LIPID ACCUMULATION IN MIXED PHOTOAUTOTROPHIC CULTURES FROM MUNICIPAL WASTEWATER

by

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DEDICATION

I would like to dedicate this thesis to my future husband, William James, for always being loving and supportive of my career and my interests.

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ABSTRACT

The growing consumption and need for sustainable forms of energy has spurred interest in biofuels and the feedstocks that offer possible solutions. Microalgae have emerged as a possible resource for the more sustainable production of biomass and biofuel, but nutrient and water demands offer challenges that limit large-scale biomass and biofuel production. Wastewater offers a potential solution to this issue as a low-quality but high-nutrient water source that could be exploited for the production of microalgal biomass. Many studies have successfully isolated or introduced algal monocultures into a wastewater environment for either lipid production or bioremediation purposes. However, recent studies have indicated increased lipid yields and nutrient removal with mixed cultures and algal consortia, but further work is needed to understand community dynamics and population networks that increase the niche landscape with compensatory interactions that promote desired functions (*e.g.*, biomass and/or lipids) in a stable manner. This thesis seeks to explore the potential of a mixed photoautotrophic population that is currently being used to treat municipal wastewater (*i.e.*, nutrient removal) for lipid producing capabilities and the impact of indigenous bacterial populations native to wastewater. In doing so we hoped to test the effects of increased biodiversity on lipid production at an interspecific level. Community dynamics, lipid profiles, and biomass productivities were monitored over a series of growth experiments utilizing filter-sterilized and non-sterile wastewater. The results from these experiments indicated substantial lipid production from communities grown in the presence of indigenous bacterial communities found in wastewater. These results suggest the prospect that wastewater possesses for biofuel production with mixed algal communities. It also indicates a more dynamic role of *in situ* community interactions in contributing to biomass and bio-oil accumulation of mixed algal communities.

CHAPTER ONE

INTRODUCTION

Alternative forms of energy have garnered much attention with rising concerns of global warming and ever increasing energy demands. Researchers have begun placing an emphasis on the types of alternative energy resources that may meet current and future energy needs. Within this research, microalgae have received much attention for their ability to produce large amounts of lipids that can be converted into biodiesel (Fields et al., 2014). Yet, limitations exist in terms of fresh water sources and nutrient content that make scale-up efforts difficult (Fields et al., 2014; Hannon et al., 2010). Wastewater offers a potential solution to some of these limitations by offering a low-quality but high nutrient water source (Fields et al., 2014; Bell et al., 2015). Many studies have documented the ability of microalgae to grow in wastewater environments as a more sustainable treatment option for inorganic nutrient and heavy metal removal, but more recent studies have also documented the ability of microalgae to produce lipid while grown in wastewater (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013; Oswald et al., 1957). Although these studies have begun to examine the ability of microalgae to produce lipid in wastewater systems, few have examined the potential relationships and effects of community dynamics between co-cultured taxa including the indigenous bacterial communities. Close relationships between microalgae and *Bacteria* have exhibited the possibility of a symbiotic relationship (Croft et al., 2005, Zhao et al., 2012; Sapp et al., 2006; Bell et al., 2015). The

nature of these relationships is still largely unexplored, but there is evidence that these associations allow algal and bacterial species to exchange important metabolites (Bell and Mitchell, 1972; Zhao et al., 2012; Sapp et al., 2006; Bell et al., 2015). Research into this area has begun to expand but there is little evidence on the effects of these relationships on algal biomass production and lipid accumulation. The purpose of the data presented in this thesis is to explore the biofuel prospect of mixed photoautotrophic communities grown in wastewater as well as the role of *in situ* community interactions in contributing to biomass and bio-oil accumulation.

Microalgae as a Biofuel Source

Microalgae are extremely efficient at fixing carbon dioxide into biomass that may later serve as possible foods, biofuels feedstock, and other high value co-products (Chisti et at., 2008; Fields et al., 2014). These capabilities have largely been overshadowed by the usage and over presence of other plant and animal based biofuels (*e.g.*, corn oil, palm oil, soybean oil). However, microalgae have demonstrated interesting biofuel and agricultural advantages over many of the resources currently being exploited. Firstly, microalgae are unique in their ability to grow in suspension with a doubling time of only a few hours (Chisti et al., 2008). By comparison, other biodiesel prospects require longer time periods to reach maturity and large land requirements that could compete with agricultural spaces and production. One study demonstrated that land requirements of traditional biodiesel crops were unfeasibly large with some crops exceeding more than 100% of total U.S. cropland (Chisti et al., 2008). The same study estimated that

microalgae were unique in requiring between 1-3% of total cropland to meet at least 50% of current U.S. transportation fuel needs (Table 1.1).

Table 1.1: Comparison of biodiesel sources with regard to needed cropland percentages needed to meet 50% of current U.S. transportation fuel needs. Chisti et al., 2008.

Crop	Oil yield (L/ha)	Land area needed (M ha) [a]	Percent of existing US cropping area [a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae [b]	136,900	2	1.1
Microalgae [c]	58,700	4.5	2.5

[a] For meeting 50% of all transport fuel needs of the United States.
[b] 70% oil (by wt) in biomass.
[c] 30% oil (by wt) in biomass.

Microalgae offer further advantages over other biodiesel sources in their ability to store larger amounts of long chain hydrocarbons commonly found in biodiesel. In fact, many studies have shown that common fatty esters found in biodiesel, palmitic acid, oleic acid, linoleic acids, and linolenic acids, are more enriched in microalgal oils than other plant based oils (Chisti et al., 2008; Lohman et al.,2014; Knothe et al., 2008). This enrichment of polyunsaturated fatty acids and fatty acid methyl esters (FAME) found in microalgae is coupled with an increased storage ability over other biodiesel sources. One study demonstrated that the algal species *Botryococcus braunii* was able to accumulate close to 50% of its dry mass as long chain hydrocarbons (Kojima et al., 1999). In part, the ability of microalgae to efficiently acquire such high lipid content is thought to be a naturally occurring process associated with nutrient limitations such as nitrogen and phosphorus (Fields et al., 2014; Lohman et al., 2014; Eustance et al., 2013). It is argued

that this process is a beneficial result of carbon dioxide fixation that allows lipid to be stored and later used when periods of stress are relieved (Hannon et al., 2010).

Scale-Up Challenges

Despite the ability of microalgae to produce and store higher contents of long chain hydrocarbons compared to other biofuel sources, there are significant challenges that scale-up attempts face in order to optimize the production of microalgal biofuels. These challenges include limitations for infrastructure, nutrients, and water as well as optimizing trade-offs between biomass levels and lipid levels.

The infrastructure used to grow and harvest microalgae are a significant area for consideration. Photobioreactors (PBR) have been considered a viable culturing method for the growth of microalgal cultures. PBRs are unique in offering a closed system resistant to contamination and better stability, but high costs of maintenance and poor gas exchange have limited scale-up opportunities in these systems (Fields et al., 2014; Pulz et al., 2001). Open raceway ponds have offered an alternative by allowing algal growth in an open system which is a more cost effective and economic alternative to closed system production. Yet, these open systems restrict the ability to control nutrient and growth parameters that may ultimately decrease lipid production compared to PBR systems (Fields et al., 2014; Norsker et al., 2011). Harvesting techniques further challenge scale-up attempts in much the same way of culture techniques and is a major economic consideration. Some methods of algal harvest have included filtration systems or centrifugation to collect biomass, but both methods are expensive especially at large

scales making them economically non-viable (Barros et al., 2015). Flocculation techniques through biotic or abiotic methods have also been researched. Abiotic techniques utilize salts, electrostatics, or polymer binding to harvest algal biomass, but limitations exist in these methods if the resulting biomass is contaminated by the chemical agent used making downstream processing or co-products unusable or more difficult to treat (Barros et al., 2015; Smith et al., 2012; Papazi et al., 2010). Biological flocculation has gained interest because it does not contaminate either the algal biomass or the remaining water with chemical precipitates or solutes allowing full recovery of water resources as well as easy downstream processing of harvested biomass (Salim et al., 2011). Among these techniques, biological flocculation using some bacterial species have been successfully performed in wastewater systems (Lee et al., 2009). The results of these microbial flocculation experiments are promising in maintaining algal cell integrity while also allowing a low cost of materials and production, but some argue that the contamination of bacterial groups from this type of technique could lead to negative effects in some pure culture systems (Salim et al., 2011).

Another area of complexity that adds to the challenge of commercial microalgae biofuel production are nutrient limitations. While algae are pervasive in many types of nutrient replete and deplete environments there are a number of key nutrients most algal species need for replication and growth. Nitrogen, phosphorus, iron, and sulfur are among the major nutrients needed for algal growth (Hannon et al., 2010). Under ideal conditions (*i.e.* adequate nutrient conditions) cellular stress is low and biomass productivities are high (Fields et al., 2014). However, while these nutrients are essential

for key cellular processes; each nutrient may not be found in appropriate concentration or a bioavailable form to support algal growth (Hannon et al., 2010). This lack of certain nutrients in different environments offers stress to the cell that limits biomass synthesis and instead increases metabolic activity toward lipid synthesis (Valenzuela et al., 2012). Commercial algal production is limited by this interplay of nutrient ratios in the optimization of either biomass productivities or lipid productivities.

A final challenge to scale-up attempts for algal biofuel production is water resources. It is plausible that any large scale production effort using algae will require a substantial amount water (Hannon et al. 2010). In an effort to increase sustainability, low-quality water resources will have to be considered in order to avoid competition for limited fresh water resources that are needed for human and agriculture needs. Algae are unique in their ability to grow in low-quality water sources while also contributing the added benefit of inorganic nutrient removal and water treatment (Fields et al., 2014; Hannon et al., 2010). These bioremediation capabilities effectively allow removal of inorganic nutrients from a multitude of environments including oceanic, dairy wastewater, industrial wastewater, and municipal wastewater (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013; Wrabel et al., 2000). The ability of microalgae to grow in such environments offers alternatives to costs associated with scale-up opportunities by cultivating microalgae in low-quality water sources that could also benefit from algal treatment to remove unwanted nutrients. A continued discussion on the bioremediation capabilities of microalgae specifically in wastewaters continues in the next section.

Microalgae as Wastewater Treatment

The chemical composition of wastewaters can be highly variable dependent upon the specific municipality and surrounding industries that feed into the water treatment system. Many municipal wastewaters tend to be high in ammonia-nitrogen, phosphorus, carbon, and some salts (Hannon et al., 2010; Eustance et al., 2013). The treatment of wastewater is an important infrastructural process that allows water to be cleaned and recycled to areas of societal need. To do so, a great deal of time and effort is spent to remove pollutants and harmful nutrients from the water before discharging into a lake or stream system. The goal of such nutrient removal is to reduce the possible negative environmental or ecological effects of water discharge into these natural systems. Two of the most important nutrients removed to avoid these effects by possible artificial eutrophication are nitrogen and phosphorus (Eustance et al., 2013; Sedlak, 1991). Typical treatment techniques in the removal of nitrogen and phosphorus utilize both chemical and biological processes to meet certain post treatment standards. While these standards may vary between municipalities, typical soluble organic nitrogen and phosphorus contents require less than 1mg/L nitrogen and 1-2 mg/L phosphorus post treatment (Sedlak, 1991). To meet these requirements, nitrogen is often removed through cellular assimilation or denitrification using microorganisms that reduce nitrate to nitrogen gas while phosphorus is typically removed using chemical precipitation (Oswald et al., 1957).

By comparison, studies using microalgae as a treatment option to these municipal systems have demonstrated close to a 99% reduction in nitrogen and phosphorus from

wastewater systems (Oswald et al., 1957). To do so, algal species assimilate large amounts of nutrients into biomass that can then be harvested from the water. Advantages to using algal bioremediation systems include lowered costs compared to traditional aeration systems in which aerobic bacteria consume organic compounds (Green et al., 1996). Instead, algal systems more efficiently consume nutrients and provide oxygen to aerobic bacteria needed to consume organic compounds through photosynthesis (Oswald et al., 1957). The potential to utilize the algal biomass produced by such remediation steps is large, especially given the challenges faced by biodiesel production efforts. Theoretically, the marriage of the bioremediation capabilities and the lipid accumulation abilities of microalgae might provide a much needed solution to scale-up attempts.

Monoculture Approaches

Typical algal cultivation techniques focus upon utilizing monoculture approaches to test lipid production and biomass accumulation (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013; Lohman et al., 2013). Specifically, these techniques often focus upon strain selection where isolates are grown in highly controlled photobioreactor (PBR) systems. While PBRs can be costly to maintain, a closed system advantageously protects algal monocultures from environmental contamination while also offering controlled nutrient influx (Pulz et al., 2001). To encourage lipid production, these culture techniques focus on key nutrient manipulations that stress algal growth and can promote high lipid production (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013; Lohman et al., 2013; Fields et al., 2014). Many studies

have focused on using the stress of nitrogen and phosphorus starvation to stimulate lipid production. Nitrogen and phosphorus are both critical cellular constituents needed for DNA replication and algal growth (Fields et al., 2014; Valenzuela et al., 2012). During nitrogen and phosphorus limited conditions algal cells are unable to replicate and continue cellular growth as normal. Cells accumulate lipid as a potential carbon or electron storage molecule that can be consumed to prolong cellular life (Valenzuela et al., 2012). Studies testing these parameters have demonstrated increases in lipid content corresponding with nitrogen and phosphorus depletion from monoculture type experiments (Figure 1.1).

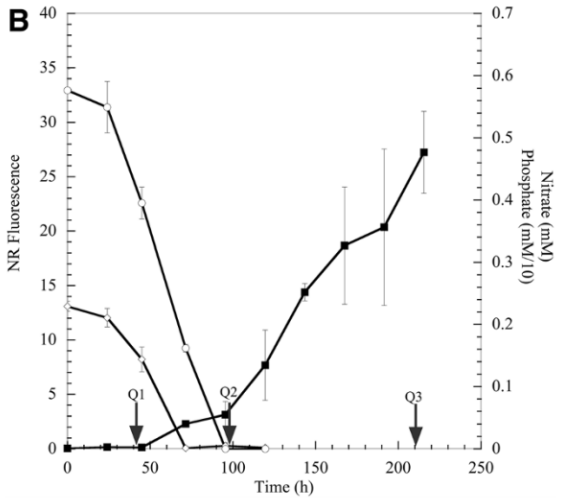

Figure 1.1 Depletion of nitrogen (○) and phosphorus (◊) along with Nile Red fluorescence intensities (■). Arrows represent time points where cells were collected for RNA sequencing (Valenzuela et al., 2012).

Consortium Approaches

While monoculture systems have provided important information regarding growth patterns of specific algal strains, there are arguments in the literature that these systems possess drawbacks in scale-up or open environments. Specifically, arguments regarding monoculture or PBR type systems point to the likelihood of accidental contamination and/or "crashes" as well as the high costs of maintaining a pure culture (Stockenreiter et al., 2012; Liu et al., 2016). Instead, some research has begun to focus on work with a consortium, where a mixture of different algal strains are grown together, or mixed communities, where algae and other taxa of *Bacteria* and/or *Archea* are grown together, in an effort to increase biodiversity and competition against potentially invasive organisms (Stockenreiter et al., 2012 & 2016; Liu et al., 2015; Bell et al., 2015. Indeed, most if not all, natural systems have evolved to operate with multiple species in which the system compartmentalizes into interacting modules (Lindemann et al., 2016). Invasive species pose a particular threat if they successfully inhabit a culture by lowering crop productivity and increase competition. Of these studies, Stockenreiter et al. (2016) conducted a unique set of experiments in a filter sterilized wastewater environment comparing monoculture lipid production to that of three different consortia of three, five, and seven algal species (Stockenreiter et al., 2016). Not only were Stockenreiter et al. (2016) able to show that the algal consortia had increased growth rates and faster inorganic nutrient removal than monoculture strains, but some consortia showed increased lipid productivity (Figure 1.2). However, attributes of the mixed cultures that contributed to increased lipid productivity were not discussed.

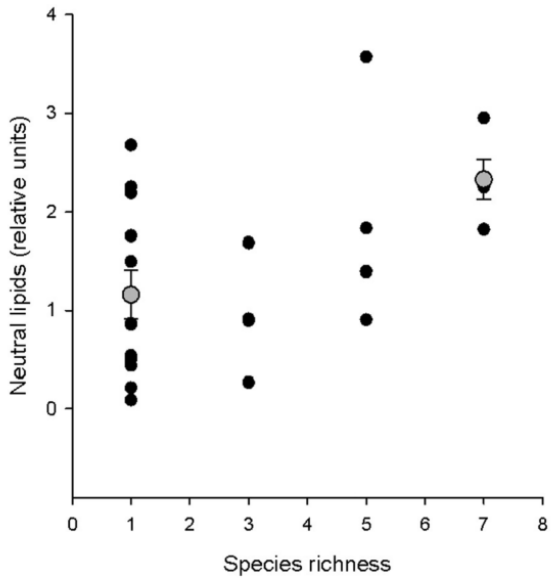

Figure 1.2: Neutral lipid content plotted against species richness at the end of growth. All monocultures were included (n=12) as well as randomly selected communities of three, five, and seven species (n=4). Grey dots represent mean values (+/- SE). (Stockenreiter et al., 2016).

This result is pertinent because it demonstrates a dynamic relationship between algal members and niche occupancy. It is also suggestive of possible lipid accumulation outside of traditional nutrient starvation so often discussed in the literature. While this study used a mixed consortium of algal strains, none of the strains selected were isolated from wastewater environments potentially belaying niche optimization of algae more fit to thrive in these environmental conditions (Stockenreiter et al., 2016). Also, this experiment utilized filter-sterilized wastewater, which could potentially misrepresent challenges to community structure and niche occupancy if an indigenous bacterial or eukaryotic community were present during inoculation and growth. Research addressing

the potential relationships between algal species and *Bacteria* are explored in the next section.

Bacterial and Algal Community Relationships

Close relationships between microalgae and *Bacteria* have long been an area of study (Bell and Mitchell, 1972). More recent work has begun to demonstrate the possibility of a symbiotic relationship between some algal and bacterial species (Croft et al., 2005, Zhao et al., 2012; Sapp et al., 2006; Bell et al., 2015). The nature of these relationships is still largely unknown, however, there is evidence to suggest that bacterial relationships within the exopolymeric area surrounding the algae or "phycosphere" allow algal and bacterial species to exchange important metabolites such as cobalamin (vitamin B_{12}), oxygen, and carbon (Bell and Mitchell, 1972; Zhao et al., 2012; Sapp et al., 2006; Bell et al., 2015). While these close relationships have been shown to exist in open and *in situ* environments, little research exists regarding the effects of these relationships on lipid production and niche occupancy potential (Zhao et al., 2012; Bell et al., 2015; Stockenreiter et al., 2012 & 2016; Liu et al., 2015).

