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Microalgae to Biofuel: An Investigation into the Role of the Native Microbial Community in the Cultivation of Algae on Wastewater

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MICROALGAE TO BIOFUEL: AN INVESTIGATION INTO THE ROLE OF THE NATIVE MICROBIAL COMMUNITY IN THE CULTIVATION OF ALGAE ON WASTEWATER

By

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I dedicate this thesis to my family and friends, especially Matt, whose unfailing support and confidence was invaluable to me.

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ABSTRACT

The growth of a locally isolated strain of green algae, *Chlorella sp.*, selected for its promise as a biodiesel feedstock, was studied in wastewater effluent from the municipal wastewater treatment plant in Tallahassee FL. Nutrient concentration and microbial community composition within the effluent were profiled at monthly intervals. Adequate nutrients for algal cultivation were observed along with a dynamic microbial community of zooplankton, green algae, diatoms, and a bacterial community that included *Proteobacteria, Bacteroidetes, Cyanobacteria, Firmicutes,* and *Actinobacteria.* The importance of this microbial community in the cultivation of *Chlorella* and the impact on biofuel production was investigated utilizing mesocosm incubations. Size exclusion was employed to examine the biogeochemical interactions between the *Chlorella* culture and the resident wastewater microorganisms. The accumulation of algal cells and chl a biomass, the production of oxygen, and the consumption of inorganic carbon and nutrients, along with changes in the microbial community composition were monitored in treatments that included the total wastewater microbial community, the wastewater bacterial community, and the *Chlorella* culture with no effluent microorganisms. The treatment that excluded the wastewater microbial community and allowed the *Chlorella* culture to grow uninfluenced, consistently demonstrated the highest abundances of algal cells. A limited abundance of algal cells and chl a biomass were observed in the treatments that contained the total wastewater microbial community, while no limitation in oxygen production or nutrient consumption was observed in these treatments. Given the presence of zooplankton that are known to graze on algal cells, it appears that a top-down control inhibits the accumulation of algal biomass in raw wastewater effluent from a municipal treatment plant. In the treatments that contained the native bacterial community, competition between the *Chlorella* culture and phototrophic bacteria, i.e. cyanobacteria, was observed. These treatments demonstrated high chl a biomass, but limited accumulation of algal cells, as well as significant consumption of nutrients, indicating that cyanobacteria may out-compete *Chlorella* for limiting nutrients. However, the heterotrophic bacterial community did appear to have a significant impact on algal growth. Treatments in which the cyanobacterial community was inhibited did not demonstrate a draw-down of limiting nutrients or a limitation in the accumulation of algal cells. In addition, shifts in the composition of the bacterial community, including a reduction in the relative abundance of *Betaproteobacteria* with a simultaneous increase in the relative abundance of

Alphaproteobacteria, Nostocophycideae, and *Oscillatoriophycideae* were observed, but these shifts occurred independently of the presence of the *Chlorella* culture, implying that there is not a strong relationship between these two groups. The limitation that occurred in the growth of *Chlorella* due to competition and top-down controls indicates that without significant manipulations of the microbial community, the cultivation of an algal monoculture in wastewater effluent from a municipal treatment plant may be unrealistic for efficient biofuel feedstock production.

INTRODUCTION

Petroleum based fuels are now widely recognized as unsustainable from both an economic and environmental perspective (8). Rising energy demand in the developing world has led to intense competition for dwindling petroleum reserves and a significant rise in fuel prices worldwide. Approximately 97% of oil imports in the United States and many other industrialized nations are liquid transportation fuels. Any disruption to fossil fuel imports would be disastrous for the economy, food supply, military and transportation systems of most developed countries (15). Additionally, there are serious concerns about climate change caused by rapidly rising atmospheric carbon dioxide concentrations and the link to the combustion of fossil fuels (52). Therefore, a carbon neutral, renewable fuel source is urgently needed to support environmental and economic sustainability in the United States and around the world.

Biofuels, organic fuels made from plants and vegetables, are emerging as a proven alternative to fossil fuels, as well as a means to decrease greenhouse gas emissions and to improve energy security (54). Currently in the United States, biofuels are produced primarily from major commercial crops, specifically corn-grain and soybean. These crops are used to produce ethanol and biodiesel, respectively (23, 61). However, biofuel produced from these feedstocks cannot realistically satisfy the existing demand for transportation fuels, as is shown in Table 1(8).

Table 1. Comparison of oil yield and land area required to produce common biofuel feedstocks.

Crop	**Oil yield (L/ha)**	**Land area needed (M ha)***	**% of existing U.S. cropping area***
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Coconut	2689	99	54
Oil palm	5950	45	24

*Land required to produce 50% of all transport fuel needs of the United States

Table 1 clearly demonstrates that the utilization of conventional biofuel feedstock crops would require impossibly large cultivation areas to meet just half of the fuel for transport needs in the U.S. Additionally there are growing concerns about the economic and environmental impacts of using agricultural crops as a fuel source. The U.S. ethanol boom of 2008 was one of many

factors that contributed to a spike in corn prices worldwide, leading to ethical questions regarding the use of the same crop for food and fuel (11). Intensive production of traditional fuel crops, such as corn and soybean, can cause detrimental environmental impacts including increased freshwater consumption, soil erosion, nutrient pollution, air pollution, and natural habitat destruction, as well as increased CO_2 emissions via the conversion of carbon-rich ecosystems to cropland (16, 61). Of particular concern is nutrient overenrichment in surrounding aquatic environments that results from the production of conventional land-based biomass crops. Production of grain-based crops requires large inputs of nitrogen, phosphorous and pesticides. These chemicals are easily leached from soils and transported to surface, ground, and coastal waters thereby causing eutrophication, loss of biodiversity and elevated levels of toxic nitrate in drinking water wells (23). A biofuel feedstock that does not compete with food crops nor create harmful environmental impacts is necessary to make biofuel a commercial reality.

Algae represent an unexploited biomass source that could provide cheap, clean, and renewable fuel. Unlike slow-growing terrestrial crops, algae have to potential to meet the transportation needs of the U.S., while being completely compatible with the current transportation fuel infrastructure (52). There are significant advantages to algae over traditional biomass sources including higher photosynthetic efficiency, higher biomass productivities, and faster growth rates (20). Due to this high productivity, algae produce significantly more biomass per unit area than terrestrial crops and therefore require a fraction of the amount of land necessary for the production of conventional biofuel feedstocks. The rapid growth rate of algae, which often display a biomass doubling time within 24 hours, allows for frequent or possibly continuous harvest, resulting in more consistent biofuel production throughout the year (9, 11). Because of these advantages, algae have the potential to generate significant quantities of oil suitable for conversion to biodiesel (41), as well as many other products such as green gasoline, aviation fuel, ethanol, methane and valuable co-products including oil, protein, and carbohydrates (52). Another significant benefit of algae is that the cultivation will not compete with food crops for land and freshwater resources. Algae can be grown in variable climates and using non-arable land that is unsuitable for agricultural purposes (20). It is therefore not necessary to utilize existing cropland, nor is it necessary to create additional cropland through hazardous habitat destruction. Algae can also be cultivated using wastewater and non-potable saline water, which is unusable for conventional agricultural or domestic purposes (52). The

ability of algae to grow on non-potable water, specifically wastewater, is an important aspect of algal cultivation because it allows for the combination of biofuel production and water remediation in a mutually beneficial partnership.

While algal feedstocks provide many advantages over their terrestrial counterparts, they still require significantly large quantities of water and fertilizer, which drive up the economic and environmental cost of algal biomass production. The input of freshwater, nitrogen, and phosphorus to algal cultivation systems accounts for approximately 10-30% of the total production costs (5, 38). The water demand for algal cultivation in open raceway ponds can be as high as 11-13 million L ha^{-1} yr^{-1}, which equates to 10 times the demand for crops such as corn, canola, and switchgrass (7, 11). Additionally, raceway ponds fed with either fresh water or sea water must be supplemented with nitrate and phosphate fertilizers to prevent nutrient limitation to algal growth (8). These fertilizers, which are produced using fossil fuels, contribute approximately 50% of the energy use and greenhouse gas emissions associated with algal biomass production (11). Commercial algal farms that utilize freshwater and fertilizer actually consume more energy, produce higher greenhouse gas emissions, and use more water than the production of terrestrial biofuel feedstocks (11, 38). The cultivation of algae on wastewater can eliminate these economic and environmental burdens associated with algal biomass production and provide a cost-effective and sustainable means of producing a biofuel feedstock.

Wastewater derived from municipal, agricultural and industrial activities is a readily available water source that can provide adequate nutrients for algal growth at reduced cost. The global annual water demand for domestic purposes during 1987-2003 was estimated at 325 billion m^3 (62), while the industrial demand exceeded 665 billion m^3 (63). The majority of this water eventually becomes wastewater effluent that is released to the environment. Chinnasamy et al (2010) estimate that if just half of this water (495 billion m^3) was used for algae production prior to its release, it could generate 247 million tons of algal biomass and 37 million tons of oil (7). Wastewater effluent contains high concentrations of both nitrogen and phosphorus, which serve as major nutrients for algal growth. The total N and P concentration in municipal wastewater is approximately 10-100 mg/L and the concentration in agricultural effluent can be as high as 1000 mg/L (41). These nutrient levels allow for the cultivation of algae without the input of expensive chemical fertilizers (5), driving down both the environmental and economic cost of biofuel production.

An important advantage to producing algal biomass on wastewater is that it not only offsets freshwater and fertilizer burdens, but it also couples biofuel production with wastewater remediation. Wastewater remediation has become progressively more important as human activities such as agricultural practices, urbanization, and industrialization have increased the entry of chemical and biological contaminants, N and P in particular, to water systems (3). Nutrient loading to rivers and coastal waters is especially problematic. Many treatment plants discharge over 10^6 L of wastewater per day with nutrient concentrations three orders of magnitude higher than the receiving water (24). Physical and chemical technologies that remove nutrients from wastewater, such as ammonia volatilization and phosphate precipitation, are energy intensive and expensive (24, 38). Approximately 60-80% of energy consumption during wastewater treatment is associated with nutrient removal. Rerouting wastewater streams through intensive algal cultivation ponds could significantly lower energy consumption during the treatment process (11). Algae have been recognized for some time as a potentially low-cost and environmentally friendly wastewater treatment option (37). Algae can effectively assimilate nutrients into cell biomass which can subsequently be removed from the wastewater through harvesting (25). Multiple studies have demonstrated that algae are capable of removing 100% of ammonia and 60-99% of phosphate from a variety of wastewater sources (3, 7, 19, 33). Combining the cultivation of algae as a cost-effective and safe means of wastewater treatment and as a source of biomass for biofuel has the potential to make both technologies environmentally and economically sustainable.

An area of research that has largely been neglected in the study of algal cultivation is the impact of the native microbial community in wastewater effluent on algal growth. Wastewater treatment plants utilize biological treatment processes, such as activated sludge, suspended growth, and lagoons, to degrade organic matter during secondary treatment (53). In addition, effluent is often stored in open ponds and reservoirs, which allow contact with natural assemblages of microorganisms (51). These aspects of the treatment process introduces a dynamic microbial community to wastewater effluent that includes zooplankton, green algae, diatoms, photosynthetic bacteria, and heterotrophic bacteria (4). The zooplankton community in wastewater is dominated by ciliates, but also includes flagellates, sarcodines, rotifers, and nematodes (1). Phototrophs that are commonly found in wastewater include algal species, such as *Chlorella vulgaris* and *Scenedesmus quadrcauda* that are typically found in environments

with high organic matter (32, 43). Other commonly found phototrophs are cyanobacteria, specifically *Phormidium, Synechococcus,* and *Synechocytis*, which tend to dominate in tertiary lagoons- shallow constructed ponds that allow sunlight, algae, bacteria, and oxygen to combine to improve water quality (53). These species thrive in tertiary lagoons because of the high nutrients, biomass, temperature, and light intensity present in the ponds (57). There is also a diverse heterotrophic bacterial community in wastewater. The most frequently observed phylum is *Proteobacteria*, which typically accounts for at least half of the bacterial gene sequences retrieved from wastewater. Phylotypes of the class *Betaprotoebacteria* often show the highest relative abundance, comprising over 25% of the total retrieved sequences. Other common phyla observed in wastewater include *Bacteroidetes, Planctomycetes, Firmicutes, Acidobacteria, Actinobacteria, Chloroflexi,* and *Nitrospirae* (45, 64). It is believed that a dynamic microbial community is necessary for stable water treatment, because a diverse and variable community can adapt to fluctuating conditions within the treatment plant (59). Thus, it follows that a dynamic microbial community will be present in wastewater effluent used as a medium for algal cultivation.

