CHARACTERIZATION AND PERFORMANCE OF ALGAL BIOFILMS FOR
WASTEWATER TREATMENT AND INDUSTRIAL APPLICATIONS

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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ABSTRACT

Characterization and Performance of Algal Biofilms for Wastewater Treatment and Industrial Applications

by

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Fundamental studies on algal biofilms grown for biological wastewater treatment and as a feedstock for biofuels production were carried out to determine: nutrient uptake capacity, biofilm growth rates, photosynthetic activity, lipid accumulation from nitrogen stress and bicarbonate addition, and algae-bacteria interactions. The algal biofilms were cultured on rotating algal biofilm reactors (RABRs) under varying conditions of light, nutrients, and species composition at bench and pilot scale.

Oxygen microsensor-based measurements and microscopy were used to characterize spatial photosynthetic zones and biofilm structure, respectively. Nutrients were analyzed using ion chromatography and TOC/TN analyzer. Lipid composition and quantification were determined using gas chromatography flame ionization detection (GC-FID) and mass spectrophotometry (GC-MS). Drip flow reactors (modified to allow for phototrophic growth) were utilized to study algae-bacteria interactions under sterile conditions.
The results showed that photosynthetic and respiration rates observed in the algal biofilms were influenced by biofilm composition, culturing conditions, orientation to the light, and nutrient availability. Nutrient removal capacity was highest during the exponential growth phase of the biofilm. A slight increase in lipid production was observed as a result of nitrogen stress and a combination of nitrogen stress with bicarbonate addition. Presence of bacteria positively influenced microalgae growth in the mixed cultures but the reverse was not true.

In conclusion, spatial photosynthetic activity and biofilm structure were successfully characterized using the methods developed in this study. Algal biofilms can potentially be utilized for nutrient uptake in wastewater but their biofuels potential will depend on the amount of biomass produced because of the low lipid yields realized. Further research on algae-bacteria interactions using species native to the wastewater grown algal biofilms is recommended.
PUBLIC ABSTRACT

Characterization and Performance of Algal Biofilms for Wastewater Treatment and Industrial Applications

Maureen Kesaano

This study was carried out on algal biofilms grown using rotating algal biofilm reactors (RABRs) with the aim of: i) characterizing their growth in terms of photosynthetic activity and morphology ii) evaluating their performance as a wastewater treatment option and a feedstock for biofuels production, and iii) examining the algal-bacteria interactions.

A review of algal biofilm technologies currently employed in wastewater treatment processes was made to compare nutrient removal efficiencies, factors that influenced algal biofilm growth, and the different bioproducts generated from algal biomass. Consequently, research efforts were directed towards addressing pertinent issues identified in literature in order to optimize these systems for wastewater treatment and bioproducts production.

Successful growth of algal biofilms in municipal wastewater and subsequent removal of nutrients from the wastewater was demonstrated. Photosynthetic and respiration rates observed with depth of the biofilm were influenced by the biofilm composition (single vs. mixed species), culturing conditions (laboratory vs. outdoor), orientation to the light, nitrogen availability (N-replete vs. N-deplete), and dissolved inorganic carbon availability (presence or absence of bicarbonate). Slight enhancement in lipid production was also observed as a result of nitrogen stress and bicarbonate addition.
However, the accumulated lipids were not as much as expected or as reported in suspended cultures. Presence of bacteria positively influenced microalgae growth in the mixed cultures but the reverse was not true.

In conclusion, photosynthetic activity and biofilm structure were characterized with methods developed for the algal biofilms in this study. For now, productivity of the algal biofilms needs to be maximized in order to fully utilize its potential as a biofuel feedstock and nutrient removal option. Further research on algae-bacteria interactions using species native to the wastewater grown algal biofilms is recommended.
DEDICATIONS

This dissertation is dedicated to my mom, Dr. Margaret Saimo-Kahwa. I am indebted to your support and encouragement through this journey.
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TERMINOLOGY

\( J \): Diffusive flux of oxygen (\( \mu \text{mol O}_2 \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \))

\( C_{O_2} \): Concentration of oxygen (\( \mu \text{mol} \cdot \text{L}^{-1} \))

\( D_e \): Effective diffusion coefficient of oxygen (typically reported in units of \( \text{cm}^2 \cdot \text{sec}^{-1} \))

\( z \): Spatial coordinate extending from surface of biofilm (\( z=0 \)) down towards the substratum

\( L_{\text{phot}} \): Depth of the photic zone (\( \mu \text{m} \))

\( t \): Time coordinate (sec)

\( P_a \): Areal rate of photosynthesis for the biofilm (\( \mu \text{mol O}_2 \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \))

\( P_{n,\text{phot}} \): Areal rate of photosynthesis for the photic zone (\( \mu \text{mol O}_2 \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \))

\( R_{\text{dark}} \): Areal respiration rate in the dark (\( \mu \text{mol O}_2 \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \))

\( P_g(z) \): Gross volumetric rate of photosynthesis (\( \mu \text{mol O}_2 \cdot \text{L}^{-1} \cdot \text{sec}^{-1} \))

\( R_{\text{light}}(z) \): Gross volumetric rate of respiration in the light (\( \mu \text{mol O}_2 \cdot \text{L}^{-1} \cdot \text{sec}^{-1} \))

\( P_g \): Gross areal rate of photosynthesis (\( \mu \text{mol O}_2 \cdot \text{cm}^{-2} \cdot \text{sec}^{-1} \))

\( \text{NADPH} \): Nicotinamide adenine dinucleotide phosphate

\( \text{ATP} \): Adenosine triphosphate

\( \text{PSII} \): Photosystem II
CHAPTER 1

INTRODUCTION

1.0 Introduction

There is a growing interest in microalgae-based systems as potential inexpensive biotechnology, which can treat wastewater, sequester CO\textsubscript{2} from the atmosphere via photosynthesis, and provide biomass for bioproducts production (Kebede-Westhead et al., 2004; Roeselers et al., 2008). Wastewater treatment facilities are required to limit the amount of nitrogen (N) and phosphorus (P) discharged into the environment because of eutrophication concerns in the receiving waters (Cai et al., 2013; Lee et al., 2013; Ruiz-Marín et al., 2010). As a result, municipal wastewaters are an inexpensive source of nutrients for microalgal cultivation, which does not compete with freshwater sources. Microalgae can use the dissolved nutrients in wastewater for plant growth and wastewater remediation will occur through subsequent harvesting of the nutrient-rich biomass (Park et al., 2011; Pizarro et al., 2006; Woertz et al., 2009). Additionally, microalgae increase medium pH through photosynthesis, which can enhance ammonia removal via volatilization and phosphorus removal via precipitation of phosphate with calcium or magnesium ions (Boelee et al., 2012; Craggs et al., 1997; Muñoz and Guieysse, 2006).

Mass cultivation of microalgae usually occurs in suspended growth systems, which makes separation of the microscopic cells from the liquid medium difficult, time consuming, and energy intensive (De la Noüé et al., 1992; Hofmann, 1998). Attached algal growth platforms have been developed to simplify harvesting and minimize downstream processing costs (Boelee et al., 2014; Gross et al., 2013; Guzzon et al., 2008). For example, Christenson and Sims (2011) designed a rotating algal biofilm
reactor (RABR) as a retrofit for a lagoon style system in order to grow and recover already existing microalgae in the Logan City wastewater lagoons. The algal biofilm successfully grew on both bench and pilot scale operations, wastewater remediation was demonstrated, and the biomass was harvested by scraping it off the growth surface (Christenson and Sims, 2012).