Summary

Based upon a review of current literature, microalgae have demonstrated the potential to contribute to a renewable energy solution. Not only are microalgae exceptional at producing long chain hydrocarbons, but they also offer other high value products for food, medicine, and bioremediation (Fields et al., 2014; Hannon et al.,

2010). There are important considerations to be accounted for when addressing scale-up projects that might utilize microalgae potential, and in the effort of reducing costs and impact low-quality but high nutrient water sources should be explored (Hannon et al., 2010). One of the most promising areas where microalgal growth could utilize such an environment is wastewater. Not only is this water source high in essential nutrients for algal growth, but microalgal growth offers the added benefit of bioremediation and water treatment that also utilizes atmospheric CO_2 (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013; Fields et al., 2014). Here, microalgal growth could be used to assimilate the nutrients (nitrogen and phosphorus) into biomass that might otherwise require more expensive removal processes. Research has shown that microalgae grown in waste environments can produce substantial amount of lipid and further studies have indicated that a consortium of algae might increase biomass and lipid yields compared to monocultures (Stockenreiter et al., 2012 & 2016; Liu et al., 2016; Bell et al., 2015). Continued research in this area is still to be done especially when considering the influence that expanded interspecific consortia and *in situ* bacterial and eukaryotic community members might have in such environments and the potential to optimize processes that have the capacity to contribute to carbon negative technologies.

CHAPTER TWO

MATERIALS AND METHODS

Mixed Photoautotrophic Community

The mixed photoautotrophic community tested in this study was provided by the company Clearas Water Recovery. Located in Missoula, Montana, Clearas Water Recovery utilizes a mixed consortium of photoautotrophic organisms to remove nitrogen and phosphorus from a municipal wastewater system. The isolated community was taken from these consortia and delivered as concentrated slurry to the Center for Biofilm Engineering. The community was refrigerated and shipped overnight. Upon arrival, the concentrated slurry was diluted in half and immediately inoculated into 500mL Erlenmeyer flasks at 10% volume. Both filter-sterilized wastewater collected from the Bozeman Water Reclamation Facility (BWRF) and Bold's Basal Media (BBM) served as media types for stock cultures. Stock cultures were maintained in an incubator at 20°C and a light:dark cycle of 14 hours of light followed by 10 hours of dark. The use of a native community has the benefit of already being adapted to the intended growth environment. Most studies look for a species that will perform well as a monoculture and then proceed to determine if lipid production is possible in a wastewater environment. Here, the environment has naturally selected for a community of microalgae and bacteria capable of growth and bioremediation in a low quality water source.

Medium Composition

The experiment tested community responses to three media types. Liquid cultures of Bold's Basal Medium (BBM), filter sterilized municipal wastewater (FILT), and non-sterilized municipal wastewater (RAW) were tested. Municipal wastewater was collected from the effluent of the primary clarifier at the Bozeman Water Reclamation Facility (BWRF) in Bozeman, Montana. The water was collected and immediately stored in large 10 L plastic containers and stored at 4°C until the time of the experiment. Sterile media of both BBM and BWRF were sterilized using a 0.2 μm polyethersulfone filter (Corning Inc., Corning, NY).

Growth Conditions

The community was maintained in an incubator at 20°C. A light:dark cycle of 14 hours of light followed by 10 hours of dark was used to replicate the conditions likely seen during longer summer days and shorter nights of environmental growth patterns. Experiments were conducted in triplicate using 250 mL Erlenmeyer flasks containing 150 mL of BBM, filter sterilized, or non-sterile BWRF water. Flasks were placed on a Labnet Orbit 1900 Heavy Duty Shaker at 125 rpm to encourage mass transfer of carbon dioxide into the medium. Samples of 3mL were taken daily from each batch culture an hour before dark cycles began. Samples were also monitored for evaporation and sterile water was added to each flask to return lost water volumes.

Anion Concentrations

Nitrate and phosphate concentrations of culture samples were determined daily using a 1mL sample on a DIONEX ICS-1100 and Chromeleon Chromatography Management System with an ASRS 4 mm suppressor. A 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate eluent mix was used.

Determination of Ammonium Concentration

Ammonium concentrations were determined as per Rhine et al. (1998) using the 2-Phenylphenol method. Briefly, 25 µL of sample were prepared in triplicate for each time point in a 96-well plate. Reagents of Citrate, 2-Phenylphenol-Nitroprusside, and buffered Hypochlorite were added and allowed to incubate at 37°C for 15 minutes. Absorbance readings were taken using a Synergy H1 hybrid reader. Gen5 microplate reader software was used to evaluate the fluorescence at an absorbance of 660nm (Rhine et al., 1998).

pH

pH was measured with a standard benchtop Oakton pH 11 Series meter. The pH meter was calibrated daily before readings using pH standards of 4.0, 7.0, and 10.0 before each use.

Cell Counts

Cell counts were used to track the growth of the community over time. Cell counts were taken using a standard hemocytometer. However, the presence of multiple organisms in the culture, specifically filamentous cyanobacteria, made accurate cell counts difficult. For this reason, chlorophyll and optical density measurements were used in conjunction to monitor growth.

Chlorophyll Measurements

Chlorophylls a, b, and carotenoids were extracted from 1 mL aliquots using the methanol extraction and optical absorption process previously described Ritchie et al. (2006). Briefly, 1 mL samples of culture were placed in 1.7 mL microcentrifuge tubes and centrifuged for 5 minutes at 16,000 x g. After centrifugation, supernatant was removed and discarded. One mL of 100% methanol was then added to the remaining pellet and re-suspended using vortex and sonication. The solution was then heated for 10 minutes in a water bath of 70°C. Finally, the solution was centrifuged again at 16,000 x g for 3 minutes before readings at 666nm, 654nm, and 470nm were taken. Chlorophyll calculations were conducted as described by Ritchie et al. (2006).

Optical Density

Optical density readings were taken during growth to monitor the accumulation of biomass over time. 200 µL of sample culture were measured in triplicate into a 96-Well plate and absorbance readings taken using a Synergy H1 hybrid reader. Gen5 microplate

reader software was used to evaluate the absorbance at 750nm. Blanks were read according to the specific media types respective to the culture. Samples of high optical density were diluted and final concentrations calculated.

Lipid Accumulation

Accumulation of lipids was tracked using the Nile Red method developed by Chen et al. (2009). 4 µL of Nile Red solution (0.25 µg/mL) were mixed in 800 µL of sample along with 200µL of DMSO. Samples were then incubated in a 96-well plate in the dark for 5 minutes, which was previously determined to be the optimal absorption time for these cultures. Nile Red fluorescence was measured using a Synergy H1 hybrid reader. Gen5 microplate reader software was used to evaluate the fluorescence at an excitation of 530 nm and emission of 575 nm. Specific Nile Red fluorescence was then calculated by dividing the fluorescent signal by cellular density for each culture. A final scaling of 10,000 was used to better represent data without decimal places and provided a scaling range of 0-100.

Biomass Collection

Biomass samples were taken during culture harvest to measure accumulation of lipids and microbial community profiles. Once a Nile Red peak was reached, each batch culture was harvested and centrifuged at 4800 x g and 4°C for 10 minutes. 200 µL of concentrated biomass was frozen separately for community analysis and the subsequent

remaining pellets were then lyophilized (Lobconco lyophilizer, Kansas City, MO) for 48 hours before being stored at -20°C prior to lipid extraction.

Lipid Extraction and Transesterification

Lipid extraction and analysis was completed as previously described by Lohman et al. (2013) before overall fatty acid methyl esters (FAME) composition was analyzed using GC-MS. Approximately 30 mg of dried biomass were transferred in duplicate for each culture type for the analysis. At the end of extraction, 1 mL of the separated organic phase was placed into a 2mL GC vial for GC-MS analysis.

FAME Analysis Using GC-MS

Using gas chromatography-mass spectrometry (GC-MS) an analysis of total cellular fatty acids (FAME) was performed as previously described by Lohman et al. (2013). FAME quantification was determined by quantifying each response peak with the nearest eluting calibration standard based on retention time, using MSD ChemStation software (Ver. D.02.00.275).

DNA Extraction

DNA was extracted using 200µL of pelleted batch culture after harvesting using a FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH) according to the manufacturer protocol. DNA extractions were also completed from 0.2 µm filters of BWRF water to assess indigenous community members in the water. 500mL of water were filtered

through each filter before extraction. The DNA was cleaned and concentrated with the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Extracted DNA was quantified with a Qubit fluorometer using a Qubit dsDNA BR detection kit (Invitrogen, Carlsbad, CA).

Gene Amplification and Sequencing

PCR primers for the SSU rRNA gene sequences for both *Bacteria* and *Eukarya* were used, and each sample was amplified in triplicate and later labeled with a unique 10 nucleotide-barcode for multiplexing. The SSU rRNA gene sequences for *Bacteria* and *Eukarya* were amplified using 25 cycles of PCR with the subsequent barcoded primers; 7F/591R (7F 5′-ACCTGGTTGATCCTGCCAG-3′ and 591R 5′-GGAGCTGGAATTACCG-3′) to target the variable region V1-V3 of *Bacteria* (Bowen De León et al., 2012) and *Eukarya* 8F/529R (8F 5′-AGAGTTTGATCCTGGCTCAG-3′and 529R 5′-CGCGGCTGCTGGCAC-3′). Amplicons were then purified using the AMPure Xp magnetic bead capture kit (Beckman Coulter, Inc., Brea, CA) before ligation of Illumina adaptors for sequencing. Amplicons were quantified using Quant-iT PicoGreen Assay Kit (ThermoFisher Scientific, Waltham, MA). Libraries from purified sample amplicons were pooled in equimolar amounts with an Illumina PhiX control library. Sequencing was then completed using an Illunima MiSeq Kit v3 600 cycle (Illumina Inc., San Diego, CA).

Community Analysis

Using the Quantitative Insights into Microbial Ecology (QIIME) software package, version 1.7.0 (Caporaso et al,. 2010b) microbial sequences were processed and analyzed. Raw sequences were demultiplexed and libraries split with respect to relative domains (*Eukarya* and *Bacteria*). Sequences smaller than 200 nt, quality scores below 25, or of chimeric identity were culled. Operational taxonomic units (OTUs) were assigned using a closed reference OTU picking protocol and clusters were referenced against a Silva 108 database and pre-clustered at a 97% sequence identity cut-off using UCLUST (Edgar, 2010).

Sequence reads matching Silva reference sequence were clustered within an OTU by a reference sequence. OTU assignments were performed for combined bacterial and eukaryotic reads. Using PyNAST (v1.1) default parameters, sequences were aligned and filtered. Centroid sequences for each cluster were selected as representative and aligned with a Silva core reference set using PyNAST (Caporaso et al., 2010a). OTUs failing to align with PyNAST or those identified as singletons were removed and discarded from further analysis. Following quality filtering and grouping, taxonomic assignments were made using the RDP Classifier program (Wang et al., 2007) with 80% confidence rating against the Greengenes database (McDonald et al., 2012) on the Silva database for phylogenetic resolution at the genus level.

Heat-maps created using the R vegan package version 2.0-10 (Oksanen, 2011) were generated from relative abundances. Given that some taxa were not present (relative abundance of 0%), 0.1 was added to all time points to allow transformation.

Distributions of taxa were then normalized using a square root transformation which helped to illustrate important co-occurrences of community members.

Statistical Analysis

A detrended coordinate analysis (DCA) was used to identify OTU distribution patterns amongst and between treatment types. Due to the unimodal nature of this data set, a DCA analysis was selected because it does not use Euclidean distances to assume linear trends between taxa but instead utilizes a χ^2 based metric that disregards any double species absence (Ramette et al., 2007). Results not only indicate potential interactions amongst taxa but also abundances of species relative to treatment types. This analysis was selected over the alternative nonmetric multidimensional scaling (NMDS) based upon the assumption that niche occupancy and existence played an important role in the tested environments (Ramette et al., 2007).

In order to identify differentially abundant OTUs between treatment types, the biomarker discovery algorithm LEfSe [linear discriminant analysis (LDA) effect size] was used. LefSe operates by first conducting a nonparametric Kruskal–Wallis sum-rank test to identify taxa with significantly different relative abundances between groups of interest. LefSe then conducts an LDA to these identified taxa ($p < 0.05$) to assess the effect size of each differentially abundant OTU. Effect sizes are then ranked to determine OTUs of greatest difference between treatment groups.

Preliminary Experiments

Preliminary experiments were performed to establish the ability of the sampled mixed photoautotrophic community to produce lipid. To address this question, the photoautroph communities were tested in two media types. The first being Bold's Basal Medium (BBM) a defined medium often used in laboratory settings for algal growth as well as sterilized primary effluent from the Clearas Recovery center (PCW). Each preliminary experiment was done with three biological replicates. Three technical replicates were also taken for each measurement of pH, cell counts, chlorophyll, Nile Red, and water chemistry.

Cultures grown in PCW had a quick but short increase in growth as was evident from optical density readings. Cultures grown in PCW began to display signs of stress based upon cessation of growth and decline in chlorophyll over time. The PCW cultures did display a spike in overall Nile Red that correlated with signs of cellular stress. While Nile Red readings were elevated, relatively low lipid production with fatty acid methyl ester (FAME) content at close to 15% (weight/weight) was observed from GC-MS analysis. The BBM cultures had a longer growth period that corresponded to small increases in biomass from optical density (OD) readings. Nile Red readings did not significantly increase in conjunction with signs of cellular stress; however, FAME content for BBM treatment of cultures did exhibit an overall increase close to 26% (weight/weight).

Data and observations from these preliminary experiments indicated some important conclusions that became the foundation for the next set of experiments. Firstly,

overall growth between the two media types demonstrated a higher biomass production in BBM over the PCW. Secondly, specific Nile Red was low for both culture conditions, and this was confirmed with GC-MS quantification. Finally, while overall FAME production between treatment types indicated BBM samples displayed higher content to that of PCW cultures; PCW cultures were shown to be capable of lipid production under the conditions tested.

With these results, further questions arose as to the nature of the community interactions taking place during growth. Experiments were set to determine if the same mixed community could produce lipid and sustain biomass in another municipality's wastewater and what, if any, interactions might be seen with an indigenous bacterial community. The results of these final experiments are the subject of the next chapter.

CHAPTER THREE

LIPID ACCUMULATION IN MIXED PHOTOAUTOTROPHIC CULTURES FROM MUNICIPAL WASTEWATER

Contribution of Authors and Co-Authors

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Contributions: Primary investigator, experimental design, and manuscript editing.

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Abstract

Microalgae have emerged as a potential resource for the sustainable production of biomass and biofuel but limitations remain with nutrient and water demands that challenge large-scale biomass and biofuel production. Wastewater offers a prospective solution to this issue as a low-quality but high-nutrient water source that might be exploited for the production of microalgal biomass. In this experiment, a mixed photoautotrophic population being used to treat municipal wastewater in Missoula, Montana for the removal of nitrogen and phosphorus was measured for biomass and lipid production. Community dynamics were simultaneously assessed using small-subunit rRNA paired-end sequencing to correlate communities, nutrient utilization, and lipid accumulation. The results of these experiments indicated substantial lipid production of close to 47% (weight/weight) from communities grown in the presence of indigenous bacterial communities found in the wastewater primary effluent. These results offer an indication of the possibilities that wastewater possesses for biofuel production with mixed algal and bacterial communities. It also suggests a more dynamic role of *in situ* community interactions in contributing to biomass and bio-oil accumulation of mixed algal communities.

Introduction

Energy consumption in the U.S. encompasses many areas of industrial, commercial, and residential sectors (www.eia.gov). Ever increasing demands for renewable energy resources have placed much attention on alternative forms of energy and their optimization. Among these alternative forms of energy, microalgae have received much attention for their ability to produce food, medicines, biofuel feedstock, and other high value products (Fields et al., 2014). However, challenges regarding nutrient and water demands have made large-scale biomass and biofuel production from microalgae limited (Fields et al., 2014; Hannon et al., 2010). Wastewater has appeared as a possible solution to some of these limitations by offering a low-quality but high nutrient

water source that might be exploited for the production of microalgal biomass (Fields et al., 2014; Bell et al., 2015).

Wastewater composition can be highly variable dependent upon surrounding municipalities, industries, and agriculture; however, current research has demonstrated the ability of microalgae to grown in a variety of wastewater environments (Eustance et al., 2013; Stockenreiter et al., 2016; Liu et al., 2016). Additional bioremediation capabilities of microalgae have demonstrated the removal of inorganic compounds offering an efficient and cost effective alternative to traditional wastewater treatments (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013; Oswald et al., 1957). Research has also shown that microalgae grown in wastewater environments can produce substantial amounts of lipid when grown as either a monoculture or algal consortia (Stockenreiter et al., 2012 & 2016; Liu et al., 2016; Bell et al., 2015).

While, traditional growth experiments have focused on monocultures of specific algal strains manipulated through nutrient starvation and stress, arguments have been made against this culture system pointing to the likelihood of contamination as well as the high costs of maintaining a pure culture (Stockenreiter et al., 2012; Liu et al., 2016). Instead, some research has begun to focus on work with a consortia of algal strains or mixed communities in an effort to increase biodiversity and competition against potentially invasive organisms (Stockenreiter et al., 2012 & 2016; Liu et al., 2016; Bell et al., 2015). Results of these studies indicated that not only did algal consortiums have increased growth rates and faster inorganic nutrient removal than monoculture strains, but

algal consortia sometimes possessed increased lipid productivities (Stockenreiter et al., 2016; Liu et al., 2016). This result is interesting not only because it argues a more dynamic relationship between algal members and niche occupancy, but also because it indicates possible lipid accumulations outside of traditional nutrient depleted conditions often seen in the literature.

While studies have begun to examine mixed consortia of algal strains and their lipid potentials in wastewater, few studies have examined the potential lipid profiles of a mixed community of algae and indigenous bacterial communities. Relationships between algae and *Bacteria* have long been an area of study, yet new work demonstrates the possibility of a symbiotic relationship between some algal and bacterial species (Croft et al., 2005, Zhao et al., 2012; Sapp et al., 2006; Bell et al., 2015; Bell and Mitchell, 1972). The nature of these relationships is still largely unknown, however, there is evidence that bacterial relationships within the exopolymeric area surrounding the algae or "phycosphere" allow algal and bacterial species to exchange important metabolites such as cobalamin (vitamin B_{12}), oxygen, and carbon (Bell and Mitchell, 1972; Zhao et al., 2012; Sapp et al., 2006). While these close relationships have been shown to exist in open and *in situ* environments, little research exists regarding the effect of these relationships on lipid production or niche occupancy (Zhao et al., 2012; Bell et al., 2015; Stockenreiter et al., 2012 & 2016; Liu et al., 2016).

Based upon these current arguments in the literature we set out to test the lipid production and biomass accumulation capabilities of a mixed photoautotrophic population currently being used to treat municipal wastewater in Missoula, Montana for

nitrogen and phosphorus content. In doing so, we hoped to test the idea of increased biodiversity attributing to higher lipid production at an interspecies level. Community dynamics were simultaneously assed throughout inoculation and growth using small-subunit rRNA paired-end sequencing to determine community changes and profiles over time. The results of this study offer an indication of the possibilities that wastewater possesses for biofuel production with mixed algal communities. It also indicates the possibility of community interactions across domains in contributing to biomass and bio-oil accumulation of mixed algal communities.

Materials and Methods

Mixed Photoautotrophic Community

The mixed photoautotrophic community tested in this study was provided by the company Clearas Water Recovery. Located in Missoula, Montana, Clearas Water Recovery utilizes a mixed consortium of photoautotrophic organisms to remove nitrogen and phosphorus from a municipal wastewater system. The isolated community was taken from these consortia and delivered as concentrated slurry to the Center for Biofilm Engineering. The community was refrigerated and shipped overnight. Upon arrival, the concentrated slurry was diluted in half and immediately inoculated into 500mL Erlenmeyer flasks at 10% volume. Both filter-sterilized wastewater collected from the Bozeman Water Reclamation Facility (BWRF) and Bold's Basal Medium (BBM) served as media types for stock cultures. Stock cultures were maintained in an incubator at 20°C and a light:dark cycle of 14 hours of light followed by 10 hours of dark. The use of a

native community has the benefit of already being adapted to the intended growth environment. Most studies look for a species that will perform well as a monoculture and then proceed to determine if lipid production is possible in a wastewater environment. Here, the environment has naturally selected for a community of microalgae and bacteria capable of growth and bioremediation in a low quality water source.