Currently, few studies have examined the microbial ecology of wastewater and the potential implications for the cultivation of algae as a biofuel feedstock. Algae are susceptible to grazing by zooplankton. The presence of zooplankton in effluent being used as a cultivation medium is likely to reduce the efficiency of algal production to low levels (38). Competing microbial groups can also inhibit algal growth through cell lysis and competition for limiting nutrients (12). Conversely, bacteria can stimulate algal growth through nutrient regeneration, and the production of vitamins and other growth factors. In order to efficiently and cost-effectively cultivate algal biomass in open raceway pond systems with wastewater as the cultivation medium, further research is needed to understand the dynamic interaction between seeded algae and the resident microbial communities.

This project investigated the dynamics of microbial communities during the cultivation of algae on secondary treated wastewater effluent from the Tallahassee T. P. Smith Wastewater Treatment Plant. The native wastewater microbial community was characterized at monthly intervals to determine seasonal variation in community composition with varying nutrient levels. A native *Chlorella* culture, enriched from a pond in the Tallahassee area, was selected for its promise as a suitable biodiesel feedstock. Size-exclusion experiments were conducted to

investigate the biogeochemical interactions between the native wastewater microbial community and the *Chlorella* species to be cultivated for biofuel. Experiments included the following treatments: 1) mesocosms containing only the bacterial community native to the wastewater, 2) mesocoms containing only a seeded monoculture of *Chlorella*, 3) mesocosms containing a combination of the native bacterial community and *Chlorella*, and 4) mesocosms containing the total native wastewater microbial community including zooplankton with and without the *Chlorella* monoculture. Algal growth and abundance as well as bacterial abundance were monitored in each mesocosm over time along with a range of biogeochemical parameters including: nutrient concentrations, nitrogen, phosphorous, dissolved oxygen, dissolved inorganic carbon, and dissolved organic carbon. The specific objectives of this research were to:

1. Characterize the seasonal dynamics of native microbial community composition in secondary treated wastewater effluent to be used as a cultivation medium for the production of algal biomass.
2. Determine the role of the native microbial community in the transformation of carbon, nutrients, and oxygen during the production of algal biomass in wastewater.
3. Evaluate the impact of the native microbial community on the production of seeded algal biomass in wastewater as the cultivation medium.

METHODS

Wastewater Effluent Description

The wastewater effluent utilized in this project was obtained from the Southeast Farm Wastewater Reclamation Facility (SEF) in Tallahassee, Florida. SEF, which is designed for agricultural reuse of wastewater, receives secondary treated effluent from the Tallahassee T.P. Smith Wastewater Reclamation Facility (TPS). TPS serves as the main municipal treatment plant for the majority of the Tallahassee area and treats up to 25.6 million gallons of wastewater per day (10). Currently TPS treats water through secondary treatment, using an activated sludge method to remove the majority of the organic matter. The clarified waster is then disinfected using a chlorination process (53). Chlorinated water is stored in open ponds for 2-3 days to allow the chlorine to degas before being piped to SEF to be reused as spray irrigation for agricultural crops.

Characterization of Wastewater Effluent over a Seasonal Cycle

Wastewater effluent was sampled at monthly intervals from SEF throughout 2011. Water samples were collected in acid-washed (rinsed with 10% hydrochloric acid) 1-L glass screw-cap bottles and returned to the laboratory for analysis. Monthly samples were collected for algal cell abundance, chl a biomass, nutrient concentrations, dissolved organic carbon (DOC) concentration, bacterial community composition, and phytoplankton community composition. See below for further details of sampling and analytical methods.

Size Exclusion Experiments

Four size exclusion experiments were performed between July 2010 and October 2011. In each experiment, five to six treatments were constructed in triplicate mesocoms to study the nutrient transformation dynamics between native wastewater microbial community and a monoculture of *Chlorella sp*, which is to be cultivated as feedstock for biofuel.

Inoculum Preparation - A monoculture of the green alga, *Chlorella,* was grown in filtered secondary treated wastewater effluent from SEF. Water was filtered through an AcroPak 1500 Capsule, containing a Supor 0.8/0.2 um prefilter and membrane, respectively. Two liters of culture were grown in a Fernbach culture flask over a period of approximately 2 weeks. The culture was grown in a temperature controlled room set at 25°C with 40W fluorescent bulbs providing light on a cycle of 14 hours light -10 hours dark. This procedure was utilized for each of the four size exclusion experiments.

Experiment 1 – July 2010

Treatments:

1. Native wastewater bacterial community, no seeded culture, exposed to natural light levels
2. Native wastewater bacterial community, no seeded culture, in the absence of light (heterotrophic condition)
3. Native wastewater bacterial community + a seeded monoculture of *Chlorella*, exposed to natural light levels
4. Native wastewater bacteria + a seeded monoculture of *Chlorella* in the absence of light
5. Seeded monoculture of *Chlorella*, no native bacterial community, exposed to natural light levels

Experimental Approach:

At SEF, 228 L of secondary treated wastewater effluent was collected and filtered through a Polypure Filter Capsule, with a pore size of 1.0 um, to exclude any large eukaryotic organisms such as other algae species and zooplankton present in the wastewater. This water was used to fill 12 18.9-L cubitainers. Half of these 12 cubitainers were inoculated with 100 mL of the *Chlorella* monoculture and 6 cubitainers (3 without inoculants and 3 with inoculants) were covered with black trash bags to prevent light exposure to these mesocosms. An additional 57 L of water was collected and filtered through an AcroPak 1500 Capsule, containing a Supor 0.8/0.2 um membrane and prefilter, to remove all microorganisms from the water. This water was used to fill 3 18.9-L cubitainers and each cubitainer was inoculated with 100 mL of the *Chlorella* monoculture. Each of the 15 cubitainers was capped with a spigot to allow for sampling with minimal exposure to the atmosphere. The cubitainers were placed in a small pool outside and a continuous flow of water was maintained to keep the temperature within the cubitainers at ambient levels. Samples for pH, dissolved oxygen (DO), dissolved inorganic carbon (DIC), nutrients (NH_4, PO_4, NO_3, NO_2), DOC, chlorophyll a, and algal and bacterial abundance were collected daily for eight days. Samples for bacterial community composition were collected after 0, 3, and 7 days of incubation. No samples for phytoplankton community analysis were taken during this experiment.

Experiment 2 – February-March 2011

Treatments:

1. Total native wastewater microbial community + a seeded monoculture of *Chlorella*

2. Total native wastewater microbial community, no seeded culture
3. Native wastewater bacterial community + a seeded monoculture of *Chlorella*
4. Native wastewater bacterial community, no seeded culture
5. Seeded monoculture of *Chlorella*, no native microbial community
6. Total native wastewater microbial community, no seeded culture, no exposure to light

Experimental Approach:

At SEF, 171 L of secondary treated wastewater effluent was collected and used, without filtering, to fill nine 18.9-L cubitainers. Three of these cubitainers were inoculated with 100 mL of *Chlorella* monoculture. Another three cubitainers were covered with black trash bags to prevent light exposure. An additional 114 L of effluent was collected and filtered through a Polypure Filter Capsule, with a pore size of 1.0 um, to exclude any large eukaryotic organisms. This water was used to fill six cubitainers, three of which were inoculated with 100 mL of the *Chlorella* monoculture. Lastly, 57 L of effluent was collected and filtered through an AcroPak 1500 Capsule, containing a Supor 0.8/0.2 um membrane and prefilter, to remove all microorganisms from the water. This water was used to fill 3 18.9-L cubitainers and each cubitainer was inoculated with 100 mL of the *Chlorella* monoculture. Each cubitainer was capped with a spigot and the water was maintained at ambient temperature by the method previously described. Samples for pH, chlorophyll a, and algal abundances were collected daily for 24 days, while samples for DO, nutrients, DIC, and DOC, were taken every other day. Samples for bacterial community composition and bacterial abundance were collected after 0, 16, and 22 days of incubation. No samples for phytoplankton community were taken during this experiment.

<u>Experiments 3 & 4 – July 2011 & October 2011</u>

Experimental Approach:

The five treatments were constructed as described in Experiment 2 with the exception of the dark treatment, which was not included. Samples for pH, DO, DIC, nutrients, DOC, chlorophyll a, and algal and bacterial abundances were collected daily for eight days during Experiment 3 and for ten days during Experiment 4. Samples for bacterial community composition and phytoplankton community composition were collected at three time points: day 0, day 4, and day 7 during Experiment 3 and day 0, day 4, and day 8 during Experiment 4.

Sample Collection and Analysis

Wastewater was collected from each cubitainer using the spigot and Tygon tubing to limit the samples' exposure to the atmosphere. Samples for DO and DIC were collected in 12 mL Exetainer vials (Labco) and were preserved immediately in the field. Water was also collected in 100-mL acid-washed glass screw cap bottles and returned to the laboratory to be sampled for algal cell abundance, chlorophyll a concentration, pH, DOC, nutrients, and bacterial abundance. Additional water was collected in autoclaved 500-mL glass screw cap bottles and returned to the laboratory to be sampled for bacterial community composition and phytoplankton community composition.

Algal cell abundance: A 0.5 mL aliquot of wastewater was added to 9.5 mL of Isoflow sheath fluid in an Accuvette. The sample was counted immediately using a Beckman Z2 Coulter Particle Count and Size Analyzer. Each sample was analyzed for the concentration of cells between the sizes of 4 and 10 µm. This size range was selected to represent the range associated with unicellular eukaryotic algae (48) and allowed for counting of the *Chlorella* species while excluding most particulates and non-algal species.

Chlorophyll a concentration: Samples were collected by filtration onto a glass microfiber filter (Whatman Grade GF/F, 25mm, pore size 7 µm). The filter was folded in half using forceps so that the chlorophyll was protected and the sample was stored in a foil packet at 0°C. Chlorophyll a was extracted from each sample by soaking the filter in 5 mL of a 2:3 mixture of DMSO (BDH, 99.9%) and 90% acetone (JT Baker, 99.9%) for 20 minutes (47). The concentration of chlorophyll extracted was determined by measuring the florescence of each sample at 685 nm using a Turner Designs Trilogy Fluorometer.

Dissolved oxygen: Samples were preserved and titrated according to the Winkler method (6).

Dissolved inorganic carbon: Samples were preserved with 15 µL of a saturated solution of mercuric chloride (7.4 g $HgCl_2$/100 mL H_2O). Samples were analyzed using a Shimadzu Total Carbon Analyzer, in comparison to a standard calibration curve generated with sodium carbonate (BDH, 99.5%).

Dissolved organic carbon: Samples were filtered through a combusted glass microfiber filter (Whatman Grade GF/F, 25mm, pore size 0.7 µm) and the filtrate was collected in a combusted glass vial and preserved with 0.01% concentrated hydrochloric acid (EMD, 37%) by volume. Filters and vials were combusted by heating at 450°C for four hours using a Thermolyne bench

top muffle furnace. Samples were analyzed for the concentration of non-particulate organic carbon using a Shimadzu Total Carbon Analyzer, in comparison to a standard calibration curve generated with acetanilide (Alfa Aesar, 98%).

Nutrients: Samples were filtered through a combusted glass microfiber filter (Whatman Grade GF/F, 25mm, pore size 0.7 µm) and the filtrate was collected in an acid-washed 30-mL plastic bottle and frozen. Samples were analyzed using a Lachat Quikchem FIA 8000+ nutrient analyzer. QuikChem methods 31-107-06-1-B, 31-115-01-1-I, 31-107-04-1-E, and 31-107-05-1-A were used to determine the concentration of ammonia, orthophosphate, nitrate, and nitrite respectively.

Bacterial abundance: Samples were collected in 15-mL centrifuge tubes (VWR polypropylene, sterile), preserved with 5% formaldehyde (J.T. Baker, Stabilized 37%) by volume and stored at 4°C. Bacterial abundance was determined using the epiflourescence direct counting method with DAPI staining (42). A 0.5 mL aliquot of sample was diluted in 2 mL of sterilized DI water (filtered through a VWR sterile syringe filter with a 0.2 um cellulose acetate membrane) and stained for 10 minutes with 0.05 mL of a DAPI working solution (0.2 mg/mL). Samples were filtered onto a black polycarbonate membrane filter (Nuclepore, 25mm, pore size 0.2 µm) using a backing filter (Whatman grade GF/C, pore size 1.2 um) for support. The membrane filter was placed in the center of a plain microscope slide (VWR VistaVision^{TM}) and covered with a cover slip. Slides were stored at 0°C prior to counting. Samples were counted using a 100x oil immersion lens on an Olympus BX51 research microscope with an X-Cite series 120Q light source. At least ten fields of view were counted per slide.

Bacterial Community Composition: Samples were collected by filtration onto a Mo-Bio sterile water filter (pore size 0.22 µm) and stored in PowerWater Bead Tubes at -80°C for subsequent analysis. Total genomic DNA was extracted using the Mo-Bio Powerwater DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's protocol. Initial analysis of the bacterial community composition was assessed by community fingerprinting using the automated ribosomal intergenic spacer analysis (ARISA) method (17). The intergenic region between the small (16S) and large (23S) subunit of the rRNA genes was amplified by PCR and was analyzed on the Agilent 2100 Bioanalyzer using the DNA 7500 Series II assay. This method allowed for a preliminary comparison of samples from different treatments to determine if any significant shift in the microbial community composition occurred. Samples that demonstrated a

shift in the community composition were selected for pyrotag sequencing. Triplicates of each treatment were pooled and the extracted DNA was sent to the DNA Services Laboratory at the University of Illinois at Chicago for sequencing.