Microalgae growth and biomass composition are not only influenced by nutrient availability but also environmental conditions such as pH, temperature, and light. Depending on the growth conditions, microalgae will produce and accumulate different compounds including carbohydrates, lipids, proteins, and pigments from which valuable products can be synthesized (Fields et al., 2014; Spolaore et al., 2006). Biofuels, nutraceuticals, animal and fish feed, fertilizers, and solvents are examples of some of the bioproducts generated from microalgae biomass (Chisti, 2007; Ellis et al., 2012; Pulz and Gross, 2004). Of interest to this study is the use of algal biofilms as feedstock for biofuels production. Current attempts to stimulate and accumulate lipids in algal biofilms involve using techniques employed in suspended microalgae cultures, such as nutrient starvation, pH stress, and chemical addition (Schnurr et al., 2013; Sharma et al., 2012). Limited research has been carried to determine the feasibility of these strategies either independently evaluated or in combination in algal biofilms.

Chapter 2 provides a detailed literature review of algal biofilm technologies currently employed in wastewater treatment. Nutrient removal efficiencies of the various algal biofilm based systems are compared, factors that influence algal biofilm growth are identified, and information on the different bioproducts generated from algal biomass is provided. Consequently, challenges faced by algal biofilm-based biotechnology as
applied to wastewater treatment both at bench scale and at pilot scale operations are highlighted and specific recommendations made to address some of the pertinent challenges.

2.0 Photosynthesis and photorespiration in microalgae cultures

Algal cultivation is not only limited by essential nutrients such as nitrogen (N), phosphorus (P), and silicon (Si) in case of diatoms, but also carbon (C) (Borowitzka and Moheimani, 2013). Carbon dioxide (CO₂) and /or bicarbonate (HCO₃⁻) are the inorganic carbon forms used by microalgae in the synthesis of biochemical compounds necessary for growth during photosynthesis, but not carbonate (CO₃²⁻)(Lin et al., 2003). As a result, pH of the medium plays an important role in the availability of inorganic carbon necessary for photosynthesis (Kumar et al., 2010; Staal et al., 2007).

During photosynthesis, light captured by photosynthetic pigments such as chlorophyll is converted to chemical energy in the form of ATP and NADPH, and used to fix carbon (CO₂) to carbohydrates (equation 1).

\[ \text{CO}_2 + \text{H}_2\text{O} \xrightarrow{\text{light}} \text{carbohydrates} + \text{O}_2 \]

Eqn1

Two distinct processes known as the light and dark reactions take place during photosynthesis. Light dependent reactions and their corresponding electron transport occur in the thylakoid membranes where the water molecule is split in the photosystem II (PSII), leading to production of oxygen (O₂), formation of reducing power (NADPH), and ATP. On the contrary, the dark (Calvin-Benson cycle) or light independent reactions occur in the stroma, where carbon is fixed through a reaction catalyzed by ribulose-1-5-bisphosphate carboxylase/oxygenase (RuBisCO) to carbohydrates (Hohmann-Marriott and Blankenship, 2012).
A competing process to carboxylation known as photorespiration also occurs essentially interfering with the Calvin cycle (Rink et al., 1998; Stephenson et al., 2011). RuBisCO combines with O$_2$ instead of CO$_2$ because it has active sites for both O$_2$ and CO$_2$. The reaction with either of these molecules is controlled by competitive inhibition i.e., high O$_2$/CO$_2$ ratios will favor photorespiration whereas low O$_2$/CO$_2$ ratios will favor carboxylation (Kühl et al., 1996; Pringault and Garcia-Pichel, 2000). In order to minimize the effects of photorespiration and enhance photosynthetic efficiency, there is a need to increase carbon fixation by effectively stripping O$_2$ or enriching CO$_2$ in microalgal cultures given that RuBisCO has a low affinity to CO$_2$ (Moroney and Somanchi, 1999).

Chapter 3 and Appendix A contain results of oxygen micro profiles from representative laboratory and field grown algal biofilm samples under different culturing conditions. The net rate of photosynthesis was determined as a measure of oxygen production. Photosynthesis, photosynthesis-coupled respiration, and dark respiration processes in the algal biofilms were determined using oxygen based microsensor measurements. This involved direct measurement of oxygen concentrations at different depths in the algal biofilm during dark and/or light conditions and analysis of the acquired data as described in supplementary section in Appendix B.

3.0 Influence of bicarbonate on algal biofilm growth, nutrient uptake and lipid accumulation

Sources of inorganic carbon for algal cultivation include atmospheric CO$_2$, dissolved inorganic carbon (DIC), CO$_2$ from bacterial degradation of organic carbon, and external sources of CO$_2$ enriched air or HCO$_3^-$ salts (Shi et al., 2007; Su et al., 2011). There are challenges associated with CO$_2$ supplementation, which include low solubility,
short retention times in aqueous medium, and difficulty in transportation (Chi et al., 2011; Wensel et al., 2014), consequently the use of HCO$_3^-$ salts is considered as a viable alternative to CO$_2$ supplementation (Gardner et al., 2012; Prins and Elzenga, 1989; Rickman et al., 2013). However, some microalgae species have biological adaptations called carbon-concentrating mechanisms (CCMs), which enable them to cope with low and variable CO$_2$ concentrations in their environments (Moroney and Ynalvez, 2007; Spalding, 2008).

Microalgae will take up HCO$_3^-$ through active carbon uptake systems and CO$_2$ by passive diffusion. With the CCMs, microalgae actively take up inorganic carbon from the external environment thereby increasing the intracellular inorganic carbon concentrations to levels higher than the external environment (Gardner et al., 2013; Guihéneuf and Stengel, 2013; Subramanian et al., 2013). The accumulated HCO$_3^-$ is then converted to CO$_2$ by carbonic anhydrase in the carboxysome of cyanobacteria or pyrenoids of eukaryotic algae respectively (White et al., 2013).

Chapter 3 contains a study carried out on laboratory grown algal biofilms to determine whether addition of 2 mM HCO$_3^-$ to the medium (synthetic wastewater) enhanced biofilm growth, nutrient uptake, lipid accumulation during nutrient deplete culturing, and photosynthetic rates with depth of the biofilm. A chlorophyte isolate referred to as WC-2B was used in this study. WC-2B was isolated from an alkaline stream in Yellowstone National Park (USA), confirmed unialgal using SSU 18S rDNA and revealed 99% alignment with Botryococcus sedeticus UTEX 2629. This species was of interest because of its tendency to form biofilms and the planktonic cultures of WC-2B
were able to accumulate lipids under high pH and nitrate deplete conditions (up to 13.9% (w/w) of extractable precursors, 7.7% (w/w) of which was TAG) in preliminary studies.

### 4.0 Algae-bacteria interactions in mixed cultures

There is need for research on the close association of microbial groups involved in the formation of algal biofilms in natural environments especially the influence of algal–bacterial interactions or predation on biofilm structure. Municipal wastewaters are no exception because they are non-sterile environments, which contain a diverse population of microorganisms. In Chapter 4, a preliminary study compared the influence of algae-bacteria interactions on the productivity of an artificial algae-bacteria co-culture growing in attached and suspended form using drip flow reactors and shaker flasks respectively. Microalgae strains *Scenedemus obliquus* and *Chlorella vulgaris* and bacteria strain *Escherichia coli* were selected for this study because of their abundance in wastewaters. Sterile conditions were maintained in these studies by autoclaving all media and apparatus used in addition to the use of antibiotics. The *E. coli* strain used had an antibiotic resistant gene encoded in a high copy plasmid so that they could survive the antibiotic treatment.