Medium Composition

The experiment tested community responses to three media types. Liquid cultures of Bold's Basal Medium (BBM), filter sterilized municipal wastewater (FILT), and non-sterilized municipal wastewater (RAW) were tested. Municipal wastewater was collected from the effluent of the primary clarifier at the Bozeman Water Reclamation Facility (BWRF) in Bozeman, Montana. The water was collected and immediately stored in large 10 L plastic containers and stored at 4°C until the time of the experiment. Sterile media of both BBM and BWRF were sterilized using a 0.2 μm polyethersulfone filter (Corning Inc., Corning, NY).

Growth Conditions

The community was maintained in an incubator at 20°C. A light:dark cycle of 14 hours of light followed by 10 hours of dark was used to replicate the conditions likely seen during longer summer days and shorter nights of environmental growth patterns. Experiments were conducted in triplicate using 250 mL Erlenmeyer flasks containing 150 mL of BBM, filter sterilized, or non-sterile BWRF water. Flasks were placed on a Labnet Orbit 1900 Heavy Duty Shaker at 125 rpm to encourage mass transfer of carbon dioxide

into the medium. Samples of 3mL were taken daily from each batch culture an hour before dark cycles began. Samples were also monitored for evaporation and sterile water was added to each flask to return lost water volumes.

Anion Concentrations

Nitrate and phosphate concentrations of culture samples were determined daily using a 1mL sample on a DIONEX ICS-1100 and Chromeleon Chromatography Management System with an ASRS 4 mm suppressor. A 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate eluent mix was used.

Determination of Ammonium Concentration

Ammonium concentrations were determined as per Rhine et al. (1998) using the 2-Phenylphenol method. Briefly, 25 μL of sample were prepared in triplicate for each time point in a 96-well plate. Reagents of Citrate, 2-Phenylphenol-Nitroprusside, and buffered Hypochlorite were added and allowed to incubate at 37°C for 15 minutes. Absorbance readings were taken using a Synergy H1 hybrid reader. Gen5 microplate reader software was used to evaluate the fluorescence at an absorbance of 660nm (Rhine et al., 1998).

pH

pH was measured with a standard benchtop Oakton pH 11 Series meter. The pH meter was calibrated daily before readings using pH standards of 4.0, 7.0, and 10.0 before each use.

Cell Counts

Cell counts were used to track the growth of the community over time. Cell counts were taken using a standard hemocytometer. However, the presence of multiple organisms in the culture, specifically filamentous cyanobacteria, made accurate cell counts difficult. For this reason, chlorophyll and optical density measurements were used in conjunction to monitor growth.

Chlorophyll Measurements

Chlorophylls a, b, and carotenoids were extracted from 1 mL aliquots using the methanol extraction and optical absorption process previously described Ritchie et al. (2006). Briefly, 1 mL samples of culture were placed in 1.7 mL microcentrifuge tubes and centrifuged for 5 minutes at 16,000 x g. After centrifugation, supernatant was removed and discarded. One mL of 100% methanol was then added to the remaining pellet and re-suspended using vortex and sonication. The solution was then heated for 10 minutes in a water bath of 70°C. Finally, the solution was centrifuged again at 16,000 x g for 3 minutes before readings at 666nm, 654nm, and 470nm were taken. Chlorophyll calculations were conducted as described by Ritchie et al. (2006).

Optical Density

Optical density readings were taken during growth to monitor the accumulation of biomass over time. 200 μL of sample culture were measured in triplicate into a 96-Well plate and absorbance readings taken using a Synergy H1 hybrid reader. Gen5 microplate reader software was used to evaluate the absorbance at 750nm. Blanks were read

according to the specific media types respective to the culture. Samples of high optical density were diluted and final concentrations calculated.

Lipid Accumulation

Accumulation of lipids was tracked using the Nile Red method developed by Chen et al. (2009). 4 µL of Nile Red solution (0.25 µg/mL) were mixed in 800 µL of sample along with 200µL of DMSO. Samples were then incubated in a 96-well plate in the dark for 5 minutes, which was previously determined to be the optimal absorption time for these cultures. Nile Red fluorescence was measured using a Synergy H1 hybrid reader. Gen5 microplate reader software was used to evaluate the fluorescence at an excitation of 530 nm and emission of 575 nm. Specific Nile Red fluorescence was then calculated by dividing the fluorescent signal by cellular density for each culture. A final scaling of 10,000 was used to better represent data without decimal places and provided a scaling range of 0-100.

Biomass Collection

Biomass samples were taken during culture harvest to measure accumulation of lipids and microbial community profiles. Once a Nile Red peak was reached, each batch culture was harvested and centrifuged at 4800 x g and 4°C for 10 minutes. 200 µL of concentrated biomass was frozen separately for community analysis and the subsequent remaining pellets were then lyophilized (Lobconco lyophilizer, Kansas City, MO) for 48 hours before being stored at -20°C prior to lipid extraction.

Lipid Extraction and Transesterification

Lipid extraction and analysis was completed as previously described by Lohman et al. (2013) before overall fatty acid methyl esters (FAME) composition was analyzed using GC-MS. Approximately 30 mg of dried biomass were transferred in duplicate for each culture type for the analysis. At the end of extraction, 1 mL of the separated organic phase was placed into a 2mL GC vial for GC-MS analysis.

FAME Analysis Using GC-MS

Using gas chromatography-mass spectrometry (GC-MS) an analysis of total cellular fatty acids (FAME) was performed as previously described by Lohman et al. (2013). FAME quantification was determined by quantifying each response peak with the nearest eluting calibration standard based on retention time, using MSD ChemStation software (Ver. D.02.00.275).

DNA Extraction

DNA was extracted using 200µL of pelleted batch culture after harvesting using a FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH) according to the manufacturer protocol. DNA extractions were also completed from 0.2 µm filters of BWRF water to assess indigenous community members in the water. 500mL of water were filtered through each filter before extraction. The DNA was cleaned and concentrated with the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Extracted DNA was quantified with a Qubit fluorometer using a Qubit dsDNA BR detection kit (Invitrogen, Carlsbad, CA).

Gene Amplification and Sequencing

PCR primers for the SSU rRNA gene sequences for both *Bacteria* and *Eukarya* were used, and each sample was amplified in triplicate and later labeled with a unique 10 nucleotide-barcode for multiplexing. The SSU rRNA gene sequences for *Bacteria* and *Eukarya* were amplified using 25 cycles of PCR with the subsequent barcoded primers; 7F/591R (7F 5′-ACCTGGTTGATCCTGCCAG-3′ and 591R 5′-GGAGCTGGAATTACCG-3′) to target the variable region V1-V3 of *Bacteria* (Bowen De León et al., 2012) and *Eukarya* 8F/529R (8F 5′-AGAGTTTGATCCTGGCTCAG-3′and 529R 5′-CGCGGCTGCTGGCAC-3′). Amplicons were then purified using the AMPure Xp magnetic bead capture kit (Beckman Coulter, Inc., Brea, CA) before ligation of Illumina adaptors for sequencing. Amplicons were quantified using Quant-iT PicoGreen Assay Kit (ThermoFisher Scientific, Waltham, MA). Libraries from purified sample amplicons were pooled in equimolar amounts with an Illumina PhiX control library. Sequencing was then completed using an Illunima MiSeq Kit v3 600 cycle (Illumina Inc., San Diego, CA).

Community Analysis

Using the Quantitative Insights into Microbial Ecology (QIIME) software package, version 1.7.0 (Caporaso et al.,2010b) microbial sequences were processed and analyzed. Raw sequences were demultiplexed and libraries split with respect to relative domains (*Eukarya* and *Bacteria*). Sequences smaller than 200 nt, quality scores below 25, or of chimeric identity were culled. Operational taxonomic units (OTUs) were assigned using a closed reference OTU picking protocol and clusters were referenced against a

Silva 108 database and pre-clustered at a 97% sequence identity cut-off using UCLUST (Edgar, 2010).

Sequence reads matching Silva reference sequence were clustered within an OTU by a reference sequence. OTU assignments were performed for combined bacterial and eukaryotic reads. Using PyNAST (v1.1) default parameters, sequences were aligned and filtered. Centroid sequences for each cluster were selected as representative and aligned with a Silva core reference set using PyNAST (Caporaso et al., 2010a). OTUs failing to align with PyNAST or those identified as singletons were removed and discarded from further analysis. Following quality filtering and grouping, taxonomic assignments were made using the RDP Classifier program (Wang et al., 2007) with 80% confidence rating against the Greengenes database (McDonald et al., 2012) on the Silva database for phylogenetic resolution at the genus level.

Heat-maps created using the R vegan package version 2.0-10 (Oksanen, 2011) were generated from relative abundances. Given that some taxa were not present (relative abundance of 0%), 0.1 was added to all time points to allow transformation. Distributions of taxa were then normalized using a square root transformation which helped to illustrate important co-occurrences of community members.

Statistical Analysis

A detrended coordinate analysis (DCA) was used to identify OTU distribution patterns amongst and between treatment types. Due to the unimodal nature of this data set, a DCA analysis was selected because it does not use Euclidean distances to assume linear trends between taxa but instead utilizes a χ^2 based metric that disregards any

double species absence (Ramette et al., 2007). Results not only indicate potential interactions amongst taxa but also abundances of species relative to treatment types. This analysis was selected over the alternative nonmetric multidimensional scaling (NMDS) based upon the assumption that niche occupancy and existence played an important role in the tested environments (Ramette et al., 2007).

In order to identify differentially abundant OTUs between treatment types, the biomarker discovery algorithm LEfSe [linear discriminant analysis (LDA) effect size] was used. LefSe operates by first conducting a nonparametric Kruskal–Wallis sum-rank test to identify taxa with significantly different relative abundances between groups of interest. LefSe then conducts an LDA to these identified taxa ($p < 0.05$) to assess the effect size of each differentially abundant OTU. Effect sizes are then ranked to determine OTUs of greatest difference between treatment groups.

Results and Discussion

Growth of Mixed Community Batch Cultures

Enrichments maintained from previous culture work were used to inoculate batch flask enrichments of sterilized (FILT) and non-sterilized (RAW) primary effluent collected from the municipal Bozeman Waste Reclamation Facility (BWRF) and Bold's Basal Medium (BBM). Each enrichment was conducted in triplicate with a 10% (volume) inoculum. Batch cultures were monitored over time at 20°C and 14h:10h light:dark cycle.

Cell counts, chlorophyll, and optical density readings (OD) were used to monitor batch growth over time. Specific Nile Red (NR) readings were also used as a proxy for lipid accumulation in batch cultures and served as the primary indicator for harvesting. Overall, cultures grown in BBM, FILT, and RAW media displayed similar growth patterns (Figure 3.1). Cell counts, OD readings, and chlorophyll measurements all indicated a quick increase in biomass followed by a more prolonged increase in all media types. Final readings also indicated a similar biomass production between the three media types with RAW and FILT samples reaching a higher overall OD reading than that of BBM samples (Figure 3.1).

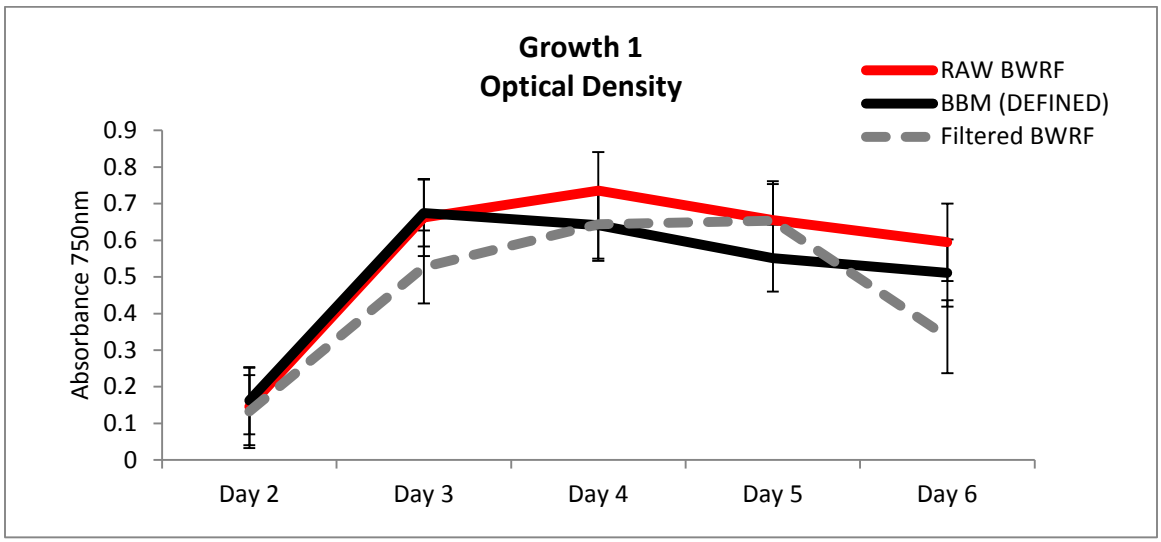


Figure 3.1: Optical density of Growth 1 batch cultures grown on unfiltered (RAW) BWRF, filter-sterilized BWRF, and Bold's Basal medium (BBM) over a six-day period.

NR readings, however, indicated larger specific NR in RAW samples compared to both FILT and BBM cultures despite similar biomass accumulations and growth patterns (Figure 3.2).

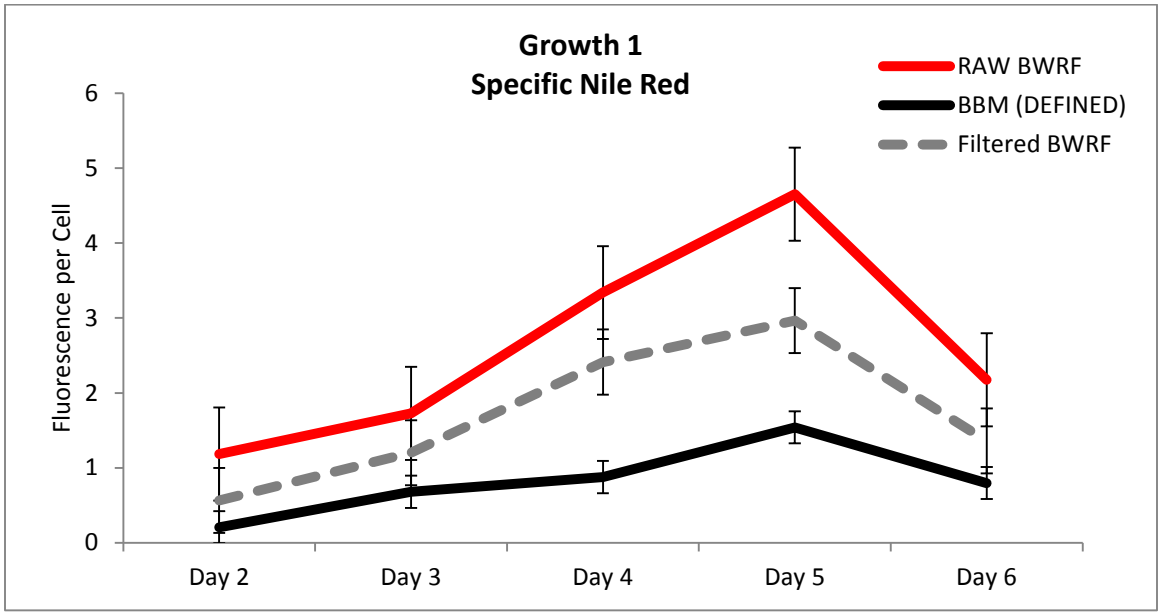


Figure 3.2: Specific Nile Red readings for Growth 1. RAW samples demonstrated higher readings than filtered and BBM cultures despite similar growth and biomass accumulations.

Repeated experiments of the conditions described above were completed at the termination of Growth 1 by returning to maintained stock cultures. Growth 2 experiments displayed higher growth and biomass accumulation patterns observed in Growth 1 experiments (Figure 3.3).

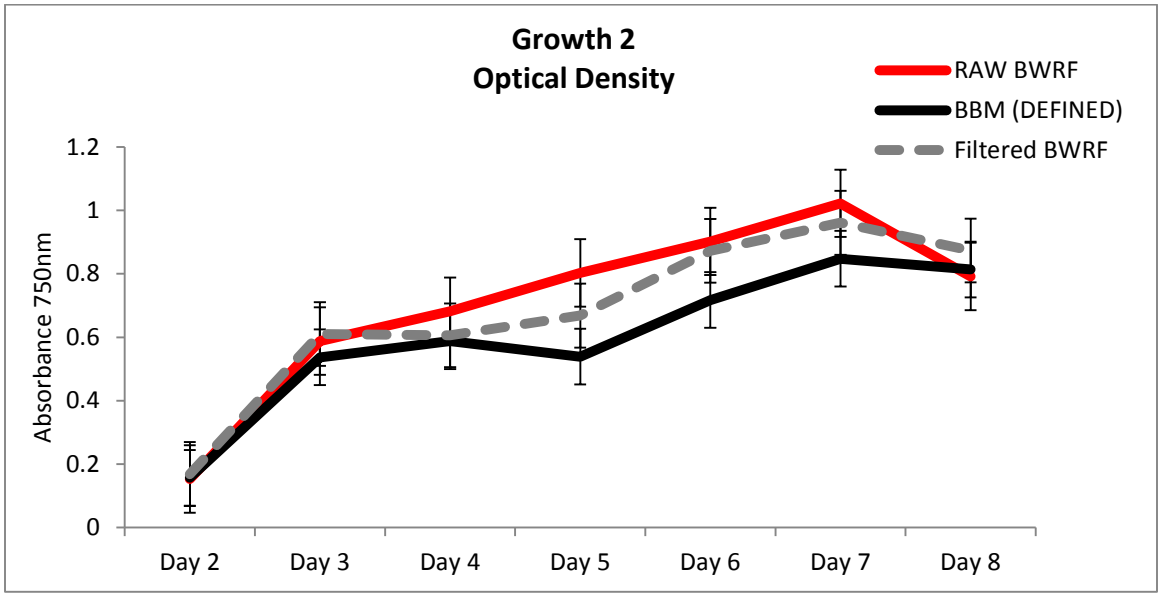


Figure 3.3: Optical density of the Growth 2 batch cultures grown on unfiltered (RAW) BWRF, Filter sterilized BWRF, and Bold's Basal medium (BBM) over an eight-day period.

RAW cultures again reached a slightly higher level of biomass than other cultures based upon OD readings. Also, NR profiles were elevated compared to culture conditions in Growth 1, but RAW samples again displayed higher specific NR readings compared to BBM and FILT treatments (Figure 3.4).