Phytoplankton and Zooplankton Community Composition: Samples were stored in 100-mL glass amber bottles and preserved with 3-mL of Lugol's solution (56). Samples were stored at 4°C until analysis, and particulates were allowed to settle over night in a 25-mL glass settling chamber. Qualitative analysis of community composition was performed using an Olympus IX71 Inverted Microscope with an Olympus TH4-100 light source.

RESULTS

Characterization of Wastewater Effluent over a Seasonal Cycle

Substantial concentrations of major inorganic nutrients and dissolved organic carbon (DOC) were observed in the wastewater effluent throughout the year (Figure 1). Nitrate concentrations ranged from 2 to 547 µM and averaged 324 µM. Orthophosphate concentrations ranged from 49 to 115 µM and averaged 80 µM. The concentration of DOC ranged from 484 to 847 µM and averaged 609 µM. A native algal community, as evidenced by cell density and chl a, persisted throughout the year (Figure 2). Algal cell densities ranged from 3.6×10^3 to 4.9×10^4 cells/mL and averaged 3.0×10^4 cells/mL. Chl a concentration averaged 2.4 µg/L.

A diverse and variable microbial community was observed in the effluent throughout the year. Qualitative microscopic analysis revealed the presence of zooplankton, specifically rotifers and ciliates, green algae, diatoms and cyanobacteria (Figure 3). Using a 97% similarity cutoff to define distinct operational taxonomic units (OTUs), the retrieved sequences revealed a diverse bacterial community. The most frequently detected bacterial phylum was *Proteobacteria*, comprising over 50% of retrieved sequences each month (Figure 4). This phylum was particularly dominant during the winter months. In December and February *Proteobacteria* comprised over 93% of the retrieved sequences. Within the *Proteobacteria* the dominant sub-phyla were *Beta-, Gamma-,* and *Alphaproteobacteria*, comprising 59, 23, and 12% of the distinct phylotypes discovered in wastewater effluent during December, respectively. In the summer months, however, the relative abundance of *Proteobacteria* sequences declined and phylotypes related to *Bacteroidetes* were more abundant. In August the phylum *Bacteroidetes* comprised over 44% of the retrieved sequences, with classes *Flavobacteria* and *Sphingobacteria* most often detected in this group. Other common phylotypes retrieved from the effluent were affiliated with the phyla *Cyanobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, Chloroflexi,* and *Lentisphaerae.*

Size Exclusion Experiments

The initial conditions for each size exclusion experiment varied (Table 2). The initial temperature ranged from 15-26 °C and the nutrient (N:P) ratios ranged from 4.4 to 9.6. The temperature changed significantly during Experiments 2 and 4, increasing by 7 °C and decreasing by 11 °C respectively.

Table 2. Initial conditions and temperature ranges for each size exclusion experiment.

Experiment	Start Date	Start Temp. (°C)	End Date	End Temp. (°C)	Nitrate Conc. (μM)	Phosphate Conc. (μM)	N:P Ratio
1	7/14/2010	26	7/26/2010	27	352.5	80.2	4.4
2	2/9/2011	15	3/3/2011	22	572.8	76.6	9.6
3	6/27/2011	25	7/4/2011	26	241.0	32.7	6.5
4	10/14/2011	27	10/23/2011	16	496.2	65.6	8.3

Algal Abundance. The maximum abundance of algal cells during Experiments 1 and 2 was observed in the treatments that contained only the monoculture of *Chlorella* and no native microbial community (Figure 5). During Experiment 1, this treatment contained an average of 3.1×10^5 algal cells per mL at its maximum growth on day 4. In the two treatments that contained the native bacterial community, algal cell densities were reduced (8.9×10^4 cells/mL) and the maximum cell density was approximately six times lower. Corresponding algal densities were observed in these two treatments, despite the fact that only one was inoculated with the *Chlorella* culture. Similar results were observed during Experiment 2. The maximum density of algal cells was observed in the *Chlorella* only treatment, which contained an average of 6.7×10^5 cells/mL at its maximum growth on day 20. Once again, reduced algal cell densities were observed in the treatments that contained the native bacterial community, which contained an average cell density that was 2-3 times lower (3.0×10^5 cells/mL) at maximum growth. The two treatments that contained the total native microbial community (unfiltered) averaged 1.0×10^5 cells/mL at maximum growth. As expected, little to no increase in algal cell density was observed in the treatments that were not exposed to light.

The results of Experiments 3 and 4 varied from the previous two experiments. During Experiment 3 similar algal cell densities was observed in the two treatments that contained the native bacterial community and the *Chlorella* only treatment, which averaged 2.5×10^5 cells/mL and 1.9×10^5 cells/mL, respectively, at maximum growth on day 4. During Experiment 4 the maximum algal cell densities were observed in the treatment that contained *Chlorella* only and the treatment that contained the native bacterial community + *Chlorella*. These treatments showed an average maximum algal cell density of 2.1×10^5 cells/mL. The treatment that contained only the native bacterial community showed no significant increase in algal cell

density. Limited algal cell density accumulation was observed in the unfiltered treatments, consistent with Experiment 2.

Chl a Concentration. In contrast to algal abundance, the highest accumulation of chl a was observed in the treatments that contained the native bacterial community in Experiments 1 and 2 (Figure 6). Although the *Chlorella* only treatments contained at least twice the algal cell densities of these treatments, a lower concentration of chl a was observed. The unfiltered treatments and the treatments that were not exposed to light showed chl a concentrations that corresponded with the algal cell densities. The accumulation of chl a during Experiments 3 and 4 paralleled accumulation of algal cell density in all treatments.

Biogeochemical Parameters. Biogeochemical parameters were monitored as an indication of algal growth and interaction between trophic levels of the native microbial community. Oxygen production, the uptake of major nutrients, pH, and carbon dynamics were indicative of phototrophic growth in all treatments that were exposed to light during Experiments 1-3. During Experiment 4, only the treatment that contained exclusively the native bacteria did not show evidence of phototrophic growth. Significant rates of oxygen production, elevation of pH, and dissolved inorganic carbon (DIC) consumption corresponded with increases in chl a concentration (Table 2). Nitrate concentrations in the wastewater were substantially depleted in all treatments that demonstrated phototrophic growth (Table 3). Nitrate concentrations in the *Chlorella* only treatments and in the treatments that contained the native bacterial community decreased by an average 193 µM, while the nitrate concentrations in the unfiltered treatments decreased by an average of 308 µM. Orthophosphate was removed completely from the system in all treatments with substantial chl a biomass accumulation. Little to no nutrient removal was observed in the treatments that were not exposed to light. Additionally, in Experiment 4 the treatment that contained only the native bacterial community showed little nutrient consumption, supporting the lack of phototrophic growth observed in this treatment. No significant change in DOC concentration was observed during Experiments 2 and 4, while an increase in DOC concentration was observed in the treatments that were exposed to light during the final days of Experiments 1 and 3.

Table 3: Rate of oxygen production, elevation of pH, and consumption of DIC, NO_3 and PO_4 during the exponential growth phase of *Chlorella*. Positive values indicate production and negative values indicate consumption.

	Experiment 1				
Treatment	O_2 (µmol L^{-1} d^{-1})	pH (ph d^{-1})	DIC (µmol L^{-1} d^{-1})	NO_3^- (µmol L^{-1} d^{-1})	PO_4^{3-} (µmol L^{-1} d^{-1})
WW* Microbial Community + *Chlorella*	-	-	-	-	-
WW Microbial Community	-	-	-	-	-
WW Bacterial Community + *Chlorella*	101	0.563	-560	-32.3	-20.5
WW Bacterial Community	83.8	0.620	-486	-30.0	-24.2
Chlorella Only	82.3	0.649	-654	-37.4	-23.8
No Light Exposure	6.2	0.012	-1.00	13.8	0.571
	Experiment 2				
WW* Microbial Community + *Chlorella*	61.96	0.350	-174	-21.92	-6.06
WW Microbial Community	53.11	0.322	-169	-17.85	-6.34
WW Bacterial Community + *Chlorella*	48.85	0.261	-219	-16.45	-7.41
WW Bacterial Community	42.06	0.290	-266	-21.14	-6.87
Chlorella Only	34.88	0.324	-194	-19.46	-5.22
No Light Exposure	2.06	0.040	-15.0	-0.990	0.581
	Experiment 3				
WW* Microbial Community + *Chlorella*	78.7	0.618	-414	-70.5	-7.83
WW Microbial Community	75.8	0.664	-394	-67.4	-8.81
WW Bacterial Community + *Chlorella*	101.1	0.606	-359	-43.9	-5.24
WW Bacterial Community	98.9	0.588	-344	-39.0	-4.78
Chlorella Only	101.3	0.695	-262	-37.5	-5.30
No Light Exposure	-	-	-	-	-
	Experiment 4				
WW* Microbial Community + *Chlorella*	68.1	0.41	-210	-25.3	-12.0
WW Microbial Community	63.9	0.42	-210	-21.7	-9.59
WW Bacterial Community + *Chlorella*	55.9	0.29	-120	-18.8	-6.37
WW Bacterial Community	13.4	0.08	-2.00	-0.47	0.42
Chlorella Only	55.3	0.31	-130	-18.1	-6.28
No Light Exposure	-	-	-	-	-

*WW indicates wastewater effluent

Table 4. Percent removal of inorganic nutrients and DOC.

Treatment	DIC	$NO3^-$	$PO4^{3-}$	DOC*
	Experiment 1			
WW Microbial Community + *Chlorella*	-	-	-	-
WW Microbial Community	-	-	-	-
WW Bacterial Community + *Chlorella*	81.5	35.8	97.5	-62.8
WW Bacterial Community	78.8	45.5	97.0	-99.8
Chlorella Only	69.8	24.9	93.7	-47.5
No Light Exposure	4.8	1.9	4.8	1.3
	Experiment 2			
WW Microbial Community + *Chlorella*	76.0	70.0	99.0	-22.0
WW Microbial Community	75.3	65.6	98.9	-14.1
WW Bacterial Community + *Chlorella*	68.4	53.2	97.8	-0.5
WW Bacterial Community	67.8	55.7	99.2	-6.8
Chlorella Only	66.3	48.7	98.2	13.2
No Light Exposure	10.7	4.2	13.6	11.1
	Experiment 3			
WW Microbial Community + *Chlorella*	66.0	98.8	97.5	-51.2
WW Microbial Community	63.0	97.4	97.2	-59.6
WW Bacterial Community + *Chlorella*	63.3	98.3	97.0	-42.5
WW Bacterial Community	60.8	99.4	96.0	-33.3
Chlorella Only	64.4	97.7	97.3	-38.1
No Light Exposure	-	-	-	-
	Experiment 4			
WW Microbial Community + *Chlorella*	67.3	48.3	97.9	4.98
WW Microbial Community	65.8	46.1	98.5	9.22
WW Bacterial Community + *Chlorella*	34.5	20.6	78.5	27.1
WW Bacterial Community	6.5	0.8	20.9	15.7
Chlorella Only	38.9	28.7	84.5	13.8
No Light Exposure	-	-	-	-

*****Negative values indicate % increase in concentration.

Microbial Community Composition. There was no indication that the cultivation of algae had a significant impact on the microbial community in wastewater. There was an observed increase in the abundance of the bacterial cells associated with the *Chlorella* culture in the *Chlorella* only treatments. However, no substantial change in the bacterial abundance was observed in the treatments that contained the native bacterial community (Figure 7). DNA fingerprinting of the bacterial community composition demonstrated a shift in the community composition over time, however no patterns could be determined between treatments (Figure 8).

DNA sequence analysis did reveal some general trends in the community composition shift (Figures 9-11). In each treatment that contained the native bacterial community and was exposed to light there was a dramatic reduction in the relative abundance of *Betaproteobacteria*, while there was simultaneous increase in the relative abundance of *Alphaproteobacteria*. Additionally these treatments displayed a significant increase in the relative abundance of cyanobacteria, in particular *Nostocophycideae* and *Oscillatoriophycideae.* Qualitative analysis of the phytoplankton and zooplankton community composition in the unfiltered treatments showed a diverse community of rotifers, ciliates, diatoms, green algae, and cyanobacteria.