### 5.0 General overview and summary of the dissertation

In chapter 5, an overview of the work done in this dissertation is given. The main goal of this research was provide fundamental information on algal biofilm communities necessary for the implementation of algal biofilm based biotechnologies in a wastewater treatment process integrated with the production of valuable bioproducts from the harvested biomass. Most of the research was carried out on bench scale with rotating
algal biofilm reactors (RABRs). The RABR was designed and developed at Utah State University as an attached growth platform for microalgae cultivation.

6.0 Justification of the study

Due to limited information on algal biofilm based systems for wastewater treatment, there is a need for fundamental studies on algal biofilm processes in terms of growth rates, nutrient removal capacity, physiological processes, and heterotrophic–autotrophic interactions. This study was carried out with the aim of providing information on algal biofilm communities in order to improve the understanding of these communities and optimize algal biofilm based biotechnology in wastewater treatment and bioproducts production. The specific aims of the study included:

1. Identifying knowledge gaps in the literature
2. Characterizing algal biofilms in terms of photosynthetic activity and structure
3. Evaluating the performance of algal biofilms as a nutrient removal option for wastewater treatment and as a feedstock for biofuels production
4. Investigating the effect of algal-bacteria interactions on productivity

7.0 Refereed publications


References


CHAPTER 2
ALGAL BIOFILM-BASED TECHNOLOGY FOR WASTEWATER TREATMENT- A REVIEW

Abstract

Widespread application of algal biofilm-based systems in wastewater treatment has been limited despite the potential benefits of a low cost nutrient removal option and a source of biomass for bioproduct production. The performance and processes involved with algal biofilm-based systems in wastewater treatment are not adequately addressed in the available literature, which hinders design and scale up of effective systems for applications to municipal, industrial, and agricultural waste streams. A critical review is presented, which examines nutrient removal trends, biomass productivity, growth requirements, and challenges for algal biofilm-based biotechnology as applied to wastewater treatment both at bench scale and at pilot scale operations. Information on algal biofilms in natural environments derived from ecology and limnology disciplines was utilized in areas of limited research with regard to wastewater treatment. This critical review identified key areas that need to be addressed for designing, building, and testing algal biofilm-based technologies that integrate both nutrient removal from wastewater and enhanced biomass production to improve the performance of engineered systems. The review identifies the need for research on factors that affect algal growth, mass transport, species selection, algal–bacterial interactions, and validation of laboratory research in field scale tests for the development of an algal biofilm based technology platform for integrating wastewater treatment and biomass production.

1.0 Introduction

Algae are ubiquitous single to multi cellular chlorophyll-containing organisms that lack true roots, stems, and leaves [1]. Biofilms are complex communities of microorganisms that grow on solid surfaces enclosed in a matrix of extracellular polymeric substances (EPS). The term “algal biofilms” therefore refers to microalgae dominated biofilm communities that colonize illuminated surfaces in the presence of moisture and nutrients [2,3]. Like bacterial biofilms, algal biofilms have the ability to adapt to changes in the environment, sustain colonies on a surface, and dissociate from a surface as a single colony or in clumps [4]. Although ubiquitous in nature, algal biofilms have mostly been studied for their detrimental effects on structures [5,6] with the aim of controlling or preventing their growth mainly due to safety and economic concerns [2]. However, recent renewed interest in algal biofilms has been driven by the need for wastewater remediation strategies for nutrient control, alternative biofuel feedstock, and effective low cost biomass harvesting techniques [7–10].

Growing algal biofilms as a nutrient removal option for wastewater treatment may provide both an effective nutrient treatment technology and a source of algal biomass for bioproduct production [11]. However, the development of algal biofilm-based wastewater treatment technologies faces challenges due to limited information on algal growth needs, biofilm area requirements, nutrient removal efficiencies, and standard operating procedures (SOPs) for either bench or field scale operations and testing [12,13]. Unlike the established heterotrophic attached growth systems used in wastewater treatment [14,15], there is a need to understand the performance and processes involved with algal biofilm-based wastewater treatment technologies in order to design and scale up effective
nutrient removal systems. Therefore the objectives of this critical review of algal biofilms include:

1. Synthesizing the current knowledge and applications
2. Evaluating nutrient removal performance
3. Characterizing environmental variables affecting growth
4. Identifying research and information needs in areas of testing and technology development

2.0 Attached growth/fixed film technologies in wastewater treatment

The use of attached growth/fixed film systems in wastewater treatment dates from 1893 with the introduction of trickling filters in England [16]. Today, there are several types of fixed film systems in use including rotating biological contactors, submerged bed bioreactors, fluidized biofilm reactors, and combinations of fixed film and suspended growth processes [15,17,18], which mostly favor growth of heterotrophic organisms with a few occurrences of autotrophs. A study by Cooke [19] described a typical trickling filter biofilm community as consisting of bacteria, fungi, protozoa, flagellates, worms, snails, nematodes, rotifers, and insect larvae. Algae species were observed in trickling filter biofilms exposed to the atmosphere under optimum temperature and sunlight conditions [19]. Interestingly, diatoms and green algae species dominated samples from high rate filters while cyanobacteria dominated samples from low rate filters [20]. However, the importance of algae in trickling filters is still debatable; some researchers consider algae a nuisance because of the clogging issues while others believe that the oxygen produced by algae is beneficial to the bacteria in the biofilm [21,22].
Wastewater treatment using fixed film systems is achieved by providing favorable growth conditions to a desired consortium of microorganisms that have the ability to metabolize the pollutant of concern. For example, organic matter in wastewater is degraded by aerobic microorganisms attached to rocks or plastic media with the tricking filter technology. Likewise, algal biofilm-based systems are designed for pollutant removal through plant uptake and subsequent harvesting of the resulting biomass [7]. Wastewater treatment facilities can take advantage of microalgae's ability to utilize nitrogen (N) and phosphorous (P) to attain stringent effluent nutrient limits and potentially use the harvested biomass for bioproducts production. The nutrient limits required of the effluent discharged from wastewater treatment plants (WWTPs) are set and regulated by the US Environmental Protection Agency (USEPA) through the National Pollutant Discharge Elimination System (NPDES) based on the available technology or need to protect receiving waters [23].

2.1 Algal biofilm-based nutrient removal option

Depending on the source, raw wastewater has sufficient nutrients for algal growth [24]. However, when algal biofilms are utilized for tertiary wastewater treatment (effluent polishing), nutrient addition may be required to optimize the molar stoichiometric ratios of carbon, nitrogen, and phosphorus (C: N: P) necessary for growth [12]. Plant assimilation accounts for the largest percent removal of N and P in algal biofilm-based systems [25–27], followed by the chemical precipitation of P with calcium and magnesium ions and ammonia volatilization due to elevated pH values driven by algal photosynthesis [28]. The N and P content (dry weight basis) of algal cells from biofilm-based systems ranged from 2.9%–7.5% N and 0.3%–2% P [29–31]. With the
exception of a study by Posadas et al. [10] where N and P content remained constant despite an increase in nutrient loading, higher nutrient content was attributed to increase in N and P loads or wastewater type [29,32]. Additionally, variability in nutrient content observed in microalgal biomass could be attributed to luxury uptake, where algal cells store N and P in excess of the amounts required for growth [13].