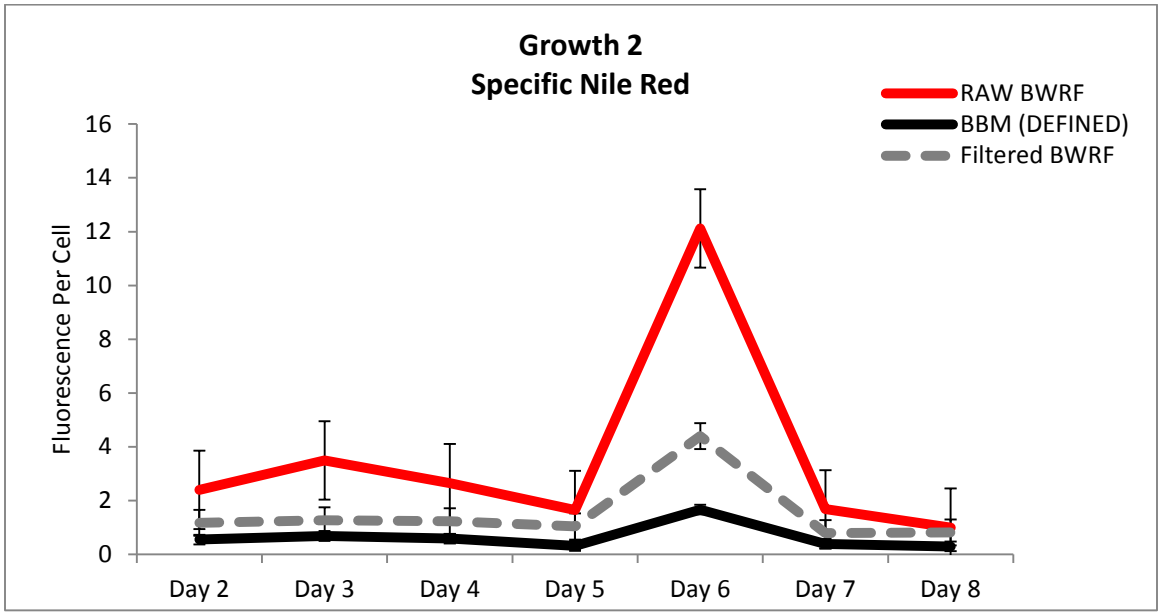


Figure 3.4: Specific Nile Red readings for Growth 2. RAW samples demonstrated higher readings than filtered and BBM cultures despite similar growth and biomass accumulations.

Analysis of Extractable Lipids from Growth

A measurement of fatty acid methyl esters (FAMEs) allows biofuel potential to be assessed from microbial cultures. To confirm NR profiles observed in experiments for Growth 1 and Growth 2, the analysis was conducted via *in situ* transesterification of biomass collected from BBM, FILT and RAW BWRF enrichments. Transesterifications and analyses were conducted as per Lohman et al. (2013), and GC-MS analysis was used to quantify FAMEs against standards (Figure 3.5).

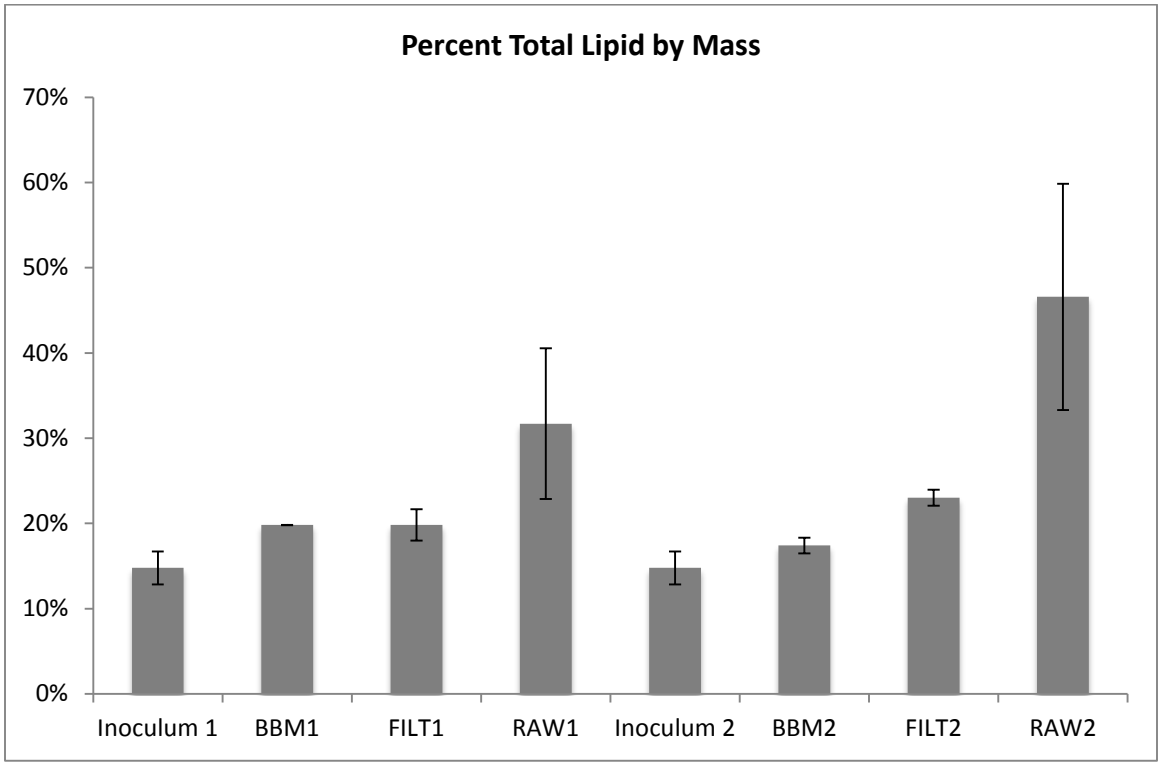


Figure 3.5: Total lipid percentages (w/w) estimated as the total amount of FAMEs for each sample estimated by in situ transesterification combined with GC-MS analysis. The highest lipid content was seen in RAW unfiltered cultures (p-value=0.07).

Generally, the major FAME compounds observed were even-numbered fatty acid chains. Overall, FAME production was relatively high as RAW BWRF cultures displayed a higher content compared to other observed algal biomass and lipid productivities in FILT and BBM treatments. The values observed in RAW cultures were the highest values observed throughout both batch cultures confirming NR profiles measured during growth (Figure 3.2 and 3.4). C16-C18 fatty acids have previously demonstrated to be most suitable for biofuel production and RAW samples had the highest total FAME primarily composed of linolenic (C18:3) and oleic (C18:1) acids (Xu et al., 2004). BBM and filter sterilized cultures displayed relatively similar FAME contents predominated by linolenic (C18:3) and palmitic (C16:0) acids.

Previously, lipid production using wastewater isolates and consortia have shown similarly large percentages, between 20% - 50%, to that observed in this study (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013). However, it is interesting that such large differences in lipid production were seen between FILT and RAW conditions given their similarities in nutrient content (Table 3.1).

Table 3.1: Characteristics of the wastewater sampled from the Bozeman Wastewater Reclamation Facility (BWRF) before and after sterilization. ND (not detected/ 0.05mg/L)

Parameters	**Primary Effluent Non-sterile (RAW)**	**Primary Effluent Filter Sterilized (FILT)**
Growth 1		
NH_4^+-N (mg/L)	45.4 ± 0.6	44.6 ± 0.2
NO_3^--N (mg/L)	ND	ND
NO_2^--N (mg/L)	ND	ND
PO_4^{-3}-P (mg/L)	5.3 ± 0.7	4.3 ± 0.1
Growth 2		
NH_4^+-N (mg/L)	44.4 ± 0.3	44.0 ± 0.5
NO_3^--N (mg/L)	ND	ND
NO_2^--N (mg/L)	ND	ND
PO_4^{-3}-P (mg/L)	4.3 ± 0.5	4.4 ± 0.3

Previous studies have shown that nutrient limitations, such as the bioavailability of nitrogen, phosphorus and CO_2, contribute to increased lipid accumulation in microalgae (Devi et al., 2012; Gardner et al., 2011; Fields et al., 2014). However, anion and cation results indicated similar depletion rates for the key nutrients phosphorus and nitrogen-ammonium between FILT and RAW communities, as well that filtering did not alter the nitrogen and phosphorus levels. Given the differences between the RAW and FILT samples, we hypothesized that the microbial communities in the RAW culture conditions were different between the samples. To address this possibility as well as the possibility of contributing community interactions, changes in community dynamics were assessed through small-subunit rRNA paired-end sequencing.

Bacterial and Eukaryotic Community Profiles

To determine the effects and identity of microbial community members from both batch culture experiments, Growth 1 and Growth 2, a comparison of BBM, FILT and RAW cultures were completed in triplicate. To this comparison, samples of BWRF water prior to the addition of the mixed community were assessed to track the state of indigenous communities over time. Total microbial DNA was extracted from both the original inocula and batch cultures at the time of harvesting followed by sequencing for both *Bacteria* and *Eukarya* community members. Quality filtered sequences were then clustered into OTUs at a 97% sequence identity resulting in the number of unique sampled OTUs found in Table 3.2.

Table 3.2: The number of unique sampled OTUs for both Growth 1 and Growth 2. Differences in OTU number between FILT and RAW treatments were significant for both domains after accounting for original OTU numbers INOC and BWRF (p-value <0.005).

	Number of Unique Bacterial OTUs	**Number of Unique Eukaryotic OTUs**
Growth 1		
BWRF Water	134	70
Inoculum	249	29
BBM	134	34
FILT	150	18
RAW	148	22
Growth 2		
BWRF Water	131	50
Inoculum	163	29
BBM	141	26
FILT	140	16
RAW	135	21

The number of unique OTUs sampled for each growth indicated a higher richness in inoculum and BWRF water samples for both *Bacteria* and *Eukarya* (Figure 3.6). Interestingly, the number of unique sampled OTUs across treatments is relatively similar (Figure 3.6).

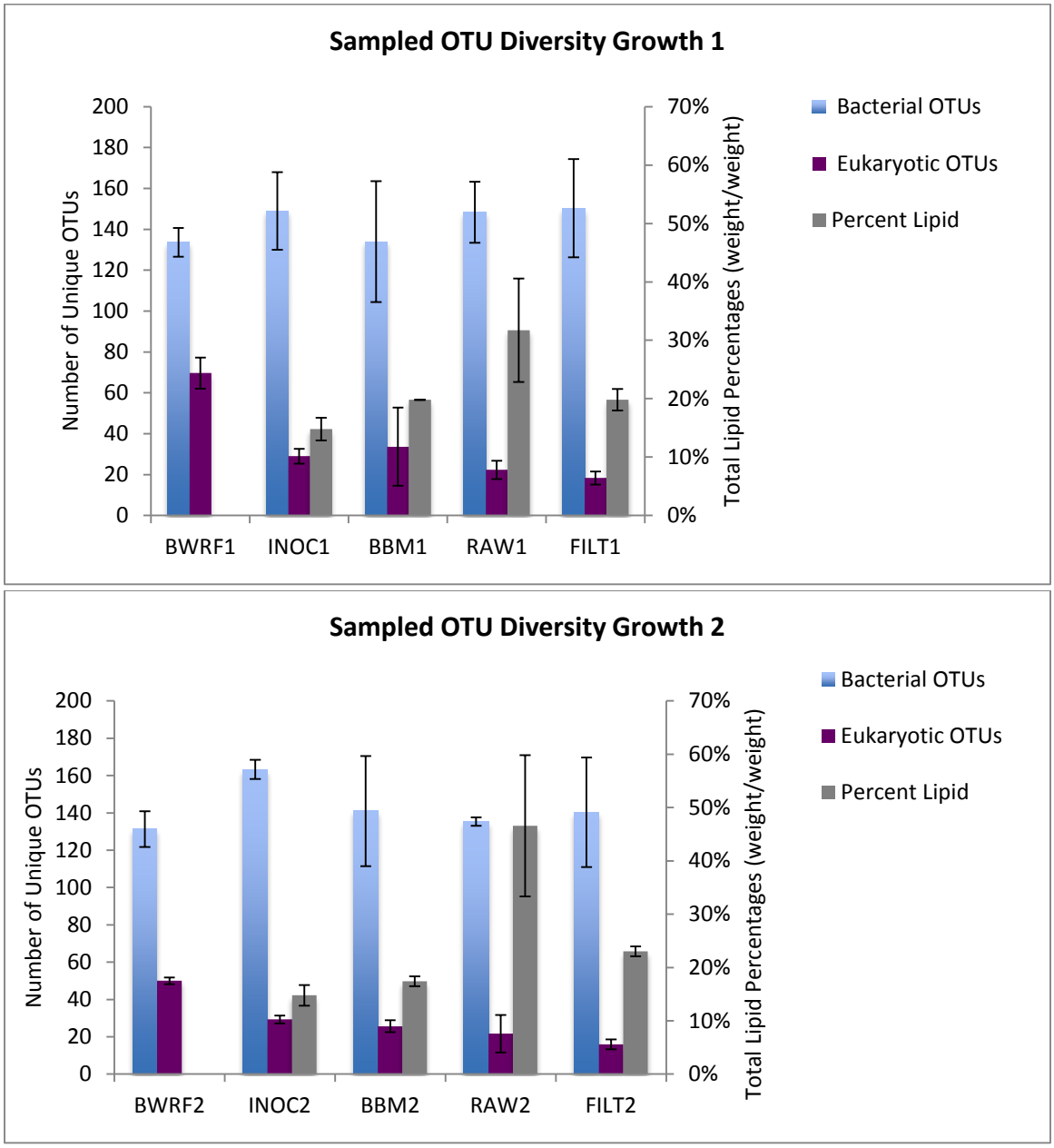
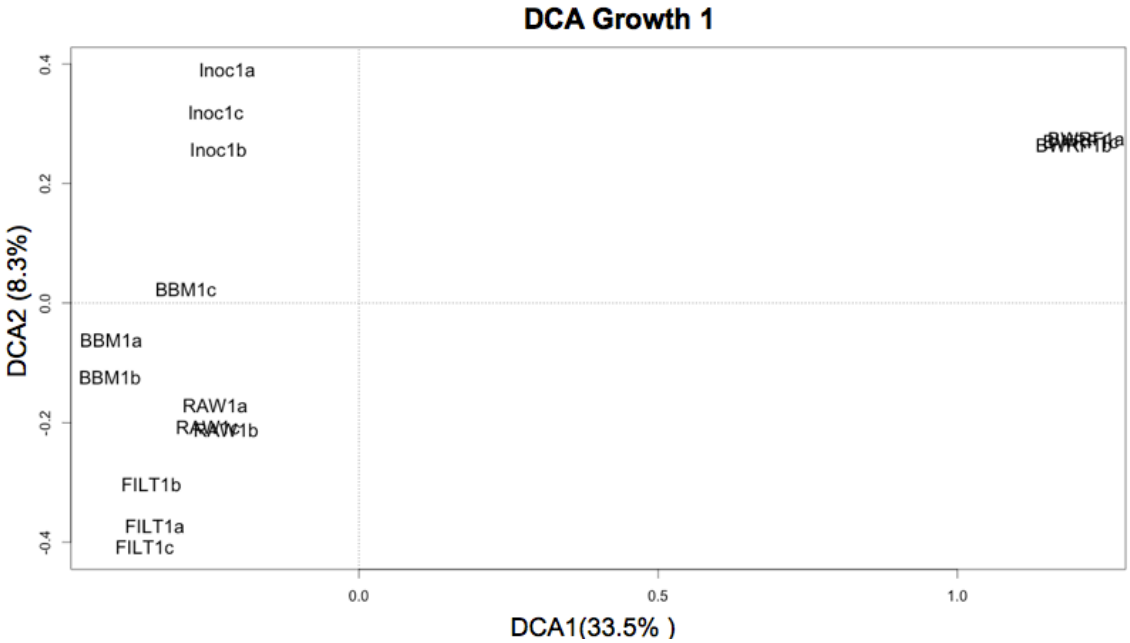


Figure 3.6: Number of unique *Bacteria* and *Eukarya* OTUs for Growth 1 and Growth 2 normalized by sequence reads. Inoculum (INOC) and BWRF water (BWRF) samples had the highest richness (p-value< 0.005). Richness between treatment types FILT, RAW and BBM were similar. Grey bars indicate total lipid percentages from GC-MS analysis (p-value=0.07).

Given the significant differences (p-value 0.07) measured with GC-MS analysis indicating higher lipid profiles in RAW samples for both Growth 1 and 2, this apparent similarity in

the number of unique OTUs sampled likely indicates that differences in lipid contents cannot completely be attributed to nutrient limitations due to increased number of taxa in unfiltered cultures compared to filtered. However, this observation does not exclude differences in relative abundances within present taxa. Specifically, DCA analysis for Growth 1 and Growth 2 suggest that the microbial profiles of each treatment type clustered separately from one another while replicates clustered more closely (Figure 3.7). This indicates that while replicates demonstrated similarities in community structure, treatment types (RAW, FILT, and BBM) possessed distinct differences in microbial community profiles. Furthermore, these results also illustrate distinct community changes from the original inocula communities (INOC) associated with treatment types BBM and FILT. Raw samples also demonstrate community changes from the original inocula, but also community structure differences from indigenous water communities (BWRF) present prior to growth (Figure 3.7).



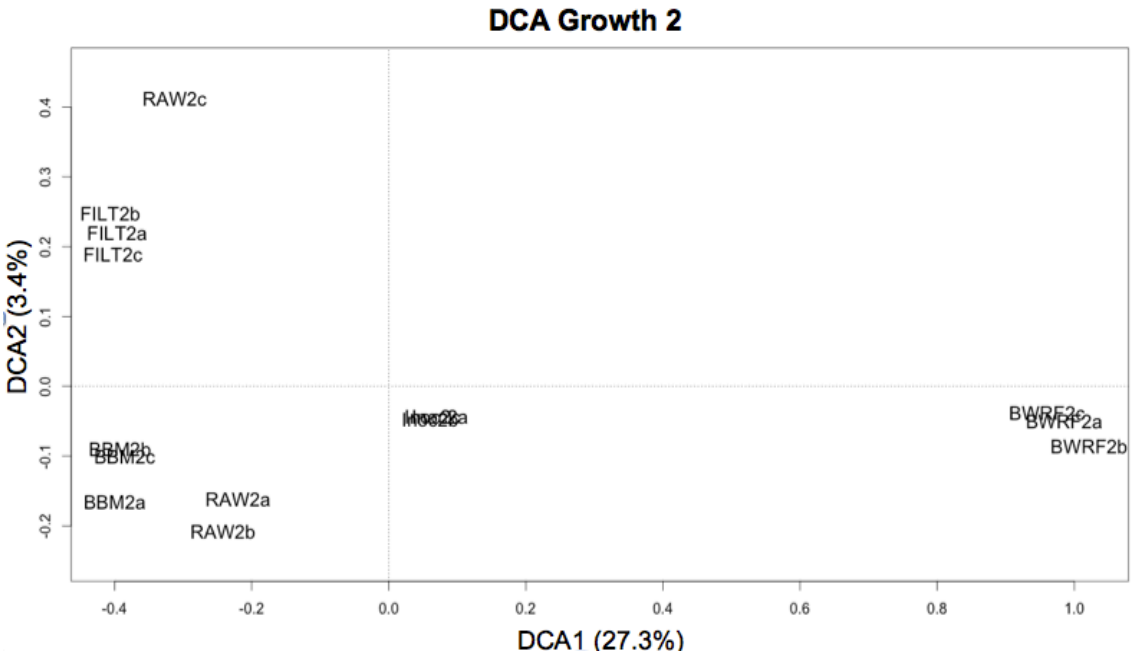


Figure 3.7: Detrended Correspondence Analysis (DCA) of Growth 1 and Growth 2 show distinct clustering of treatment types and close clustering of replicates. This indicates community distinctions between treatments.