DISCUSSION

Characterization of Wastewater Effluent over a Seasonal Cycle

Characterization of the wastewater effluent from the Tallahassee T.P. Smith Wastewater Treatment Plant (TPS) revealed that it contained elevated nutrient concentrations when compared with natural freshwater systems (22). These nutrient concentrations were suitable for algal cultivation without the addition of chemical fertilizers (2). However, the effluent also supported a dynamic microbial community that may have a significant impact on algal production. Algal cell density and chl a biomass analysis indicated the presence of a persistent native phototrophic community. Additionally, qualitative analysis of the overall community revealed the presence of rotifers, ciliates, green algae, diatoms, and cyanobacteria. Ciliates dominate the zooplankton community in wastewater treatment plants due to their ability stabilize the biological treatment process by balancing bacterial production (39). Although they are primarily bacterivores in wastewater treatment, ciliates are also known to graze small (<6 um) algal cells (30, 46). In addition, rotifers are efficient herbivores that are common in wastewater, specifically in effluent ponds and reservoirs which have lower ammonia levels and high densities of algal cells, similar to the conditions found in raceway ponds for algal cultivation (44). The presence of these zooplankton could significantly reduce biomass accumulation during algal cultivation. Green algae, diatoms, and cyanobacteria are phototrophs that are currently adapted to effluent conditions and may compete for limiting nutrients with algal species cultivated for biofuel. Cyanobacteria in particular are known to compete well at low N:P ratios (50). The typical N:P ratio in the effluent from TPS is 5.4, below the suggested optimal ratio for the growth of eukaryotic algae in freshwater environments (6.8 – 10; (35). The ability to assimilate nitrogen species at low concentrations, as well as the ability to tolerate low irradiance and high pH (21, 31), may allow cyanobacteria to outcompete eukaryotic or green algae in wastewater effluent.

Within the planktonic community, a diverse bacterial community was observed in effluent from TPS. Microbial community composition of effluent from TPS was consistent with previous studies of wastewater at the phylum and class level of taxonomic resolution. In agreement with previous work, members of the phylum *Proteobacteria* were consistently found to dominate the sequence diversity of the heterotrophic bacterial community in TPS effluent. The relative abundance of operational taxonomic units (OTUs) classified within this phylum ranged

from 40% to 70% in studies of wastewater microbial communities (45, 64). *Betaproteobacteria* were the dominant class observed in this study, averaging a relative abundance of 50% in the retrieved sequences, which is also corroborated by past studies in wastewater treatment plants (WWTPs; (59). Wagner et. al. (2002) observed members of the *Betaproteobacteria* that are involved in phosphorous uptake and that others, such as *Nitrosomonas eutropha* and *Nitrosococcus mobilis,* are important ammonia oxidizers (58). Other phyla common to WWTPs were observed in this study including: *Bacteroidetes, Chloroflexi, Planctomycetes, Firmicutes, Acidobacteria, Nitrospirae, Actinobacteria,* and *Verrucomicrobes.*

Water within a treatment plant wastewater goes through several stages, such as clarification and chlorination, as well as extensive outside storage prior to release as effluent. The variation in the stage of the treatment process is likely to impact microbial community compositions. In contrast to past studies, *Cyanobacteria* were observed to be 5-10 % of the sequences retrieved from wastewater, whereas this group was largely absent in previous work. Previous studies had examined the microbial community composition of water within a treatment plant, typically during secondary treatment with activated sludge, whereas, this study examined the microbial community composition of effluent after it was exposed to outside storage in reservoirs. Further studies that employ next generation sequencing technologies, such as those used here, should be applied to elucidate the structure-function relationships of microbial community in different stages of wastewater treatment.

Competition by Native Wastewater Phototrophic Organisms.

Evidence from the growth of phytoplankton communities as well as biogeochemical parameters in size exclusion experiments demonstrates that the growth of seeded algae is suppressed by the native microbial community resident in wastewater effluent. Although the importance of native eukaryotic algae cannot be completely excluded, these were not observed in abundance by microscopy or detected by Coulter counter. Strong shifts in the abundance and composition of planktonic organisms between treatments indicated that this suppression was likely due to competition for available nutrients between the seeded algae and other photosynthetic organisms native to the wastewater. Additionally a significant reduction in algal biomass accumulation was attributed to the presence of grazers. The heterotrophic bacterial community, however, did not appear to substantially impact the growth of algae.

The growth of *Chlorella* was significantly limited by the presence of native photosynthetic organisms in the wastewater, in particular during the first two size exclusion experiments. During Experiments 1 and 2 the highest density of algal cells was observed in the C*hlorella* only treatment, while significantly lower algal cell densities were observed in treatments that contained the native wastewater microbial community. The treatments that contained the native bacterial community clearly demonstrated the competition between the *Chlorella* culture and other photosynthetic organisms. No significant difference in algal cell density was observed between the treatment that was inoculated with the *Chlorella* culture and the treatment that was not. In addition both treatments demonstrated the highest concentration of chl a biomass, despite lower algal cell densities than the *Chlorella* only treatment. The presence of high chl a concentration with low algal cell density indicates that small photosynthetic organisms, such as cyanobacteria, were abundant in these treatments. Unicellular cyanobacteria, typically 0.2 – 2 um in size (41), would be able to pass through the filter utilized to remove eukaryotic organisms. Cyanobacteria contain chl a, but the small size of these picoplankton would exclude them from the coulter counter analysis of algal cell density. The fact that there was no increase in algal cell density in the treatment that contained the bacterial community + *Chlorella* indicates that the presence of cyanobacteria in these treatments inhibited the growth of *Chlorella*. Visual evidence from microscopic analysis corroborated these conclusions. Small unicellular phototrophs (< 2 um in diameter) were abundant in TPS effluent (Figure 3) and these picoplankton outnumbered cells of green algae in the treatments with *Chlorella.*

Biogeochemical parameters in the size exclusion experiments indicated competition between cyanobacteria and seeded algae for nutrients. Phototrophic metabolism as evidenced by substantial oxygen production, elevation of pH, and the consumption of dissolved inorganic carbon (DIC) was observed repeatedly in treatments that contained only the native bacterial community. Rates of oxygen production and nutrient uptake were similar in the presence and absence of *Chlorella* (Table 2), both in the presence of the whole community (unfiltered treatments) and the bacterial community alone (1 um filtered treatments). Trends between treatments were also supported by the extent to which nutrients and inorganic carbon were depleted in the effluent medium (Table 3). Photosynthetic activity was not observed in the treatments that contained the native bacterial community, but were not exposed to light. This observation supports the conclusions that other phototrophs, likely cyanobacteria, are responsible

for the observed oxygen production and nutrient consumption in the absence of seeded algae. The nutrient consumption and algal cell densities in treatments that contained the native bacterial community during Experiments 1 and 2 illustrates that cyanobacteria became dominant in these treatments and prevented the growth of *Chlorella* through competition for limiting nutrients. Orthophosphate was removed completely in these treatments as well as in all treatments where photosynthesis occurred. However, due to the low N:P ratio in the wastewater, it is unlikely that orthophosphate was removed biologically. An analysis of metal concentrations demonstrated a removal of iron from the system (data not shown). It is therefore likely that the elevated pH caused by photosynthetic activity triggered an abiotic reaction that removed orthophosphate through precipitation as ferric phosphate (18).

The resilience of cyanobacteria is well documented in past studies of nutrient-rich systems. Cyanobacteria thrive in warm, stable, eutrophic aquatic environments and can account for nearly the entire phytoplankton biomass in hypertrophic lakes (57, 60). Such eutrophic conditions are often mimicked during wastewater treatment, particularly subsequent to the secondary treatment stage, in maturation ponds and high rate algal ponds (HRAPs). Vasconcelos et. al. (2001) discovered that cyanobacteria comprised 66.5% of the phytoplankton community in maturation ponds, and a study of HRAP performance by Cromar et. al. (1997) found that cyanobacteria were continuously dominant in ponds with high organic matter content during summer months (13, 57). This study also found that during the summer, ponds with lower organic matter would shift from algal dominance to cyanobacterial dominance with longer retention times, but the colder temperatures during the fall experiments eventually favored Chlorophyceae (13).

Cyanobacteria contain a number of physiological characteristics that allow them to succeed in wastewater, including the ability to outcompete other phytoplankton members for nitrogen uptake under condition of low N:P ratios (21). Chinnasamy et. al. (2010) found that cyanobacteria were dominant in treated carpet mill effluent with a N:P ratio of 0.83:1 (7). In addition, cyanobacteria can utilize CO2 at a higher rate than Chlorophyceae and can tolerate high pH, which is beneficial when the pH in wastewater is often between 8 and 9 (31). These organisms are also well equipped to maintain their growth rates at low irradiance, which can explain the observations of this experiment and the experiments by Cromar et al (1997). High light attenuation caused by high organic matter and high phytoplankton biomass favors

cyanobacteria, allowing them to become dominant as retention time increases (21). Due to these physiological advantages, cyanobacteria are well adapted for algal cultivation as a biofuel feedstock raceway ponds and photobioreactors. Strategies for biomass feedstock production in wastewater should address the contribution of cyanobacteria, either by preventing the growth of native phototrophs or by using cyanobacteria as an alternative biomass source.

Top-Down Control of Algal Biomass Accumulation.

The impact of grazing organisms was evidenced by the decreased accumulation of algae concurrent with the maintenance of photosynthetic activity in unfiltered treatments of raw wastewater effluent. Although algal cell densities and chl a concentrations were substantially lower in unfiltered treatments, rates of O_2 production and nutrient uptake were comparable to other treatments that contained phototrophic growth. Additionally, the highest percentage of nitrate removal was observed in the unfiltered treatments. These biogeochemical factors indicate phototrophic growth was not inhibited by limited access to nutrients, but rather that the accumulation of algal cells was reduced through a top-down control, such as grazing.

Observations of top down control are supported by microscopic analysis of the zooplankton community in TPS effluent. Ciliates and rotifers were observed in the unfiltered treatments. These zooplankton are common in wastewater and are known to limit algal cell density (55). Studies of algal populations in eutrophic lakes have shown that zooplankton control the density of green algae, and conversely, the removal of this grazing pressure allows for the rapid buildup of a dense algal population (14, 26). This phenomenon also occurs in HRAPs for wastewater treatment. Grazing in wastewater treatment was first reported by Uhlmann et al. (1971) who noted that grazing in sewage ponds could suppress phytoplankton growth to such an extent that nutrients remained unused (55). Lincoln et. al. (1983) discovered that an infestation of rotifers and cladocerans drastically reduced algal densities in HRAPs and Mesple et. al.(1995) determined that models of growth rates in HRAPs were significantly improved by the inclusion of zooplankton grazing (28, 34). Estimation of grazing rates demonstrated that ciliates were capable of a clearance rate of 3.3 +/- 2.0 ul $cell^{-1}$ h^{-1} when grazing on *Chlorella* and that a single rotifer is capable of ingesting 2000 *Chlorella* cells per hour (44, 46). At this grazing rate a relatively small population of zooplankton, especially rotifers or other metazoan, in wastewater effluent could dramatically reduce algal cell density and limit biomass accumulation for biofuel production.

An interesting observation was that there was no evidence for cyanobacterial abundance in the unfiltered treatments. The significant presence of ciliates may account for this shift in phytoplankton community. While ciliates will graze algae they are typically bacterivores (39). A study of protozoa grazing rates in a eutrophic reservoir found that the grazing rates of ciliates ranged from 23-4200 bacteria $cell^{-1}$ h^{-1} and 2-560 cyanobacteria $cell^{-1}$ h^{-1} depending on the species (49). This same study determined that some taxa of ciliates can survive exclusively on picoplankton when biomass is significantly high and that these ciliates rarely consume phytoplankton >2 um in size. Another study of a naturally eutrophic lake found that ciliate grazing rate on bacteria and autotrophic picoplankton (<3 um) was four orders of magnitude higher than ciliate grazing rate on nanoplankton (3-6 um; (65). These studies suggest that ciliates preferentially graze on picoplankton, i.e. cyanobacteria, over larger phytoplankton. This preference may become important to algal cultivation in wastewater. Rotifers and other metazoan are highly efficient grazers, but they are sensitive to ammonia concentrations. Lincoln et. al. (1983) minimized the rotifer and cladocerans population in HRAPs by increasing the ammonia concentration and found that the algal cell densities returned to pre-grazing levels (28). Removing the grazing pressure of metazoans, through increased ammonia concentrations or another method, while allowing ciliates to remain to control the population of cyanobacteria, may increase algal cell densities in wastewater. Manipulating the phytoplankton dynamics in wastewater effluent may be necessary to obtain sufficient algal biomass for biofuel production.

Activity of the Heterotrophic Bacterial Community.

The native heterotrophic bacterial community did not have a significant impact on the growth of *Chlorella*. During Experiment 4, there was an absence of cyanobacterial abundance in the treatments that contained the native bacterial community, which significantly altered the results of the experiment. In previous experiments the treatment that contained only the native bacterial community demonstrated high chl a concentrations and low algal cell densities. However, during Experiment 4 this treatment demonstrated no increase in either algal cell density or chl a concentration. The treatment that contained the native bacterial community + *Chlorella* had previously shown algal cell densities approximately one third the densities found in the *Chlorella* only treatment. During Experiment 4 no significant difference was observed in the maximum algal cell density and maximum chl a concentration in these treatments. The results of Experiment 4 dictate that when cyanobacteria are not abundant in the system, the

growth of *Chlorella* is not adversely affected by the presence of the native wastewater bacterial community.

