) and removal of nitrate and organic matter from the wastewater.

## 4.2 Materials and Methods

### 4.2.1 Intoduction

Experiments were conducted at the Norwegian University of Life Sciences (UMB), Ås, Norway. Biomass production for energy feedstock was investigated using recirculating aquaculture system (RAS) wastewater. The first consideration is made for the feed and its ability to support algal biomass (Section 4.2.2). Secondly, algal system performance was compared under axenic and non-axenic conditions for an indigenous algae consortium and two pure algae cultures (*Chlorella* and *Scenedemus*). A control (treatment with no algae and only aquaculture wastewater) was compare nitrogen and organic removal system performance.
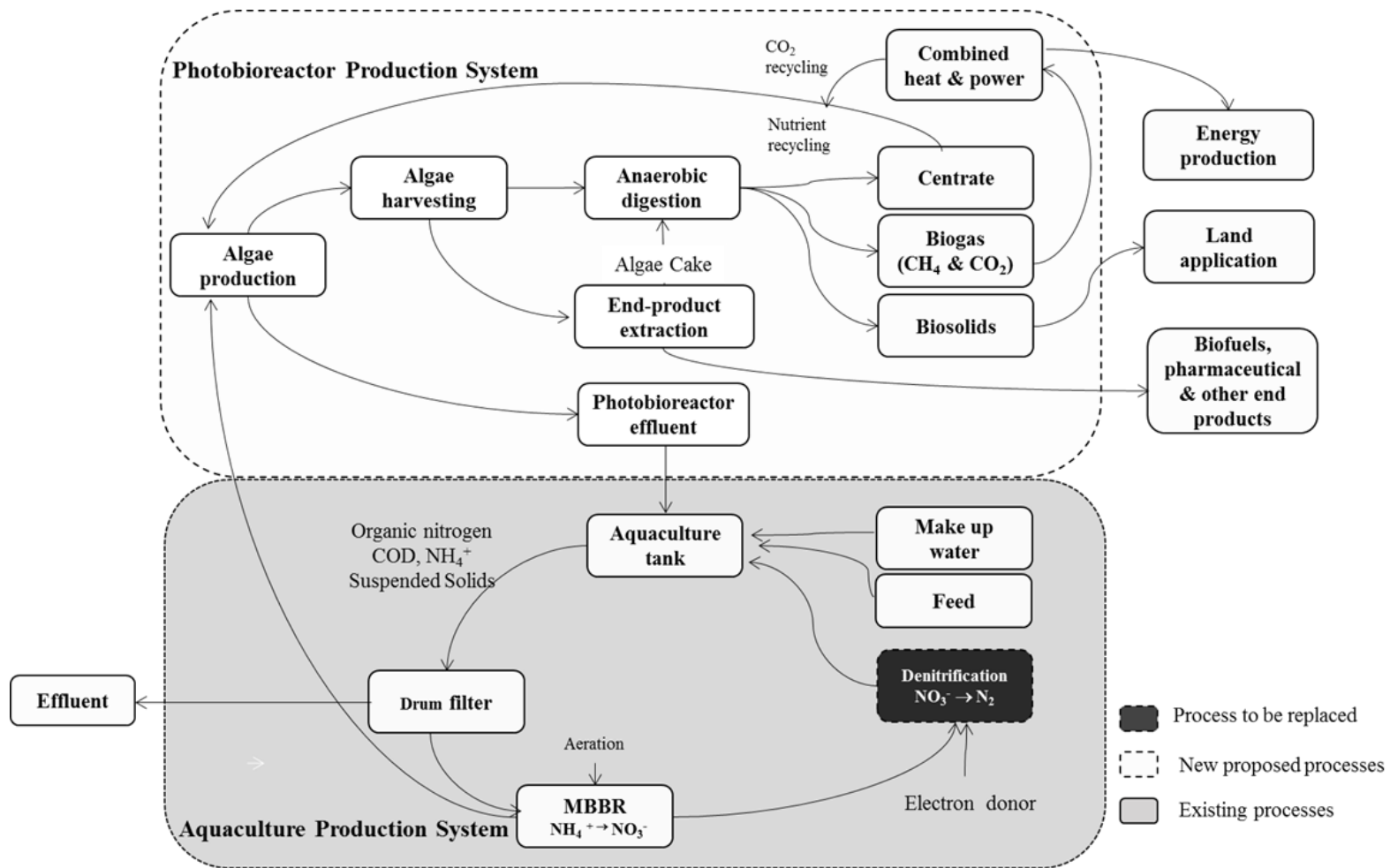
Figure 4.1: Proposed integration of algae co-cultivation with aquaculture

*4.2.2 Aquaculture Wastewater Feed*

Approximately 10 L of wastewater was collected from a UMB campus tilapia RAS, which has a

total volume of 4,200 L.  The flow rate in the RAS was approximately 150 L/min, with 98-99%

recirculation. The RAS included a drum filter with a screen mesh size 40 micron (Hydrotech

HDF 501) and a moving bed bioreactor (MBBR) containing extruded plastic media for

nitrification.  The mean annual tilapia biomass produced was 300 kg/ year. Tilapia are fed Aller

37/10 (Appendix H) daily, which has a protein content of 37%. For the axenic treatments,

aquaculture wastewater was filter sterilized using a 0.2 µm glass fiber filter (AP 1504700).  In

order to maintain N rather than P limited conditions (discussed below), 15 mg/L of phosphorous

was added to the feed in the form of $K_2HPO_4$.

*4.2.3 Algae Cultures*

Three different algae cultures were used in this study were an indigenous mixed species

consortium [123], *Chlorella sp* (NIVA CHL-137) and *Scenedesmus quadricauda* (NIVA-CHL

7). The indigenous algae were harvested from the surface of a secondary clarifier at the Howard

F. Curren Advanced Wastewater Treatment Facility in Tampa, Florida.  The consortium was

identified and enumerated by the Environmental Biotechnology Laboratory in the Department of

Soil & Water Science at the University of Florida. The primary genera within the consortium

identified included: *Chlorella* (95.2%), *Chlamydomonas* (3.1%), and *Stichococcus* (1.1%). Pure

cultures of *Chlorella* and *Scenedesmus* were acquired from the Norwegian Institute for Water

research (NIVA) culture collection. All three algae cultures were initially grown using an

aseptically prepared synthetic medium, a light irradiance of $153.3 \pm 18.8$ µmol/m$^2$/sec and a

temperature of 25ºC (controlled using a water bath).  The medium consisted of 1,000 mg of a

balanced agricultural fertilizer (Superex gronnsak) in tap water, resulting in the following

41

approximate composition (mg/L): $NO_3^-$-N (90), Ca (30), P (50), K (310), Mg (20), S (30), Mn (0.90), B (0.30), Zn (0.25), Cu (0.12), Mo (0.05), Co (0.01). The algae were grown under aseptic conditions in a 300 mL photobioreactor (described below) for 4 days. A 10.0 mL aliquot of the algae stock culture was centrifuged using an Eppendof Model # 5810 (Horsholm, Denmark) centrifuge. The supernatant was decanted and 5.0 mL of phosphate buffered dilution water was added to the centrifuge tubes to gently resuspend the algae. This process of washing to remove residual nutrients from the growth medium was repeated. Phosphate buffered dilution water was prepared by adding the following to 1.0 L of deionized water (mg/L): $KH_2PO_4$ (3,500), $KHPO_4$ (4,300) and NaCl (8,500). The pH of the dilution water was measured and adjusted to $7.2 \pm 0.5$ using 1N sodium hydroxide, if needed, and autoclaved at a pressure and temperature of 103.4 kPa and 115 °C.

### 4.2.4 Reactor Setup and Operation

Photobioreactors consisted of cylindrical glass tubes with tapered bottoms, a diameter of 4.12 cm, a height of 31.2 cm and an overall volume of 300 mL. A 280.0 mL aliquot of wastewater, filtered or unfiltered, was added to each photobioreactor. Washed algae (described above) were added to the respective photobioreactor. Unfiltered RAS wastewater without added algae was used as an uninoculated control. Experiments were performed in triplicate, for a total of 21 reactors. Algal growth conditions for all treatments included: light irradiance of $153.3 \pm 18.8$ $\mu mol/m^2/sec$ (using daylight fluorescent tubes), a temperature of 25°C (controlled using a water bath) and a filtered 1% $CO_2$- air mixture (provided using gas diffusers). A 10.0 mL sample was collected from each photobioreactor every 6-8 hours for the duration of the experiment and tests were conducted as described below to determine biomass, end-product productivity, and nutrient and organic compound removal.

*4.2.5 Analytical Methods*

The optical transmissivity of the RAS wastewater was determined at 256 nm. Samples were

analyzed in accordance with *Standard Methods* [124] for the following parameters: pH ($4500H^+$-

B), total suspended solids (TSS) (2540B), total nitrogen (TN) (4500-N), nitrate ($NO_3^-$ -N) (4500-

$NO_3$ B), Chemical Oxygen Demand (COD) (5220 D), phosphate ($PO_4^{3-}$) ($4500$-$KMnO_4$), and

heterotrophic plate counts (HPC) (9215). The starch content of the algae biomass was measured

using a Megazyme total starch (AA/AMG) kit (catalog # K-TSTA), which follows Association

of Official Agricultural Chemists (AOAC) Method 996.11. The method was modified to allow

for smaller sample volumes. The final lipid content (%) was determined using the method of

Bligh and Dyer [122]. Total chlorophyll was determined using the method described by Franco

et al. [99]. Total chlorophyll was calculated using Liechtenthaler equations [100]. Particle

counts > 2 μm were measured using a Multisizer 4 Coulter Counter (Brea, CA). Elemental

analyses of algal biomass and aqueous samples was carried out using a Perkin Elmer (Waltham,

Massachusetts) Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Optima

5300 DV) for: total phosphorous (TP), K, Ca, Na, S, Mg, Fe, Zn, Cu, Mn and Al. Samples were

decomposed by adding $HNO_3$ at 10 % (v/v ) before oxidation with peroxidisulfate during

autoclaving at 250 °C for 1.5 hr. A light microscope (Leica DM 5000B) equipped with a camera

(Leica DFC 425) was used to periodically monitor algae growth and physiological changes.

Different filters and magnifications (10, 40,100 X) were used to obtain the best visual analysis.

*4.2.6 Statistical Analyses*

One-way analysis of variance was used to determine whether differences in means for different

algal cultures were significant. T-tests were used to determine whether the differences between

axenic and non-axenic conditions within a given algal species were significant. These tests were done in Microsoft Excel. A p- value of $< 0.05$ was considered statistically significant.

## 4.3 Results and Discussion

### 4.3.1 Aquaculture Wastewater as a Feed

A summary of the initial aquaculture wastewater feed characteristics for both axenic and non-axenic treatments is shown in Table 4.1. The observed TN values (17.9 and 18.5 mg/L) were slightly lower than values reported by other authors (between 20 to 40 mg/L) for a RAS with a denitrification process [1]. The observed TN concentrations should be able to support an algal biomass concentration of approximately 285 mg/L in a batch reactor, assuming algal biomass has a chemical formula of $C_{106}H_{263}O_{110}N_{16}P$ [38]. In this study, experiments were conducted under batch conditions to maintain axenic algal treatments; however, higher biomass densities are possible if cultures are grown using the proposed process (Figure 4.1), where nitrified effluent from the MBBR and recovered nutrients from anaerobic digestion are continuously circulated through the photobioreactor, which replaces the denitrification process. Most ($>97\%$) of the initial TN was in the form of $NO_3^-$ (Table 4.1). Although algae utilize ammonia in preference to $NO_3^-$ as a growth substrate [34], high ammonia concentrations ($> 34$ mg/L), such as those found in many municipal and agricultural waste streams are a toxicity concern, as free (unionized) ammonia dissipates transmembrane proton gradients in algae [61,46,62,126]. Therefore utilizing RAS wastewater with $NO_3^-$ concentrations such as those observed in this study is favorable as a feed.

The observed TP concentrations (2.0 and 2.5 mg/L prior to supplementation) were lower than typical values seen in RAS, which have been shown to range between 6.2 and 37 mg/L [127]. The observed N/P ratio of approximately 9 was within the range (7 to 10 gN/gP) that has been

shown to be optimal for algal growth [70]. Additional P was provided (15 mg/L added); however, to ensure that the algal system in this study was N rather than P limited to favor lipid accumulation [128,114,129-131]

Light transmissivity at 256 nm was 99.0% and 97.8%, for filtered and unfiltered samples, respectively (Table 4.1), indicating that the presence of particles in the unfiltered wastewater would not hinder light transmission to an algae culturing system. This is a very high light transmissivity, when compared to some other waste streams, such as municipal sludge centrate, which has a low light transmittance (ranging from 0.1% to 21%) with no pretreatment [97]. Using aquaculture wastewater as a growth media is therefore less challenging when considering this characteristic.

pH values were similar under both axenic and non-axenic conditions. This was probably attributed to the RAS system being well buffered.  A pH between 6.5 and 7.5 is considered optimal for most green algae species [112].  The mean COD concentration was slightly higher under non-axenic conditions, most likely due to the presence of particulate COD.  COD in aquaculture wastewater is attributed to the undigested feed and fish fecal inputs [132].  The presence of COD in the wastewater can provide a source of organic carbon and result in increased growth in mixotrophic algae such as *Chlorella* [133,134].  As expected, HPCs were below detection limits in the filter sterilized feed.

Concentrations of elements (K, Ca, Na, S, Mg, Fe, Zn, Cu, Mn, Al) determined by ICP-OES are also shown in Table 4.1.  Most of concentrations were within the range observed by Martins et al. [135] for RAS wastewaters. Cu concentrations were within the optimal growth range for *Scenedesmus*; however, Zn concentrations were much higher than the optimal range reported in

Knauer et al. [136]. Sulfur concentrations were at optimal levels for the growth of *Chlorella vulgaris* based on the Liang et al. [41] and also should not present concerns based on American Society for Testing and Materials biodiesel standards [137].

Table 4.1: Aquaculture wastewater feed characteristics

| Mean concentrations | Axenic | Non-axenic |
|---|---|---|
| TN (mg/L) | 17.9 | 18.5 |
| $NO_3^-$ (mg/L) | 17.6 | 18.1 |
| COD (mg/L) | 238 | 253 |
| TP* (mg/L) | 17.0 | 17.5 |
| $PO_4^{3-}$-P* (mg/L) | 16.9 | 17.1 |
| pH | 6.94 | 6.97 |
| Transmissivity (%) | 99.0 | 97.8 |
| HPC (CFU/100 mL) | 0 | 183 |
| Potassium (K) (mg/L) | 66 | 65 |
| Calcium (Ca) (mg/L) | 62 | 64 |
| Sodium (Na) (mg/L) | 21 | 21 |
| Sulfur (S) (mg/L) | 15 | 16 |
| Magnesium (Mg) (mg/L) | 10 | 11 |
| Iron (Fe) (mg/L) | 0.016 | 0.069 |
| Zinc (Zn) (mg/L) | 0.011 | 0.022 |
| Copper (Cu) (mg/L) | 0.006 | 0.007 |
| Manganese (Mn) (mg/L) | 0.002 | 0.003 |
| Aluminum (Al) (mg/L) | < MDL | 0.006 |

*TP and $PO_4^{3-}$-P concentrations given after supplementation with 15 mg/L of TP; MDL= method detection limit

*4.3.2 Biomass Production and Intercellular Products*

The range of heterotrophic counts during different experimental phases is shown in Table 4.2. As expected, HPCs were below detection limits throughout the experiment for the axenic treatments (data not shown). Under non-axenic conditions, the HPCs increased to more than $10^3$ CFU/100 mL within 14 to 38 hours in treatments containing algae. After 38 hours, HPCs declined in all algae treatments, and were below the detection limit (30 CFU/100 mL) in the indigenous algal culture. Although the control photobioreactor that was not inoculated with algae

maintained HPCs above 30 CFU/100 mL throughout the experiment, there were higher counts

within the first 49 hours, after which the counts declined.

Table 4.2: Heterotroph bacterial population viability under non-axenic conditions (HPCs were < 30 CFU/100 mL for all samples under axenic conditions).

| Time (Hours) | Viability under non-axenic conditions | | | |
|:---:|:---:|:---:|:---:|:---:|
| | Indigenous | *Chlorella* | *Scenedesmus* | No algae |
| 0 | + | + | + | ++ |
| 14 | ++ | ++ | ++ | ++ |
| 25 | ++ | ++ | ++ | ++ |
| 38 | ++ | ++ | ++ | ++ |
| 49 | - | + | + | ++ |
| 72 | - | - | - | + |

- HPC < 30 CFU/100 mL; +HPC > 30 CFU/100 mL; ++HPC > $10^3$ CFU/100 mL.