The results of preliminary sequencing profiles and DCA analysis indicate that while the numbers of OTUs present for each treatment type were similar, there are distinct differences between community members across each treatment type.

Phylogenetic Community Analysis

Sequence analysis was performed with Quantitative Insights into Microbial Ecology (QIIME) software package, and the R vegan package in order to monitor community changes over time. Relative abundances for Growth 1 and Growth 2 indicated additional differences between treatment types (Figure 3.8 and 3.9). Replicates displayed consistent community member profiles as RAW, FILT, and BBM samples were all predominated by two Unknown bacterial OTUs (Figure 3.8). Interestingly, community

dynamics between BBM samples and the original Inoculum (INOC) were more similar than either RAW or FILT treatments. Furthermore, it is interesting to note the changes in bacterial community dynamics between the initial BWRF water community members present in the BWRF and those present at the end of experimental growth (RAW). Specifically, community members *Trichococcus*, *Pseudomonas,* and *Leptotrichia* experience a marked decrease in abundance after inoculation (Figure 3.8). Other studies have found high abundances of these community members in urban sewer systems (VandeWalle et al., 2012), especially in primary effluent where these populations seem more adept to change in the presence of oxygen. Other gut microbes typically introduced in primary effluent are strictly anaerobic and quickly decrease in abundances (VandeWalle et al. 2012). Yet, the decreases seen here argue that while *Trichococcus*, *Pseudomonas,* and *Leptotrichia* are better adept than other gut microbes to the sewer environment, they are still out competed by other populations in the conditions tested.

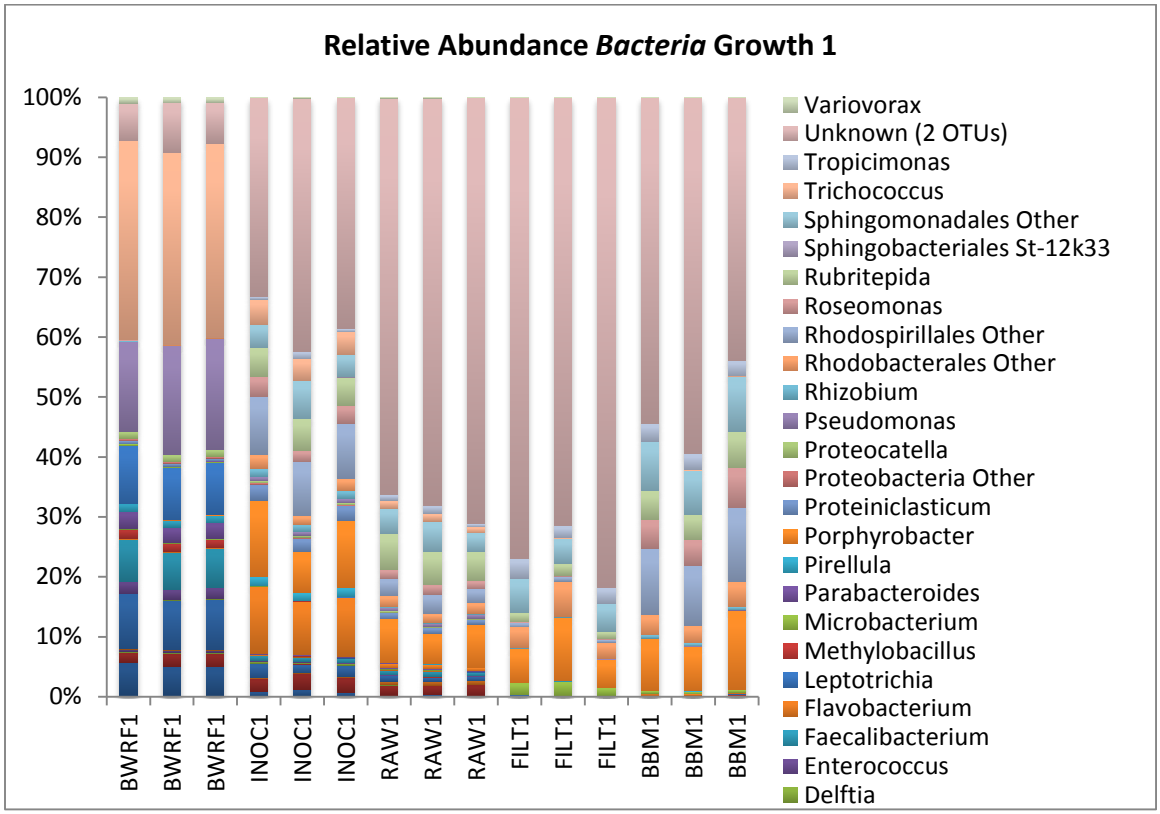


Figure 3.8: Percent relative abundance of unique *Bacteria* OTUs present above 1% for each culture condition in Growth 1 as well as the original inocula (INOC) and Bozeman Reclamation wastewater (BWRF). Dominant OTUs included two Unknown bacterial OTUs in all samples except BWRF water samples that were predominated by *Trichococcus.*

Eukaryotic OTUs showed less diversity than the bacterial population (Figure 3.9). RAW samples showed a decrease in Fungi sequences of *Ascomycota* and *Saccharomyces*. However, all samples experienced dominance in the green algae *Chlorophyceae*, three Unknown *Eukarya* OTUs, and an unknown *Coccidia* OTU (Figure 3.9). *Coccidia* is a spore forming intracellular parasite often found in animal intestinal tracts. Members of *Coccidia* often have unique life cycles that allow replication both sexually and asexually (Hutchinson et al., 1971). Infected animals produce spores, termed oocyst, in their stool that later mature through multiple rounds of asexual replication to become sporulated

oocysts that infect the next host (Hutchinson et al., 1971). It is possible that the appearance of *Coccidia* correlates to the open nature of the sewer systems tested and the dominance of *Coccidia* in each sample is likely due to rounds of asexual replication within each flask. For the purposes of data representation, *Coccidia* OTUs were removed from Figure 3.9.

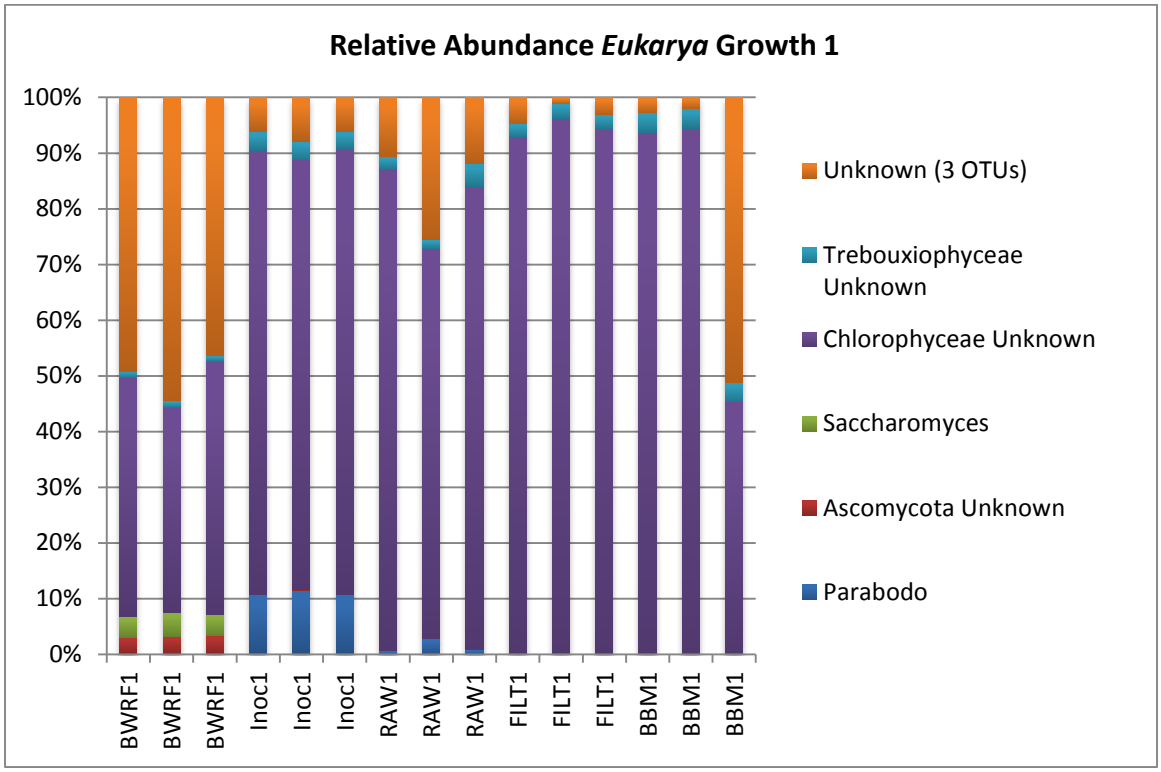


Figure 3.9: Relative abundance of unique *Eukarya* OTUs present above 1% for Growth 1. Each culture condition as well as the original inocula (INOC) and Bozeman Reclamation wastewater (BWRF) were characterized. Dominant OTUs included *Chlorophyceae* and three Unknown OTUs in all samples.

Repeated experiments of the conditions described previously were completed and microbial community dynamics were again tracked via small-subunit rRNA paired-end sequencing. Growth 2 demonstrated similar tends to Growth 1 where replicates were seen to display consistency in community members across biological replicates. RAW, FILT, and BBM samples were again predominated by two Unknown bacterial OTUs present in

high abundances in INOC samples, but also an increased relative abundance of *Porphyrobacter* sequences across all growth conditions (Figure 3.10). An aerobic anoxygenic phototrophic member of α-proteobacteria, *Porphyrobacter* increases were interesting given the documented symbiotic relationships exhibited by some algal species and α-proteobacteria (Shimada, 1995; Bell et al., 2015; Croft et al., 2005; Zhao et al., 2012).

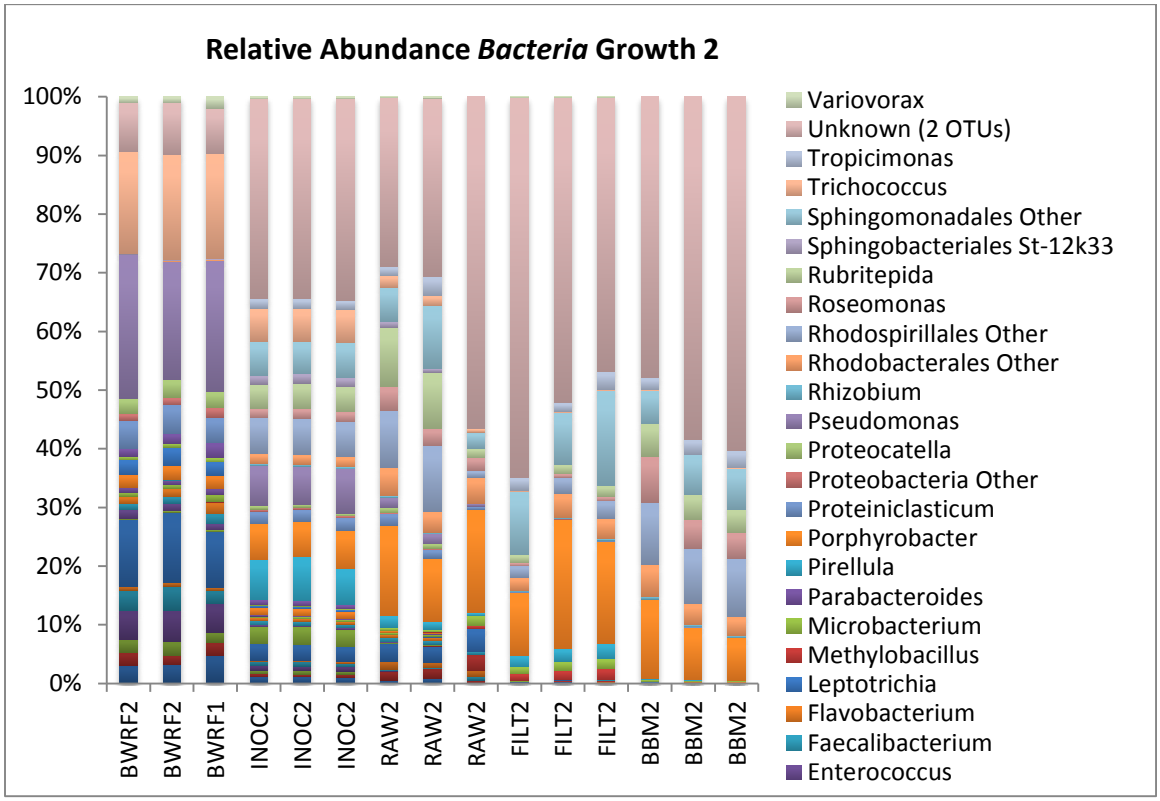


Figure 3.10: Percent relative abundance of unique *Bacteria* OTUs present above 1% for each culture condition of Growth 2 as well as the original inocula (INOC) and Bozeman Reclamation facility water (BWRF). Dominant OTUs again included two Unknown bacterial OTUs in all samples as well as *Porphyrobacter* not previously observed in Growth 1.

Community dynamics between RAW samples and the original Inoculum (INOC) were more similar than either BBM or filtered treatments. This result is interesting in

comparison to results seen in analysis from Growth 1 where BBM samples and Inoculum communities were most similar. Again, changes in the bacterial community were noted between the initial unfiltered community members present in the Bozeman Reclamation Facility wastewater (BWRF) and those present at the end of experimental growth (RAW). Specifically, *Trichococci* seemed to experience a decrease in relative abundance while *Pseudomonads* increased in BWRF samples as compared to relative abundances observed in the first growth experiment (Growth 1, Figure 3.8). However, after inoculation, *Trichococci*, *Pseudomonads*, and *Burkholderiales* experienced decreases in relative abundances (Figure 3.10). Again, these populations have been documented to be abundant in such sewer systems (VandeWalle et al. 2012), but competitive growth among other more adept populations within the inocula or wastewater may explain decreases after mixed community growth.

Eukaryotic communities for Growth 2 were similar to those of Growth 1 including a high presence of *Chlorophyceae*, three Unknown OTUs, and an Unknown *Coccidia* OTU (Figure 3.11).

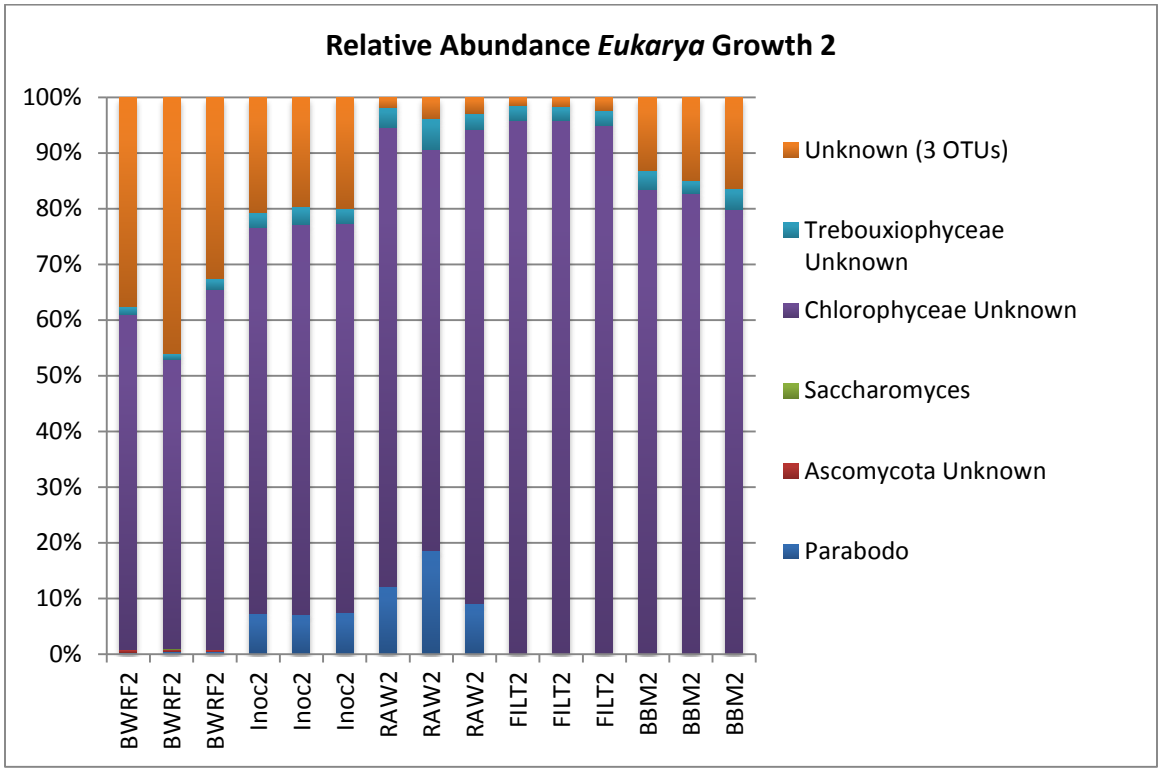


Figure 3.11: Relative abundance of unique *Eukarya* OTUs present at above 1% for each culture condition in Growth 2 as well as the original inocula (INOC) and Bozeman Reclamation wastewater (BWRF). Dominant OTUs again included *Chlorophyceae* and three Unknown eukaryotic OTUs in all samples.

Again *Coccidia* sequences were removed to facilitate better data visualization. However, despite the many similarities to Growth 1, Growth 2 did demonstrate some differences in BWRF samples where initial abundances of fungal sequences were low compared to Growth 1 (Figure 3.9). This decrease may be related to BWRF storage between Growth 1 and Growth 2 experiments at a low temperature (20°C) that could potentially have affected these populations.

A comparison of co-occurrences shown by the heat map in Figure 3.12, demonstrate further associations between both bacterial and eukaryotic occurrences per sample for both Growth 1 and Growth 2 (Figure 3.12).