The biogeochemical parameters that were monitored during Experiment 4 indicated that without the presence of cyanobacteria, the bacterial community did not impact the ability of *Chlorella* to access nutrients and perform photosynthesis. Both the *Chlorella* only treatment and bacterial community + *Chlorella* treatment produce oxygen and utilize DIC at the same rate and the percent consumption of nitrate is not significantly different. The growth of *Chlorella* in the treatment that contained that bacterial community was not inhibited nor supported by the presence of the heterotrophic bacterial community. This inference is supported by the treatments in Experiments 1 and 2 that were not exposed to light. There is minimal consumption of nitrate and orthophosphate in these treatments, indicating that the heterotrophic bacterial community is not competing for limiting nutrients. Additionally, there is no production of nutrients due to organic carbon respiration, so it is unlikely that the bacterial community is supporting algal growth via nutrient regeneration.

Studies of wastewater treatment in HRAPs have shown that under the right conditions algae and heterotrophic bacteria can form a mutually beneficial relationship. In this relationship algae utilize DIC and inorganic nutrients produced through aerobic bacterial degradation of organic matter to perform photosynthesis and produce oxygen. This oxygen is then used by the bacterial community to continue the degradation of organic matter and recycle DIC and nutrients (4). Oron et. al. (1997) observed that stably operating ponds fed with raw sewage contained a algae/bacteria ratio of at least 1:100 and theorized that ideally a ratio of 1:300 is necessary for adequate DIC regeneration (36). However, in this system the algae/bacteria ratio during Experiment 4 decreased rapidly to approximately 1:10 during algae growth. In addition, the ponds in Oron et. al. (1997) were fed with raw sewage, meaning there was a significant concentration of labile organic matter for aerobic bacterial degradation. The wastewater utilized in this experiment had previously been through extensive biological treatment and the remaining DOC was likely recalcitrant and not readily degradable by aerobic bacteria. The low concentration of bacterial cells and the limited availability of labile organic matter would have prevented the heterotrophic bacterial community from having a significant impact on algal cultivation.

Microbial Community Composition.

The analysis of bacterial cell abundances and community composition did not indicate that the cultivation of algae significantly impacted the resident bacterial community composition in wastewater effluent. Bacterial abundance remains relatively constant during the algal growth phase in all treatments, with the exception of the *Chlorella* only treatment. The low bacterial cell abundance observed in this treatment at the start of the experiment implies that exclusion of the native wastewater bacterial community through filtration was successful. However, a bacterial community associated with the *Chlorella* culture was introduced to the treatment during inoculation. The cultivation of *Chlorella* did not appear to stimulate the growth of the native wastewater bacterial community. Bacterial abundance usually increases during the senescence of a phytoplankton bloom when algal cells typically exude large quantities of organic matter (40). An increase in DOC did occur in most of the treatments during the final days of sampling. However, low nutrient concentrations and high pH likely limited the growth of bacterial cells.

DNA fingerprinting analysis of the bacterial community composition showed a shift in the community during the size exclusion experiments, but did not display an apparent pattern between treatments. Pyrosequencing provided for a higher resolution of characterization and revealed pronounced shifts in bacterial community composition. During Experiments 2 and 3 each treatment that contained native wastewater organisms and was exposed to light demonstrated a similar shift in bacterial community composition (Figures 9 and 10).The relative abundance of cyanobacterial taxa (*Synechococcophycideae, Oscillatoriophycidae, Nostocophycida*) increased with incubation time during the period of active phototrophic growth in all treatments, except those that contained *Chlorella* only. This observation further supports the contribution of cyanobacteria to phototrophic metabolism. A high relative abundance of cyanobacteria was also observed in the *Chlorella* only treatments. However, due to the low bacterial cell abundances in these treatments caused by filtration, the actual abundance of cyanobacteria was likely low. The treatment that contained the native bacterial community + *Chlorella* in Experiment 4 did not demonstrate the same increase in relative abundance of cyanobacteria as observed in other treatments (Figure 11). While the relative abundances are within the bacterial community only and cannot be directly related to other observations, such as chl a concentration, the fact that this treatment did not see an increase in the relative abundance

of cyanobacteria supports the conclusion that these organisms were not important in this treatment.

A dramatic shift in the *Proteobacteria* was observed with time in the size exclusion experiments. This shift included a dramatic decrease in the relative abundance of *Betaproteobacteria* with a corresponding increase in the relative abundance of *Alphaproteobacteria. Betaproteobacteria* was typically the dominant class within each treatment at the start of each experiment, usually comprising at least 50% of the retrieved sequences. At the final day of sampling this class was a much smaller fraction of the bacterial community, often as low as 5% of the retrieved sequences. A significant percentage of OTUs within the *Betaproteobacteria* are closely related to ammonia oxidizing bacteria, such as *Nitrosomonas.* The low concentrations ammonia present in the effluent may have selected against ammonia oxidizing *Betaproteobacteria* and allowed *Alphaproteobacteria*, another group often highly abundant in wastewater treatment and eutrophic systems, to become dominant. *Alphaproteobacteria* have been associated with high temperature and long retention times, conditions that were present during the size exclusion experiments (29). Additionally, some groups within the *Alphaproteobacteria,* in particular *Sphingomonadales* which was highly abundant in this system, have been shown to increase in abundance with increase in pH (27). These characteristics may have contributed to the shift in bacterial community observed in the size exclusion experiments.

The shift in the relative abundance of *Beta-* and *Alphaproteobacteria* occurred in all treatments that were exposed to light during Experiments 2 and 3, with the exception of the *Chlorella* only treatment, regardless of the presence or absence of the *Chlorella* inoculant. The inhibition of significant *Chlorella* growth caused by the presence of wastewater microorganisms likely limited the effect the cultivation of *Chlorella* would have on the bacterial community composition. However, during Experiment 4, the treatment that contained native bacterial community + *Chlorella* demonstrated a shift in community composition that was more consistent with the *Chlorella* only treatments. The *Chlorella* only treatments had a bacterial community composition that was dominated by *Betaproteobacteria* on the final day of sampling, which is also observed in the treatment that contained the native bacterial community + *Chlorella* in Experiment 4. This fact indicates that the cultivation of *Chlorella* did impact the bacterial community composition when *Chlorella* was not inhibited by other microorganisms.

CONCLUSIONS

This study aimed to evaluate the impact of the microbial community currently residing in wastewater effluent on the cultivation of algae as a feedstock for biofuel. A local algal strain of *Chlorella* was selected for its growth capability on wastewater as a promising biofuel feedstock, and the cultivation of this alga on wastewater effluent was adversely affected by the presence of a dynamic microbial community. Wastewater has been proposed as an effective medium for algal cultivation due to its wide availability and ample nutrient concentrations, however, a diverse and variable microbial community may limit its usefulness. This study documents a diverse and dynamic microbial community in municipal wastewater effluent that includes rotifers, ciliates, green algae, cyanobacteria, and a dynamic heterotrophic bacterial community. The accumulation of seeded *Chlorella* cells was often significantly inhibited by the presence of these microorganisms, in particular the presence of rotifers, ciliates, and cyanobacteria.

The variation in phototrophic growth and biogeochemical interactions when trophic levels are experimentally excluded from the effluent microbial community demonstrated competition for limiting nutrients and top-down controls restricted the accumulation of seeded *Chlorella*. No significant increase in the abundance of phototrophs was observed when raw, nutrient-rich effluent, containing the complete wastewater microbial community, was inoculated with the *Chlorella* culture. Given that biogeochemical parameters indicated there was no limitation in the consumption of nutrients or the production of oxygen, it appears that the presence of zooplankton that are known to graze on algal cells, created a top-down control on algal cell accumulation. When the *Chlorella* culture was introduced to a system that contained exclusively the wastewater bacterial community, competition between the *Chlorella* culture and phototrophic bacteria, i.e. cyanobacteria, was observed. In some instances no increase in algal cell density was observed, despite high levels of chl a biomass, nutrient consumption and oxygen production. Cyanobacteria, which have been shown to be highly abundant in hypertrophic systems, are capable of out-competing the *Chlorella* culture for limiting nutrients and becoming the dominant organism in this system.

The heterotrophic bacterial community, which under certain conditions could have supported algal growth through regeneration of nutrients and inorganic carbon, did not appear to significantly impact the cultivation of *Chlorella* in this study. Low bacterial cell densities and limited availability of labile organic matter would have inhibited the ability of the heterotrophic

community to support algal growth. Additionally, community composition analysis indicated that shifts in the microbial community occurred independently of the presence of *Chlorella* in most cases. Under the conditions present in this system, the heterotrophic bacterial community did not have a strong relationship to the cultivated algal species.

An inhibition of algal biomass accumulation due to the presence of native microorganisms in wastewater effluent indicates limitations in the ability of this system to efficiently cultivate algae as biofuel feedstock. The cultivation of a monoculture of a selected algal strain on effluent would require manipulation of the native microbial community. Grazing pressure would need to be reduced through filtration or changes in the water chemistry and the impact of competitors would need to be lessened. However, large scale manipulation of wastewater effluent is unrealistic as a cost-effective means of biofuel feedstock production. A more feasible technique may be to stimulate the growth of the natural assemblage of organisms within effluent. Further research is necessary to determine the optimal strategy for cultivation of eukaryotic algae or other phototrophs, such as cyanobacteria, as a biofuel feedstock on wastewater.

FIGURES

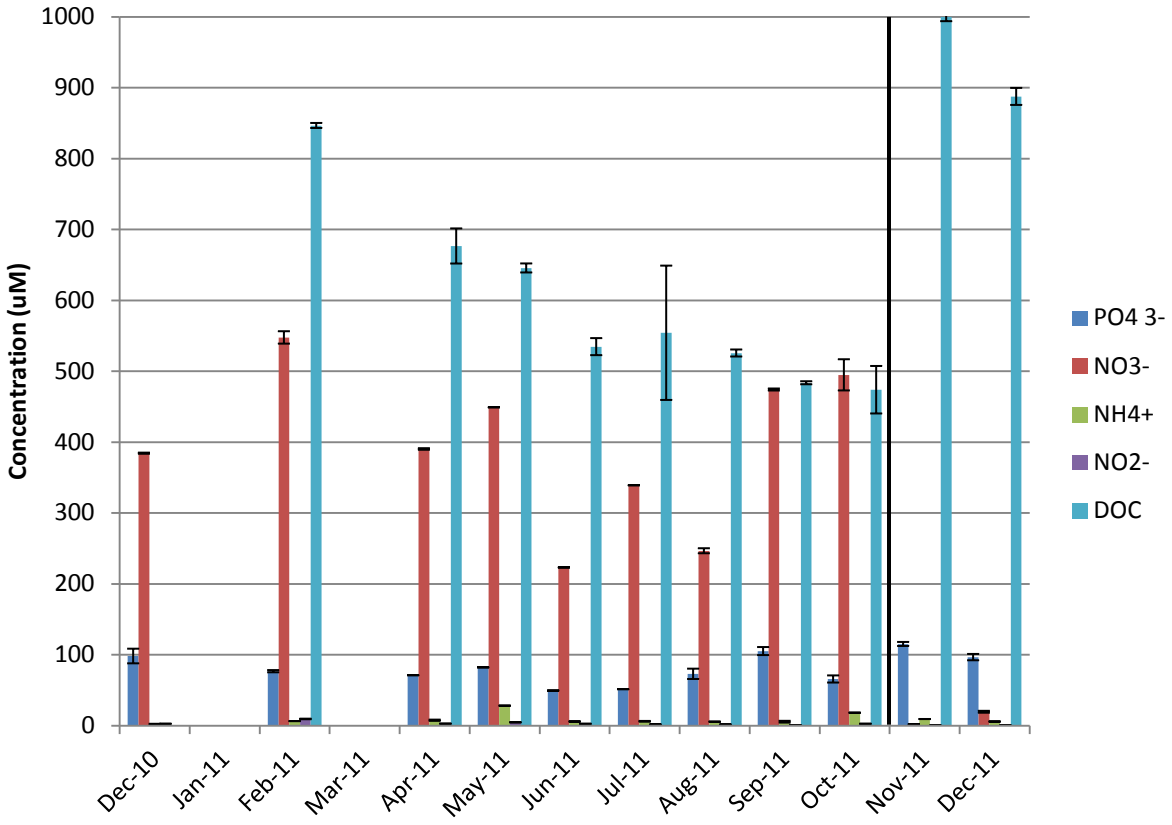


Figure 1: Concentration of nutrients and dissolved organic carbon in wastewater effluent from SEF throughout 2011. Black line indicates the installation of a nutrient removal system at the Tallahassee T.P. Smith Wastewater Treatment Plant.