Growth curves for *Scenedesmus* under both axenic and non-axenic conditions are shown in

Figure 4.2.  A maximum mean biomass concentration of 384 mg/L was achieved for

*Scenedesmus* after 72 hours, with no significant differences between the two treatments.  This

exceeds the amount predicted by the TN concentrations (Section 4.3.1), possibly due to initial

inoculum addition or the algae having a different elemental composition than suggested by the

general formula. Similar growth curves were obtained for the indigenous consortium and

*Chlorella* (data not shown).  Particle counts were slightly higher for *Scenedesmus* under axenic

conditions (Figure 4.2b), possibly because the presence of microorganisms facilitated auto-

flocculation.  Microscopic photographs of *Scenedesmus* (Figure 4.3) show dispersed cell growth

under axenic conditions and the presence of well-defined aggregates under non-axenic

conditions.  The presence of indigenous aquaculture microorganisms may have increased

S*cenedesmus* autoflocculation by facilitating extracellular polymeric substance (EPS)

production. Although no EPS measurements were made in this study, Guo et al. and Manheim

[138,139] noted the influence of EPS on algae flocculation. Cell aggregates were not observed

with the other cultures; however, which had similar particle counts under axenic and non-axenic conditions (data not shown).
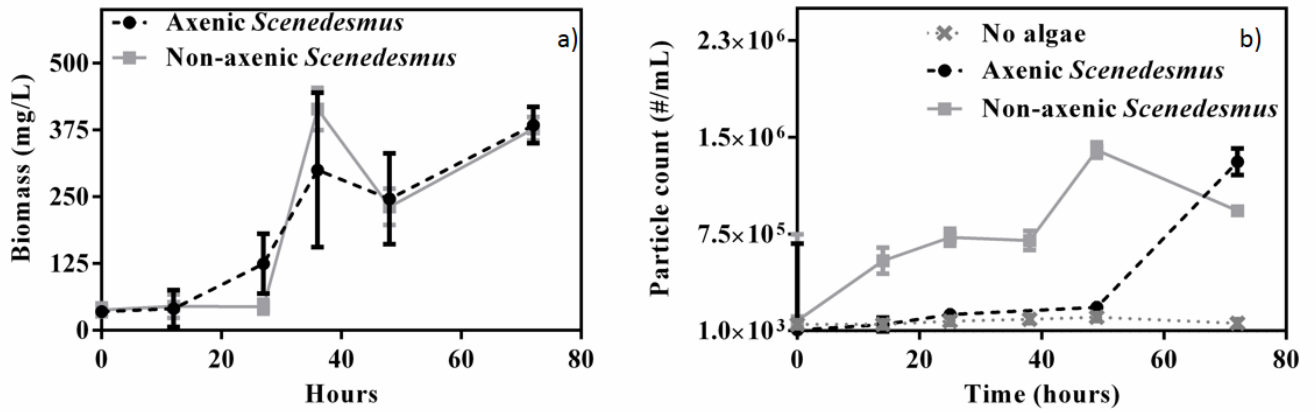


Figure 4.2: Biomass (a) and particle counts (b) for *Scenedesmus*.
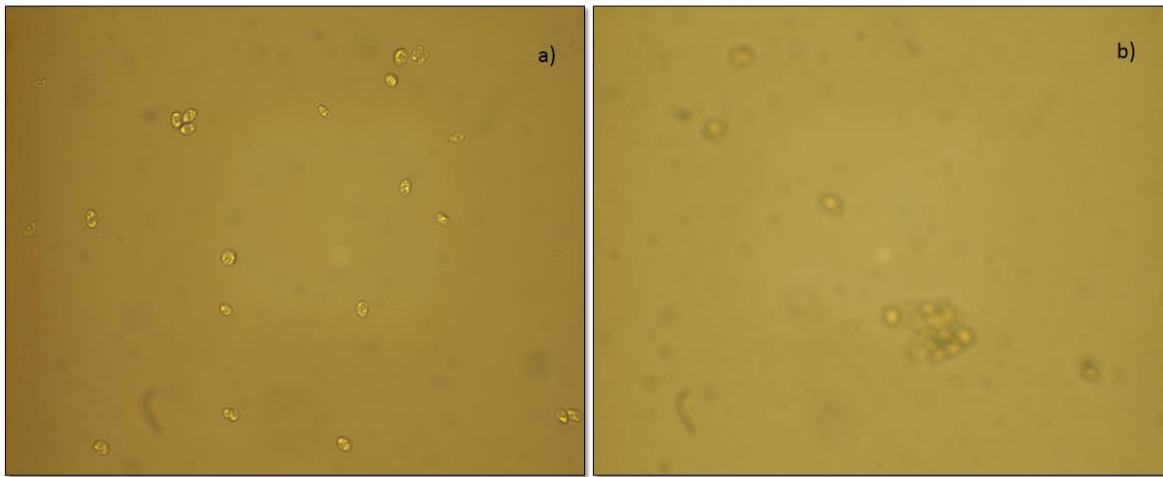


Figure 4.3: Microscopic observations (100 x magnification) for *Scenedesmus*, under axenic (a) and non-axenic (b) conditions.

Maximum mean algal biomass productivity ranged from 4.9 to 11.6 mg/L/hr, with no significant differences in productivity between axenic and non-axenic conditions within a single culture, as shown in Figure 4.2. *Scenedesmus* had the lowest mean maximum biomass productivity (5.3 mg/L/hr average of both axenic and non-axenic cultures), while the indigenous algal consortium

had moderate productivity (5.9 mg/L/hr) and *Chlorella* had the highest productivity (9.2 mg/L/hr).  Rodolfi et al. obtained similar productivities for both *Scenedesmus* and *Chlorella* cultures of 7.9 mg/L/hr and 7.1 mg/L/hr, respectively [140], most likely due to similar temperature ($25^{\circ}C$) and continuous illumination (100 μmol/m$^2$/sec).

No negative effects were observed when operating algal systems under non-axenic conditions using aquaculture wastewater, possibly due to the short experimental duration and the small scale at which experiments were conducted.  Other researchers have observed negative consequences associated with microbial contamination. Theegala et al. [141] noted that outdoor cultures usually last for only short periods of time and continuous systems rarely exceed a few weeks. Mitchell and Richmond [142] showed that the rotifers depleted *Monoraphidium minutum* populations, but only became a problem after four days. Smith and Crews [17] noted that algal species richness increased with water surface area, especially where algal systems were grown under natural, open conditions.  Algal ponds were susceptible to contamination and the number of invading species was positively correlated with the physical size of the cultivation system.

No significant differences were observed in chlorophyll contents (mg/g) between axenic and non-axenic conditions within a single culture.  *Scenedesmus* produced a slightly higher total chlorophyll content under non-axenic than axenic conditions, as shown in Figure 4.4.  For the indigenous and *Chlorella* cultures, the maximum total chlorophyll content was slightly higher under axenic conditions. The chlorophyll content (mg/g) for all algal cultures was between 12 and 48 mg/g, as shown in Table 4.3.  In treatments without any inoculated algae, chlorophyll contents ranged from 0.1 to 2.6 mg/g, indicating that some indigenous algae may have been present in the aquaculture wastewater.
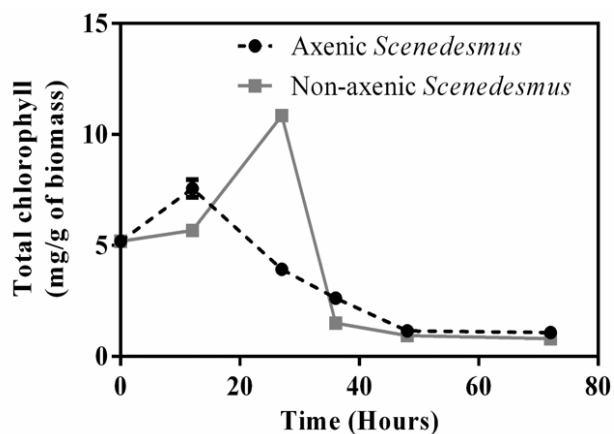
Figure 4.4: Total chlorophyll content over a 72 hour period for *Scenedesmus* under axenic and non-axenic conditions.

Comparisons of starch and lipid content values for all three algal cultures under axenic and non-axenic conditions are shown in Table 4.3. *Chlorella* produced the highest overall starch content compared with the other two cultures under both axenic (16.8%) and non-axenic (10.7%) conditions. Final lipid contents for indigenous and *Chlorella* cultures were significantly higher under non-axenic conditions. Microscope images and fluorospectrocopy in Appendix D confirmed that the lipid content increased with dramatically with nitrogen deprivation. Although *Scenedesmus* had a significantly higher overall lipid content than the other two cultures, differences observed between axenic and non-axenic conditions were not significant.

$NO_3^-$-N and starch concentrations over time are shown in Figure 4.5. $NO_3^-$-N concentrations were reduced to less than 10 mg/L within the first 24 hours. N limited (< 10 mg/L) and N starvation (< 1 mg/L) conditions have been shown to result in higher lipid contents as final storage products [128,114,129-131], with most of the total lipids as TAG (triacylglycerides) produced under N deprived conditions [143]. The results obtained in this study were generally consistent with other studies. In many cases, starch is formed as an intermediate storage

compound [144], and hence the timing of harvesting is important if the process is to be optimized for lipid production. Wang et al. [145] showed that the lipid bodies in a wild type *Chlamydomonas reinhardtii* increased 15 fold after a 48 hour period of N starvation. In this study, starch analyses were performed for each sampling point and used to determine the timing of starch storage depletion and the beginning of lipid accumulation [143,86]. Due to sample size requirements, only final lipid content was measured. For *Scenedesmus* under axenic conditions, the peak starch content (7.5%) was observed at 25 hours (Figure 4.5a). Under non-axenic conditions; however, the maximum starch content (14.1%) was observed at time zero and steadily decreased over 38 hours, after which it remained constant (Figure 4.5b). The initial high starch content for *Scenedesmus* under non-axenic conditions can be attributed to the presence of microorganisms and EPS production. When *Scenedesmus* started to grow exponentially between 25 and 38 hours, most of the carbon was probably used for growth and not EPS storage [146].
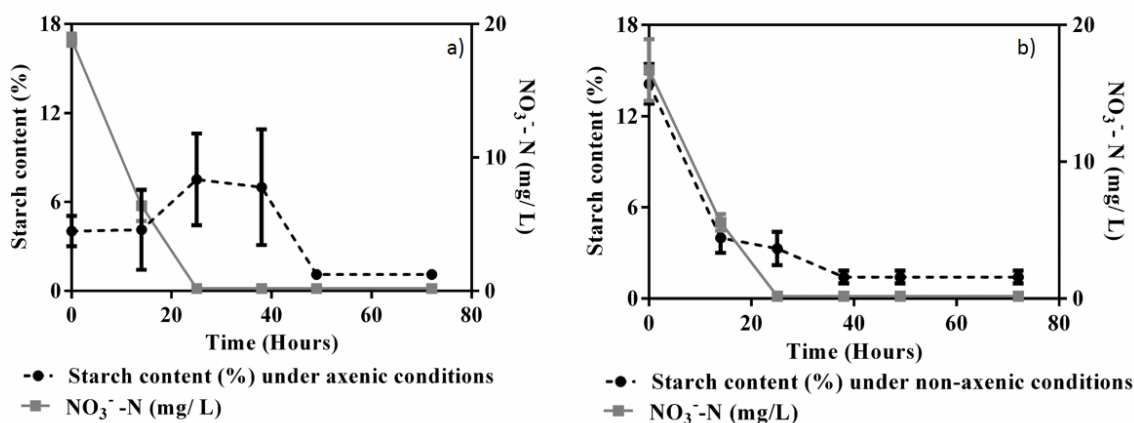


Figure 4.5: Starch content and $NO_3^-$-N concentrations over time for *Scenedesmus* under axenic (a), and non-axenic (b) conditions.

Gross calorific values varied from 20.2 to 26.5 MJ/Kg, as shown in Table 4.3. The indigenous algal consortium had the lowest calorific value (20.2 MJ/kg), whereas *Scenedesmus* under non-axenic conditions had the highest calorific value (26.5 MJ/kg). Although the calorific values

were slightly higher for all cultures under non-axenic conditions, these differences were not

significant.  There is a strong correlation between algal lipid content and calorific value [128].

Lipids are largely comprised of long-chain TAGs, which have an energy value 2.25 times greater

than starch on a weight basis [143].  The presence of other microorganisms may have increased

algal physiological stress, under already nutrient limited and starvation conditions, and resulted

in a shift in algal storage compounds from starch to lipids between 25 to 48 hours. Most

researchers focus on lipid and TAG production, as more valuable biofuel derivatives can be

produced from this fraction [147].

Table 4.3**:** Summary of gross calorific content, mean biomass, chlorophyll, starch and lipid
production

| | | | Culture | |
| --- | --- | --- | --- | --- |
| | **Conditions** | **Indigenous** | *Chlorella* | *Scenedesmus* |
| Mean biomass productivity (mg/L/hr) | Axenic | $5.80 \pm 0.30$ | $11.6 \pm 3.80$ | $5.70 \pm 0.20$ |
| | Non-axenic | $5.90 \pm 0.20$ | $6.70 \pm 0.30$ | $4.90 \pm 0.20$ |
| Max. chlorophyll (mg/g of biomass) | Axenic | $6.20 \pm 0.03$ | $7.12 \pm 0.03$ | $7.57 \pm 0.40$ |
| | Non-axenic | $4.10 \pm 0.07$ | $4.59 \pm 0.05$ | $10.85 \pm 0.19$ |
| Maximum starch content (%) | Axenic | $9.30 \pm 7.50$ | $16.8 \pm 2.80$ | $7.50 \pm 5.10$ |
| | Non-axenic | $9.10 \pm 3.60$ | $10.7 \pm 3.60$ | $6.85 \pm 4.70$ |
| Final lipid content (%) | Axenic | $5.70 \pm 2.40$ | $12.5 \pm 5.6$ | $58.6 \pm 10.7$ |
| | Non-axenic | $23.4 \pm 3.40$ | $50.4 \pm 7.6$ | $85.4 \pm 0.40$ |
| Calorific content (MJ/KG) | Axenic | $20.2 \pm 0.60$ | $22.0 \pm 1.0$ | $24.3 \pm 0.70$ |
| | Non-axenic | $22.1 \pm 0.6$ | $23.6 \pm$ | $26.5 \pm 4.60$ |

N.B.- Biomass productivity (calculated as: $\Delta X / \Delta t$, where X was the TSS concentration) for all
algal cultures under axenic and non-axenic conditions.

Timing of harvesting algae should correspond with the maximum production of the targeted end-product. If pigments are the desired end product, harvest time should correspond with the peak chlorophyll content. Some processes, such as pyrolysis, are optimized using algae with higher carbohydrate or starch contents, which were observed during the middle of the growth period. Since the primary activity of most algal cells is photosynthesis, there was little accumulation of starch and lipids in the young cells [148], indicating that harvesting should be delayed if lipids are the desired end product.

*4.3.3 Nitrogen and Organic Matter Removal*

Since 97% of the initial TN was in the form of $NO_3^-$, (Table 4.1) only $NO_3^-$ was measured during the algal growth experiments. For all treatments with algae, $NO_3^-$ concentrations were reduced to less than 10 mg/L within the first 14 hours (N depletion) and to less than 1.0 mg/ L within 24 hours (N starvation). Overall $NO_3^-$ removal efficiencies ranged from 96.4 to 99.4% for all systems inoculated with algae, as shown in Table 4.4, with no significant differences between algal cultures or treatments. The removal efficiency for the treatment that was not inoculated with algae had a $NO_3^-$ removal efficiency of only 17.6%, indicating that the presence of algae was needed for N removal in aquaculture wastewater under these conditions. The TN removal rate was moderate (129 mg $TN/m^2/$ day) when compared to other studies using different technologies, including membrane, integrated plant, wetland and algal treatment systems. Denitrification membrane systems tend to be more compact, and have higher removal efficiencies, but there are no useful end-products derived from the process [77]. Wetland and aquaponic systems had very similar TN removal rates of approximately 520 -560 mg $TN/m^2/day$ [149,150].

Removal of COD over time for *Scenedesmus* under both axenic and non-axenic conditions are

shown in Figure 4.6a. Overall COD removal efficiencies are shown in Figure 4.6b. Under

axenic conditions, approximately 25 % COD removal was observed in algal treatments, most

likely due to mixtrophic growth of algae. Prior studies have shown that lipid production is

increased for green algae under mixotrophic and heterotrophic conditions [137,40,151];

however, due to the use of real RAS wastewater no comparisons could be made on lipid

production with or without COD in this study. COD removal (74.4 to 99.7%) was significantly

higher under non-axenic conditions for all cultures (Figure 4.6b), indicating that the

microorganisms present in the aquaculture wastewater were needed to achieve high COD

removal efficiencies required for wastewater treatment.

Table 4.4: Summary of $NO_3^-N$ removal efficiency (%) for the different treatments

| | | Culture | | | |
| | Conditions | Indigenous | *Chlorella* | *Scenedesmus* | No algae |
|---|---|---|---|---|---|
| $NO_3^-N$ removal efficiency (%) | Axenic | 99.4 ± 0.8 | 98.1± 0.3 | 98.7 ± 0.5 | NA |
| | Non-axenic | 96.4 ± 0.1 | 96.3 ± 0.3 | 99.0 ± 2.0 | 17.6 ± 0.8 |
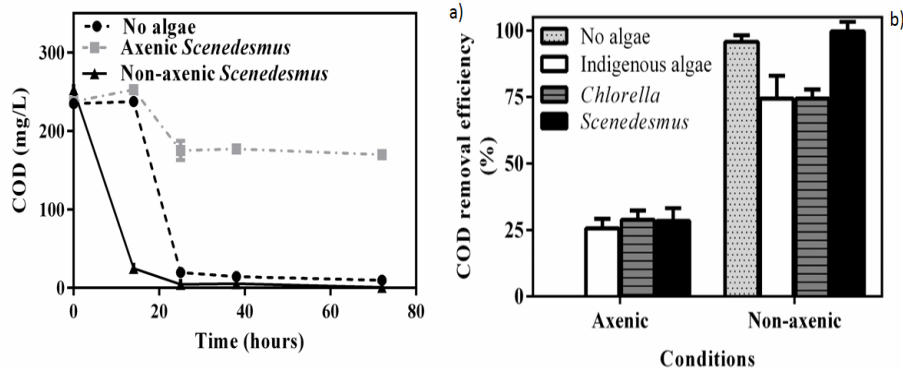


Figure 4.6: a) COD removal for *Scenedesmus* under axenic and non-axenic conditions. b) COD removal efficiency for all algal cultures under axenic and non-axenic conditions as well as RAS wastewater with no inoculated algae.