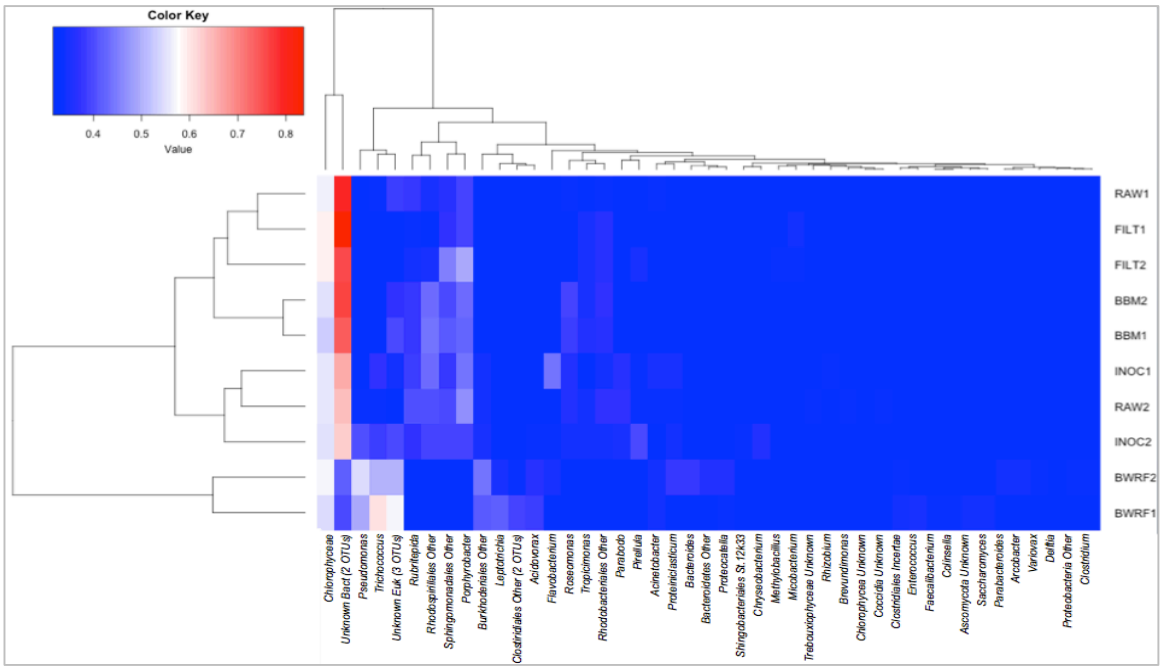


Figure 3.12: Heat map of relative abundances of unique Bacterial and Eukaryotic OTUs present above 1%. Co-occurrences for each sample from Growth 1 and Growth 2 clustered according to relatedness. RAW, FILT, and BBM cultures demonstrated a decrease in the presence of OTUs found in Inoculum and BWRF samples. Increases in *Rubritepida* sequences were determined in RAW samples and increases in *Chryseobacterium* sequences in FILT samples.

From this comparison, specific OTUs were seen to cause clustering of treatments. Specifically, two Unknown bacterial OTUs increase in all samples during growth (Figure 3.12). While abundances of these two OTUs are low in BWRF water, the inocula display a higher prevalence that increases in abundance over all treatments. Instead, BWRF samples are characterized by larger abundances of *Pseudomonas* and *Trichococcus* sequences and three Unidentified eukaryotic OTUs (Figure 3.12). RAW samples; however, displayed higher abundances in four α-proteobacteria OTUs, such as *Rubritepida* and *Rhodospirillales,* compared to FILT samples that had higher Flavobacterial OTUs such as *Chryseobacterium* (Figure 3.12). The presence and increase of α-proteobacteria OTUs

is interesting, given evidence that some specific bacterial groups and microalgae have been shown to exhibit a symbiotic relationship in the acquisition and exchange of crucial metabolites within the phycosphere (Zhao et al., 2012; Sapp et al., 2007; Bell and Mitchell, 1972). Specifically, bacterial members of α-proteobacteria have been isolated and identified to exhibit such relationships with microalgae in *in situ* and open environments (Bell et al., 2015; Croft et al., 2005; Zhao et al., 2012).

While co-occurrences are useful for drawing potential biological inferences they lack statistic validity. To address this issue, an analysis using the LDA Effect Size (LEfSe) algorithm was completed to determine community biomarkers between samples as well as determine their effect size (Segata et al., 2010). In doing so, identified taxonomic markers between FILT and RAW treatment types pointed to some potentially key community differences (Figure 3.13).

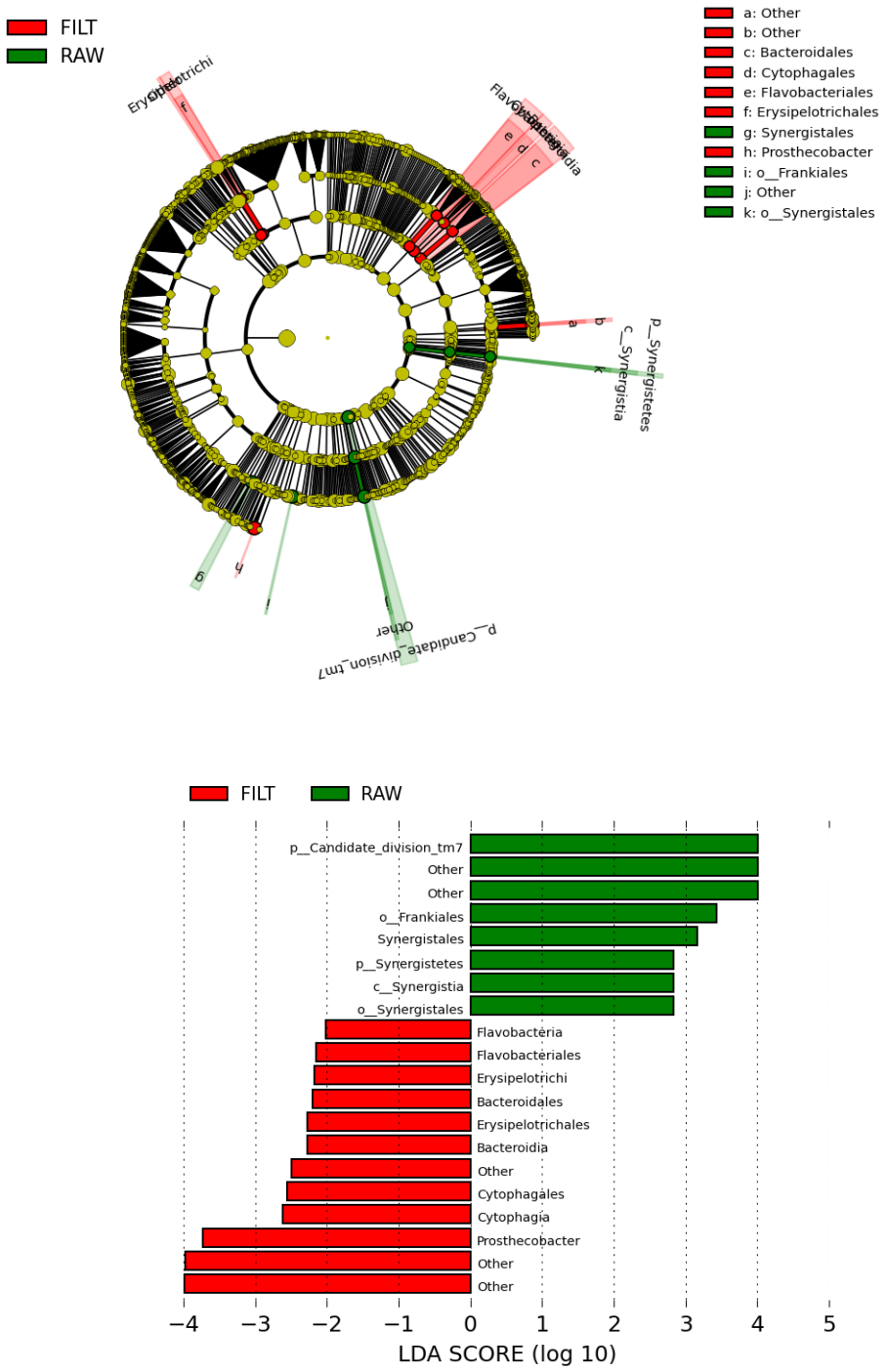


Figure 3.13: Comparison of RAW and FILT bacterial biomarkers indicating an enrichment of eight *Bacteria* OTUs in RAW samples. A.) Ranked LDA scores ($p<0.05$) from LEfSe analysis indicating statistically significant biomarkers. B.) Taxonomic representation of differences between RAW and FILT treatments. Differences are represented by treatment color where circle size indicates abundance.

As indicated in Figure 3.13, RAW treatments demonstrated enrichment in eight bacterial OTUs while FILT treatments demonstrated enrichment in twelve. RAW treatments possessed high abundances in three novel OTUs, but also high abundances in the phyla *Synergistestes* and *Actinobacteria*. *Synergitestes* (including class *Synergistia*) are known anaerobes found in a plethora of environments including the gut of animals as well as anaerobic wastewater lagoons. Specifically, in wastewater environments, these microbes have been documented to contribute to nitrogen release in the fermentation of amino acids (Baena et al., 1998). *Actinobacteria* OTUs identified here (including order *Frankiales*) are commonly found in soil with the oligotrophic ability to degrade organic matter as well as nitrogen fixation (Normand et al., 2012).

FILT samples were characterized by twelve OTUs with a higher prevalence of phyla *Firmicutes* and *Bacteroidetes* than RAW samples. *Bacteroidetes* OTUs (including *Bacteroidia*, *Cytophagia*, and *Flavobacteria*) include strict and facultative anaerobes commonly found in the gut microbiome (Turnbaugh et al., 2006; Devillard et al., 2007). Three OTUs of the phylum *Frimicutes* (including *Eryspelotrichales*, *Eryspelotrichi*, and one unclassified) were indicated in high abundances as well in FILT samples. Interestingly, the *Roseburia* species among this phylum have been indicated in obesity studies for their ability to metabolize linoleic type fatty acids (Turnbaugh et al., 2006; Devillard et al., 2007). LEfSe analysis further indicated a large abundance of the oligotrophic *Prosthecobacter* in FILT samples compared to RAW (Staley et al., 1980).

Eukaryotic LefSe analysis further indicated biomarker differences between RAW and FILT treatments (Figure 3.14).

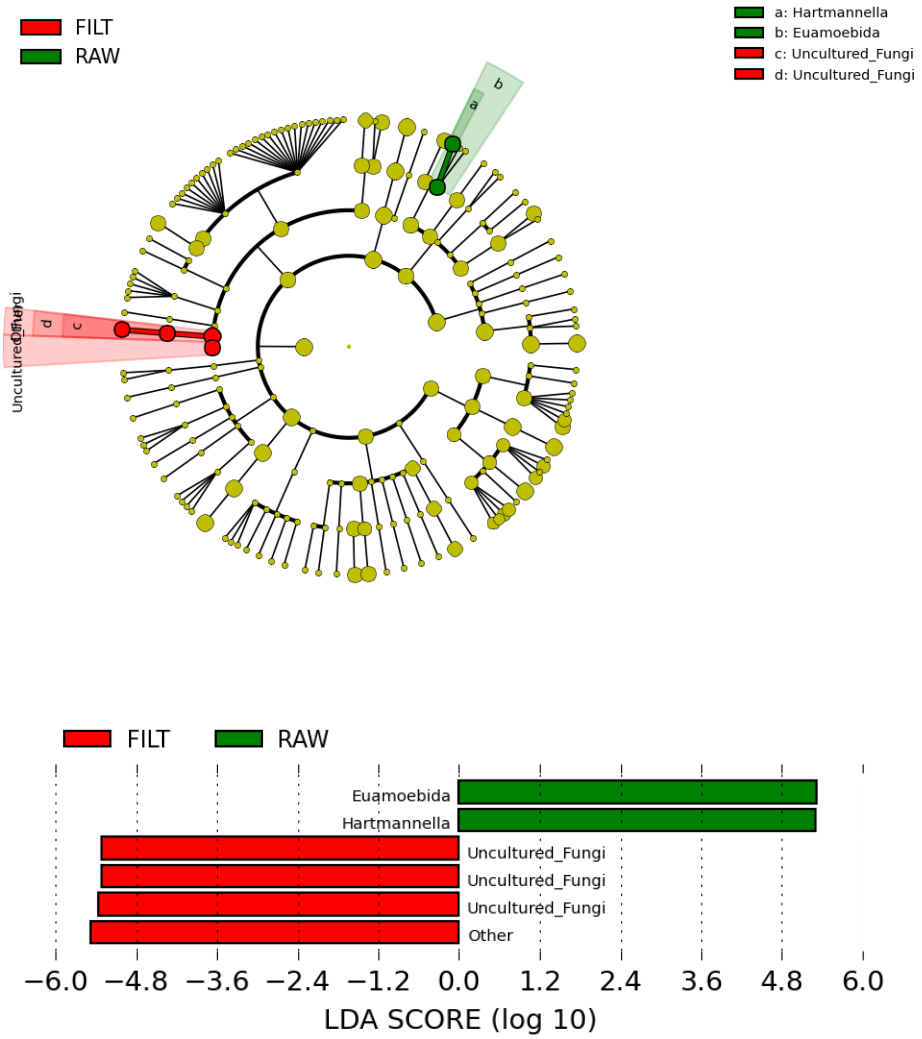


Figure 3.14: Comparison of RAW and FILT bacterial biomarkers indicating an enrichment of two *Eukarya* OTUs in RAW samples. A.) Ranked LDA scores ($p<0.05$) from LEfSe analysis indicating statistically significant biomarkers. B.) Taxonomic representation of differences between RAW and FILT treatments. Differences are represented by treatment color where circle size indicates abundance. Yellow indicates non-significant taxa.

RAW samples were characterized by an enrichment in two OTUs in the phyla *Amoebozoa* including *Hartmanella* and *Euamoebia.* Amoebas are known to occupy a variety of niches either as predators, scavengers, or parasites. However, some naked

amoebas, such as *Euamoebida*, have demonstrated the ability to consume *Bacteria* in wastewater environments thereby contributing to nutrient regeneration for other niche holders (Ramirez et al., 2014). Alternatively, FILT samples were characterized by enrichments in three unclassified fungal OTUs and one unclassified eukaryotic OTU. With limited classification it is difficult to speculate about potential interactions or roles that fungal groups in wastewater might perform. While research is limited on fungal groups and metabolisms in general, there is some evidence to suggest that fungal groups of both filamentous and non-filamentous morphology can occupy and replicate in wastewaters (More et al., 2010). These fungal groups are interesting not only because they have similar nutrient requirements and metabolic activities similar to some algae (i.e., nitrogen assimilation), but also because they have potential generalist capabilities that might serve well in low nutrient environments (i.e. nitrification) (More et al., 2010). It is possible that while nutrient levels remain high fungal groups compete with fewer niches for resource allocation, but as nutrients deplete these fungal groups have the metabolic capacity to expand into other niches and offer further competition.

Summary

In summary, a mixed photoautotrophic community being used as a primary source of nitrogen and phosphorus removal from a municipal wastewater plant, was shown to generate biomass and a substantial amount of lipid in another municipal wastewater environment. Interestingly, the community grown in the presence of the indigenous wastewater community achieved a higher percentage of lipid, 47% w/w, compared to growth in a filter sterilized and control condition. Community analysis utilizing small-

subunit rRNA paired-end sequencing of both eukaryotic and bacterial community members indicated differential clustering between treatment types. Further analysis using LEfSe, indicated an enrichment of ten bacterial and eukaryotic clades in RAW samples where FILT samples were enriched for sixteen. Of those OTUs represented in RAW samples with high abundance, *Synergistestes* and *Actinobacteria* indicated bacterial community members with potentially influential metabolic activities in the degradation of amino acids and other organic matter. This is significant not only in the context of carbon release and utilization but also potential denitrification by algal members to sustain algal growth and cell functions. Amoeba clades in RAW samples offer further evidence to the potential turnover of organic material into bioavailable form for utilization by other community members. Alternatively, FILT samples indicated an enrichment in *Firmicutes*, *Prosthecobacter*, *Bacteroidetes*, and fungal clades. While, specific contributions in a wastewater environment are difficult to attribute, the oligotrophic nature of many of these organisms indicates a preference for generalist type organisms compared to RAW samples. This may indicate that while RAW samples possess fewer biomarker OTUs than FILT samples, those organisms present within RAW samples are uniquely specific for the niche that they occupy or prefer a niche landscape with more competition amongst OTUs. Unlike FILT samples, RAW communities represent the selection of community members from both inocula and those indigenous to the wastewater. This perhaps offers further support to arguments seen in other consortia experiments of phytoplankton and algae where increased biodiversity and competition necessitates a more efficient use of resources between taxa (Ptacnik et al., 2008;

Corcoran et al., 2012; Stockenreiter et al., 2011 and 2016). These relationships are demonstrated by the diversity of metabolic properties represented in biomarker clades isolated from RAW samples as well as literature evidence of the close relationships between some algal and bacterial species (Bell and Mitchell, 1972; Zhao et al., 2012; Sapp et al., 2006; Bell et al., 2015).

While further work on this community is needed to establish the nature of the relationships of the organisms here in, the results achieved argue a unique perspective on biomass and bio-oil accumulation beyond traditional nutrient limitation and monoculture studies. These results allude to the complexity and dynamics inherent to an *in situ* community while also suggesting the prospect that wastewater possesses for biofuel production with mixed algal communities. Overall, further efforts towards optimization and scale-up of micoalgae biofuels needs to consider alternative perspectives when working with mixed communities; one that incorporates the dynamic interplay of organisms and niches contributing to biomass and bio-oil accumulation.

CHAPTER FOUR

CONCLUSIONS AND FUTURE WORK

The growing consumption of fossil fuels and the need for sustainable forms of energy has spurned much interest in biofuels and the feedstocks that might potentially offer solutions to this crisis. Microalgae are positioned uniquely among these options not only for their ability to generate large amounts of biomass and bio-oil but also for their ability to do so while coupling with other high yield processes necessary to society. Amongst these potential processes wastewater treatment offers the infrastructure and design to sustain a microalgae approach to both bioremediation and lipid production. While, traditional culturing methods in these environments have focused on monoculture production of lipid through nutrient limitation, this thesis argues the possible lipid production and accumulation of mixed communities of algal and bacterial taxa.

There is substantial evidence in the literature that wastewater offers a nutrient rich but low-quality water source that algal species are capable of growth and lipid production (Chinnasamy et al., 2010; Zhou et al., 2011; Devi et al., 2012; Eustance et al., 2013; Oswald et al., 1957). Further studies have demonstrated the ability of mixed consortia to more efficiently allocate resources and harness niche optimization resulting in larger production of biomass and higher lipid yields (Stockenreiter et al., 2016; Liu et al., 2016). However, attention should be paid to the consortium in question given the heterogeneous nature of wastewaters and their nutrient fluctuations. This latter point argues the necessity to select microalgae and bacterial communities that simultaneously complement

one another but are also adept at survival in that particular waste environment if scale-up efforts are pursued.

Furthermore, the nature of the relationships between community members and metabolite exchange need to be explored if mixed communities are to be used as a solution. This thesis and other works have demonstrated the complex and dynamic nature of microbial relationships given a laboratory setting, *in situ* dynamics are no doubt going to prove even more complex in understanding (Croft et al., 2005, Zhao et al., 2012; Sapp et al., 2006; Bell et al., 2015; Bell and Mitchell, 1972). Still, even with the challenges of this complexity, determining the interplay of organismal dependence could prove essential for selecting and optimizing a microbial community for maximized production of biofuels.

A pilot study using this mixed community has begun to address this issue utilizing new selection techniques paired with next generation sequencing to identify community members co-localized in space and time. To do so, we have begun utilizing a microfluidic platform. Specifically, this technique offers the ability to utilize a high-throughput selection process on a small sample volume. Tiny drops generated by oil emulsion encase single cells and can be co-flowed with enzymes, PCR primers, and fluorescent dyes before a selection event isolates drops of interest (Spencer et al., 2015; Hanson et al., 2008; Rotem et al., 2015; Romero et al., 2015; Macosko et al., 2015). For our purposes, Nile Red fluorescence offers the ability to select for high lipid producing algal cells from a mixed community setting. Isolated algal cells along with their closely associated bacterial partners can then be amplified and sequenced for identification. This

technology may prove extremely valuable not only in identifying bacterial algal relationships in the phycosphere, but also the specific exchange of metabolites characterizing this co-localization.