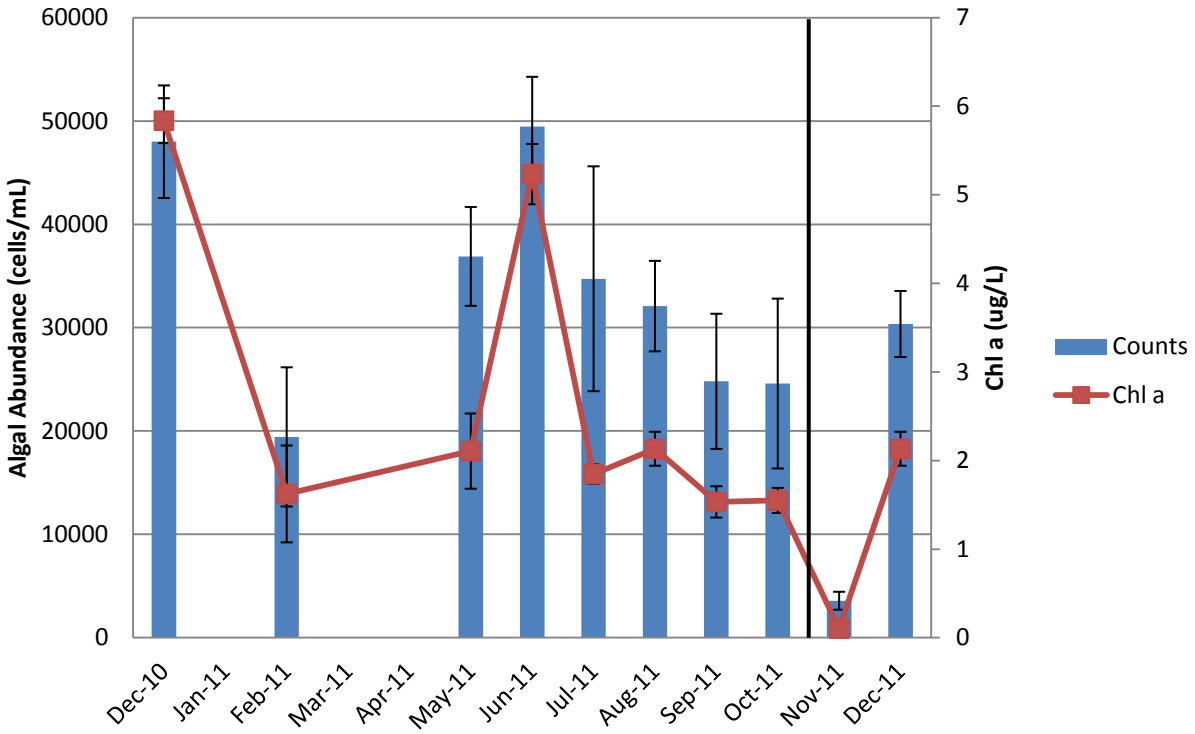


Figure 2: Algal abundance and chl a biomass in wastewater effluent from SEF throughout 2011. Black line indicates the installation of a nutrient removal system at the Tallahassee T.P. Smith Wastewater Treatment Plant.

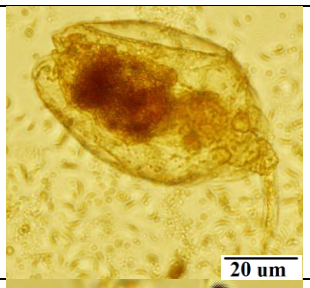
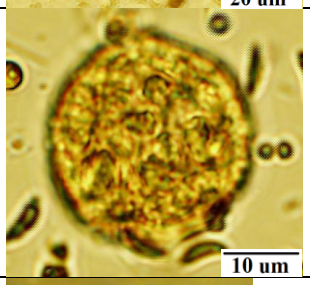
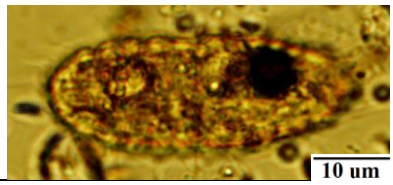
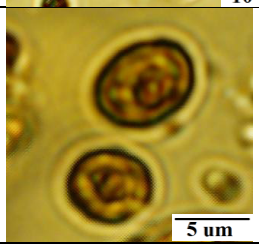
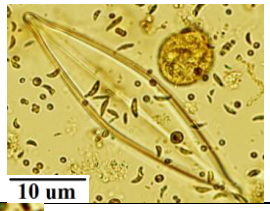
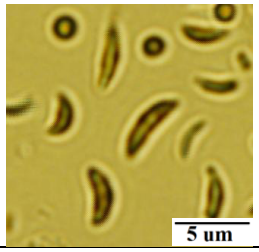
Rotifer	
Ciliates	 
Green Algae	 
Diatoms	  
Cyanobacteria	 

Figure 3. Qualitative analysis of the phytoplankton community in wastewater effluent from SEF.

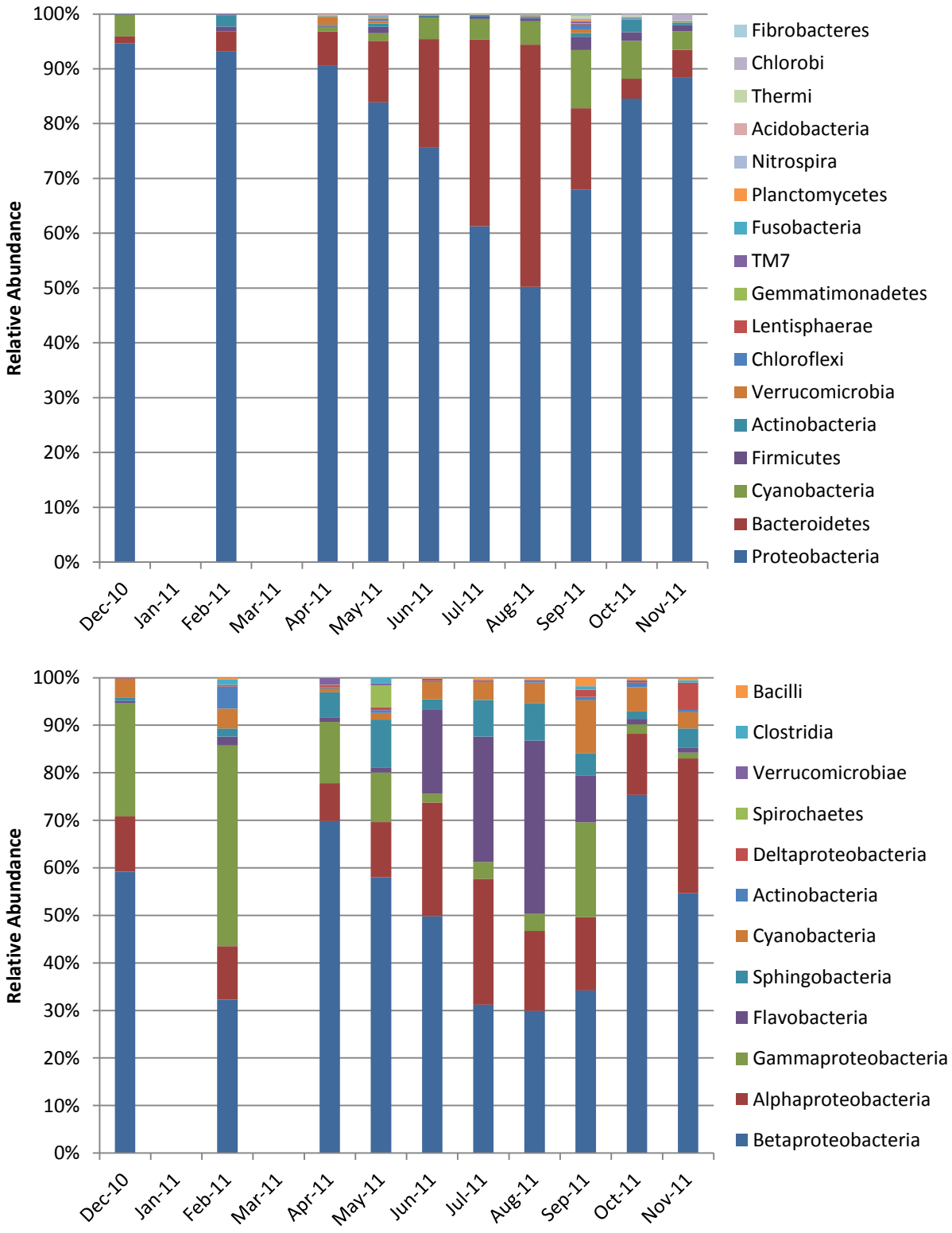


Figure 4: Histograms of the relative abundance of operational taxonomic units (OTUs) retrieved from wastewater effluent from SEF throughout 2011. Top: Phylum level relative abundances. Bottom: Class level relative abundances - minimum level for inclusion was an average of 0.25% of the retrieved sequences.

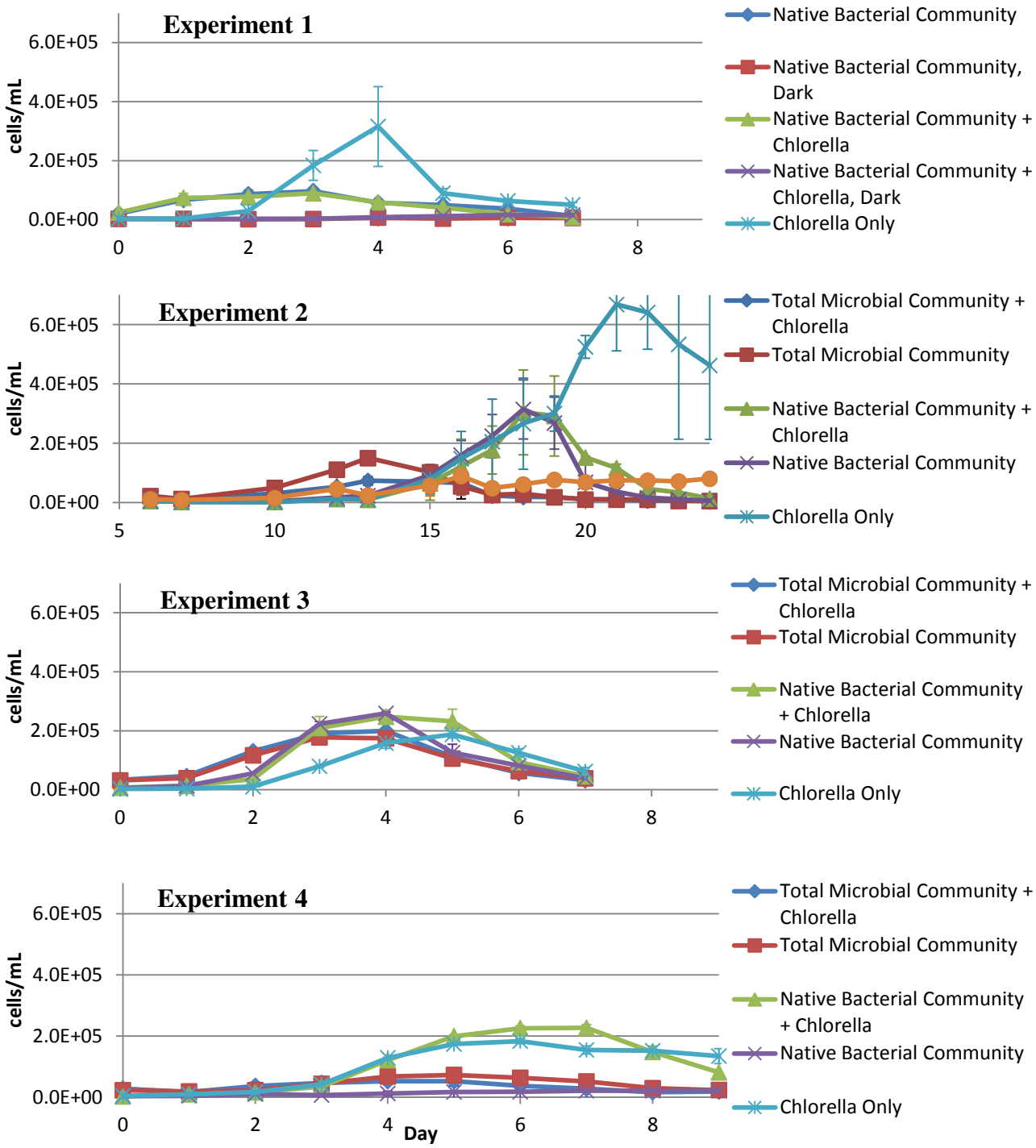


Figure 5: Algal cell abundance during each size exclusion experiment over time.