Nutrient removal trends mirrored the biofilm growth pattern where low uptake capacities were observed at the start of the growth phase, which increased as growth peaked and the death phase was characterized by a reduction in nutrient uptake capacity [29]. Low nutrient removal efficiencies observed at the start of the growth phase were attributed to insufficient establishment and acclimation of the algal biofilm community [26] and at the death phase to a loss of algal biofilm integrity due to sloughing [29]. Increase in nutrient loading rates correlated to higher N and P uptake rates and mean algal productivity [26,30,33], although the trend ceased when the maximum uptake capacity was reached [29]. Posadas et al. [10] reported 30–50% ammonium (NH₄⁺-N) removal due to nitrification in algal biofilms treating both municipal and centrate wastewater. Babu [34] also associated the presence of algal biofilms to the significant improvement in nitrification rates observed in stabilization ponds studied. He hypothesized that the algal biofilms provided a protective barrier that prevented wash out of the nitrifying bacteria since growth rates of nitrifying bacteria are relatively low compared to microalgae [35].

Similarly, Mulbry and Wilkie [36] attributed the increase in effluent nitrate (NO₃⁻) concentrations above influent concentration in treated dairy wastewater to high nitrification rates associated with algal biofilms (Table 2-1). NO₃⁻ uptake by algae in the
biofilm was said to be inhibited because of preferential uptake of ammonium ions (NH$_4^+$) and low levels of soluble carbon in the wastewater. In this case, the high nitrification rates were not desirable and control of nitrification in the system was recommended by either lowering the N loading rate and/or increasing the retention time [31]. However, a follow-up study by Kebede-Westhead et al. [30] found that nitrification was controlled under high irradiance levels and bubbling of carbon dioxide (CO2), which enhanced algal photosynthesis. On the contrary, growth of nitrifying bacteria could be affected by diurnal fluctuations of oxygen concentrations, competition for dissolved inorganic carbon (DIC), and competition with heterotrophic bacteria as a consequence of microalgae altering the biofilm chemical microenvironment via photosynthesis and respiration [37,38].
Table 2-1: Nutrient removal efficiencies using algal biofilm-based treatment systems for animal and municipal wastewater

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Influent mg/l</th>
<th>Effluent mg/l</th>
<th>Percent removal</th>
<th>Retention time, d</th>
<th>Wastewater type</th>
<th>Mode of operation</th>
<th>Refs</th>
</tr>
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<tbody>
<tr>
<td>TP</td>
<td>770.0</td>
<td>205.0</td>
<td>73.4</td>
<td>6.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
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<tr>
<td></td>
<td>770.0</td>
<td>76.7</td>
<td>90.0</td>
<td>10.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>770.0</td>
<td>54.0</td>
<td>93.0</td>
<td>15.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>^a^0.1</td>
<td>92.4</td>
<td>3.0</td>
<td>Municipal</td>
<td>Batch</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>303.0</td>
<td>3.0</td>
<td>99.0</td>
<td>N/A</td>
<td>Animal</td>
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<td>36</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>^a^0.4</td>
<td>69.7</td>
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<td>130</td>
</tr>
<tr>
<td></td>
<td>^a^2.1</td>
<td>1.6</td>
<td>23.8</td>
<td>0.25</td>
<td>Municipal</td>
<td>Continuous</td>
<td>9</td>
</tr>
<tr>
<td>TN</td>
<td>517.0</td>
<td>198.5</td>
<td>61.6</td>
<td>6.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>517.0</td>
<td>118.0</td>
<td>77.2</td>
<td>10.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>517.0</td>
<td>110.3</td>
<td>78.7</td>
<td>15.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>11.7</td>
<td>5.6</td>
<td>52.1</td>
<td>3.0</td>
<td>Municipal</td>
<td>Batch</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>1210.0</td>
<td>28.0</td>
<td>97.7</td>
<td>N/A</td>
<td>Animal</td>
<td>Continuous</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>18.5</td>
<td>^a^11.0</td>
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<td>91.1</td>
<td>19.1</td>
<td>79.0</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>91.1</td>
<td>34.1</td>
<td>62.5</td>
<td>5.2</td>
<td>Municipal</td>
<td>Continuous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>91.1</td>
<td>63.1</td>
<td>30.7</td>
<td>3.1</td>
<td>Municipal</td>
<td>Continuous</td>
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<td>49.0</td>
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<td>75.5</td>
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<td>Continuous</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>^a^4.5</td>
<td>1.1</td>
<td>75.6</td>
<td>0.25</td>
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<td>Continuous</td>
<td>9</td>
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<tr>
<td>NH₄⁺-N</td>
<td>309.0</td>
<td>17.6</td>
<td>94.3</td>
<td>6.0</td>
<td>Animal</td>
<td>Batch</td>
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<td></td>
<td>309.0</td>
<td>9.4</td>
<td>97.0</td>
<td>10.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>309.0</td>
<td>4.0</td>
<td>98.7</td>
<td>15.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>^a^0.2</td>
<td>96.3</td>
<td>3.0</td>
<td>Municipal</td>
<td>Batch</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>306.0</td>
<td>0.4</td>
<td>99.9</td>
<td>N/A</td>
<td>Animal</td>
<td>Continuous</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>^a^3.0</td>
<td>44.4</td>
<td>2.0</td>
<td>Municipal</td>
<td>Continuous</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>32.0</td>
<td>1.5</td>
<td>95.3</td>
<td>4.0</td>
<td>Municipal</td>
<td>Continuous</td>
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<tr>
<td>NO₃⁻-N</td>
<td>&lt;1</td>
<td>12.2</td>
<td>-1120.0</td>
<td>N/A</td>
<td>Animal</td>
<td>Continuous</td>
<td>36</td>
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<tr>
<td></td>
<td>5.57</td>
<td>^b^2.2</td>
<td>60.5</td>
<td>0.7</td>
<td>Municipal</td>
<td>Continuous</td>
<td>29</td>
</tr>
<tr>
<td>PO₄³⁻-P</td>
<td>17.7</td>
<td>9.2</td>
<td>48.0</td>
<td>6.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>17.7</td>
<td>3.8</td>
<td>78.5</td>
<td>10.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>17.7</td>
<td>3.6</td>
<td>79.7</td>
<td>15.0</td>
<td>Animal</td>
<td>Batch</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>0.97</td>
<td>^⁹^0.2</td>
<td>88.5</td>
<td>0.7</td>
<td>Municipal</td>
<td>Continuous</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>1.0</td>
<td>85.7</td>
<td>10.4</td>
<td>Municipal</td>
<td>Continuous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>3.0</td>
<td>57.1</td>
<td>5.2</td>
<td>Municipal</td>
<td>Continuous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>5.0</td>
<td>28.6</td>
<td>3.1</td>
<td>Municipal</td>
<td>Continuous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>0.8</td>
<td>63.6</td>
<td>4.0</td>
<td>Municipal</td>
<td>Continuous</td>
<td>131</td>
</tr>
</tbody>
</table>