Despite the challenges facing energy production from mixed communities, there is a growing research base to argue that optimization of mixed community biofuel production is possible and worthy of further efforts. Using the knowledge that has been obtained in municipal and other types of wastewater, mixed communities may offer a marriage of green processes with cost efficiency more so than other forms of biofuel or even other monoculture systems. In doing so, much needs to be learned regarding the nature and abilities of these communities but early indications demonstrate positive influences of mixed culture systems in wastewater environments for the production of value-added products.

REFERENCES CITED

Baena, S., Fardeau, M., Labat, M., Ollivier, B., Thomas, P., Garcia, J., & Patel, B. K. C. (1998). Aminobacterium colombiense gen . nov . sp . nov ., an Amino Acid-degrading Anaerobe Isolated from Anaerobic Sludge. *Anaerobe*, (an980170), 241–250.

Barros, A. I., Gonçalves, A. L., Simões, M., & Pires, J. C. M. (2015). Harvesting techniques applied to microalgae: A review. *Renewable and Sustainable Energy Reviews*, *41*, 1489–1500. http://doi.org/10.1016/j.rser.2014.09.037

Bell, T. A S., Prithiviraj, B., Wahlen, B. D., Fields, M. W., & Peyton, B. M. (2015). A Lipid-Accumulating Alga Maintains Growth in Outdoor, Alkaliphilic Raceway Pond with Mixed Microbial Communities. *Frontiers in Microbiology*, *6*(January), 1480. http://doi.org/10.3389/fmicb.2015.01480

Bell, W., & Mitchell, R. (1972). Chemotactic and Growth Response of Marine Bacteria to Algal Extracellular Products. *The Biological Bulletin*, *143*(2).

Bowen de León, K., Ramsay, B. D., & Fields, M. W. (2012). Quality-Score Refinement of SSU rRNA Gene Pyrosequencing Differs Across Gene Region for Environmental Samples. *Microbial Ecology*, *64*(2), 499–508. http://doi.org/10.1007/s00248-012-0043-9

Bruckner, C. G., Rehm, C., Grossart, H. P., & Kroth, P. G. (2011). Growth and release of extracellular organic compounds by benthic diatoms depend on interactions with bacteria. *Environmental Microbiology*, *13*(4), 1052–1063. http://doi.org/10.1111/j.1462-2920.2010.02411.x

Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L., & Knight, R. (2010). PyNAST: A flexible tool for aligning sequences to a template alignment. *Bioinformatics*, *26*(2), 266–267. http://doi.org/10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Walters, W. a. (2011). NIH Public Access, *7*(5), 335–336. http://doi.org/10.1038/nmeth.f.303.QIIME

Chen, W., Zhang, C., Song, L., Sommerfeld, M., & Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods*, *77*(1), 41–47. http://doi.org/10.1016/j.mimet.2009.01.001

Chinnasamy, S., Bhatnagar, A., Hunt, R. W., & Das, K. C. (2010). Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresource Technology*, *101*(9), 3097–3105. http://doi.org/10.1016/j.biortech.2009.12.026

Chisti, Y. (2008). Biodiesel from microalgae. *Trends in Biotechnology*, *26*(3), 126–131. http://doi.org/10.1016/j.tibtech.2007.12.002

Corcoran, A. a., & Boeing, W. J. (2012). Biodiversity Increases the Productivity and Stability of Phytoplankton Communities. *PLoS ONE*, *7*(11), 1–9. http://doi.org/10.1371/journal.pone.0049397

Croft, M. T., Lawrence, A. D., Raux-Deery, E., Warren, M. J., & Smith, A. G. (2005). Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature*, *438*(7064), 90–3. http://doi.org/10.1038/nature04056

Devillard, E., McIntosh, F. M., Duncan, S. H., & Wallace, R. J. (2007). Metabolism of linoleic acid by human gut bacteria: Different routes for biosynthesis of conjugated linoleic acid. *Journal of Bacteriology*, *189*(6), 2566–2570. http://doi.org/10.1128/JB.01359-06

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. http://doi.org/10.1093/bioinformatics/btq461

Eustance, E., Gardner, R. D., Moll, K. M., Menicucci, J., Gerlach, R., & Peyton, B. M. (2013). Growth, nitrogen utilization and biodiesel potential for two chlorophytes grown on ammonium, nitrate or urea. *Journal of Applied Phycology*, *25*(6), 1663–1677. http://doi.org/10.1007/s10811-013-0008-5

Fields, M. W., Hise, A., Lohman, E. J., Bell, T., Gardner, R. D., Corredor, L., … Gerlach, R. (2014). Sources and resources: Importance of nutrients, resource allocation, and ecology in microalgal cultivation for lipid accumulation. *Applied Microbiology and Biotechnology*, *98*(11), 4805–4816. http://doi.org/10.1007/s00253-014-5694-7

Gardner, R., Peters, P., Peyton, B., & Cooksey, K. E. (2011). Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the chlorophyta. *Journal of Applied Phycology*, *23*(6), 1005–1016. http://doi.org/10.1007/s10811-010-9633-4

Green, F. B., Bernstone, L. S., Lundquist, T. J., & Oswald, W. J. (1996). Advanced integrated wastewater pond systems for nitrogen removal. *Water Science and Technology*. http://doi.org/10.1016/0273-1223(96)00356-3

Green, F., Lundquist, T., & Oswald, W. (1995). Energetics of advanced integrated wastewater pond systems. *Water Science and Technology*, *31*(12), 9–20. http://doi.org/10.1016/0273-1223(95)00488-9

Hannon, M., Gimpel, J., Tran, M., Rasala, B., & Mayfield, S. (2010). Biofuels from algae: challenges and potential. *Biofuels*, *1*(5), 763–784. http://doi.org/10.4155/bfs.10.44

Hanson, J. a, Chang, C. B., Graves, S. M., Li, Z., Mason, T. G., & Deming, T. J. (2008). Nanoscale double emulsions stabilized by single-component block copolypeptides. *Nature*, *455*(7209), 85–8. http://doi.org/10.1038/nature07197

Knothe, G. (2008). " Designer " Biodiesel : Optimizing Fatty Ester Composition to Improve Fuel Properties, 1358–1364.

Kojima, E., & Zhang, K. (1999). Growth and hydrocarbon production of microalga Botryococcus braunii in bubble column photobioreactors. *Journal of Bioscience and Bioengineering*, *87*(6), 811–815. http://doi.org/10.1016/S1389-1723(99)80158-3

Lee, A. K., Lewis, D. M., & Ashman, P. J. (2009). Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel. *Journal of Applied Phycology*, *21*(5), 559–567. http://doi.org/10.1007/s10811-008-9391-8

Lindemann, S. R., Bernstein, H. C., Song, H.-S., Fredrickson, J. K., Fields, M. W., Shou, W., Beliaev, A. S. (2016). Engineering microbial consortia for controllable outputs. *The ISME Journal (in Press)*, 1–8. http://doi.org/10.1038/ismej.2016.26

Liu, J. (2016). Interspecific biodiversity enhances biomass and lipid productivity of microalgae as biofuel feedstock. *Journal of Applied Phycology*, 25–33. http://doi.org/10.1007/s10811-015-0535-3

Lohman, E. J., Gardner, R. D., Halverson, L., Macur, R. E., Peyton, B. M., & Gerlach, R. (2013). An efficient and scalable extraction and quantification method for algal derived biofuel. *Journal of Microbiological Methods*, *94*(3), 235–244. http://doi.org/10.1016/j.mimet.2013.06.007

Macosko, E. Z., Basu, a, Satija, R., Nemesh, J., Shekhar, K., Goldman, M., McCarroll, S. a. (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell*, *161*(5), 1202–1214. http://doi.org/10.1016/j.cell.2015.05.002

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for

ecological and evolutionary analyses of bacteria and archaea. *Isme J*, *6*(3), 610–618. http://doi.org/10.1038/ismej.2011.139

Medicine, T. (1971). The Life Cycle of the Coccisdian Parasite, Tozoplasma Gondii in the Domestic Cat. Department of Toxoplasmosis and Viral Diseases , Statens Seruminstitut , Copenhagen , Denmark In previous year, *65*(3).

More, T. T., Yan, S., Tyagi, R. D., & Surampalli, R. Y. (2010). Potential use of filamentous fungi for wastewater sludge treatment. *Bioresource Technology*, *101*(20), 7691–7700. http://doi.org/10.1016/j.biortech.2010.05.033

Normand, P., Gury, J., Pujic, P., Chouaia, B., Crotti, E., Daffonchio, D., Gtari, M. (2012). Genome sequence of radiation-resistant modestobacter marinus strain BC501, a representative actinobacterium that thrives on calcareous stone surfaces. *Journal of Bacteriology*, *194*(17), 4773–4774. http://doi.org/10.1128/JB.01029-12

Norsker, N. H., Barbosa, M. J., Vermue, M. H., & Wijffels, R. H. (2011). Microalgal production - A close look at the economics. *Biotechnology Advances*, *29*(1), 24–27. http://doi.org/10.1016/j.biotechadv.2010.08.005

Oksanen, J. (2015). Multivariate Analysis of Ecological Communities in R, 1–40. http://doi.org/10.1016/0169-5347(88)90124-3

Oswald, W. J., Asce, A. M., & Gotaas, H. B. (1957). Photosynthesis in Sewage Treatment, 73–105.

Papazi, A., Makridis, P., & Divanach, P. (2010). Harvesting Chlorella minutissima using cell coagulants. *Journal of Applied Phycology*, *22*(3), 349–355. http://doi.org/10.1007/s10811-009-9465-2

Prathima Devi, M., Venkata Subhash, G., & Venkata Mohan, S. (2012). Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phases: Effect of nutrient supplementation. *Renewable Energy*, *43*, 276–283. http://doi.org/10.1016/j.renene.2011.11.021

Ptacnik, R., Solimini, A. G., Andersen, T., Tamminen, T., Brettum, P., Lepisto, L., Rekolainen, S. (2008). Diversity predicts stability and resource use efficiency in natural phytoplankton communities. *Proceedings of the National Academy of Sciences*, *105*(13), 5134–5138. http://doi.org/10.1073/pnas.0708328105

Pulz, O. (2001). Photobioreactors: Production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology*. http://doi.org/10.1007/s002530100702

Ramette, A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiology Ecology*, *62*(2), 142–160. http://doi.org/10.1111/j.1574-6941.2007.00375.x

Ramirez, E., Robles, E., Martinez, B., Ayala, R., Sainz, G., Martinez, M. E., & Gonzalez, M. E. (2014). Distribution of free-living amoebae in a treatment system of textile industrial wastewater. *Experimental Parasitology*, *145*(S), S34–S38. http://doi.org/10.1016/j.exppara.2014.07.006

Rhine, E. D., Mulvaney, R. L., Pratt, E. J., & Sims, G. K. (1998). Improving the Berthelot Reaction for Determining Ammonium in Soil Extracts and Water. *Soil Science Society of America Journal*, *62*(2), 473. http://doi.org/10.2136/sssaj1998.03615995006200020026x

Ritchie, R. J. (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis Research*, *89*(1), 27–41. http://doi.org/10.1007/s11120-006-9065-9

Romero, P. a., Tran, T. M., & Abate, A. R. (2015). Dissecting enzyme function with microfluidic-based deep mutational scanning. *Proceedings of the National Academy of Sciences*, *112*(23), 201422285. http://doi.org/10.1073/pnas.1422285112

Rotem, A., Ram, O., Shoresh, N., Sperling, R. a., Schnall-Levin, M., Zhang, H., … Weitz, D. a. (2015). High-throughput single-cell labeling (Hi-SCL) for RNA-Seq using drop-based microfluidics. *PLoS ONE*, *10*(5), 1–14. http://doi.org/10.1371/journal.pone.0116328

Salim, S., Bosma, R., Vermuë, M. H., & Wijffels, R. H. (2011). Harvesting of microalgae by bio-flocculation. *Journal of Applied Phycology*, *23*(5), 849–855. http://doi.org/10.1007/s10811-010-9591-x

Sapp, M., Schwaderer, A. S., Wiltshire, K. H., Hoppe, H. G., Gerdts, G., & Wichels, A. (2007). Species-specific bacterial communities in the phycosphere of microalgae? *Microbial Ecology*, *53*(4), 683–699. http://doi.org/10.1007/s00248-006-9162-5

Sedlak, R. (1991). *Phosphorus and Nitrogen Removal from Municipal Wastewater: Principles and Practice*.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. http://doi.org/10.1186/gb-2011-12-6-r60

Sheehan, J., Dunahay, T., Benemann, J., & Roessler, P. (1998). A look back at the U. S. Department of Energy's aquatic species program: biodiesel from algae. *National Renewable Energy Program*, *328*, 291 p. http://doi.org/10.2172/15003040

Shimada, K. (1995). Anoxygenic Photosynthetic Bacteria. In R. E. Blankenship, M. T. Madigan, & C. E. Bauer (Eds.), (pp. 105–122). Dordrecht: Springer Netherlands. http://doi.org/10.1007/0-306-47954-0_6

Smith, B. T., & Davis, R. H. (2012). Sedimentation of algae flocculated using naturally-available, magnesium-based flocculants. *Algal Research*, *1*(1), 32–39. http://doi.org/10.1016/j.algal.2011.12.002

Spencer, S. J., Tamminen, M. V, Preheim, S. P., Guo, M. T., Briggs, A. W., Brito, I. L., Alm, E. J. (2015). Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers. *The ISME Journal*, 1–10. http://doi.org/10.1038/ismej.2015.124

Staley, J. T., Marshall, K. C., & Skerman, V. B. D. (1980). Budding and prosthecate bacteria from freshwater habitats of various trophic states. *Microbial Ecology*, *5*(4), 245–251. http://doi.org/10.1007/BF02020332

Stockenreiter, M., Graber, A.-K., Haupt, F., & Stibor, H. (2011). The effect of species diversity on lipid production by micro-algal communities. *Journal of Applied Phycology*, *24*(1), 45–54. http://doi.org/10.1007/s10811-010-9644-1

Stockenreiter, M., Haupt, F., Seppälä, J., Tamminen, T., & Spilling, K. (2016). Nutrient uptake and lipid yield in diverse microalgal communities grown in wastewater. *Algal Research*, *15*(APRIL), 77–82. http://doi.org/10.1016/j.algal.2016.02.013

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, G. J. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest.pdf. *Nature*, *444*, 21–28.

Valenzuela, J., Mazurie, A., Carlson, R. P., Gerlach, R., Cooksey, K. E., Peyton, B. M., & Fields, M. W. (2012). Potential role of multiple carbon fixation pathways during lipid accumulation in Phaeodactylum tricornutum. *Biotechnology for Biofuels*, *5*(1), 40. http://doi.org/10.1186/1754-6834-5-40

VandeWalle, J. ., Fomby, P., & Cherlin, A. J. (2012). Acinetobacter, Aeromonas, and Trichococcus populations dominate the micobial community within urband sewer infastructure. *Evironmental Microbiology*, *14*(9), 181–204. http://doi.org/10.1038/nature13314.A

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. http://doi.org/10.1128/AEM.00062-07

Wrabel, M. L., & Peckol, P. (2000). Effects of bioremediation on toxicity and chemical composition of No. 2 fuel oil: Growth responses of the brown alga Fucus vesiculosus. *Marine Pollution Bulletin*, *40*(2), 135–139. http://doi.org/10.1016/S0025-326X(99)00181-2

Zhao, G., Du, J., Jia, Y., Lv, Y., Han, G., & Tian, X. (2012). The importance of bacteria in promoting algal growth in eutrophic lakes with limited available phosphorus. *Ecological Engineering*, *42*, 107–111. http://doi.org/10.1016/j.ecoleng.2012.02.007

Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., & Ruan, R. (2011). Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. *Bioresource Technology*, *102*(13), 6909–6919. http://doi.org/10.1016/j.biortech.2011.04.038

APPENDICES

APPENDIX A

SUPPORTING MATERIAL FOR CHAPTER TWO MATERIALS AND METHODS

Growth and Lipid Analysis for Preliminary Studies

Preliminary experiments were performed to establish the ability of the mixed photoautotrophic community tested in this thesis to produce lipid in its natural wastewater environment. To address this question, the community of photoautrophs were tested in two media types. The first being Bold's Basal Medium (BBM) a defined media often used in laboratory settings for algal growth as well as sterilized primary effluent from the Clearas Recovery center (PCW) where the community was harvested. Each preliminary experiment was done with three biological replicates. Three technical replicates were also taken for each measurement of pH, optical density (OD), cell counts, chlorophyll, Nile Red, and water chemistry. Repeated growth experiments were conducted at the termination of Growth 1 by returning to stored slurry inocula at 20°C.

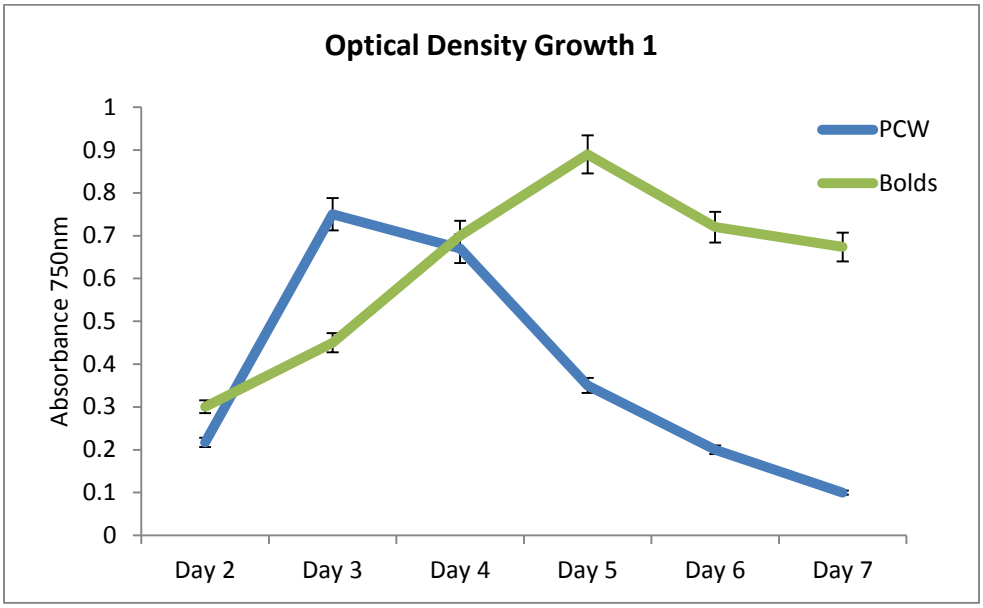


Figure A.1: Optical density of Growth 1 batch cultures grown on primary clarifier effluent (PCW) and Bold's Basal Medium (BBM) over a seven-day period. Initial PCW growth rates were high but later exceeded by BBM cultures that exhibited higher biomass yields.