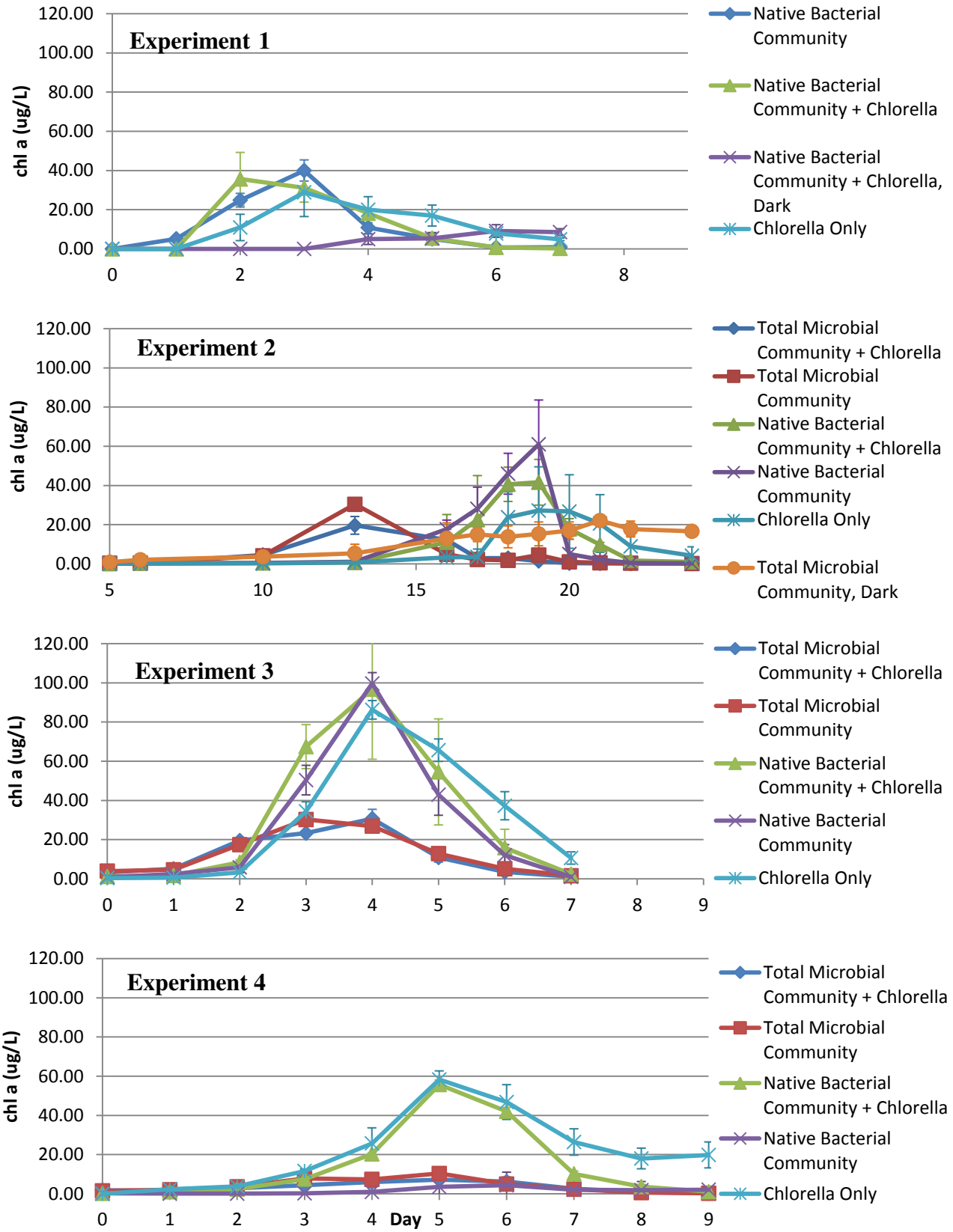


Figure 6. Chl a biomass during each size exclusion experiment over time.

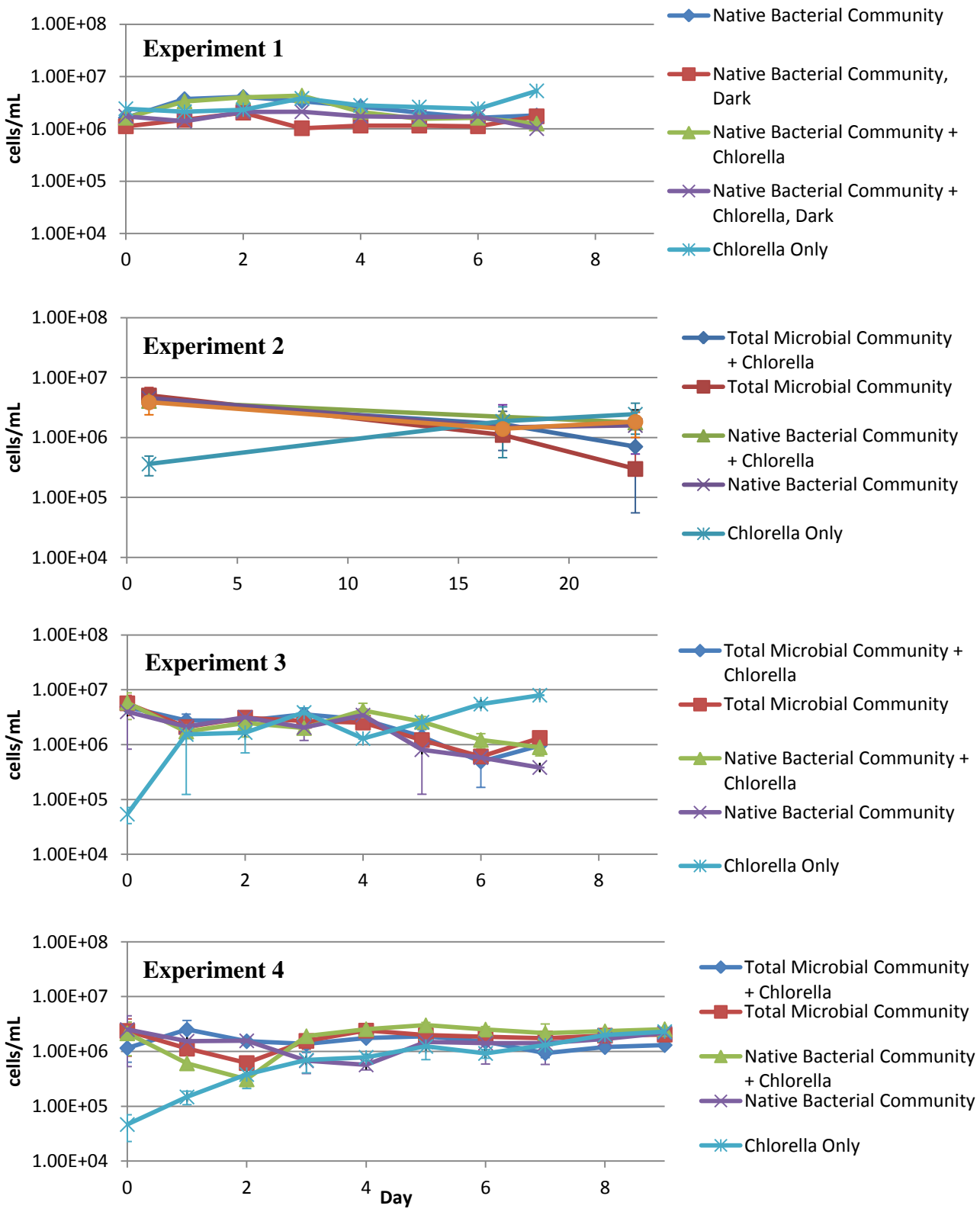


Figure 7. Bacterial cell abundances during each size exclusion experiment over time.

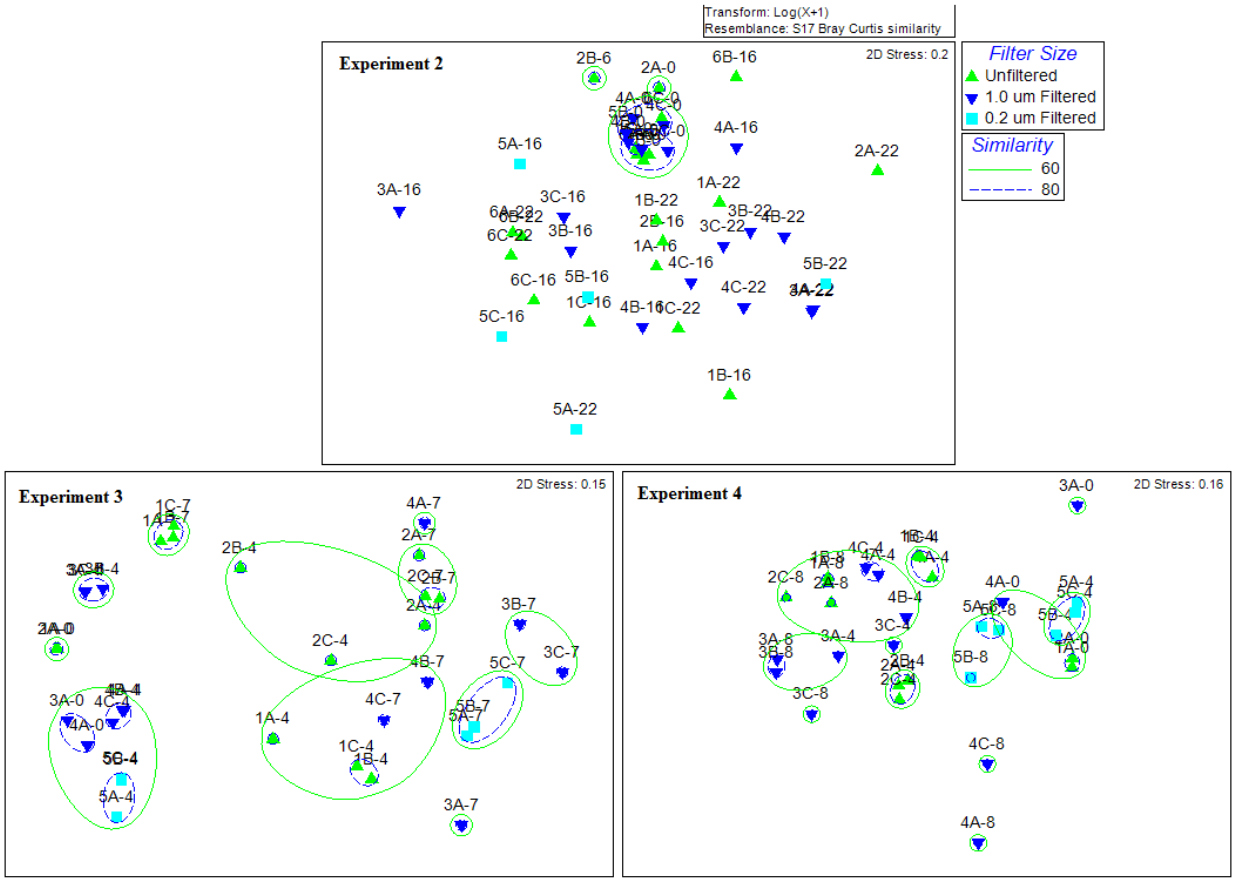


Figure 8. Bray Curtis similarity plot of bacterial community composition fingerprinting. Number-letter combination indicates treatment and triplicate, second number indicates sampling day. Treatment 1: Total microbial community + *Chlorella*. Treatment 2: Total microbial community. Treatment 3: Native bacterial community + *Chlorella*. Treatment 4: Native bacterial community. Treatment 5: *Chlorella* only. Treatment 6: Total microbial community, dark.