**4.4 Conclusions**

Algae and fish co-cultivation has the potential to improve water quality and fish health, while producing a feedstock for onsite energy production and/or feed supplementation. However, maintaining large-scale algal cultivation systems under axenic conditions is impractical. Results from this study showed that biomass and lipid productivity are improved under non-axenic conditions. Final lipid content for all cultures was significantly higher under non-axenic conditions, most likely due to competition for N by indigenous microorganisms. In addition, the presence of both indigenous RAS microorganisms and algae produced a treated wastewater effluent with low N and COD concentrations. Algae alone removed N, while microorganisms alone removed COD. Negative consequences of contamination of algal cultures with RAS microorganisms were not observed. This may have been due to the short growth period (72 hours) in the batch system.

## CHAPTER 5: AUTHENTIC SCIENCE RESEARCH AMONG HIGH SCHOOL STUDENTS

### 5.1 Background and Rationale

Authentic science experiences have been described in the K-12 science education literature as activities that are as similar as possible to the daily activities of scientists and engineers [152]. Scientific learning and inquiry are quite complex, and traditional classroom environments and didactic instruction does not lend itself to higher-level scientific inquiry [153,154]. In contrast, students participating as authentic contributors to a research project experience real-world representations of the scientific enterprise.

The Next Generation Science Standards (NGSS) were developed on the Framework for K-12 Science Education developed by the National Research Council (NRC). These standards were developed to favor the inquiry based approach to learning science and argues that these experiences increase scientific understanding and knowledge. Scientific inquiry refers to the Science, Technology, Engineering and Math (STEM) engagement and understanding of the nature of science [155]. With early adoption by 26 states in the US and integration within some school districts despite statewide non-adoption, curriculum is being developed and piloted for the NGSS. Agencies such as the National Science Foundation fund research and education programs to broaden participation in STEM and sites such as teachengineering.org are repositories and resources for STEM curriculum that interfaces with research areas at the university level.

Energy research is viewed as important by policy makers, stakeholders in the energy sector and the broader scientific community. However, there is a misconceived notion that HS students in

low-income urban communities[4], such as East Tampa, are not interested in STEM. One reason given for this is that they believe it does not connect to their everyday experiences or interests. Chapman [157] stated that:

*"Most students from low-income urban families envision scientists as white men,*

*such as Einstein, wearing lab coats and safety goggles."*

An individual's life experiences are important in yielding useful, powerful and transferable knowledge. The inadequacies HS students display in science should not be seen as the sole reason for their disengagement in science, since knowledge construction is a socially, politically and culturally defined process [158]. As researchers, we should be advocates and vehicles for social and educational reform.

Multiple pedagogical strategies need to be employed to maintain the interest of all students and engage women and men of color and thereby create a multicultural, diverse scientific community that mimics the demographics of society [159,160]. Sadler et al. [161] noted that research programs that emphasize hands-on authentic science experiences, such as the one described in this study, can increase retention of undergraduates in science majors, particularly African-American students. In addition, Scholz et al. [162] showed that a 15 week internship not only improved environmental science high-school students' credentials, but there was a notable enhancement in students' analytical thinking, report writing, and presentation skills. The University of South Florida (USF) is a scientific center and research platform for the local community. University researchers have the potential to facilitate scientific inquiry with HS

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[4]Approximately 79%  or eight out of ten children had 'reduced or free lunches' in this HS, while the mean for the Hillsborough District was approximately 59% or six out of ten [156].

students serving as novice researchers while contributing to authentic research. This algal research-based project was executed at a magnet HS in East Tampa during spring and fall 2012 semesters in agricultural biotechnology and marine science classes. Although, this was a magnet science and engineering HS, the FCAT scores suggested that many students were disengaged from the STEM field. In the 2009 academic year, the mean pass rate among the Hillsborough County School District (SDHC) for Florida Comprehensive Assessment Tests (FCAT) was 68% for mathematics. However, the statistics for students who passed mathematics at this HS were lower (53%) than the district's average.

**5.2 Methods**

Formal and informal methods were used throughout my doctoral tenure to communicate scientific concepts related to algal feedstock generation and wastewater treatment. Informal methods used included an open mic poetry recital (Appendix F) and Earth Expo Events. This chapter highlights the formal methods used in an East Tampa HS. Section 5.2.1 highlights the preparation done prior to the HS experimentation by university researchers. Section 5.2.2 highlights the research design of the HS experiments and protocols developed. Section 5.2.3 highlights the methods used in gauging success of the authentic science experience. Most of the assessment was qualitative.

*5.2.1 Preparation of Inoculum by University Researchers*

Indigenous algae were harvested from the surface of a secondary clarifier at the Howard F. Curren Advanced Wastewater Treatment Facility in Tampa, Florida. The consortium was identified and enumerated by the Environmental Biotechnology Laboratory in the Department of Soil and Water Science at the University of Florida. The primary genera within the indigenous consortium identified included: *Chlorella* (95.2%), *Chlamydomonas* (3.1%), and *Stichococcus*

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(1.1%). The culture was initially grown using an aseptically prepared standard algal growth medium (Bold medium [163]), a light irradiance of 67.5 μmol/m$^2$/sec and a temperature of 25°C in a temperature controlled room. This algae was then used to inoculate the reactors used in the HS experiments.

*5.2.2 Experimental Design*

HS students were given an initial lecture by the professor (Dr Ergas) on the background and goals of the research. The reasons why we wanted them to conduct experiments to determine how feed composition, mimicking municipal high- strength and aquaculture wastewater feeds influenced algae growth rates, was also explained. Each group constructed three photobioreactors using commonly available materials including 3.0 L clear soda bottles, aquarium pumps and tubing (Figure 5.1). All students also learned to conduct basic laboratory measurements including total solids, pH and light intensity. Researchers also stressed proper recording of data in lab notebooks and Excel spreadsheets.

Two rounds of experiments were conducted.  In the first round, students were given two different synthetic wastewater feeds (swine and aquaculture). These feeds mimicked actual compositions of wastewater observed by the Ergas research group. It was assumed that most of the nitrogen in nitrified aquaculture wastewater was in the form of nitrate ($NO_3^-$) and most of the nitrogen in swine wastewater is in the ammonium ($NH_4^+$) form. HS students conducted these experiments in groups. Each group was responsible for conducting algal growth experiment using one feed mixture in triplicate (Table 5.1). Data from all of the teams was pooled to draw conclusions on the effect of wastewater type on algal growth rates.

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Prior to starting the second set of experiments, students discussed their results and ideas for another round of experiments. Brainstorming, creating mental maps and input from the engineering researchers on novel research questions were used to design the course of action for the second round of experiments. An example of a mental map is shown in Appendix I. In the second experiment, all but one group examined at the effect of different variables. One group was asked to repeat on of the initial experiments without any changes (a control group). The following variables were examined by the other groups: 1) addition of an artificial light source, 2) addition of baking soda (an inorganic carbon source), and 3) use of a higher gas flow rate. The effect of each treatment on biomass productivity was compared to the control.
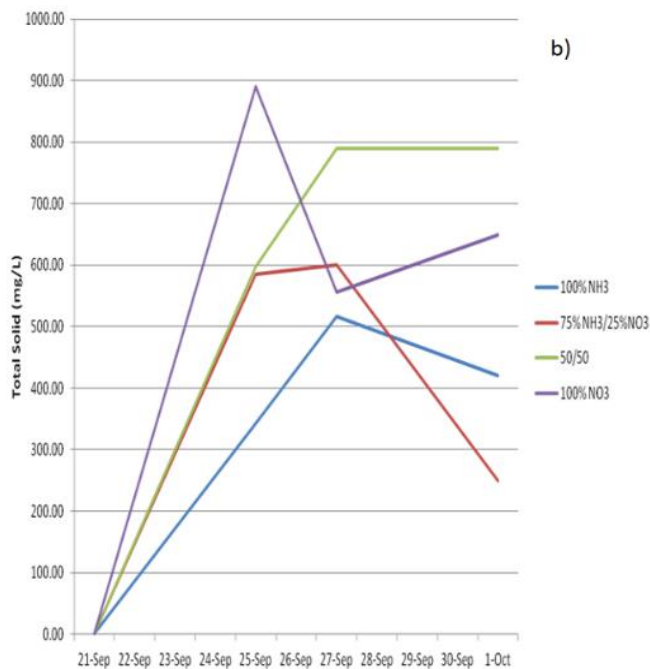


Figure 5.1: Initial setup and productivity achieved. a) One of four groups set up their photobioreactors. Photo credit: Angela Chapman b) One of the graphs produced and presented by a student group in a final presentation to show results from the 1st experiment, examining the effect of nitrogen form on biomass productivity.

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The success of this authentic science experience was assessed in several ways:

1. Personal observations and journaling. A journal was maintained by the graduate student researchers to record the events and progress during the experience.

2. Skills gained by graduate and HS students. Both groups of students were expected to be able to design experiments, analyze data, present their results, participate in discussions and answer open ended questions posed by the researchers.

3. Pre and post evaluation assessment carried out by a College of Education graduate student [157].

Table 5.1**:** Feed composition of swine and aquaculture waste treatments

| Chemical formula | Concentration (mg/L) | | | |
|---|---|---|---|---|
| | Group 1 100% $NH_4^+$- N (mg/L) | Group 2 50% $NH_4^+$ -N and 50% $NO_3^-$-N (mg/L) | Group 3 75% $NH_4^+$ -N and 25% $NO_3^-$ - N (mg/L) | Group 4 100% $NO_3^-$ N (mg/L) |
| $NH_4HCO_3$ | 400 | 208 | 312 | 0 |
| $NaNO_3$ | 0 | 224 | 112 | 448 |
| $KHCO_3$ | 100 | 100 | 100 | 100 |
| $NaHCO_3$ | 400 | 400 | 400 | 400 |
| $CaCl_2\ 2H_2O$ | 25 | 25 | 25 | 25 |
| $MgSO_4\ 7H_2O$ | 64 | 64 | 64 | 64 |
| $K_2HPO_4$ | 24 | 24 | 24 | 24 |
| $K_2SO_4$ | 4 | 4 | 4 | 4 |
| NaCl | 25 | 25 | 25 | 25 |
| Trace metal* | - | - | - | - |
| Vitamin B12* | - | - | - | - |

*Trace metal and Vitamin B12 is provided based on Bold medium[163].

**5.3 Results and Discussion**

*5.3.1 Personal Observations and Journal Notes*

Over the course of the project, HS students became increasingly engaged and proactive in class discussions and execution of research tasks. Their engagement probably increased due to increased familiarity with the project, methods and the graduate students. One of the students told me:

*"For the first time I feel like a scientist."*

To me, it was important to demonstrate to HS student groups that high algal productivity could be achieved using soda bottle reactors and aquarium supplies. This was important in demonstrating that science experiments can be conducted using easily accessible materials. This experience was also important to me and other novice graduate students in designing protobioreactors and algae experiments. We learned hands-on skills in how to connect the air tubing, adjust air flow rate, and manipulate growth variables.

Although the students were able to understand key concepts of algal growth, I felt that students were not able to fully understand how this algae grown on wastewater can be used to make biofuel. They saw that the contents of the reactor was green, but did not gather enough knowledge of downstream processing to understand how algae can be made into fuel.

*5.3.2 Experimental Design and Discussion*

HS student groups' results showed that nitrogen form influenced algal growth. The feed using 100% $NO_3^-$ showed the highest initial productivity, approximately 890 mg/L on day 5 (September 25$^{th}$) (Figure 5.1b). However, the highest overall productivity, of approximately 795

mg/L, was achieved and sustained using 50% $NH_4^+$- N and 50% $NO_3^-$. HS students were then able to understand the idea of optimal growth and ammonia toxicity.

The university-based algae research group was able to obtain some useful preliminary data for the indigenous algae's productivity under different feed conditions. The second round of experiments conducted by the groups demonstrated scientific problems that scientists face and how different factors influence biomass production. For example, one group investigated the effect of supplemental lighting by adding artificial lighting (Figure 5.2). Their results showed that treatments without additional lighting had higher productivity than cultures with additional lighting. Based on the discussion, it was clear that the HS students understood that this may have been due to photo-inhibition in cultures with additional lighting.  A high light intensity is toxic to algae.
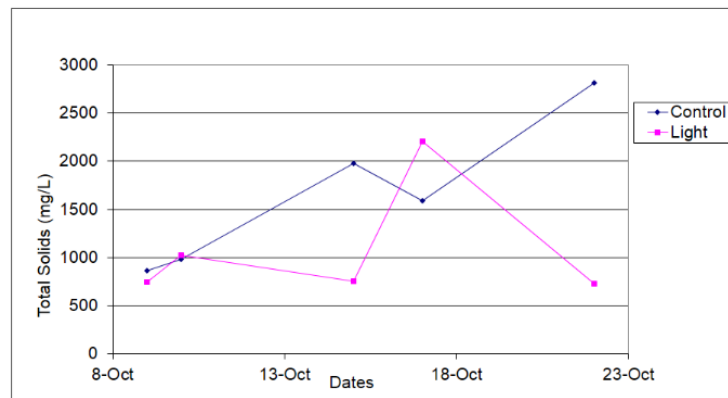


Figure 5.2: HS student produced graph showing treatments with no additional lighting (control) and treatments with additional lighting (light).

### 5.3.3 Skills Gained by Graduate and HS Students

HS students collected samples for total solids (TS) analyses, recorded data, and analyzed the data for their triplicates and determined the mean and standard deviations. Each group was asked to

set up their respective treatments in triplicate and learned that replicates were important in ensuring quality assurance and control (Figure 5.1 a). They were able to input data into Excel spreadsheets and calculated mean and standard deviations using the data they obtained. In addition, HS students communicated their scientific findings from their experiments using a PowerPoint presentation in front of university professors, the district science supervisor and HS teachers (Figure 5.1 b).

*5.3.4 Pre and Post Evaluation Assessment*

Chapman [157] conducted a pre and post test to determine HS understanding and appreciation of the research experience. HS students had a greater understanding of scientific theories related to algae derived biofuel and photosynthesis. When students were asked the open ended question:

*"What are the benefits obtained from growing algae?"*

There was a 35% decrease in the number of HS students that said, "I don't know." or answered incorrectly. In addition, there was an increase in the understanding of photosynthesis and that algae can be used as provide an alternative energy source.

**5.4 Conclusions**

Having a university-based algal project with involvement of University of South Florida (USF) researchers, teachers and high school (HS) students increased scientific understanding and skills among HS students. Graduate students gained greater in-depth practical understanding as these students had to learn skills, such as designing a photobioreactor, while simultaneously teaching HS students how to construct photobioreactors, design and conduct experiments, and gather scientific data. HS students gained a greater understanding of key biological and chemical processes, such as photosynthesis. In addition, they learned important skills, such as calculating

mean and standard deviation, using Excel, orally communicating scientific concepts and preparation of a PowerPoint presentation. From personal observations, HS students engagement increased over the course duration as they had an increased familiarity of the project, theory and methods.

## CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

Algal research is the central theme of this dissertation (Figure 6.1). This research examined: 1) the biomass and lipid production, and removal efficiencies on municipal centrate and aquacultures wastewaters, 2) the effect of irradiance on biomass production, 3) the effect of indigenous microbes on algal resilience, and 4) the facilitation of greater understanding of scientific principles and interest in science among HS students through authentic science research on biofuel production. The major findings of this research were:

1. Bioenergy feedstock production

   In this study, an indigenous algae consortium was cultivated on municipal sludge centrate, a high-strength wastewater. Mean biomass productivity if 5.2 g m$^{-2}$ day$^{-1}$, which was relatively high compared with other studies carried out with high ammonia strength wastewaters. This study was one of the first to co-cultivate algae with aquaculture products to facilitate energy and cost savings, while producing useful biomass feedstocks and end-products.

   Non-axenic conditions had no effect on overall starch and chlorophyll production; however, significantly higher lipid contents were achieved under non-axenic conditions. The higher algal lipid content under non-axenic conditions may have been due to competition with bacteria for nutrients and nitrogen limited conditions.

   A simple irradiance-based model was developed from the fundamental Michaelis-Menten photosynthesis-irradiance (PI) response for photosynthetic organisms. A good fit to the

experimental data was obtained with the irradiance-based model ($R^2=0.96$), indicating that the system was light limited.

Appendix B [123] is a preliminary study that considers co-location of wastewater treatment plants, including HFC AWTP and algal production facilities, and scenarios considering biofuel for vehicle use and biogas for residential use. Further research could explore and integrate biorefineries into wastewater treatment and aquaculture facilities.

2. Wastewater treatment

More than 65% total nitrogen (TN) and 72.6% total phosphorus (TP) was removed from both waste streams investigated in this research. COD removal was only 8% when centrate was treated, most likely because most of the biodegradable COD has already been removed during anaerobic digestion. Investigations examining the effects of axenic conditions on wastewater treatment showed the presence of bacteria in aquaculture wastewater was required for effective removal of organics, while effective nitrogen removal was observed in all systems containing algae.