^a^ Influent /effluent values estimated from graphs, ^b^ Removal efficiency calculated from initial concentration to recommended target effluent value, ^L^ lab study, ^P^ pilot study, N/A- data not available, TP = Total phosphorus, TN = Total nitrogen
Table 2-2: Statistical analysis system (SAS) output of two-sample t-test for nutrient removal influenced by factors and levels

| Factors                | Levels | No. of observation | \(^a\)TP, % removal | Pr > |t| | \(^a\)TN, % removal | Pr > |t| |
|-----------------------|--------|--------------------|---------------------|------|---------------|---------------------|------|---------------|
| Wastewater type       | Animal | 3                  | 85.5 ± 10.6         | 0.1904 | 72.5 ± 9.5                          | 0.2649 |
|                       | Municipal | 8                  | 63.2 ± 25.8         | 0.4359 | 59.6 ± 17.5                          | 0.5384 |
| Mode of operation     | Batch  | 4                  | 87.2 ± 9.3          | 0.0596 | 67.4 ± 12.8                          | 0.5384 |
|                       | Continuous | 7                  | 59.0 ± 24.7         | 0.0596 | 60.6 ± 18.7                          | 0.5384 |
| Residence time        | 0 – 7  | 8                  | 62.1 ± 25.1         | 0.1013 | 57.4 ± 15.8                          | 0.0533 |
|                       | 8 – 15 | 3                  | 89.6 ± 3.7          | 0.1013 | 78.3 ± 1.0                           | 0.0533 |

\(^a\) data includes values reported for phosphate and nitrate concentrations in absence of TP and TN values for the studies considered

3.0 Generation of algal biomass for bioproducts production

Algal biomass has a potential to be used for a variety of bioproducts including biofuels, bioplastics, nutraceuticals, animal feed, fertilizers, and personal care products [39–41]. Mass cultivation of algae involves the use of suspended cultures in open ponds or closed photobioreactors with fresh or marine water as the growth media [42–45]. Suspended algal systems are designed to minimize attachment or settlement of cells onto bioreactor surfaces, while attached algal systems promote cell adhesion to a surface with a polymeric matrix [7]. Unfortunately, wastewater-based algal biofilm studies typically focus on nutrient removal (Table 2-1), often disregarding aspects of growth and biomass accumulation for bioproduct production. Furthermore, among the institutions that produce microalgae on a large scale, very few use attached growth systems much less a combination of attached growth with wastewater as the medium (Table 2-3).
Table 2-3: Organizations involved in large-scale algal cultivation using attached growth systems

<table>
<thead>
<tr>
<th>Organization</th>
<th>Bioreactor</th>
<th>Treatment emphasis</th>
<th>Products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydromentia</td>
<td>Algal Turf Scrubber® - ATS™</td>
<td>Nutrient removal from point and non point sources</td>
<td>Compost, animal feed, biofuels</td>
<td><a href="http://www.hydromentia.com">http://www.hydromentia.com</a></td>
</tr>
<tr>
<td>OneWater Inc.</td>
<td>Algaewheel</td>
<td>Nutrient removal from municipal and onsite decentralized treatment facilities</td>
<td>N/A</td>
<td><a href="http://www.onewaterworks.com">http://www.onewaterworks.com</a></td>
</tr>
<tr>
<td>GreenShift Corp.</td>
<td>GS CleanTech CO₂ bioreactor</td>
<td>Carbon capture</td>
<td>N/A</td>
<td><a href="http://www.greenshift.com">http://www.greenshift.com</a></td>
</tr>
<tr>
<td>European Commission</td>
<td>Algadisk</td>
<td>Carbon capture</td>
<td>Biomass</td>
<td><a href="http://www.algadisk.eu">www.algadisk.eu</a></td>
</tr>
<tr>
<td>Utah State University</td>
<td>Rotating Algal Biofilm Reactor (RABR)</td>
<td>Nutrient removal from municipal wastewater</td>
<td>Biofuels</td>
<td>9</td>
</tr>
<tr>
<td>Iowa State University</td>
<td>Revolving Algal Biofilm cultivation system (RAB)</td>
<td>N/A</td>
<td>Biomass</td>
<td>132</td>
</tr>
<tr>
<td>Qingdao institute of Bioenergy and Bioprocess Technology</td>
<td>Algal “disk”</td>
<td>N/A</td>
<td>Biofuels</td>
<td>63</td>
</tr>
</tbody>
</table>

Proponents of algal biofilm-based systems within a wastewater treatment process argue that these systems provide an alternative growth platform that addresses issues of nutrient supply limitations, biomass harvesting, and land and water availability.
commonly associated with mass algal cultivation [9,46–49]. Also, surface colonization by microalgae has been observed within 24 h [4,50] and long term stability demonstrated in continuous studies despite the dynamic nature of algal biofilms (Table 2-4). However, lack of culture control, contamination, inconsistent nutrient composition, potential occurrence of pathogenic organisms in the biomass, and public perceptions are some of the issues that limit the use of wastewater in cultivation of microalgae for valuable bioproducts production [12,51]. There are guidelines and regulatory standards that have been developed so far to streamline development, production, and commercialization of bioproducts from algal biomass [52–54].

Theoretical algal yields based on nutritional availability (mg/L N or P) and/or N:P ratios in different types of wastewater calculated by Christenson and Sims [11] ranged from 0.1 g to 42.8 g biomass/L. Higher nutrient (N and P) concentrations in industrial and animal wastewater accounted for higher calculated algal biomass yields compared to municipal wastewater. Algal biofilm productivity values from bench to pilot scale operations ranged from 0.6 to 31 g/m²/d with municipal and animal wastewater and values greater than 50 g/m²/d using nutrient growth media (Table 2-4). Hydromentia using Algal Turf Scrubber®-ATS™ has successfully implemented the use of wastewater as a growth medium for microalgae, and converted the harvested biomass into commercial products including compost and feed (Table 2-3). The success of the ATS is attributed to collaborative research efforts between scientists and industry both on bench [25,30,32,36] and pilot scale levels [33].
Table 2-4: Comparison of algal biofilm growth conditions and biomass productivity values from different studies