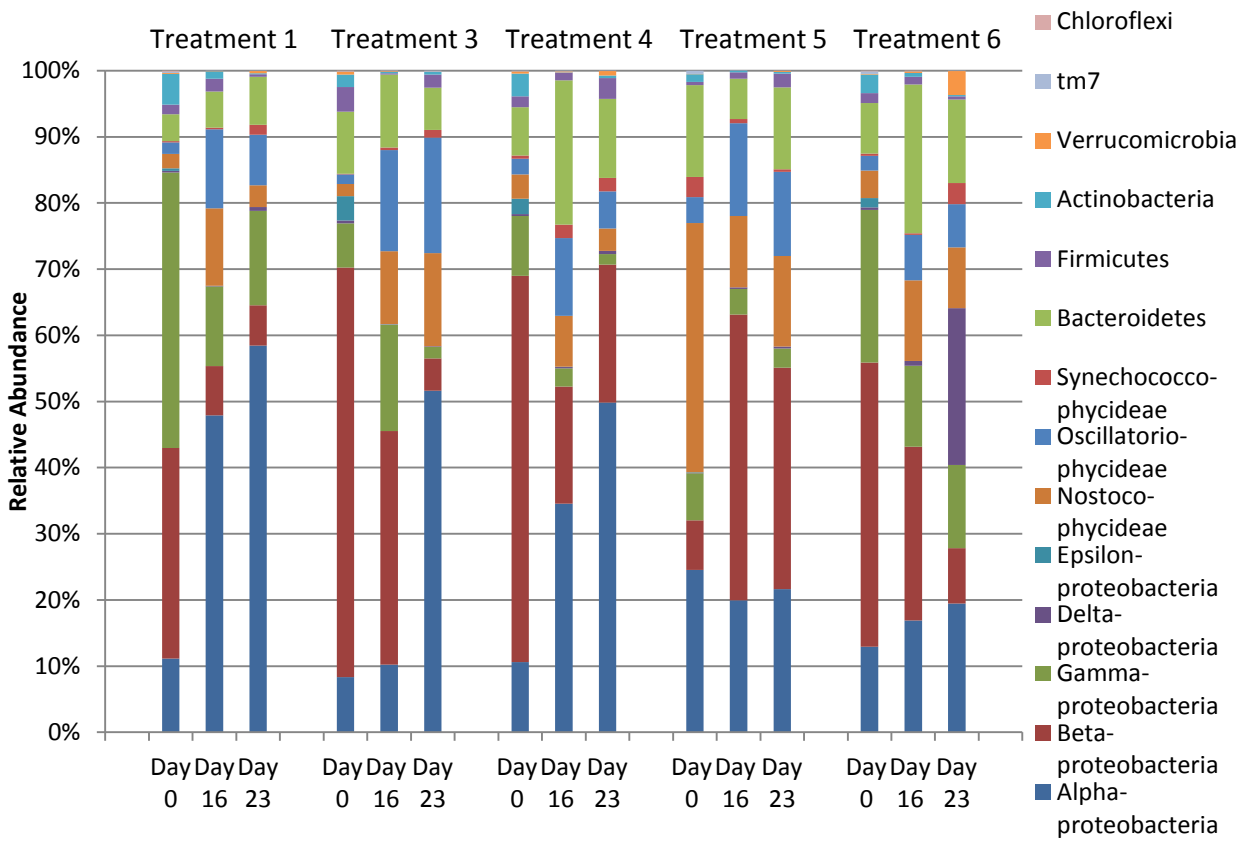


Figure 9. Histograms of the relative abundance of OTUs retrieved from each treatment during Experiment 2. Treatment 1: Total microbial community + *Chlorella*. Treatment 3: Native bacterial community + *Chlorella*. Treatment 4: Native bacterial community. Treatment 5: *Chlorella* only. Treatment 6: Total microbial community, dark.

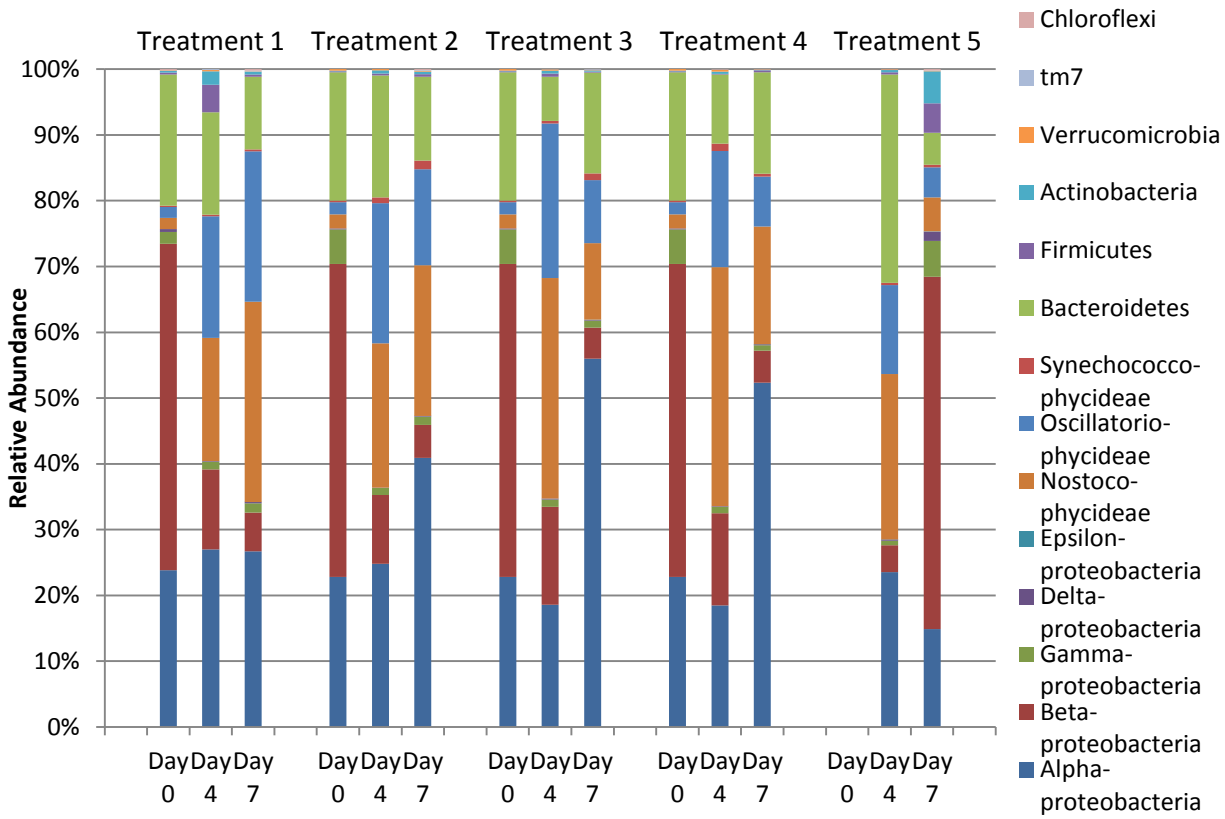


Figure 10. Histograms of the relative abundance of OTUs retrieved from each treatment during Experiment 3. Treatment 1: Total microbial community + *Chlorella*. Treatment 2: Total microbial community. Treatment 3: Native bacterial community + *Chlorella*. Treatment 4: Native bacterial community. Treatment 5: *Chlorella* only. DNA concentrations for Treatment 5, Day 0 were too low for successful sequence analysis

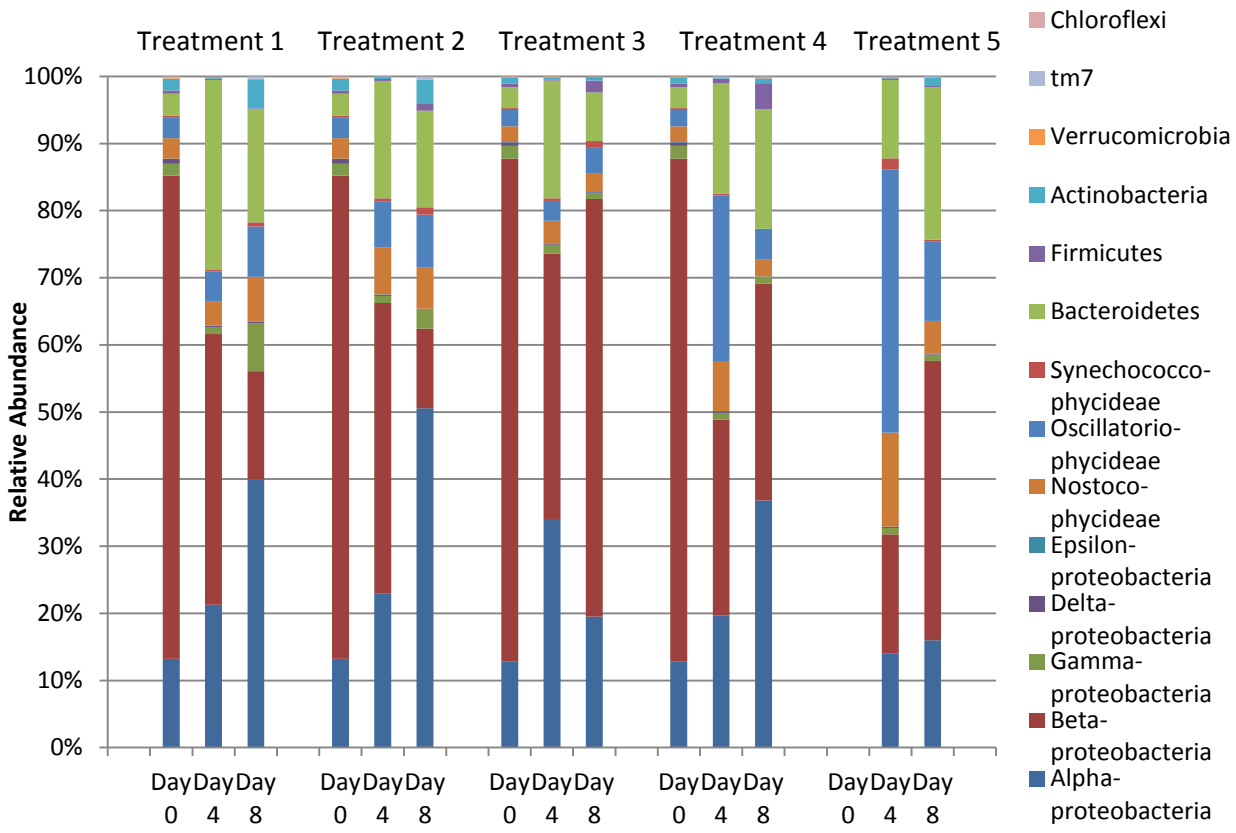


Figure 11. Histograms of the relative abundance of OTUs retrieved from each treatment during Experiment 4. Treatment 1: Total microbial community + *Chlorella*. Treatment 2: Total microbial community. Treatment 3: Native bacterial community + *Chlorella*. Treatment 4: Native bacterial community. Treatment 5: *Chlorella* only. DNA concentrations for Treatment 5, Day 0 were too low for successful sequence analysis.

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BIOGRAPHICAL SKETCH

CLAIRE SMITH

EDUCATION

Master of Science, Earth, Ocean, and Atmospheric Sciences
August 2009-present
The Florida State University, Tallahassee, FL 32306

THESIS: "Microalgae to Biofuel: An Investigation into the Role of the Native Microbial Community in the Cultivation of Algae on Wastewater."

Bachelor of Science, Chemistry
August 2005-May 2009
Ursinus College, Collegeville, PA 19426

THESIS: "Hydroboration of Single-Walled Carbon Nanotubes."

PROFESSIONAL EXPERIENCE

The Florida State University — January 2010-present
Researcher – Tallahassee, FL

Studied the cultivation of algae on wastewater effluent as a biofuel feedstock and investigated the impact of the native microbial community in wastewater on algal growth. Utilized analytical chemistry techniques to monitor biogeochemical parameters including oxygen production, nutrient uptake, and carbon dynamics. Examined the microbial community using microscopy and molecular techniques.

The Florida State University — August 2009-December 2009
Research Assistant – Tallahassee, FL

Investigated the importance of the microbial community on the degradation of carbon in peat lands. Analyzed the production of methane and carbon dioxide using gas chromatography in peat land samples with and without an inhibited microbial community.

Ursinus College — June 2008-December 2008
Research Fellow – Collegeville, PA

Studied the hydroboration and the amination of carbonyl functional groups on single-walled carbon nanotubes using ultraviolate light to facilitate the reaction. Utilized Infrared spectroscopy and UV/VIS spectroscopy to analyze results. Completed the synthesis of graphene utilizing graphite as a starting material.

Imperial College — January 2008-April 2008
Research Intern – London, UK

Synthesized lipid membranes and investigated membrane/protein interactions. Utilized a fluorescent probe and fluorescent spectroscopy to study this interaction.

Ursinus College August 2007-December 2007
Research Assistant – Collegeville, PA

Completed the synthesis of a porphyrin, utilizing column chromatography as a purification process. Analyzed results using mass spectrometry.

Ursinus College August 2006-May 2009
General Chemistry Teaching Assistant/Tutor – Collegeville, PA

Assisted students in performing laboratory experiments successfully. Answered specific questions on homework problems and lab report calculations. Worked individually with students to aid their understanding of chemistry theory.

PAPERS AND PRESENTATIONS

M. D. Ellison, L. K. Buckley, G. G. Lewis, C. E. Smith, E. M. Siedlecka, C. V. Palchak, and J. M. Malarchik. **Photochemical Hydroboration-Oxidation of Single-Walled Carbon Nanotubes,** *Journal of Physical Chemistry C*, vol. 113, p. 18536, October 29, 2009

"Microalgae to Biofuel: An Investigation into the Role of the Native Microbial Community in the Cultivation of Algae on Wastewater," presented at Florida Energy Systems Consortium Summit, Orlando, 2010.

"Hydroboration of Single-Walled Carbon Nanotubes," presented at the National Conference of the American Chemical Society, Philadelphia, 2008.

SELECTED UNIVERSITY ACTIVITIES AND AWARDS

The American Chemical Society Annual Award for Outstanding Scholastic Achievement in the ACS Approved Department of Chemistry, Philadelphia Section
W. W. Smith Scholar and Prize Winner
CRC Press Freshman Chemistry Achievement Award
Beardwood Chemical Society – Vice President