3. Educational outreach

A collaboration was formed with a faculty member and graduate student in the USF College of Education to design, implement and evaluate an authentic science research experience of HS students. A background on algal research and two experiments were conducted with local HS students and teachers to investigate algal growth in photobioreactors under varying conditions. Using authentic science experiences increased the understanding of core chemistry and biology concepts identified by the Next Generation Science Standards and practices, and at the same time stimulate STEM (Science Technology, Engineering and Mathematics) interests and generate useful data

for the university based researches, as graduate students gained hands-on experience in

experimental design.
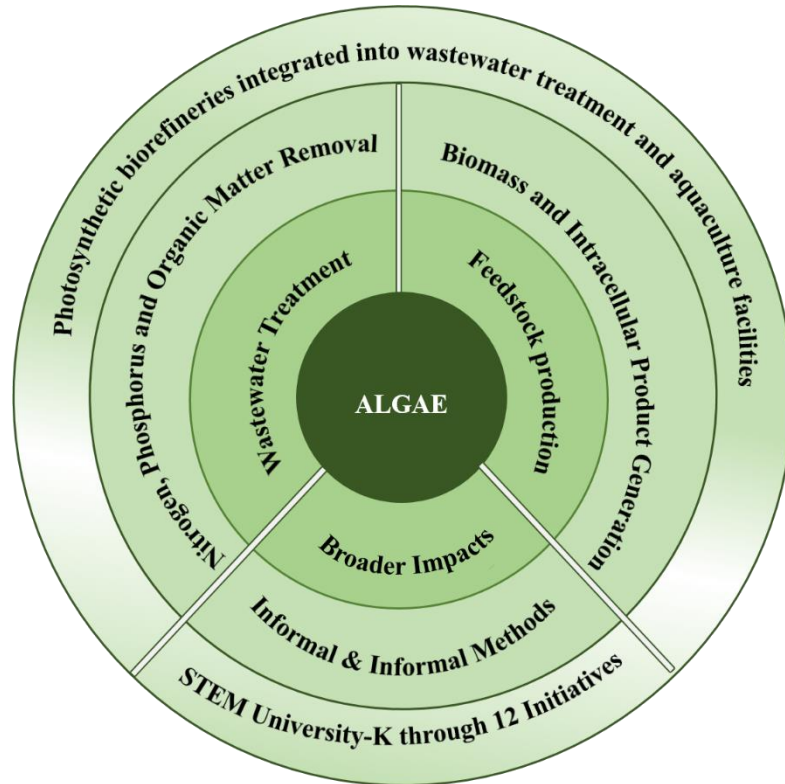


Figure 6.1: The research completed during my doctoral tenure focused on: wastewater treatment, feedstock production and educational outreach

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# APPENDIX A: EXPERIMENTAL DESIGN AND PROTOCOLS DEVELOPED

## A.1 Chlorophyll Analyses

2.0 mL of the original sample was pipetted into a 2.0 mL centrifugation tube. The sample was then centrifuged using Eppendorf centrifuge 5415R (serial # 0011120) at 5,000 rpm for 10 minutes and 20$^o$C. The supernatant was then disposed of and the algae pellet was then stored at -20$^o$C.

A formal thawing was not required as samples stored at -20$^o$C quickly thawed at room temperature. Equal portions of cell disruption beads 0.5 mm (Scientific Industries S1-BG 05) and 0.1mm (Scientific Industries S1-BG 01) were added until a total volume of 0.5mL was achieved. 1.5mL of methanol was then added. The samples were then shaken using a cell disrupter for a duration of 10minutes at 30,000rpm. The tubes were then centrifuged for 10 minutes at 20$^o$C and 13,000rpm. 0.75ml of the supernatant was then pipetted into a 1.5mL disposable polystyrene cuvettes and then measured at 665, 652 and 470nm wavelengths. Total chlorophyll was determined using the method described by Franco et al. [99].  Total chlorophyll was calculated using Liechtenthaler equations [100].

## A.2 Starch Analyses

Similar initial sample preparation steps were taken for chlorophyll and starch analyses. 2.0 mL of the original sample was pipetted into a 2.0mL micro- centrifugation tube. The sample was then centrifuged at 5,000 rpm for 10 minutes and 20$^o$C. The supernatant was then disposed of and the algae pellet was then stored at -20$^o$C. Equal portions of cell disruption beads 0.5mm and 0.1mm

were added until a total volume of 0.5mL was achieved. 1.5mL of methanol was then added. The samples were then shaken using a cell disrupter for a duration of 10minutes at 30,000 rpm. The tubes were then centrifuged for 10 minutes at 20°C and 13,000rpm. The supernatent was then poured into a hazardous container. Any excess methanol was allowed to evaporate under the fume hood.

Megazyme total starch (AA/AMG) kit (catalog # K-TSTA), which follows Association of Official Agricultural Chemists (AOAC) Method 996.11 Standard Method was used and modified to allow for smaller sample volumes. 0.2µL of 80% ethanol was added to the micro-centrifugation tube. 200µL of DMSO solution was then added and the mixture was vortexed well for 2minutes and then put on a hot plate at 100°C for 5mins and shaked at 650rpm. 0.3mL of amylase- sodium acetate solution buffered at pH 5 (Solution 1) was added at again heated at 100°C for 6 mins. 04mL sodium acetate, 10 µL amyloglucosylase solution buffered at pH 9.5 was added and then voretexed lightly and then heated at 50°C for 30minutes. 70µL deionized water was then added. The mixture was then vortexed at 14,000rpm for 10minutes at 20°C.  In new micro-centrifuge tubes, 33.4µL of the supernatent was then added to 1.0mL of GOPOD solution. Duplicates for each sample was prepared. To ensure quality assurance, 2 blanks and 2 check standards were used. For the blank, 33.4µLdeionized water was added to 1.0mL of GOPOD solution. For the check standard, 33.4µL of the check standard was added 1.0mL of GOPOD solution. All samples, including blanks containing the deionized water was then transferred to 1.5 mL disposable polystyrene cuvettes and then measured at 510 nm. Total starch (%) was then calculated.
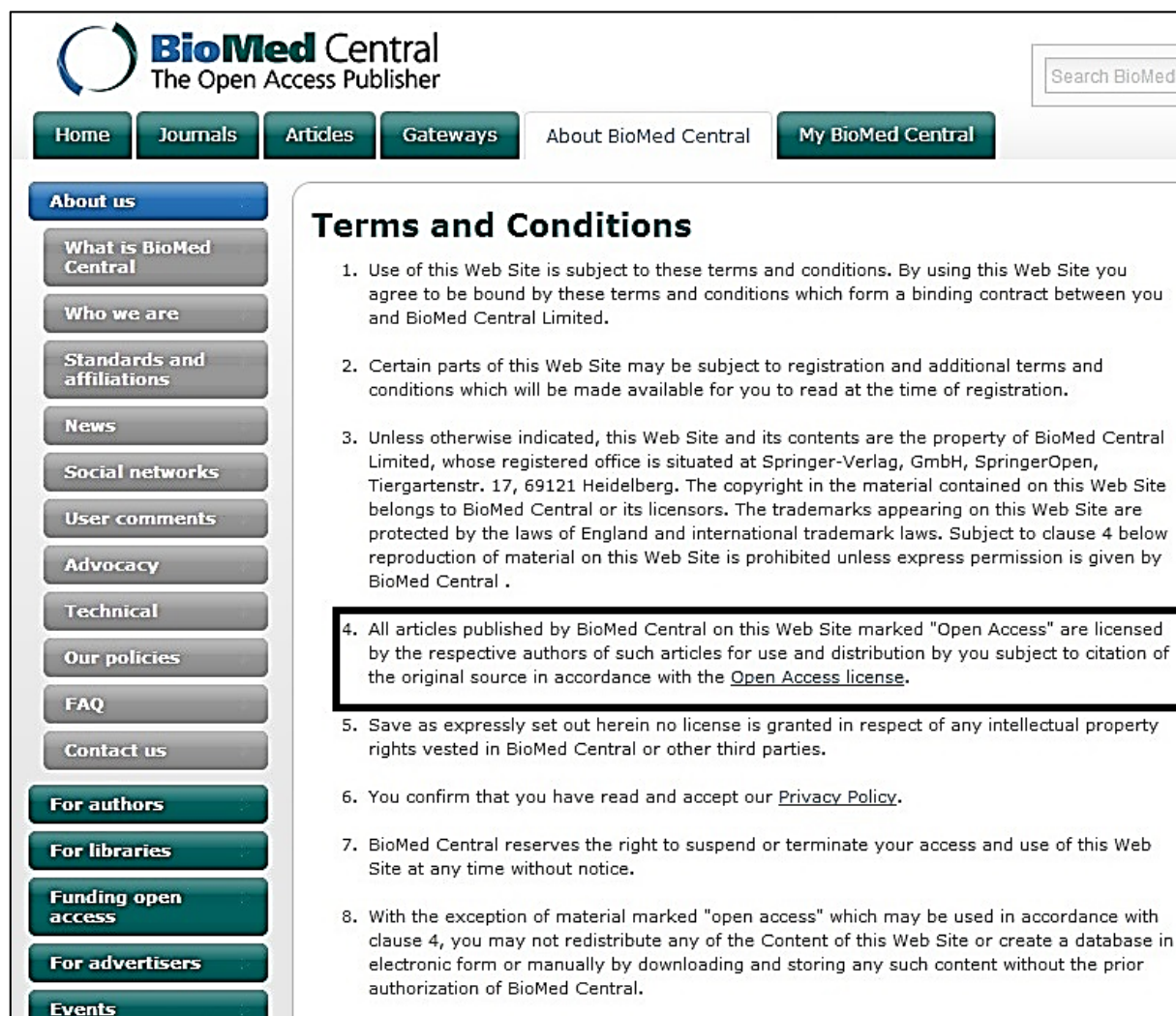
## A.3 Final Lipid Analyses

The algal lipid content (%) was determined using the by Bligh and Dryer method. A sample of algae suspension was centrifuged at 3,800 rpm for 10 minutes to obtain a concentrated algae paste. Algae pellets stored at -20°C and in 50mL tubes with known wet weights were defrosted and then vortexed to homogenize. The dry weight ($w_d$) of the pellet was determined gravimetrically after drying it at 60°C. 3.0mL of a 2:1 methanol/chloroform solution was added to a 15mL tube. The suspension then vortexed for 2 minutes and left for 24 hours. Thereafter, 1.0 mL of chloroform was added and mixture and vortexed for 2 mins. 2.0 mL of water was then added and the mixture was again agitated for 2 min. The layers were separated by centrifugation at 2,000 rpm for 10 min. The lower layer was extracted with a glass syringe and filtered through a Whatman no. 1 filter into a previously weighed glass vessel ($w_1$). The solvent was dried in a water bath at 98°C and the vessel was weighed again ($w_2$) to obtain the lipid content of the sample as;

$$lipid\ content = \frac{w_2 - w_1}{w_d} \times 100\% \qquad \text{(Eq. A.1)}$$

# APPENDIX B: PUBLISHED PAPER

The permission for this paper is below.

# Wastewater use in algae production for generation of renewable resources: a review and preliminary results

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## Abstract

Microalgae feedstock production can be integrated with wastewater and industrial sources of carbon dioxide. This study reviews the literature on algae grown on wastewater and includes a preliminary analysis of algal production based on anaerobic digestion sludge centrate from the Howard F. Curren Advanced Wastewater Treatment Plant (HFC AWTP) in Tampa, Florida and secondary effluent from the City of Lakeland wastewater treatment facilities in Lakeland, Florida. It was demonstrated that a mixed culture of wild algae species could successfully be grown on wastewater nutrients and potentially scaled to commercial production. Algae have demonstrated the ability to naturally colonize low-nutrient effluent water in a wetland treatment system utilized by the City of Lakeland. The results from these experiments show that the algae grown in high strength wastewater from the HFC AWTP are light-limited when cultivated indoor since more than 50% of the outdoor illumination is attenuated in the greenhouse.

An analysis was performed to determine the mass of algae that can be supported by the wastewater nutrients (mainly nitrogen and phosphorous) available from the two Florida cities. The study was guided by the growth and productivity data obtained for algal growth in the photobioreactors in operation at the University of South Florida. In the analysis, nutrients and light are assumed to be limited, while $CO_2$ is abundantly available. There is some limitation on land, especially since the HFC AWTP is located at the Port of Tampa. The temperature range in Tampa is assumed to be suitable for algal growth year round. Assuming that the numerous technical challenges to achieving commercial-scale algal production can be met, the results presented suggest that an excess of 71 metric tons per hectare per year of algal biomass can be produced. Two energy production options were considered; liquid biofuels from feedstock with high lipid content, and biogas generation from anaerobic digestion of algae biomass. The total potential oil volume was determined to be approximately 337,500 gallons per year, which may result in the annual production of 270,000 gallons of biodiesel when 80% conversion efficiency is assumed. This production level would be able to sustain approximately 450 cars per year on average. Potential biogas production was estimated to be above 415,000 kg/yr, the equivalent of powering close to 500 homes for a year.

## Introduction

The United States (US) imports about 57% of the petroleum it consumes. Among all sectors, transportation accounts for 72% of all petroleum consumption [1]. As energy consumption increases, the US dependence on foreign oil will also increase and compete heavily with the energy demands of rapidly growing economies such as China, India and Brazil. This will place tremendous pressure on global oil production and may decrease energy security. In addition, the wide and sustained use of petroleum-based fuels has been implicated as a major cause of increased atmospheric greenhouse gases, which may contribute to global climate change [2]. These challenges have sparked the quest for alternative energy sources to serve as viable replacements to reduce dependence on fossil fuels and improve environmental sustainability. Among the many options, microalgae are receiving enormous attention as a source for the

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production of biofuels. Model estimates from Pacific Northwest National Laboratory have suggested that algal biofuels (particularly biodiesel) have the potential to meet as much as 17% of the transportation fuel demand [3]. Microalgae oil production per unit area of land far exceeds other oil crops such as corn, soybean, coconut, and oil palm by as much as 2–3 orders of magnitude [4]. Furthermore, they do not compete for arable land and can be produced year-round in suitable climates. They also grow much faster than traditional crops (doubling time can be as fast as 24 hours) and are likely to recover more quickly from adverse effects [5,6].

Large-scale commercial production of algae, however, is potentially more costly than traditional crop production. Algae cultivation requires significant quantities of energy and water and the use of off-site generated carbon dioxide. One energy intensive process, for example, is the harvesting of the algal biomass, which can account for as much as 30% of the total cost of production [7-11]. In addition, water and nutrients are among the most critical variables in algal production [10,12]. Fortunately, algae can be grown in both fresh water and seawater depending on species, but nutrient costs can be substantial. The main nutritional requirements for algal growth are nitrogen, phosphorous, and a number of micronutrients including potassium [5]. Algae take up these nutrients along with $CO_2$ and produce biomass via photosynthesis. Various combinations of fertilizers maybe used, including common field crop N-P-K fertilizer, but the associated costs can sometimes exceed the value of the final algae products [10].

For algal biofuels to achieve their full potential, inputs to algal cultivation must be inexpensive allowing for the economical mass production of feedstock. A convenient and cheap source of nutrients is municipal, industrial and agricultural wastewaters. Nutrient removal is an important aspect of wastewater treatment because rich nutrient streams discharged into natural water bodies can result in eutrophication. Furthermore, centrate (a nutrient-rich effluent stream from the anaerobic digestion process) is generally recycled to the head of the wastewater treatment plant and can increase the cost and destabilize the overall treatment process due to phosphorus accumulation. Since algae are known to grow in wastewater, a possible synergistic solution is to co-locate and integrate algal production with treatment of nutrient-rich wastewater and utilization of $CO_2$ from power plant flue gas. This approach essentially reduces the cost of algal production, while preventing eutrophication and mitigating $CO_2$ emissions [13-16].

Florida, and particularly the Tampa Bay area, has been identified as an ideal location for the development of algal feedstock and biofuel production because it receives significant sunshine, and demonstrates a relatively uniform

seasonal evaporation loss compared to many other areas of the country [3]. The latter is particularly important for open pond cultivation systems that lose significant amounts of water via evaporation. In this study, wastewater use for algae production is reviewed, particularly for renewable energy generation. A preliminary assessment of the potential to produce algal feedstock from wastewater is presented for two Tampa Bay cities. These include the City of Tampa and the City of Lakeland. All the wastewater from the City of Tampa is treated at the Howard F. Curren Advanced Wastewater Treatment Plant (HFC AWTP). HFC AWTP has a designed average daily flow capacity of 96 million gallons per day (MGD) and employs high-purity oxygen aeration for biochemical oxygen demand (BOD) removal followed by nitrification and denitrification. Lakeland's municipal wastewater is treated by two traditional wastewater treatment plants and the secondary effluent is released into a 1,400-acre wetland treatment system (WTS) to achieve permissible nutrient reduction levels. The average daily flow rate into the wetland is 5.2 MGD. The WTS consist of a series of wetland cells connected by engineered discharged structures. Effluent from the WTS is discharged to the Alafia River. A wide cross-section of freshwater algal species thrives in the WTS.

Most of the electricity supplied to the Bay Area comes from Tampa Electric Company (TEC), which has a power plant located about 15 miles south of the Lakeland WTS and another plant across from the HFC AWTP. Together, these two power plants emit approximately 5.5 million metric tons of $CO_2$ annually. Further, to lessen the burden on scarce freshwater resources, TEC and the City of Lakeland entered into a reclaimed water agreement in 2009 that allows TEC to use reclaimed effluent from the WTS commencing at the end of 2012. TEC will install a water treatment system to ensure that the effluent meets its cooling water standards.

The location of these facilities presents a potentially viable opportunity to explore synergy for algal feedstock production using wastewater and industrial $CO_2$. A preliminary assessment was made to determine the quantity of algal feedstock that can be generated. The analysis was guided by experimental work on the growth of algae in enclosed bench-scale photobioreactors. The aim was to assess algae growth rate, nutrient uptake and lipid production using anaerobic digestion centrate from HFC AWTP and the Lakeland WTS.

## Experimental methods
### Inoculum collection and scale-up
Wild-type algae were harvested from a secondary clarifier at the HFC AWTP in Tampa, Florida. Samples were transferred to 1-L flasks and bubbled with 2% $CO_2$ in air during an 18-hr light/dark cycle under artificial light

conditions of 310 µmol m$^{-2}$ sec$^{-1}$. Anaerobic digestion sludge centrate from the same facility was used as the scale-up medium after removal of suspended matter with a filter cloth. There were no nutrient additions to the centrate. Inoculum was grown until the culture biomass was 2 g dry wt L$^{-1}$ as determined by total suspended solid (TSS) analysis with 5 mL algae suspension according to the standard method [13]. University of Florida Environmental Biotechnology Laboratory analyzed samples and determined that the main algal species were *Chlorella* sp. and *Scenedesmus* sp.