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Light (µmol/m²/s), Photoperiod (h)</th>
<th>Temp (°C)</th>
<th>Bioreactor/Substratum</th>
<th>Species</th>
<th>Production rates (g/m²/d)</th>
<th>Period (d)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw and anaerobically digested dairy manure</td>
<td>40 – 140 16: 8 L/D</td>
<td>22</td>
<td>Lab ATS units with polyethylene mesh, (&quot;1m²&quot;)</td>
<td>Algal consortia</td>
<td>5.0</td>
<td>63</td>
<td>36</td>
</tr>
<tr>
<td>Raw and anaerobically digested dairy manure</td>
<td>Outdoors</td>
<td>Outdoors</td>
<td>Pilot scale ATS units with nylon mesh, (&quot;30m²&quot;)</td>
<td>Algal consortia</td>
<td>2.5 – 25</td>
<td>270</td>
<td>33</td>
</tr>
<tr>
<td>Anaerobically digested dairy manure</td>
<td>270 – 390 23: 1 L/D</td>
<td>19 – 24</td>
<td>Lab ATS units with polyethylene mesh, (&quot;1m²&quot;)</td>
<td>Algal consortia</td>
<td>5 – 23</td>
<td>N/A</td>
<td>30</td>
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<tr>
<td>Dairy manure</td>
<td>110 – 120 24</td>
<td>20</td>
<td>Polystyrene foam, loofah sponge, polyethylene fabric, cardboard, (8 x 17cm)</td>
<td><em>Chlorella sp.</em></td>
<td>0.58 - 2.57</td>
<td>15</td>
<td>110</td>
</tr>
<tr>
<td>Swine manure</td>
<td>240 – 633 23:1 L/D</td>
<td>23 – 26</td>
<td>Lab scale ATS units with polyethylene mesh, (&quot;1m²&quot;)</td>
<td>Algal consortia</td>
<td>7.1 – 9.6</td>
<td>N/A</td>
<td>32</td>
</tr>
<tr>
<td>Centrate and raw municipal wastewater</td>
<td>72 – 104 16: 8 L/D</td>
<td>18 – 27</td>
<td>5 mm thick foam PVC, (&quot;0.5 m²&quot;)</td>
<td>Mixed culture</td>
<td>0.5 – 3.1</td>
<td>220</td>
<td>10</td>
</tr>
<tr>
<td>Municipal wastewater</td>
<td>Outdoor conditions 9.6 - 19.2</td>
<td></td>
<td>Cotton rope around aluminum wheels, 4.62 m² reactor area</td>
<td>Mixed culture</td>
<td>20– 31</td>
<td>12 - 20</td>
<td>9</td>
</tr>
<tr>
<td>Municipal wastewater</td>
<td>2800 lx 6: 6 L/D</td>
<td>20 – 22</td>
<td>Polymethyl methacrylate, (PMMA) reactor with cylindrical polypropylene fiber bundle carriers, (2600 – 3000 m²/m³)</td>
<td><em>Scenedesmus Sp.</em></td>
<td>N/A</td>
<td>3a 91b</td>
<td>130</td>
</tr>
<tr>
<td>Municipal wastewater</td>
<td>100 24</td>
<td>23 – 27</td>
<td>PMMA cased flow cell with 2.5 x 2.5 x 0.5 cm coupons</td>
<td><em>S. Obliquus</em> and <em>C. Vulgaris</em></td>
<td>7</td>
<td>5.5</td>
<td>50</td>
</tr>
<tr>
<td>Condition</td>
<td>Light (lx)</td>
<td>Temperature (°C)</td>
<td>Medium</td>
<td>Algae Species</td>
<td>Culture</td>
<td>Notes</td>
<td>Notes</td>
</tr>
<tr>
<td>---------------------------------</td>
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</tr>
<tr>
<td>Municipal and synthetic wastewater</td>
<td>230</td>
<td>24</td>
<td>Flow cell with 1 mm PVC plastic sheet, (0.018 m²)</td>
<td>Mixed culture</td>
<td>2.1 - 7.7</td>
<td>N/A</td>
<td>6&lt;sup&gt;a&lt;/sup&gt; 24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Synthetic wastewater</td>
<td>10</td>
<td>24</td>
<td>Filter paper</td>
<td>Trentepohlia aurea</td>
<td>N/A</td>
<td>40</td>
<td>133</td>
</tr>
<tr>
<td>Modified BG11</td>
<td>15, 30, 60 and 120 16: 8 L/D</td>
<td>20, 30</td>
<td>Flow-lane Incubator with 76 x 25 x 1 mm polycarbonate slides</td>
<td>Mixed culture</td>
<td>0.02 – 2.9</td>
<td>30</td>
<td>61</td>
</tr>
<tr>
<td>BG 11</td>
<td>12.8 – 134.5 24</td>
<td>28 – 32</td>
<td>Glass chamber containing multiple glass plates with algal “disks”, (0.001 m²)</td>
<td>S. obliquus</td>
<td>70.9</td>
<td>9</td>
<td>63</td>
</tr>
<tr>
<td>BG 11</td>
<td>492.2 Outdoor conditions</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>50 – 80</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>BG 11</td>
<td>55</td>
<td>24 – 26</td>
<td>8 mm thick concrete slab, (0.275 m²)</td>
<td>B. Braunii</td>
<td>0.71</td>
<td>35</td>
<td>47</td>
</tr>
<tr>
<td>Soda lake water</td>
<td>N/A</td>
<td>12: 12 L/D</td>
<td>N/A</td>
<td>Aquarium containing perspex rack with Perspex discs or polished quartz glass</td>
<td>Mixed culture</td>
<td>N/A</td>
<td>2 – 5</td>
</tr>
<tr>
<td>Clean stream water</td>
<td>12 – 88 14: 10 L/D</td>
<td>16 – 17</td>
<td>Continuous mats of unglazed ceramic tiles</td>
<td>Diatom dominated culture</td>
<td>N/A</td>
<td>10</td>
<td>64</td>
</tr>
</tbody>
</table>

<sup>a</sup>batch study  <sup>b</sup>continuous study  L/D-Light/Dark cycle, <sup>c</sup>surface area per cultivation module, N/A-data not available, <sup>d</sup>Culture from stream dominated by Microspora willeana Lagerh, Ulothrix zonata, Ulothrix aequalis Kütz, Rhizoclonium hieroglyphicum Kütz, and Oedogonium spp.

### 4.0 Characterization of algal biofilm growth conditions

Although there are documented success in using algal biofilms as a nutrient removal option in wastewater treatment at bench and pilot scale levels (Table 2-1), optimization of growth under different environmental conditions still remains a major challenge. The transition from bench to full-scale operations is hindered by limited research on wastewater remediation using algal biofilms and variability in growth
conditions (Table 2-4). The economics of algal biofilm based systems are also unknown given the lack of empirical data on installation, operation, and maintenance costs. However, there is consensus that the generation of bioproducts derived from algal biomass would considerably reduce costs associated with these systems in wastewater treatment [49,55,56]. Therefore, with limited information regarding applicability of algal biofilms in wastewater treatment, ecological and limnological studies were also utilized in assessing the contributions of the most important factors in the formation and survival of algal biofilms.

4.1 Light

Light is the energy source for algal photosynthesis, and is essential for microalgal growth. However, algal growth inhibition can occur as a result of too much light (photoinhibition) in the upper layers of the biofilm and/or too little light (photo limitation) in the lower shaded parts of the biofilm [37,57]. Microalgae have the ability to adapt their photosynthetic apparatus to changes in light intensities in order to optimize photosynthetic efficiency or to prevent photo damage [58–60]. The specific value at which photoinhibition or light limitation occurred varied with biofilm community and prevailing growth conditions. Guzzon et al. [61], Hill et al. [62], and Liu et al. [63] reported light saturation of algal biofilms at 60, 100, and 150 µmol m$^{-2}$ s$^{-1}$ respectively, whereas Hill and Fanta [64] considered algal biofilms growing between 12 - 88 µmol m$^{-2}$ s$^{-1}$ light limited.

Kinetic expressions relating specific growth rates to light intensity and models fitted from experimental data are widely applied to suspended algal cultures to predict algal productivity [65–67]. Such modeling applications are limited in algal biofilm
studies probably due to the heterogeneity and complexity of biofilm communities [37]. However, illuminated biofilm communities were reported to have increased algal density compared to biofilms grown in the dark [68]. Green algae were the dominant early surface colonizers under high light and heterotrophic bacteria under low light conditions [69]. Diatoms acclimated better to low light than green algae thereby dominating light limited algal biofilms [61, 70]. Guzzon et al. [61] reported an increase in cellular P content in algal biofilms across measured light intensities of 15, 30, 60, and 120 µmol m$^{-2}$ s$^{-1}$ and accumulation of P in algal cells at 120 µmol m$^{-2}$ s$^{-1}$. Algal biofilms grown under a similar light range (40–140 µmol m$^{-2}$ s$^{-1}$) showed higher cellular nutrient content of 7.1% N and 1.47% P [36] compared to 3.6% N and 0.65% P in algal biofilms studied at two higher light regimes averaging 270 and 390 µmol m$^{-2}$ s$^{-1}$ respectively [30]. The enhanced nutrient accumulation observed at the lower incident light intensities was associated to slower growth rates [30].