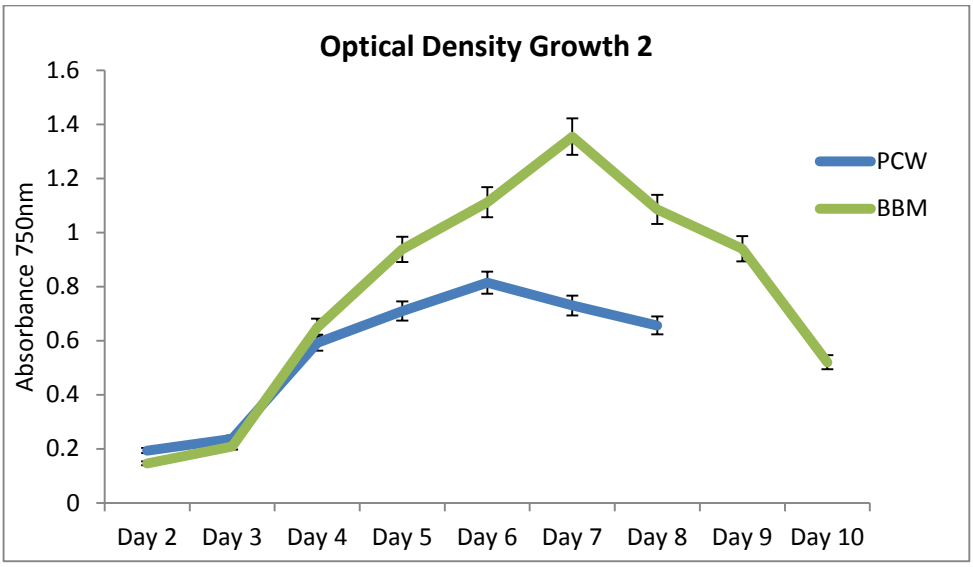
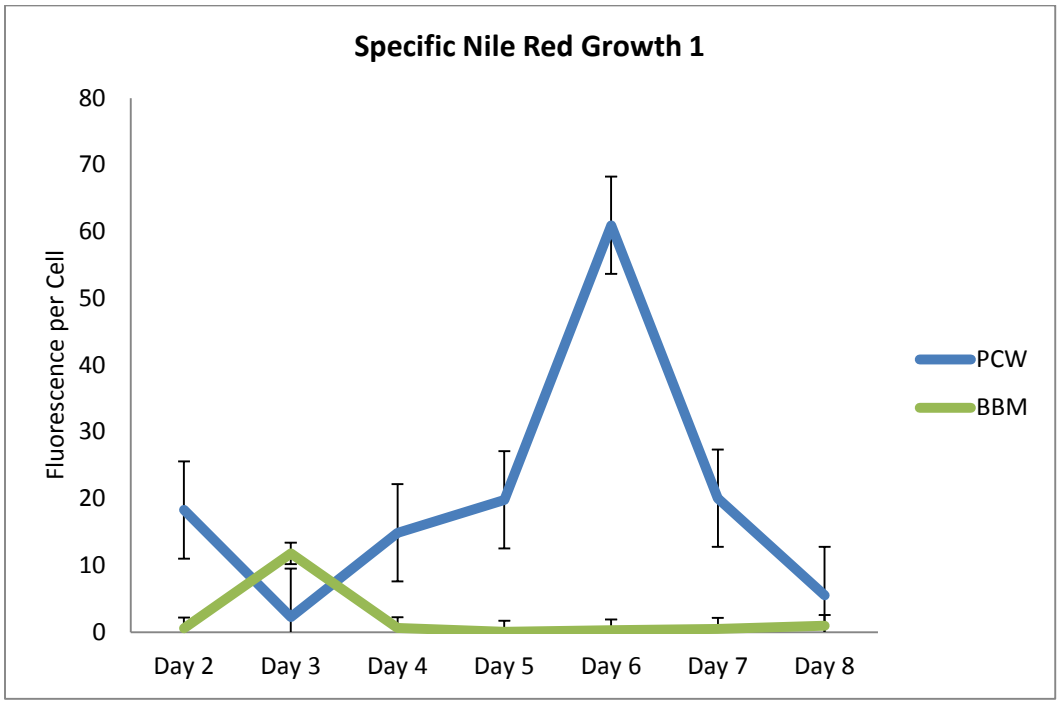


Figure A.2: Optical density of Growth 2 batch cultures grown on primary clarifier effluent (PCW) and Bold's Basal Media (BBM) over a ten-day period. Although initial growth rates were similar, BBM cultures exhibited higher biomass yields.



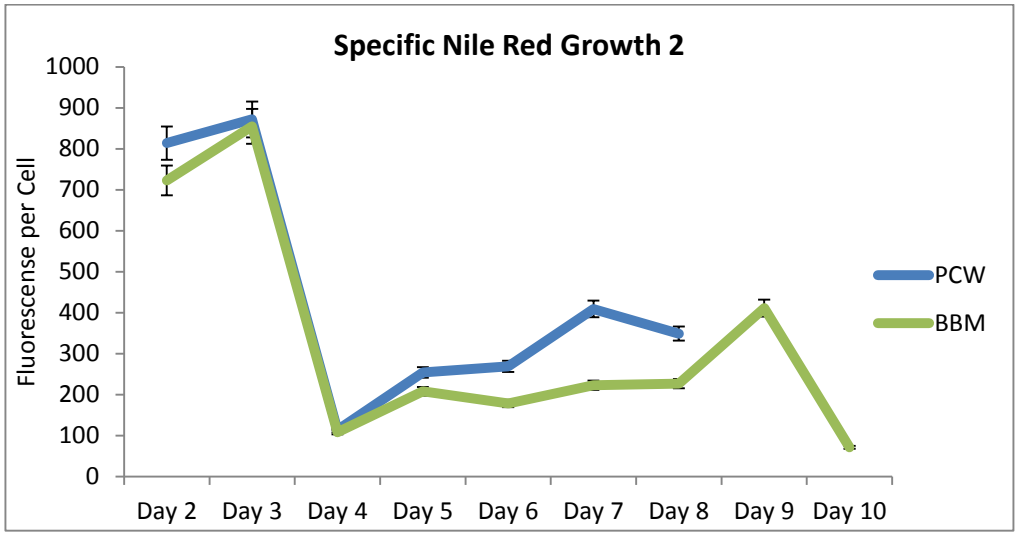


Figure A.3: Specific Nile Red readings for batch cultures (Growth 1 and Growth 2) after five minutes of exposure. Nile Red readings were relatively low for both PCW and BBM cultures.

Cultures grown in PCW had a quick but short increase in growth as was evident from optical density readings for both Growth 1 and Growth 2 (Figure A.1 and A.2). Cultures grown in PCW began to display signs of stress based upon stop in growth and decline in biomass over time. The PCW cultures did display a spike in overall Nile Red for both experiments that correlated in conjunction with signs of cellular stress (Figure A.3).

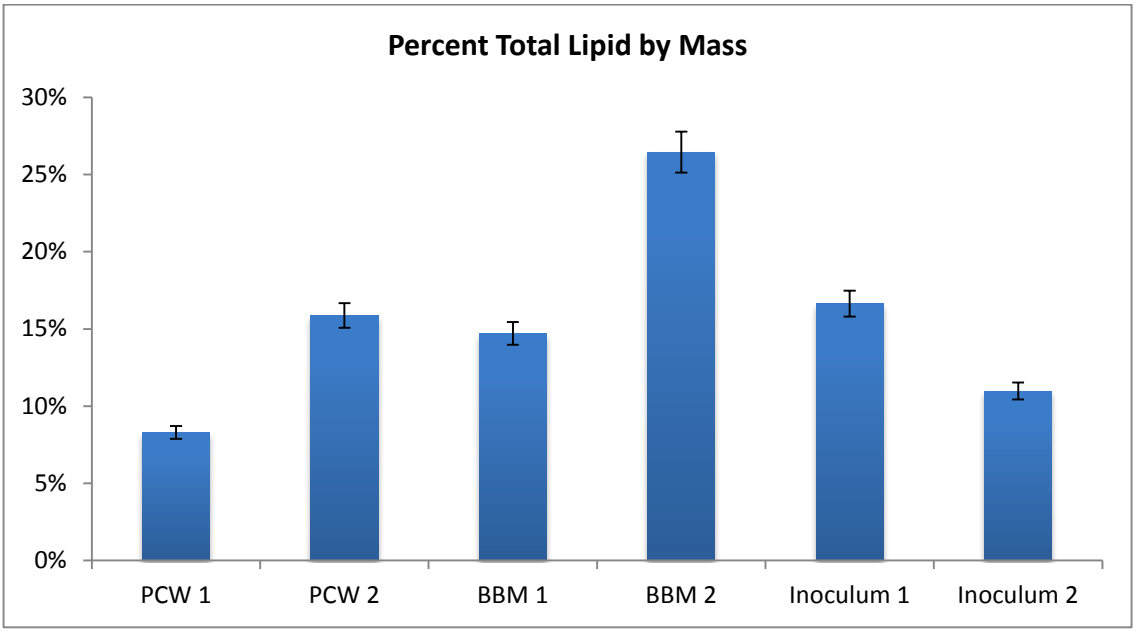


Figure A.4: Total lipid percentages (weight/weight) estimated as the total amount of FAMEs for each sample estimated by in situ transesterification combined with GC-MS analysis. The highest lipid content was observed in BBM cultures, however PCW cultures did demonstrate the ability to produce lipid.

These lower Nile Red readings seen in Figure A.2 correlated with relatively low lipid production with fatty acid methyl ester (FAME) content at close to 15% (weight/weight) (Figure A.3). The BBM cultures had a longer growth period that corresponded to small increases in biomass. However, FAME content for BBM treatment of cultures did exhibit an overall increase at close to 26% (weight/weight).

Data and observations from these preliminary experiments indicated some important conclusions that became the foundation for our final experiments. Firstly, overall growth between the two media types demonstrated a higher biomass production in BBM over the PCW. Secondly, specific Nile Red was low for both culture conditions, and this was confirmed with GC-MS quantification. Finally, while overall FAME production between treatment types indicated BBM samples displayed higher content to that of PCW

cultures; PCW cultures were shown to be capable of lipid production under the conditions tested.

Bold's Basal Medium Content

Table A.1: Bold's Basal Medium.

Bold's (1 liter)	
Reagent	
KH_2PO_4	175 mg
$CaCl_2*2H_2O$	25 mg
$MgSO_4*7H_2O$	75 mg
$NaNO_3$	250 mg
K_2HPO_4	75 mg
NaCl	25 mg
H_3BO_3	11.42 mg
Microelements (ME)	1 mL
S1	1 mL
S2	1 mL
S3	1 mL
B12	0.5 mL

Microelement solution.

ME (1 liter)	g
$ZnSO_4*7H_2O$	8.82
$MnCl_2*4H_2O$	1.44
$MoO_3Na2*2H_2O$	0.71
$CuSO_4*5H_2O$	1.57
$Co(NO_3)_2*6H_2O$	0.49

Solution 1.

S1 (1 liter)	g
Na_2EDTA	50
KOH	3.1

Solution 2.

S2 (1 liter)	g
$FeSO_4*7H_2O$	4.98
H_2SO_4 (con)	1 (mL)

Solution 3.

S3 (1 liter)	g
Inositol	5
Thymine	3
Thiamine*HCl (B1)	0.5
Nicotinic acid (niacin)	0.1
Ca pantothenate	0.1
p-Aminobenzoic acid	0.01
Biotin (vitamin H)	0.001
Folic Acid	0.002

B12 Vitamin Solution.

B12 (1 liter)	g
Cyanocobalamin (B12)	0.004

APPENDIX B

SUPPORTING MATERIAL FOR CHAPTER THREE EUKARYOTIC ABUNDANCES

Relative Sequence Abundance Growth 1 and Growth 2

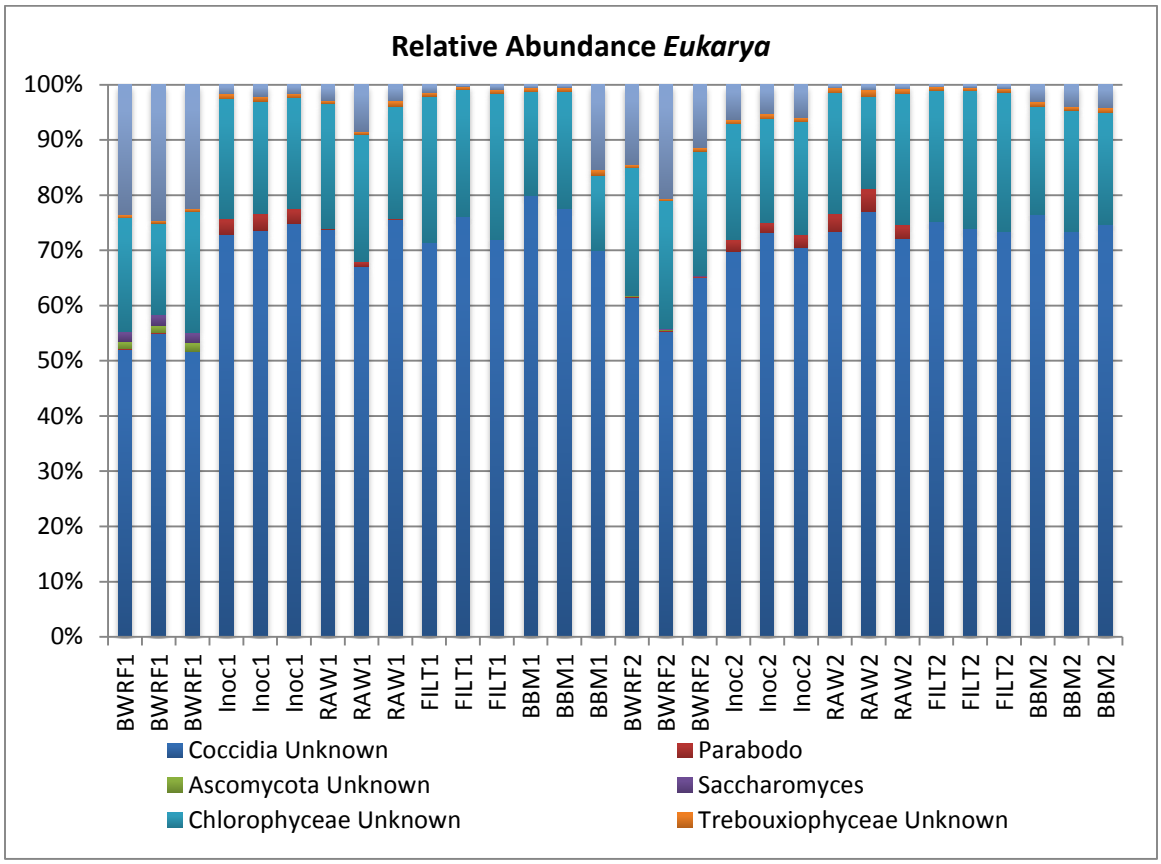


Figure B.1: Percent relative abundance of unique *Eukarya* OTUs present above 1% for each culture condition in Growth 1 and Growth 2. Coccidian dominance in all samples can be seen at greater than 70%.

APPENDIX C

SUPPORTING MATERIAL FOR CHAPTER FOUR FUTURE WORK

Microfluidic Platform and Protocols

The studies completed by our group and others have indicated the potential importance in mix-community algal growth, however, determining high lipid producing members of these communities can be challenging. The desire to physically separate individual algal cells from other community members is increasingly coveted in such systems. Using a microfluidics platform previously employed in High-throughput Single-Cell labeling (Rotem et al, 2015), we began exploring the possibility of isolating high producing algal cells from a mixed community of *Eukarya* and *Bacteria*. Pairing these techniques with next generation sequencing to identify key community as well as members co-localized in space and time could provide insight into community relationships and metabolite exchange.

To do so, tiny drops are generated by oil emulsion to encase single cells that can be co-flowed with enzymes, PCR primers, and fluorescent dyes before a selection event isolates drops of interest (Spencer et al., 2015; Hanson et al., 2008; Rotem et al., 2015; Romero et al., 2015; Macosko et al., 2015). For our purposes, Nile Red fluorescence offers the ability to select for high lipid producing algal cells from a mixed community setting (Figure C.1).

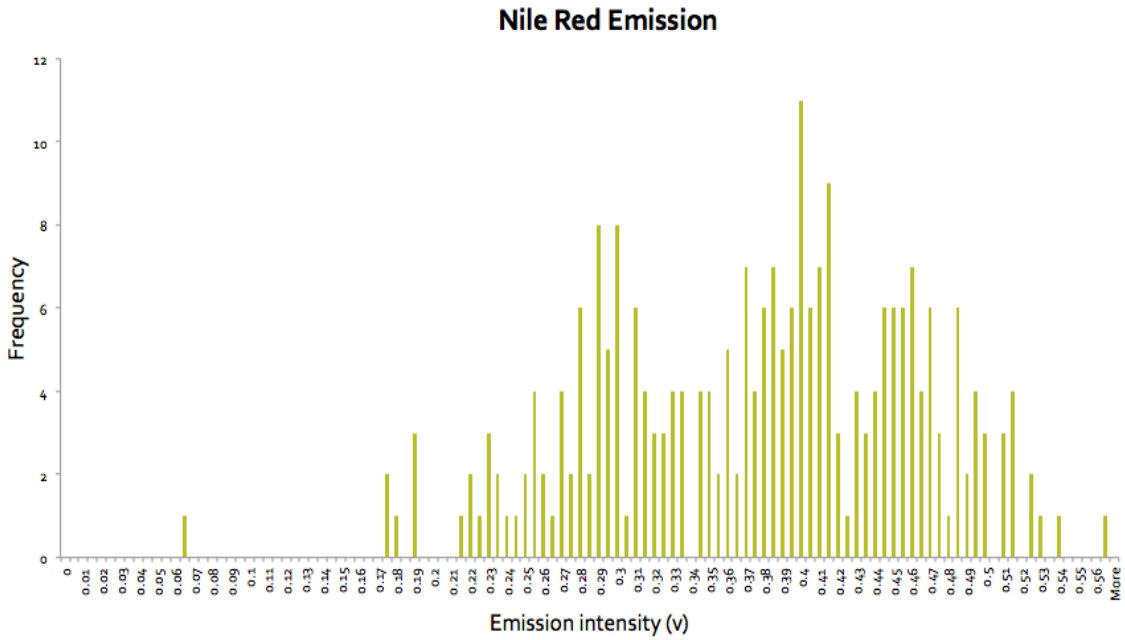


Figure C.1: The histogram illustrates our principle for selection. Each emission peak indicates an individual drop exhibiting by Nile Red fluorescence. Parameters within the microdroplet platform allow selection thresholds of interest as individual drops pass detection. Robert Schaefer, 2015.

Isolated algal cells from these selection events can then be processed downstream for sequencing, PCR, or growth. Closely associated bacterial partners might also be detected given variation in drop sizes used to house algal cells. This technology may prove extremely valuable not only in identifying bacterial algal relationships in the phoycosphere, but also the specific exchange of metabolites characterizing this co-localization.

Sample Preparation

Preliminary experiments tested mixed communities in non-sterile municipal wastewater. Growth was monitored according to traditional protocols of OD, Nile Red, and Chlorophyll. Once a peak in Nile Red fluorescence was determined before

harvesting, algal cells were harvested in 2mL aliquots. Cellular concentration were diluted to anywhere between 10^5-10^6 cells/mL. To this solution 8μL of Nile Red (0.25 mg Nile red/ml of acetone) was added and placed into a 3mL syringe. Samples were incubated for 5 minutes and then processed in the dark to protect the integrity of the dye before drops were finally placed into a 1.7mL microcentrifuge tube. Collected drops were then frozen at -20°C before DNA extraction.

DNA Extraction

DNA was extracted using 200μL of the aqueous layer from lysed microdroplets using a FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH) according to manufacturer protocols. The DNA was cleaned and concentrated with the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Extracted DNA was quantified with a Qubit fluorometer using a Qubit dsDNA BR detection kit (Invitrogen, Carlsbad, CA). Unfortunately, DNA concentrations were below detection limits even after re-concentration efforts.