## Photobioreactor setup and operation

The algae cultivation setup consisted of three tubular polyethylene photobioreactors (obtained from the Norwegian University of Life Sciences, Norway), which were housed in a greenhouse at the Botanical Gardens of the University of South Florida in Tampa, Florida. Figure 1 shows the setup of the photobioreactors, which began operation in February 2011. The reactors were 237.13 cm high with a diameter of 12.32 cm. They were each operated at a volume of 7 liters. Air containing 2% $CO_2$ was bubbled through the reactor using coarse bubble diffusers to provide inorganic carbon for photoautotrophic growth, as well as mixing. The gas flow rate was maintained at 0.5 L min$^{-1}$. The

reactors were operated on a semi-continuous basis with a mean cell retention time of 7 days.

Each day, 1 L of the reactor volume was replaced with centrate collected from the HFC AWTP. The nutrient content of the centrate was analyzed prior to feeding the reactors. A data-logger (Onset® HOBO U12) was used to record irradiance, ambient temperature, culture temperature and relative humidity every 15 minutes.

A 1-L batch reactor was also operated with wetland water from the City of Lakeland WTS. The WTS contained a native population of algae, whose diversity was previously analyzed by GreenWater CyanoLab (Palatka, FL) and shown to include *Bacillariophyta*, *Chlorophyta* and *Cyanobacteria* groups. Air with 2% $CO_2$ was fed to the reactor in like manner as the plastic reactors. A low-nutrient media was maintained by semi-continuous addition of 50 mL of 22.5 mg L$^{-1}$ $K_2HPO_4$ and 60.71 $NaNO_3$ to the batch reactor. The batch was operated for 3 weeks. Similar nutrient analyses were performed as previously described. All nutrients used in the study were obtained from Sigma Aldrich (St. Louis, MO).

## Pretreatment and wastewater characterization

Anaerobic digestion sludge centrate was collected once weekly and filtered with a fabric to remove coarse bio-solids. Total nitrogen (TN), ammonia and total
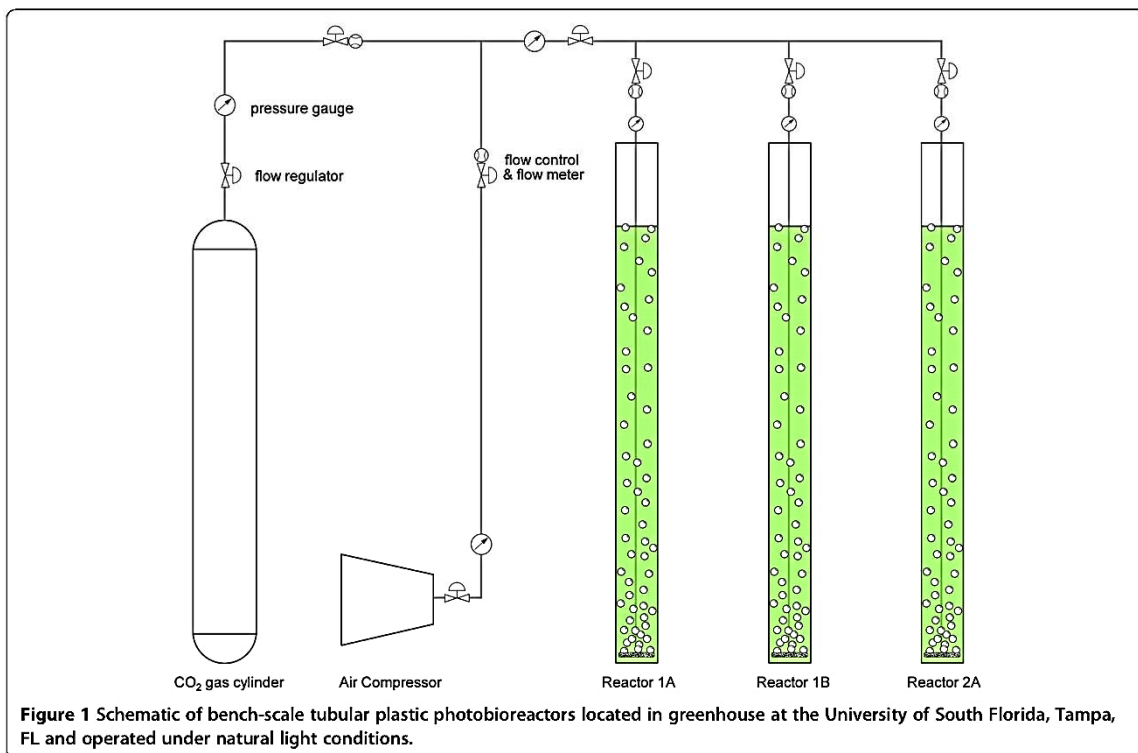


**Figure 1** Schematic of bench-scale tubular plastic photobioreactors located in greenhouse at the University of South Florida, Tampa, FL and operated under natural light conditions.

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phosphorous (TP) content were determined. To avoid death of the culture when the centrate nutrient content was very low, the TN concentration in the feed was maintained between 200–250 mg $L^{-1}$ by addition of $(NH4)_2SO_4$. Table 1 provides details of the nutrient content of the centrate.

*Biomass and nutrient monitoring*
Measurements of TSS and pH were performed daily. Nutrient removal analyses were performed every week for TN, ammonia ($NH_3$), nitrate ($NO_3^-$), TP and chemical oxygen demand (COD) according to Standard Methods [17]. TSS was determined by filtering a 5-mL algae suspension followed by drying in an oven for 24 hours.

*Lipid content analysis*
The algal lipid content was determined according to the method by Bligh and Dryer [18]. A sample of algae suspension was centrifuged at 3,800 rpm for 10 minutes to obtain a concentrated algae paste. The dry weight ($w_d$) of the paste was determined gravimetrically after drying at 60°C. A 2-mL sample of algae solution was mixed with 4 mL of a 2:1 methanol/chloroform solution in a glass vessel. The suspension was left for 24 hours. Thereafter, 1 mL of chloroform was added and the solution was mixed on a vortex for 1 min. 2 mL of water was then added and the mixture was again agitated for 2 min. The layers were separated by centrifugation at 2,000 rpm for 10 min. The lower layer was extracted with a glass syringe and filtered through a Whatman no. 1 filter into a previously weighed glass vessel ($w_1$). The solvent was dried in a water bath at 98°C and the vessel was weighed again ($w_2$) to obtain the lipid content of the sample as;

$$lipid\ content = \frac{W_2 - W_1}{W_d} x 100\% \tag{1}$$

## Results
### Light conditions
The experiment was conducted in the summer from May 7, 2011 to September 30, 2011 in greenhouse conditions at the University of South Florida, Tampa, Florida. An evaporative cooling system kept peak daily ambient temperatures in the greenhouse below 40°C. Figure 2 shows

**Table 1 Nutrient content of centrate used as growth media for mixed algae species**

| Parameter | Range |
|---|---|
| Total nitrogen | 200 – 250 mg $L^{-1}$ |
| Total phosphorous | 2 – 75 mg $L^{-1}$ |
| Ammonia | 100 – 250 mg $L^{-1}$ |

the instantaneous PAR and daily integrated insolation for the period of cultivation. Daily insolation was highest in the early summer months (May-July) averaging 12 mol-photons $m^{-2}$ $d^{-1}$. During the latter period of cultivation (August-September), mean daily insolation fell to 10 mol-photons $m^{-2}$ $d^{-1}$. Daily peak instantaneous PAR was *ca.* 600 μmol-photons $m^{-2}$ $s^{-1}$.

### Temperature and pH
Culture temperature for the duration of the experiment is shown in Figure 3. Mean culture temperature was 29.2°C. Peak daily culture temperatures remained mostly below 40°C. Diurnal temperature changes were on average 13°C for the period of cultivation. Changes in pH are shown in Figure 4 and were more variable ranging between 6 and 9. There was an excursion of pH above 9 from days 20 to 30.

### Biomass development and production rates
Microalgae biomass development in the photobioreactors is shown in Figure 5. Standing biomass concentration during the first 80 days of operation was 0.75 g dry wt $L^{-1}$. Air diffusers were replaced on day 80 and resulted in improved mixing and an associated doubling in the standing biomass. Steady state dry biomass concentration remained below 2 g dry wt $L^{-1}$. Photobioreactor areal production $P$ (g dry wt $m^{-2}$ $d^{-1}$) was calculated based on illuminated surface area $A$ ($m^{-2}$) according to equation 2.

$$P = \frac{QC}{A} \tag{2}$$

where Q is the daily flow rate (L $d^{-1}$) and C the algae biomass concentration (g dry wt $L^{-1}$). The mean production rate for the first 80 days was 2.5 g dry wt $m^{-2}$ $d^{-1}$, which increased to 4.5 g dry wt $m^{-2}$ $d^{-1}$ for the last 45 days. The maximum sustained production rate was 7 g dry wt $m^{-2}$ $d^{-1}$ for one week. The areal productivity of the batch culture with Lakeland WTS algae was approximately 0.5 g dry wt $m^{-2}$ $d^{-1}$ (data not shown). For the tubular reactors, the most active growth period occurred from days 87–100, after the diffusers were replaced.

### Nutrient uptake
The fraction of nutrients taken up is illustrated in Figure 6. Nutrient uptake was determined from the difference between filtered and unfiltered samples. The latter was diluted before analysis. TN uptake was just below 60%, while 72% ammonia was taken up. Phosphorous removal was greater than 85%.
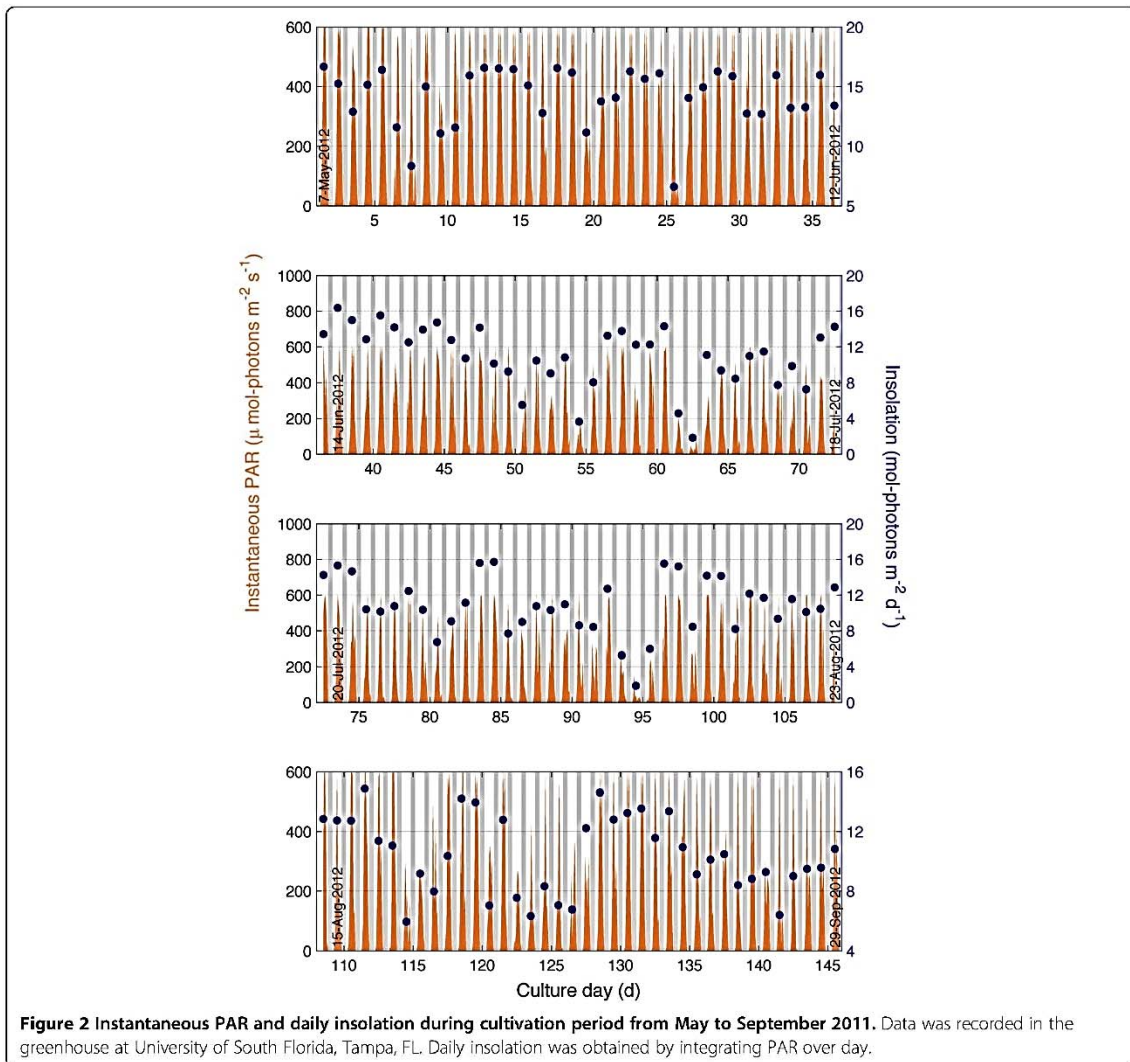
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**Figure 2 Instantaneous PAR and daily insolation during cultivation period from May to September 2011.** Data was recorded in the greenhouse at University of South Florida, Tampa, FL. Daily insolation was obtained by integrating PAR over day.

### Lipid content

Algae grown on the high strength centrate had very low lipid content (<10%) compared to the 65% lipid content of Lakeland WTS algae consortium.

### Discussion

#### Algae biomass production potential from wastewater resources

This study was conducted to assess the potential of cultivating algae using wastewater as a nutrient medium. The consortium of algal species, including *Scenedesmus* sp. and *Chlorella* sp. grew favorably on anaerobic sludge centrate from the HFC AWTP. There was relatively high nutrient uptake for phosphorous and ammonia. Total nitrogen uptake was much lower because organic nitrogen

was most likely not assimilated by the culture. The mean productivity obtained for the entire cultivation period was 3.3 ±1.5 g dry wt m$^{-2}$ d$^{-1}$. These results are similar to Woertz et al. [19] who report an algae production rate of 3 g dry wt m$^{-2}$ d$^{-1}$ for *Chlorella* sp. grown on wastewater. Li et al. [20] report a biomass production rate of 13 g dry wt m$^{-2}$ d$^{-1}$ for algae grown on centrate. Their results showed that by the end of a 14-day batch culture 94% ammonia, 89% TN and 81% TP was removed. Their system was continuously operated at 50% daily harvesting rate, compared to 14% used in this study. Zhou et al. [21] also grew algae on full strength anaerobic sludge centrate and obtained a biomass production rate of 12.8 g dry wt m$^{-2}$ d$^{-1}$. The lipid content reported by Li et al. [20] was ca. 11%, similar to these results. This is a

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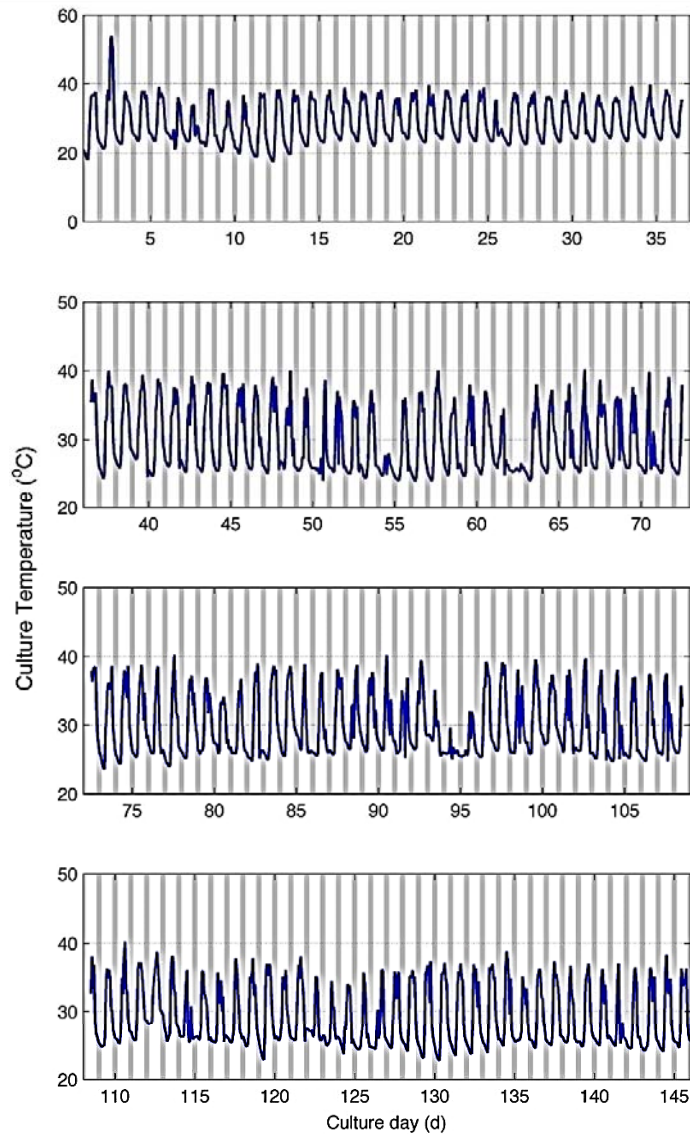
**Figure 3** Culture temperature profile recorded from May-September 2011 during cultivation of wild algae grown on anaerobic sludge centrate in Tampa, FL.

downside of growing algae, especially *Chlorella* sp., in high strength nitrogen media. The caloric content which is linked to lipid production is significantly reduced [22]. In general, high lipid content is achieved when the organisms are "starved" of nitrogen [4,22,23].