Provision of artificial light to algal biofilms treating large quantities of wastewater is not an economically viable option compared to harnessing natural sunlight. However, temperate climates have variable solar irradiances often exceeding 2000 µmol m$^{-2}$ s$^{-1}$ in the summer and below 100 µmol m$^{-2}$ s$^{-1}$ in the winter months. Attempts to optimize light utilization in algal biofilm-based systems have been directed to bioreactor design modification. Biofilm-based photobioreactors are typically designed with high surface area to volume ratios for efficient light penetration. However, immobilized algal cultures on the reactor surfaces are vulnerable to photo damage from constant exposure to high irradiances [71]. Christenson and Sims [9] designed a rotating biofilm reactor that allowed alternate exposure of different sections of the algal biofilm community to light.
Liu et al. [63] achieved spatial light dilution through a biofilm reactor design consisting of multiple cultivation modules (glass plates) inserted inside a glass chamber. Genetic engineering of microalgae species is another area of research that is being developed to improve light utilization efficiency [72,73], but its application in wastewater based algal biofilms is yet to be realized.

Light affects the nutrient removal performance of algal biofilm based systems by directly influencing algal growth, therefore effective utilization of available light and avoidance of photo inhibition is essential for successful application of algal biofilms in wastewater treatment.

4.2 Temperature

The performance of an algal biofilm-based system will be affected by temperature fluctuations like any biological wastewater treatment system [74,75]. Comparing algal biofilm-based systems to trickling filters, Schroepfer et al. [76] described a transient microbial community on trickling filters that readily adjusted to changing temperatures. The amount of film observed on the filters varied with temperature, mainly due to changes in microbial and grazing activities [77]. Similarly, temperature effects on algal growth rates, species composition, and grazing activity have been reported in algal biofilm communities [78]. Tuchman and Blinn [79] reported an increase in algal densities with temperature up to a maximum standing crop density; algal densities were higher on slides with periphyton communities from a hot pond site (30.5 °C), than at a weir (27.5 °C) or intake (23.1 °C) site, respectively. The Arrhenius relationship describes the effect of temperature on algal growth rates under constant light intensity and optimal nutritional conditions [80,81]. In natural biofilm communities, the response of algae species to
temperature changes is variable (species dependent) and often coupled with light and nutritional effects [58] limiting the model application to laboratory studies. However, De Nicola [82] described a general trend in natural ecosystems where diatoms dominated at temperatures 5–15 °C, green algae at 15–30 °C, and cyanobacteria above 30 °C. A natural marine site at 28.5 °C favored settlement of macrofoulants (Barnacle cyprids and juvenile bryozoans), which severely grazed on microalgae species unlike a modified site (condenser outfall area of a power plant) at 35.7 °C [78]. Similarly, algal productivity values greater than 20 g/m2/d were obtained from pilot scale algal turf scrubber (ATS) units in the spring months but not the summer months, because the high water temperatures in the summer months favored the snail population, which grazed on the algal biofilm [33].

Biofilm photobioreactors are particularly vulnerable to high temperature changes and evaporation rates due to the high surface to volume ratios. Although algal biofilm-based systems have less water requirements compared to open pond systems, most of the water is lost to evaporation [47]. Concerns over evaporative losses arise from the need to conserve water, maintain water chemistry, and prevent cell desiccation. A thermal model developed for an algal biofilm photobioreactor, without any active cooling, predicted evaporative losses of about 6.0, 7.3, 3.4, and 1.0 L/m²/d in spring, summer, fall, and winter months, respectively [83]. Similarly, evaporation rates reported in actual studies ranged from 1 to 5 L/m²/d [10, 47], which is comparable to open pond systems for suspended algal growth [84, 85].

On the contrary, operational performance of algal biofilm-based systems in below freezing temperatures is a concern that has not been adequately addressed. Cultivating
algal biofilms in a greenhouse or under a cover may mitigate effects caused by below freezing temperatures, but the feasibility of this concept on large scale facilities needs to be investigated. Unlike trickling filters where a design modification from shallow uncovered rocks to deep covered plastic media units significantly reduced temperature fluctuations to less than 2 °C in winter months [86], algal biofilm growth is restricted to shallow bioreactors for efficient light penetration. Thermal control in wastewater-based algal biofilm systems should be considered to minimize temperature fluctuations due to extreme weather conditions, facilitate algal growth, and maintain the desired microbial population.

4.3 Nutrients

Availability of nutrients to a biofilm community influence algal growth, type of biofilm formed, succession, and species composition [30,68,87]. Environments with high levels of biodegradable organic matter favor heterotrophic biofilms [88], while phototrophic biofilms grow in response to light and inorganic nutrients [89]. Nutrients in the bulk solution are transported to algal cells across a concentration boundary layer by diffusion [90]. Monod's kinetic expression has been applied in algal biofilm studies to describe algal growth with respect to nutrient concentration [62,64] and predict the rate of substrate utilization [91,92]. For example, Hill et al. [62] determined a growth saturation threshold of 25 µg/L soluble reactive phosphorus (SRP) for algal growth in stream biofilms. Sekar et al. [68] associated successional changes in microalgae species during biofilm development to nutrient changes (N: P ratios) in the growth media. Green algae species initially dominated the biofilm community followed by diatom species and finally cyanobacteria in the N-deficient conditions.
Microalgae utilize dissolved inorganic carbon in the form of carbon dioxide (CO$_2$ (aq)) and bicarbonate (HCO$_3^-$) present in wastewater [10,26]. Other available sources of carbon include atmospheric CO$_2$ [11,93] and bacterial degradation of organic carbon [13,27]. For a typical medium strength wastewater having total alkalinity of 120 mg/L CaCO$_3$, total suspended solids (TSS) of 210 mg/L, pH of 7 (HCO$_3^-$ as the dominant species) at 25 °C [94], the total theoretical CO$_2$ concentration calculated using standard method 4500-CO$_2$ D [95] is at least 106 mg CO$_2$/L. Bioavailability of inorganic carbon is influenced by pH gradients within the biofilm and bulk solution [96–98], therefore algal biofilms tend to be carbon limited with a significant increase of carbonate (CO$_3^{2-}$) with respect to CO$_2$ (aq) and HCO$_3^-$ driven by high pH conditions. As a measure against growth inhibition due to carbon limitations in algal biofilms, some researchers supplied CO$_2$ from an external source [30,63]. Utilization of waste CO$_2$ from industrial processes has been proposed as an inexpensive source of CO$_2$ [12,99], however this also requires close proximity of WWTPs to the carbon source.

Carbon, nitrogen, phosphorus, and silicon (in the case of diatoms) are key elements in microalgae growth. Cellular nutrient ratios of C: N: P are often utilized as indicators of nutrient limitation in algal communities [100,101], with the Redfield ratio (106:16:1 molar basis) considered typical for optimally growing phytoplankton [102]. Matching microalgae C: N: P ratios to that of the media is one of the ways in which nutrient limitations are predicted and minimized in algal growth studies. Christenson and Sims [9] and Boelee et al. [13] both reported adjusting for nutrients in effluent wastewater by the addition of sodium nitrate (NaNO$_3$) and industrial grade urea respectively to obtain the desired N:P ratio. However, cellular nutrient ratios were not
reliable in predicting nutrient limitations in algal biofilm communities growing under low nutrient concentrations [103] or influenced by temporal changes (seasonality) [104]. Additionally, variability in elemental composition of microalgae resulting from either environmental conditions or species composition has been reported [105,106]. Boelee et al. [13] summarized C: P ratios ranging from 34:1 to 418:1 and N: P ratios from 3.5:1 to 38:1 of different microalgae species in literature. Therefore, the use of cellular nutrient ratios in determining nutrient limitations of algal biofilms should be validated with field studies in the presence of other potentially limiting factors.