### Potential application to large-scale algal production
Photobioreactor optimization can potentially increase biomass production, as observed from improving only air bubbling in this study. Improved air delivery was achieved by changing from spherical to cylindrical ceramic diffusers, resulting in better mixing. Work by Richmond [24], Richmond and Zou [25] and Qiang and Richmond [26] indicates that highly productive and efficient enclosed algal systems can be obtained by optimizing cell density and mixing rate in relation to photon flux density, particularly when nutrients are not limited. In addition, better aeration promotes increased mass transfer allowing for the removal of oxygen, which can become inhibiting at high concentrations [19].

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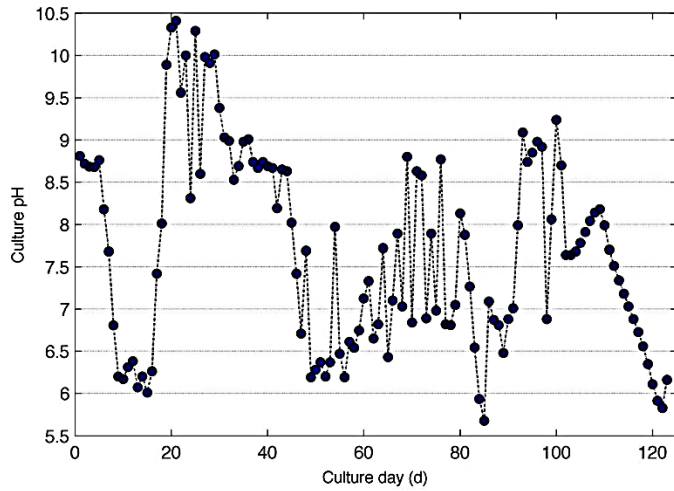
**Figure 4 Daily culture pH for algae grown on anaerobic sledge centrate from the Howard F.** Current Advanced Wastewater Treatment Plant, Tampa, FL. pH measurement was taken once a day and therefore do not capture any diurnal variations.

However, there are limits to the photosynthetic conversion of sunlight energy into algal biomass in large-scale outdoor cultures. Under light-limited growth, there is an upper limit for light conversion efficiency of a large-scale culture. In practice, this usually translates to a maximum potential yield of 30–40 g dry wt $m^{-2}$ $day^{-1}$ under ideal outdoor sunlight conditions for short periods and considerably less for longer durations. This indicates that the non-optimized operation in this preliminary assessment was able to achieve 10% of the maximum.



**Figure 5 Growth (top) and areal biomass productivity (bottom) of mixed algal species grown on anaerobic sludge centrate for the period May to September 2011 in Tampa, FL.** Productivity calculations are based on reactor illuminated surface area.

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**Figure 6** Fraction of nutrients taken up by mixed algae species grown in enclosed photobioreactor from centrate medium. Nutrient uptake was determined from the difference between filtered and unfiltered samples.

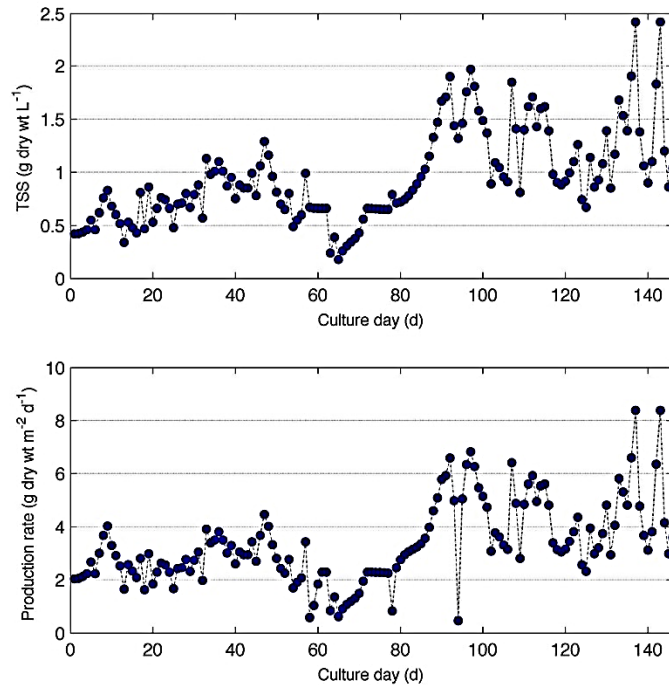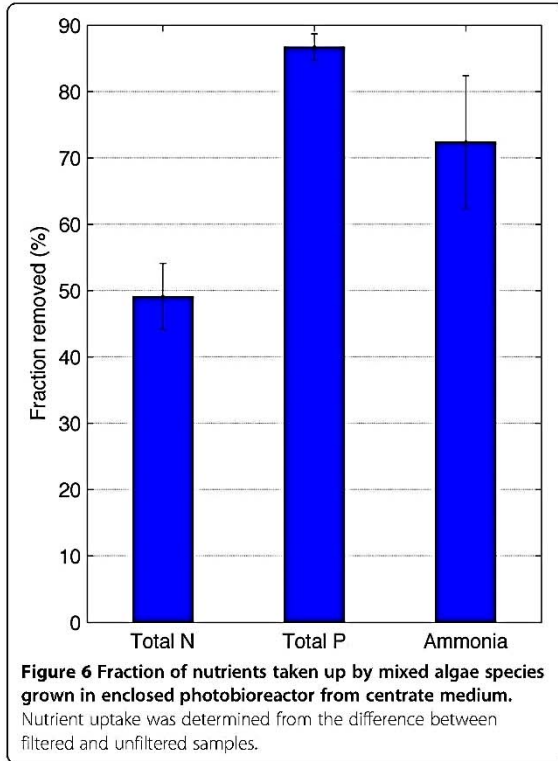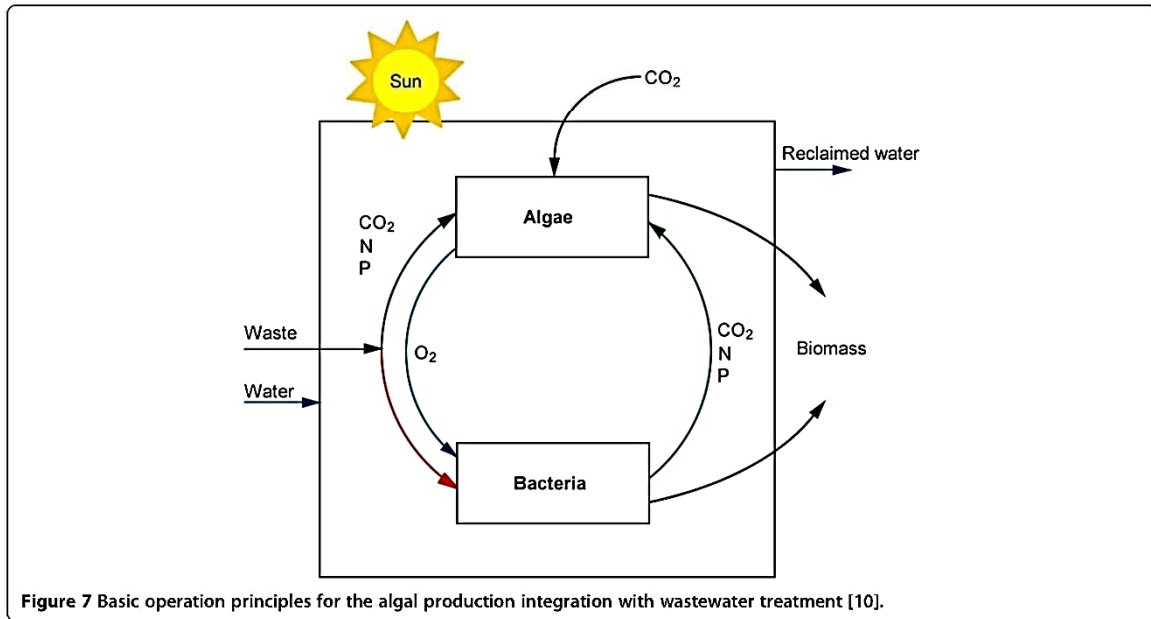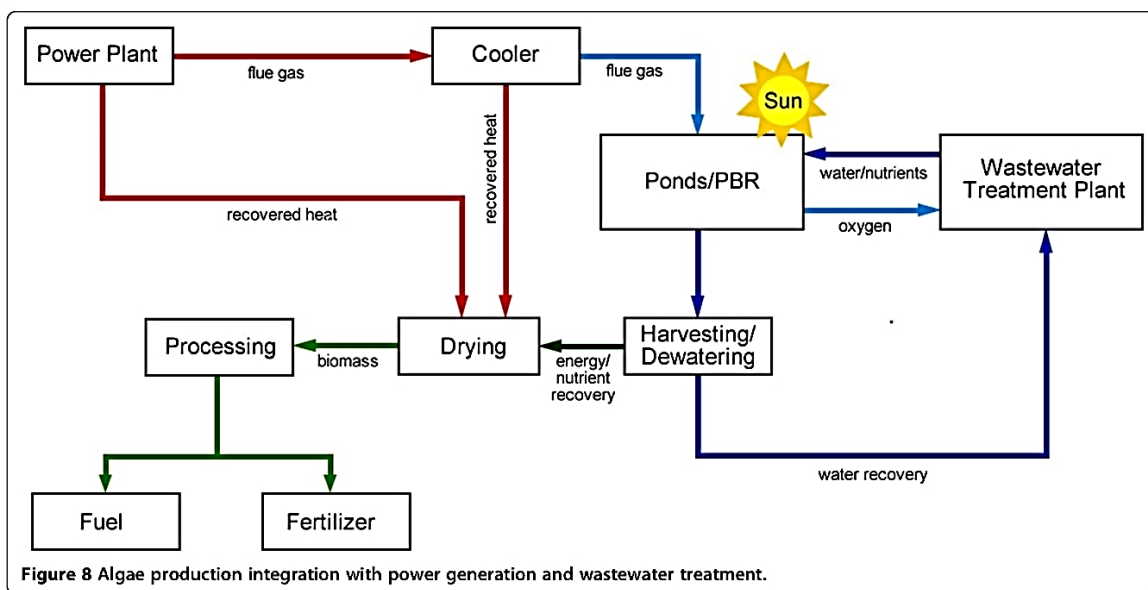However, the cultures were grown under conditions of reduced light. It is possible to cultivate algae outdoor and improve light utilization through vertical reactor orientation, while keeping peak temperature down due to mutual shading of reactors [27].

Production in high rate algal ponds (HRAP) is possible and has shown commercial production rates as high as 40 g dry wt $m^{-2}$ $d^{-1}$ [28]. Craggs et al. [29] provide a good summary of production in HRAP. There is a wide variability of production rates achieved based on wastewater source, type, location and culture conditions. Algae growth in HRAPs has also been shown to achieve greater than 75% nutrient removal [30]. Production was shown to improve with $CO_2$ addition from 10.6 to 15.2 g dry wt $m^{-2}$ $d^{-1}$. Li et al. [20] and Zhout et al. [21] scaled up their wastewater-grown algal with 25-L BIO-COIL reactors and obtained net biomass productivity of 13 and 12.8 g dry wt $m^{-2}$ $d^{-1}$ respectively.

The basic principles and a schematic behind the operation for algal integration with wastewater facilities and power plants are shown in Figure 5 and Figures 7 and 8. While the challenges associated with algal harvesting, species control, and fuel conversion must be solved for large-scale production, the harvestable yields of algal biomass (g dry wt $m^{-2}$ $d^{-1}$) helps to determine the potential of algal systems for energy and fuel production. These yields depend largely on nutrient availability and lighting conditions. In this section, the nutrient removal efficiency and observed areal productivity for the bench-scale photobioreactors are used to determine the size of the algae production facility.



**Figure 7** Basic operation principles for the algal production integration with wastewater treatment [10].

**Figure 8** Algae production integration with power generation and wastewater treatment.

Microalgae biomass results mainly from photosynthesis, which utilizes inorganic compounds (including $CO_2$). In simple terms, algal biosynthesis can be described by the following chemical equations where ammonium and nitrate are the nitrogen sources respectively [31,32];

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^-$$
$$+ HPO_4^{2-} \xrightarrow{hv} C_{106}H_{263}O_{110}N_{16}P + 106O_2 \qquad (2)$$

$$16NO_3^- + 124CO_2 + 140H_2O$$
$$+ HPO_4^{2-} \xrightarrow{hv} C_{106}H_{263}O_{110}N_{16}P + 138O_2$$
$$+ 18HCO_3^- \qquad (3)$$
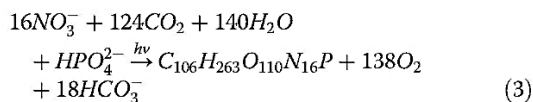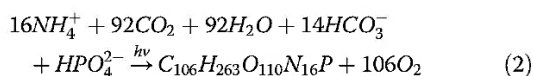
In the above equations, the chemical formula $C_{106}H_{263}O_{110}N_{16}$ represents algal biomass [32]. According to the stoichiometry, 1 g of ammonia-nitrogen ($NH_3$-N) or nitrate-nitrogen ($NO_3^- - N$) produces about 15.8 g of biomass and consumes 18.1 and 24.34 g of $CO_2$ in the process, respectively. In addition to nutrient availability, algal biomass production also depends on light energy ($hv$). In the absence of nutrient limitation, photosynthesis increases with increasing irradiance until the maximum algal growth rate is attained as described my

Michaelis-Menten kinetics [24-26]. A condition known as photoinhibition can occur when the irradiance is increased beyond the saturation point resulting in damage to algal light receptors and a decrease in the photosynthetic rate and productivity [24,25].

The total amount of algal biomass produced may be estimated by considering the total flows of nitrogen. Nitrogen is assumed to be the limiting nutrient since phosphorous is generally considered to be an abundant nutrient in Tampa due to the numerous phosphate deposits. The annual production estimates for algal production based on the concentrations of nitrogen in wastewater from the HFC AWTP and the Lakeland WTS are shown in Table 2. These calculations include the average flow rate of water passing through each plant. The required area to facilitate production is estimated based on the observed productivity for algae grown on centrate and the Lakeland WTS water. The growth rate and lipid production for algae grown on wastewater with moderate nitrogen levels (~30 mg/L) were adopted from Woertz et al. [19] as 3 g dry wt m$^{-2}$ d$^{-1}$ and 30% lipids by dry weight respectively.

Algal production is restricted by available land close to the HFC AWTP. Approximately 200 hectares of suitable

**Table 2** Potential biomass production estimates for algae grown on wastewater nutrients in the Tampa Bay area, FL

| Description | Source | Flow rate (MGD) | Nitrogen (mg L$^{-1}$) | Algae biomass (tons yr$^{-1}$) | CO$_2$ consumed (tons yr$^{-1}$) | Indoor area (ha) | Outdoor area (ha) |
|---|---|---|---|---|---|---|---|
| Wastewater | HFC AWTP | 3.0 | 30 | 1,965 | 3,026 | 179 | 179 |
| Centrate | HFC AWTP | 0.5 | 427 | 4,660 | 7,179 | 182 | 80 |
| Wastewater | WTS | 5.0 | 10 | 1,091 | 1,681 | 598 | 598 |
| **Total** | | | | **7,716** | **11,889** | **959** | **857** |

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**Table 3 Annual biofuel production estimates derived from algae growth in wastewater nutrients in the Tampa Bay area, FL**

| Description | Source | Algae biomass (tons yr⁻¹) | Biofuel (gal yr⁻¹) | Total revenue (US$ yr⁻¹)[1] |
|---|---|---|---|---|
| Wastewater | HFC AWTP | 1,965 | 112,833 | 451,332 |
| Wastewater | WTS | 1,091 | 156,712 | 626,848 |
| **Total** | | **3,056** | **269,545** | **1,078,180** |

[1]Biodiesel cost $4.05 per gal taken from the Center for Agricultural and Rural Development (CARD), http://www.card.iastate.edu/research/bio/tools/hist_bio_gm. aspx, Iowa State University, Ames, Iowa.

land area is available onsite and is located within close vicinity to where centrate is generated. Therefore, the flow rate has been chosen to reflect the land restriction for the indoor production. It is assumed that algae grown on moderate and low strength nutrient are nutrient limited and hence, their productivities are not affected by increasing light beyond a certain value. However, for algae grown on high strength centrate, the outdoor production area can be reduced since the algae are not nutrient limited.
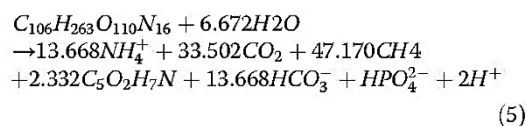
### Energy production and revenue potential
#### Liquid biofuels
For biofuel production, algae need to have a lipid content exceeding 20% [10], some researchers even suggest 40% [33]. This means that high strength wastewater would not be suitable for cultivating algae for lipid production. Realistically, the best algae for lipid production are those from the Lakeland WTS or algae grown on low strength wastewater. Usable lipids were assumed to be 20% and 50% of the algae dry wt. for moderate strength wastewater and low strength pond water, respectively. An algal oil-to-biofuel conversion efficiency of 80% was used, which is similar to that obtained for vegetable oil [34]. The biofuel potential for the various algae are shown in Table 3. The total potential volume of biofuel obtained is approximately 269,545, which can, on average, fuel 450 cars per year (assuming 15,000 miles yr⁻¹ with an average of 25 miles per gallon).

#### Biogas generation
Algae biomass may be anaerobically digested to produce methane, especially biomass which may be considered unsuitable for liquid biofuel production due to low lipid content. The stoichiometric relationships for this process are illustrated in Equation (5), which were developed from half reactions assuming that ammonia is the

nitrogen source [35]. The fraction of electrons towards energy production ($f_e$) was estimated to be 0.89 based on the work by Yuan et al. [36].

$$C_{106}H_{263}O_{110}N_{16} + 6.672H2O$$
$$\rightarrow 13.668NH_4^+ + 33.502CO_2 + 47.170CH4$$
$$+2.332C_5O_2H_7N + 13.668HCO_3^- + HPO_4^{2-} + 2H^+$$

$$(5)$$

According to equation 5, 1 mole of algae biomass produces 47.17 moles of methane. However, previous research has shown that algal biomass is not particularly easy to digest having a biogas yield of 29.5% [36,37]. Therefore, 1 g of algae dry wt. is estimated to generate 62.7 mg methane. The estimated production of biogas and the derived energy are shown in Table 4 assuming that the energy content of methane is 55 MJ kg⁻¹ for the HFC AWTP.

The above calculations assumed that the total production of algae goes toward digestion. It is also possible to extract lipids and attempt to derive biogas from spent biomass. The combination of algae production on the wastewater nutrient sources shows the potential for energy generation that can power close to 500 homes.

### Conclusions
This work shows that there are important benefits to be derived from integrating algal production systems with nutrient-rich waste streams. The feedstock potential of the HFC AWTP and the Lakeland WTS is estimated to be approximately 71 tons ha⁻¹ yr⁻¹ of algal biomass, 270,000 gal hr⁻¹ of liquid biofuel, and 415,000 kg yr⁻¹ of methane. Renewable energy derived from algae will play a significant role in providing energy security while important services such as water treatment can be synergistically achieved by these systems. Even though the

**Table 4 Biogas production estimates for anaerobic digestion of algae biomass grown on wastewater nutrients**

| Description | Source | Algae biomass (tons yr⁻¹) | Biogas production (kg yr⁻¹) | Total energy (MJ yr⁻¹) | Households powered[1] |
|---|---|---|---|---|---|
| Wastewater | HFC AWTP | 1,965 | 123,215 | 6,776,838 | 144 |
| Centrate | HFC AWTP | 4,660 | 292,294 | 16,076,165 | 342 |
| **Total** | | **6,625** | **415,509** | **22,853,003** | **486** |

[1] Assuming average energy consumption of 653 kWh per month and thermal efficiency of natural gas turbine of 60% with waste heat recovery.

analysis has been preliminary, it shows that there is good potential for algal feedstock production in the Tampa Bay area. However, there are many important factors to be considered to assess whether algal production systems would be competitive. These include analysis of energy and cost associated with harvesting and extraction for example. It is hoped that with further research many of these challenges can be overcome.

## Competing interests
The authors declare that they have no competing interests.

## Authors' contributions
OKD, TH, IU and BG designed, constructed and operated the photobioreactors and carried out all algae and nutrient analysis related to the centrate studies OKD and TH conducted the studies based on the Lakeland algae samples, carried out lipid analysis and drafted the manuscript. SE, JW and QZ were co-PIs on this project and advised the research assistants on this project. All authors read and approved the final manuscript.

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# APPENDIX C: HIGH SCHOOL (HS) EXPERIMENTAL DETAILS

Table C.1: Feed composition used HS students in experiments

| Inoculation date | Experiment | Treatment # | $NO_3^-$ -N (mg/L) | $NH_4^+$- N (mg/L) | N form and contribution |
|---|---|---|---|---|---|
| 09.21.12 | 1 | 1 | 750 | 0 | 100%$NO_3^-$N, 0%$NH_4^+$-N |
| | | 2 | 565 | 183 | 75% $NO_3^-$N, 25%$NH_4^+$-N |
| | | 3 | 375 | 375 | 50% $NO_3^-$N, 50%$NH_4^+$-N |
| | | 4 | 0 | 750 | 0%$NO_3^-$N, 100%$NH_4^+$-N |
| 09.25.12 | 2 | 5 | 343 | 0 | 100%$NO_3^-$N, 0%$NH_4^+$-N |
| | | 6 | 438 | 146 | 75% $NO_3^-$N, 25%$NH_4^+$-N |
| | | 7 | 299 | 299 | 50% $NO_3^-$N, 50%$NH_4^+$-N |
| | | 8 | 0 | 890 | 0%$NO_3^-$N, 100%$NH_4^+$-N |
| 09.27.12 | 3 | 9 | 517 | 0 | 100%$NO_3^-$N, 0%$NH_4^+$-N |
| | | 10 | 450 | 150 | 75% $NO_3^-$N, 25%$NH_4^+$-N |
| | | 11 | 395 | 395 | 50% $NO_3^-$N, 50%$NH_4^+$-N |
| | | 12 | 0 | 557 | 0%$NO_3^-$N, 100%$NH_4^+$-N |
| 10.01.12 | 4 | 13 | 420 | 0 | 100%$NO_3^-$N, 0%$NH_4^+$-N |
| | | 14 | 188 | 62 | 75% $NO_3^-$N, 25%$NH_4^+$-N |
| | | 15 | 395 | 395 | 50% $NO_3^-$N, 50%$NH_4^+$-N |
| | | 16 | 0 | 650 | 0%$NO_3^-$N, 100%$NH_4^+$-N |

# APPENDIX D: PRELIMINARY CULTIVATIONS AND FLUOROSPECTROSCOPY EXPERIMENTS CONDUCTED AT UNIVERSITY OF FLORIDA (UF)

## D.1 Aim

To grow the indigenous algae on municipal centrate and monitor fluorescence at 490 and 680nm

The initial set-up and growth considerations for the indigenous algae are described in Section 3.2.1 before the culture was shipped to University of Florida, BEST Algae Lab. BEST Algae Lab received samples on July 3rd, 2012. Below shows a summary of the methods and preliminary results.

## D.2 Methods

The methods were divided into three sections: 1) cultivation conditions, 2) observation of algae growth and lipid production by fluorospectrocopy and microscopy.

### D.2.1 Cultivation Conditions

Algae (USF-2012.7) were cultivated in a 250ml Erlenmeyer flask using autoclaved centrate (from USF) as the growth medium at an inoculation of 10% (v/v). Sparging with 0.45μm-filtered air provided mixing. Algae were illuminated by 300μmol photons/$m^2$/s provided by full spectrum fluorescent lights (T5 Plantmax™) on a 20: 4 (light: dark cycle). Initial pH of the culture was 9.15.

### D.2.2 Observation of Algae Growth and Lipid Production by Fluorospectrocopy

Algae growth was monitored by in-vivo chlorophyll fluorescence at 490/680nm (excitation/emission) on a Nanodrop fluorospectrometer (ND 3300, Thermo Scientific). Staining

algae aliquots with 2% (v/v) Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one, MP

Biomedicals, LLC., Solon, OH) dissolved in acetone (250μg/ml) was used to qualitatively

monitor lipid production over time.  Fluorescence values of stained algae were measured on a

Nanodrop fluorospectrometer at 490/585nm (ex/em).

*D.2.3 Microscopy and Photography*

Photographs of cells were taken at initiation of experiment (T0) and on the last day of the

experiment (T168).  Cells were stained with Nile Red for lipid observation as described

previously.  Samples were centrifuged to a cell paste at 15,000rpm for 10sec (Eppendorf 5414

Hamburg, Germany). The resultant cell paste was mounted on a glass microscope slide and

viewed under a Nikon Labophot (Nikon Corporation Tokyo, Japan) equipped with epi-

fluorescent illumination, 50w mercury halide illuminator and a 490nm excitation and 520nm

long pass emission filter. Images were taken with a Spot Insight color mosaic digital camera

(Diagnostic Instruments Inc., Sterling Heights, MI).  Nile Red fluoresces yellow under

hydrophobic conditions (within oil droplets), red auto-fluorescence of chlorophyll was observed.

**D.3 Preliminary Results**

Growth of algae culture USF-2012.7 peaked at 48 hours with chlorophyll auto-fluorescence

measured at 61,300 RFU 680nm.  The culture slowly declined in chlorophyll auto-fluorescence

from 48 to 144 hours and then began a rapid decline at 168 hours.  Lipids followed an inversed

time course when compared to chlorophyll auto-fluorescence, initially low (258 RFU 585nm)

but began to rise at 48h, plateaued from 96 to 144 hours and then increased dramatically at 168

hours.  Initial (Figure D.2) and final (Figures D.3 and D.4) photographs show the dramatic

change in the culture state from chlorophyll auto-fluorescence (red) to Nile Red-stained lipid fluorescence (yellow), dominated by *Chlorella*.
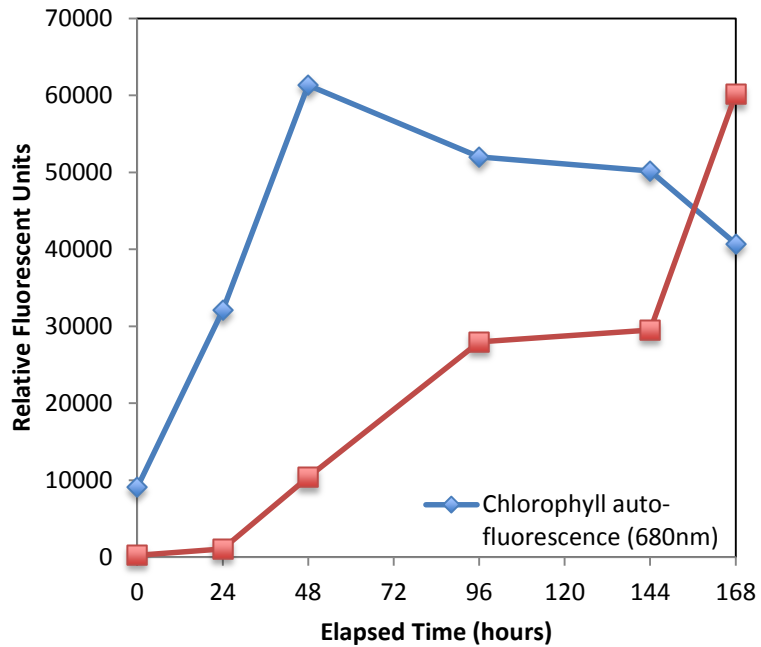


Figure D.1:  Time course of algal growth (USF-2012.7) showing chlorophyll auto-fluorescence (680nm) and lipid content after staining with Nile Red (585nm).
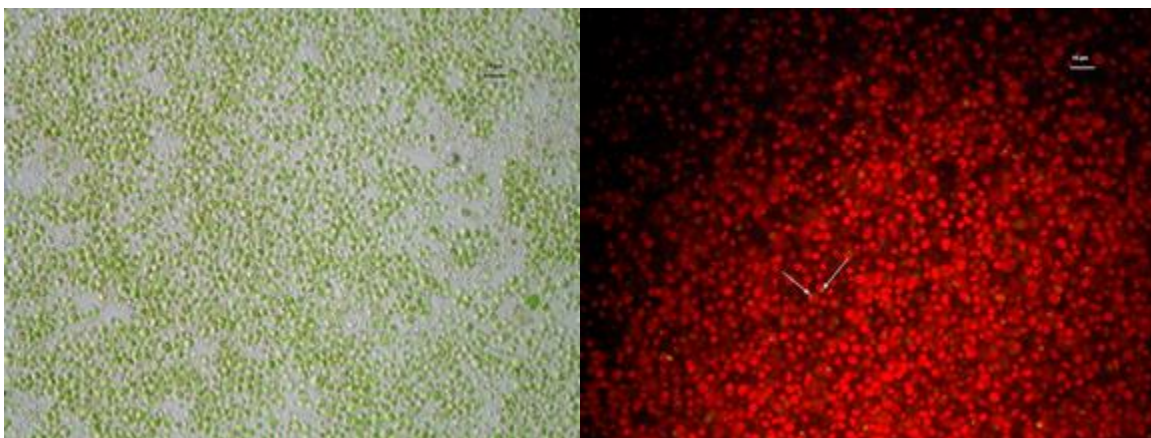


Figure D.2: Algae at T-0hour, a): brightfield illumination and *b)*: epi-fluorescent illumination stained with Nile Red, arrows indicate lipid droplets (yellow), red is chlorophyll autofluorescence.
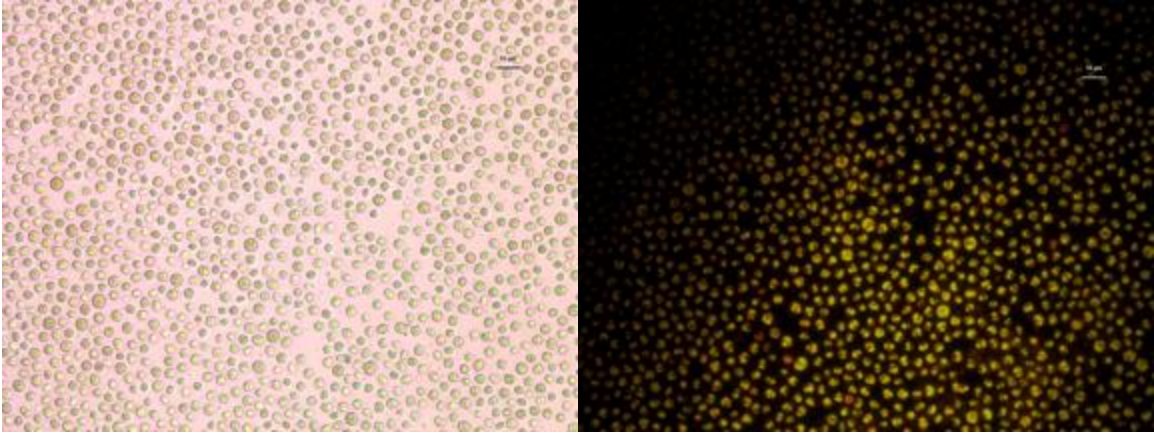
Figure D.3: Algae at T-168hour, *a)*: brightfield illumination and *b)*: epi-fluorescent illumination stained with Nile Red, lipid droplets throughout (yellow), red is chlorophyll autofluorescence.



Figure D.4: Algae at T-168hour under higher magnification (1250x), *a)*: brightfield illumination and *b)*: epi-fluorescent illumination stained with Nile Red, lipid droplets throughout (yellow), red is chlorophyll autofluorescence.
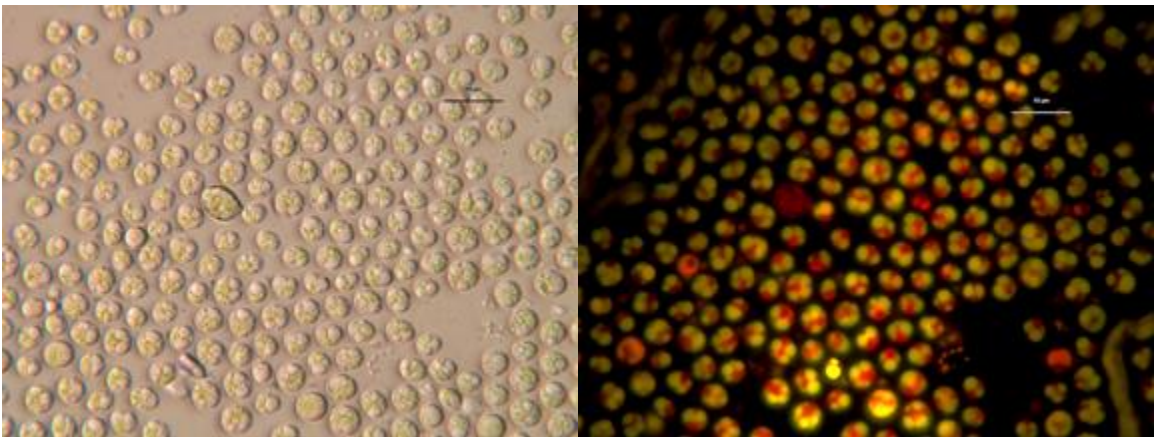
# APPENDIX E: SUMMARY OF THE OPERATING CONDITIONS FOR THE THREE PHASES

Table E.1: Operating conditions for all three phases

| Operating Condition | Phase | | |
|---|---|---|---|
| | I<br>Pilot scale experiments | II<br>High school experiments | III<br>Bench-scale experiments |
| Reactor design | Tubular reactors. Each reactor was 237.23cm in height and had a diameter of 12.32cm. | 3-L cylindrical plastic soda bottles. The diameter was 12.7cm. | 1-L pyrex glass cylinders with a outer diameter of 4.12cm and an operational height of 32.0cm. |
| Inoculum | indigenous consortium | Indigenous consortium | *Chlorella* and *Scenedesmus* monocultures and indigenous algae consortium |
| Feed description | Two reactors with different feeds were used. One received 100% centrate, whereas the second reactor received an aquaculture-centrate mixture (ACM) of 50% TN adjusted centrate and 50% synthetic aquaculture wastewater. The TN concentration in the centrate was adjusted to 200-300 mg/L, as needed, by addition of $(NH_4)_2SO_4$. The synthetic aquaculture wastewater contained 200 mg/L $NO_3$-N ($KNO_3$) and 25 mg/L TP ($KH_2PO4$). | Reactors received varying concentrations of aquaculture and swine waste mixtures | RAS wastewater from a Tilapia unit. |
| Operating conditions | Semi-continuous continuously mixed flow reactors (CMFRs). | Batch reactor. | Batch reactor. |
| Mean cell residence time (MCRT) or growth period | 7 days | 11 days | 3 days |
| Gas flow rate and partial pressure | All reactors had a flow rate of 500mL/ min. One reactor had Both 2% with centrate feed and ACM received a 2% mix of $CO_2$ and air. The other received 5% $CO_2$. | 500mL/ min and ambient air. | 1% $CO_2$. |
| Temperature | The temperature ranged from 25-32ºC in the greenhouse. | Unknown. | 25ºC temperature control room. |
| Light intensity and duration conditions | Natural lighting (Figures 4-5). | Natural lighting. | 130μmol/m²/ sec light intensity. |

## APPENDIX F: POEM PERFORMED AT AN OPEN MIC RECITAL IN OSLO, NORWAY

This video can also be viewed on youtube:

 https://www.youtube.com/watch?v=nPuWXZu8CSw.

I am married or so it says on facebook.

Apparently that makes it official.

My husband may be invisible to most,

But I see him daily.

You ask, "Trina, who is *Chlorella vulgaris*? Who really has a name like that?"

*Chlorella vulgaris* is my amazing superhero.

He is very microscopic but like Mighty Mouse can achieve great things.

He is an algae species. He tends to be very introverted but if you listen closely he would tell you,

" I am trying to save the world by providing a source of clean, renewable fuel. I could save the

world for your kids. All I need is sunlight, nutrients from wastewater, carbon dioxide and my

wife to talk to me sometimes."

While other wives go home and worry about cooking dinner, my husband loves the left overs

and waste.

He even grows exponentially using toilet water.

He is amazing and is able to clean the most toxic industrial gases.

And is the only one who can produce petroleum-based substitutes.

You may see an environmentalist or a hippie;

But I see a person who believes that environmental degradation is self-destructive and an injustice to all mankind.

Many think that climate change is a hoax or natural phenomenon, but I say, "There is no wisdom in acting like there is no tomorrow."

What if climate change is catapulted by human activities and we are ensuring that our children will not have enough food, clean water or are homeless,

Will we laugh?

Or cry?

The bees are pivotal for the sustenance of life. They are dying, yet we pay no mind.

Will we have food to feed the 7 billion?

Will Pakistan be the next Waterworld? I don't think Kevin Costner lives there.

Is there relief? Is there hope?

We either remediate or adapt.

 Remediating or reducing greenhouse gas emissions requires us to change lifestyles. Are we willing to compromise?

Do we understand the interconnectedness of human behavior and the web of life.

Gaia!

**APPENDIX G: EXPERIMENTS EXAMINING THE EFFECT OF ALGAL DIVERSITY**

The experimental procedure and protocols for these experiment was the same as described in

Chapter 4. These experiments considered the effects of polycultures (more than one species of

algae present in a culture). The experimental design consideration and results are shown below.

Table G.1: Experimental design showing inoculation (# of cells)

| Treatment description | Starting cell # of *Scenedesmus* | Starting cell # of *Chlorella* | Starting cell # of indigenous culture | Total cell count (#/ mL) |
|---|---|---|---|---|
| High cell density of *Scenedesmus* and *Chlorella* | $1.22*10^6$ | $1.20E*10^6$ | - | $2.42*10^6$ |
| High cell density of *Chlorell*a and indigenous cultures | - | $4.30*10^6$ | $5.44E*10^6$ | $9.74*10^6$ |
| High density *Scenedesmus* and indigenous cultures | $1.49*10^6$ | - | $1.44*10^6$ | $2.93E*10^6$ |
| Low density *Chlorella* and indigenous cultures | - | $1.20*10^6$ | $1.15*10^6$ | $2.35*10^6$ |
| Low density *Scenedesmus* and indigenous cultures | $1.22*10^6$ | - | $1.15*10^6$ | $2.37*10^6$ |
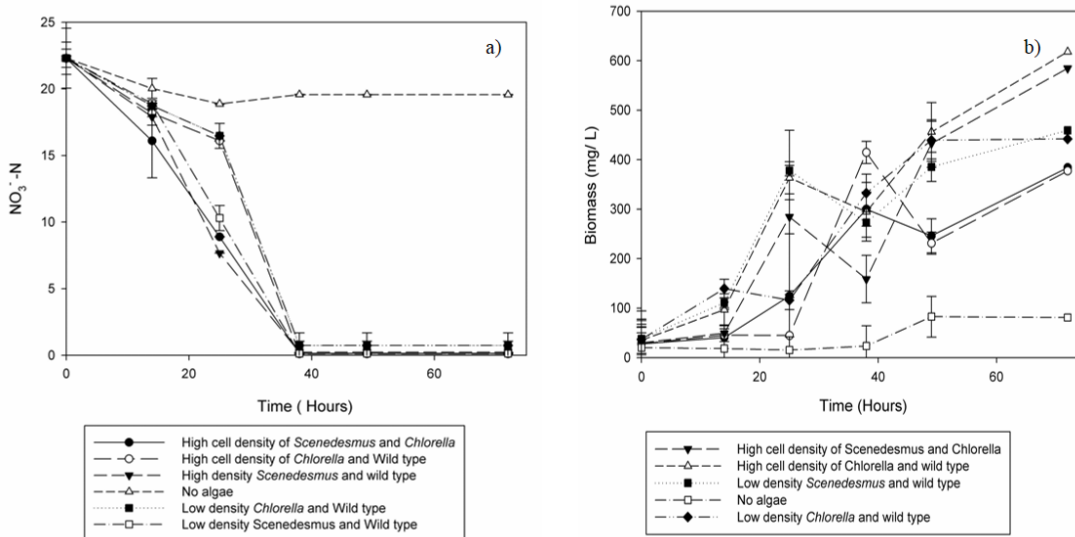
Figure G.1: a) Nitrate removal (mg/L) and b) biomass production of polycultures (mg/L)
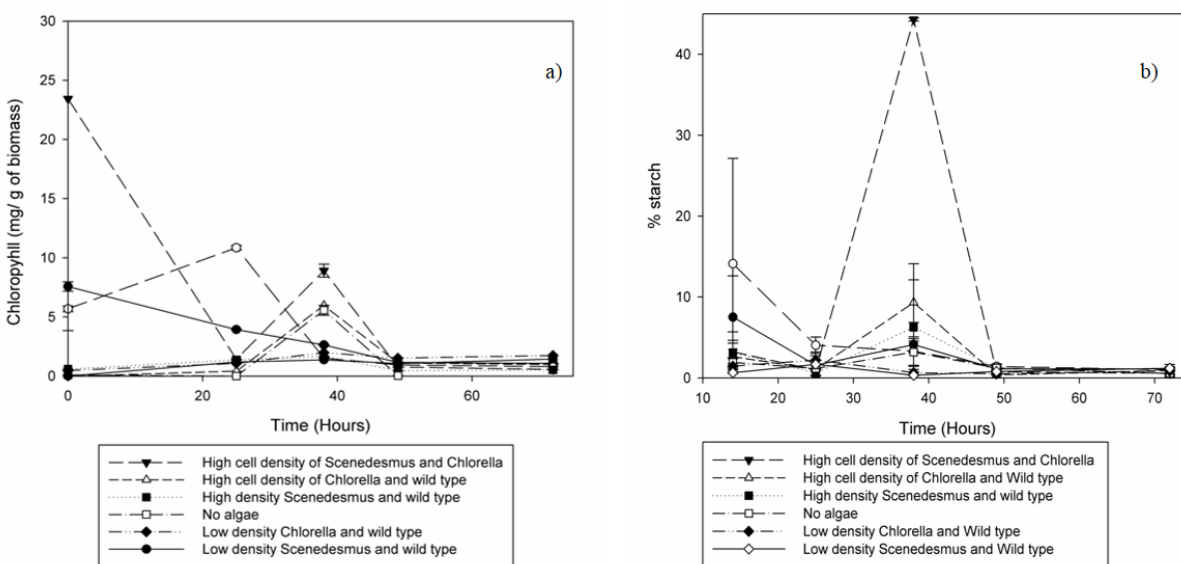


Figure G.2: a) Chlorophyll (mg/g) and b) Starch content (%)

Table G.2: Treatment and the final lipid content (%) after 72 hours

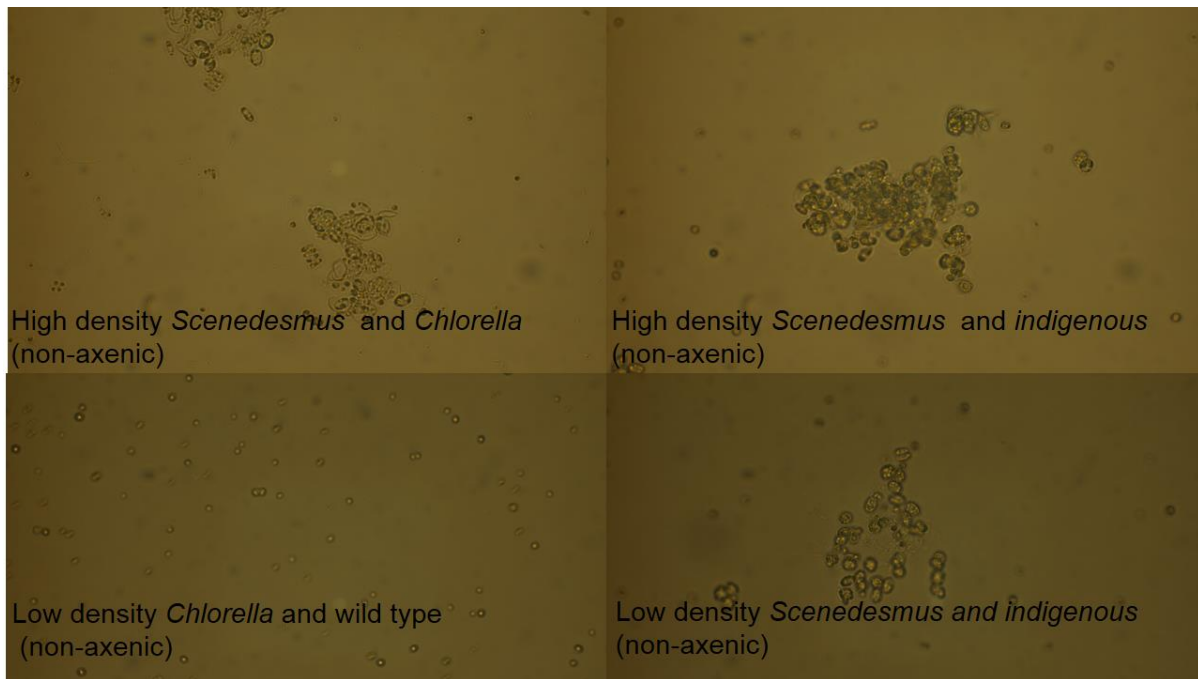| Treatment description | Lipid content (%) |
|---|---|
| High cell density of *Scenedesmus* and *Chlorella* | 44.0 |
| High cell density of *Chlorella* and indigenous cultures | 64.2± 10.6 |
| High density *Scenedesmus* and indigenous cultures | 81.5± 4.9 |
| Low density *Chlorella* and Wild type | 61.2± 0.6 |
| Low density *Scenedesmus* and Wild type | 42.6 |



Figure G.3: Microscope images (100 x magnification) of polycultures with high and low initial cell density.

Table G.3: Summary of irradiance parameters and determination of photoefficiency

| | |
|---|---|
| Daily light (mols/m$^2$/day) | 11.23 |
| # of days | 3 |
| Surface area (m$^2$) | 0.086 |
| Total light dose for reactor (mol/day) | 2.90 |
| Biomass concentration (mg/L) | 0.69 |
| Photoefficiency | 0.24 |

## APPENDIX H: TILAPIA FEED USED AT UMB ON-CAMPUS FACILITY

Table H.1: Composition of major constituents in feed (Aller 37/10 FLOAT)

|  | 2mm | 3mm | 4.5mm | 6mm |
|---|---|---|---|---|
| Crude protein (%) | 37 | 37 | 37 | 37 |
| Crude fat (%) | 10 | 10 | 10 | 10 |
| NFE (%) | 36.7 | 36.7 | 36.7 | 36.7 |
| Ash (%) | 6.9 | 6.9 | 6.9 | 6.9 |
| Fibre (%) | 4.4 | 4.4 | 4.4 | 4.4 |
| Phosphorus (%) | 1.2 | 1.2 | 1.2 | 1.2 |
| Digestible energy (MJ) | 17.4 | 17.4 | 17.4 | 17.3 |

Table H.2: Vitamins in the feed per kg

| Vitamin A (IU) | 10.0 |
|---|---|
| Vitamin D3 (IU) | 1.0 |
| Vitamin E (mg) | 200 |

# APPENDIX I: MENTAL MAP OF IDEAS AND CONCEPTS
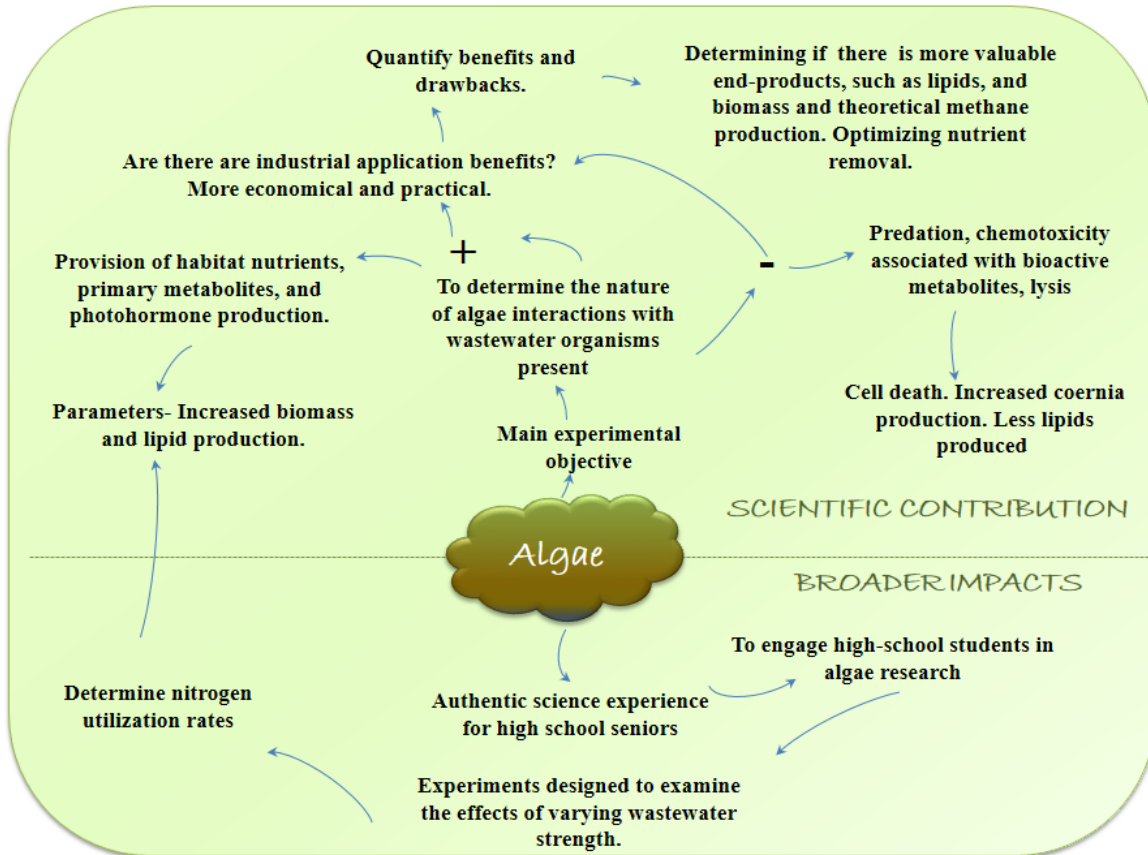


Figure I.1: Mental map

## APPENDIX J: LIST OF NOTATIONS

Table J.1: List of notations

| Terms | Description | Units |
|---|---|---|
| $A$ | Reactor surface area | $m^2$ |
| $a$ | Light attenuation constant (modified Beer-Lambert equation) | $m^{-1}$ |
| $b$ | Light attenuation constant (modified Beer-Lambert equation) | g-DW $m^{-3}$ |
| $B$ | Biomass concentration | g-DW$m^{-3}$ |
| $B_{tp}$ | Biomass prior to the time of harvest | g-DW$m^{-3}$ |
| $B_{ta}$ | Biomass concentration after harvest | g-DW$m^{-3}$ |
| $d_{eff}$ | Effective path length of the photobioreactor | m |
| $E_k$ | Light saturation constant | $\mu$mol-photon $m^{-2}\,s^{-1}$ |
| $I$ | Irradiance at a given depth | $\mu$mol-photon $m^{-2}\,s^{-1}$ |
| $I_o$ | Incident irradiance | $\mu$mol-photon $m^{-2}\,s^{-1}$ |
| $P_{net}$ | Net photosynthetic carbon fixation rate | $\mu$mol-C $m^{-2}\,s^{-1}$ |
| $P_m$ | Maximum photosynthetic carbon fixation rate | $\mu$mol-C $m^{-2}\,s^{-1}$ |
| $P_z$ | Gross carbon photosynthetic rate | $\mu$mol-C $m^{-2}\,s^{-1}$ |
| $r$ | Algae growth rate | g-DW $m^{-3}\,s^{-1}$ |
| $R_B$ | Biomass dependent respiration rate | $\mu$mol-C $m^{-2}\,s^{-1}$ |
| $R_o$ | Specific biomass respiration rate | $\mu$mol-C g-DW$^{-1}\,s^{-1}$ |
| $V$ | Reactor working volume | $m^3$ |
| $V_H$ | Harvest volume | $m^3$ |
| $z$ | Depth | m |

# APPENDIX K: LIST OF ACRONYMS AND ABBREVIATIONS

Table K.1: List of acronyms and abbreviations

| Acronym | Term |
|---------|------|
| AWRS | Algal Wastewater Reactor Systems |
| COD | Chemical oxygen demand |
| HS | High school |
| TN | Total nitrogen |
| TP | Total phosphorus |
| TSS | Total suspended solids |

**ABOUT THE AUTHOR**

Trina Cassandra Halfhide completed her BS in Environmental and Natural Resource Management and Environmental Biology from the University of the West Indies, St. Augustine, Trinidad in 2005. Directly after completion of the BS, Trina then worked in the environmental engineering industry and governmental agencies until 2010. She embarked upon the MS in Environmental Science and Policy from the University of South Florida (USF), Tampa Florida and graduated in 2009. She was then directly enrolled in the PhD Engineering Sciences Program in the Department of Civil and Environmental Engineering at USF. She engaged in teaching and research assistantships, while serving as President in the school's chapter of Engineers for a Sustainable World (ESW) for two years. Trina is a returning 2013 United States- Norway Fulbright Fellow and has coauthored many peer-reviewed journal papers.