4.4 Substratum

Most algal adhesion studies are focused on determining the influence of surface characteristics and material composition on biofilm formation in order to promote cell attachment and sustain biofilm growth [107–109]. Surface texture is one of the factors that influence microalgae attachment to different substrata. Rough or porous surfaces were generally associated with higher cell attachment due to increased surface area and protection against hydraulic shear forces [34]. The downside to porous materials such as polyurethane foam, loofah, and nylon sponge was the difficulty in harvesting algal biomass growing in the pores [110]. Tuchman and Blinn [79] observed that the composition of periphyton communities on aluminum slides was more representative of the community found on natural substratum (plant material) than on glass slides in the same study. The aluminum slides with cuts and grooves had a more comparable microtopography to that of natural vegetation. Cao et al. [108] also reported better adhesion of *Scenedesmus dimorphus* cells on stainless steel sheets with micro scale laser textured surface than the regular steel surface used as a control.
Material properties and composition are another area of interest in algal biofilm adhesion studies. Christenson and Sims [9] investigated algal attachment to different substrata including cotton rope, low thread and high thread cotton, polyester, jute, nylon, polypropylene, and acrylic and realized better growth on cellulose based natural polymers surfaces than synthetic polymer surfaces. Sekar et al. [107] noticed greater attachment of *Chlorella vulgaris*, *Nitzschia amphibian*, and *Chroococcus minutes* species to hydrophobic surfaces (titanium, perspex, and stainless steel) with the exception of copper and its alloys compared to hydrophilic glass. Copper and its alloys (aluminum and admiralty brass) hindered cell attachment because of toxicity issues. Although, hydrophobic surfaces promoted higher cell densities, surface hydrophobicity had very little effect on adhesion strength of algal biofilms [109]. Conversely, Irving and Allen [50] found no correlation between surface hydrophobicity and cell adhesion density of *Scenedesmus obliquus* and *C. vulgaris* biofilms grown in wastewater. Their study showed that material properties of a substratum had a less significant influence on algal biofilm formation compared to species selection and control.

Currently, there are no standard materials recommended for use in algal biofilm growth studies (Table 2-4). However, factors such as cost, durability, availability, and reliability should be considered when selecting materials to grow algal biofilms. Other than screening of materials that can sustain biofilm growth, there is little material testing under field/operational conditions outside bench scale studies, and this makes it difficult to deduce which materials are suitable for growing algal biofilms in wastewater treatment scale up operations.
4.5 Extracellular polymeric substances (EPS)

EPS is composed of polysaccharides, proteins, nucleic acids, lipids, and humic acids [111,112], which determine its physical and chemical properties. The roles of EPS in algal biofilm communities include: assisting cell movement [113], preventing cell desiccation [6], protecting cells against toxic substances [114], and providing stability as an adhesive material [115]. EPS production is influenced by biofilm age, nutrient availability, species composition, response to stress [112,115,116] and also indirectly linked to temperature and light via algal photosynthesis and growth [117].

High EPS to biomass ratios were observed in young and severely grazed algal biofilms probably due to EPS production as a survival mechanism [116]. Alternately, possible contribution of EPS from the initial process of cell attachment in young biofilms has been suggested [111]. Wolfstein and Stal [118] observed maximum EPS production at 15 and 25 °C during the early stationary growth phase (about 8 d) after which, temperature effects on EPS production diminished with culture age. Cyanobacteria and diatom biomass were positively correlated to EPS produced in wastewater-based algal biofilms [112]. The close coupling of photosynthetically fixed carbon to EPS production highlights the role of light in its production [116]. However, the absence of light does not completely inhibit EPS synthesis. Underwood and Smith [113] showed that axenic monocultures of *Cylindrotheca closterium*, *Navicula perminuta*, and *Nitzschia sigma* were able to secrete significant amounts of EPS by utilizing previously stored glucan as a carbon source in order to facilitate migration from the dark.

Algal biofilms in wastewater environments are susceptible to grazers and frequent changes in growth conditions due to seasonal and diurnal fluctuations; therefore
screening species for EPS production and minimizing carbon limitations could sustain biofilm growth.

4.6 Species interactions

Natural biofilm communities are comprised of a number of microbial cells including fungi, algae, protozoa, flagellates, and bacteria [2,112,119]. Species distribution and dominance are affected by growth limiting factors, biofilm age, grazers, and different microenvironments in the biofilm [78,116,120]. Interactions between autotrophic and heterotrophic organisms exist in biofilm communities [69,121,122], despite the limited information on their respective contribution to the structure and function. The extent and specificity of algal–bacterial interactions in biofilm communities is also not well defined. Irving and Allen [50] observed that a large proportion (>70% total mass) of C. vulgaris and S. obliquus species remained in suspension when grown under sterile axenic conditions, but quickly formed biofilms in non-sterile wastewater. Bacteria were said to provide vitamins and inorganic carbon to algae, which in turn supplied organic carbon and oxygen to bacteria [123]. Also, surfaces initially colonized by bacteria had faster algal biofilm formation [69,124]. However, there are bacterial strains that prevent algal growth through the production of inhibitory compounds or cell-to-cell contact [125]. Rivas et al. [126] showed both enhanced growth of Botryococcus braunii biofilms in the presence of Rhizobium sp. and reduced growth in the presence of Acinetobacter sp. at 20 °C.

Productivity of algal biofilms and species diversity is influenced by grazer activity. A grazer population of Chironomids, Gastropods, Trichopteran larvae, Ephemeroptera larvae, and crustaceans significantly reduced the algal biomass across all
seasons with summer being the most affected [87]. Hillebrand et al. [89] found out that filamentous species (*Ulothrix* and *Spirogyra* spp.) and chain-forming diatoms (*Melosira* *sp.*) were more susceptible to grazing than single celled algae species. However, laboratory grown algal biofilms are often pre-treated to minimize the effect of grazers on the biofilm structure and function leading to biased results that rarely depict field conditions. Mulbry et al. [127] reported adding *Bacillus thuringiensis* larvicide to control Chironomid larvae and Guzzon et al. [61] stored freshly inoculated BG 11 medium at −20 °C for 24 h to remove grazers. There is a need to understand the close association of microbial groups involved in algal biofilm formation especially the influence of algal–bacterial interactions and predation on biofilm structure and function for successful implementation of algal biofilm-based systems.

**5.0 Interaction of environmental and biological factors**

The scope of this review is limited to single factors that influence algal biofilm formation and development in natural/wastewater environments. However, there are situations where interaction between two or more factors has a greater influence on biofilm formation than any single factor. Research on algal biofilms has demonstrated the difficulty in separating combined effects of environmental variables on algal growth rates [64,87,128], EPS production [117,118], colonization rate [79,129], and species composition [50,58]. The need to take into account the interactions of environmental variables such as light intensity, temperature and nutrient concentration was also emphasized in algal growth modeling efforts [80]. Therefore, understanding how the different environmental and biological factors interrelate will eliminate bias introduced
by independent study of single factors on algal biofilm growth thereby easing the transition from controlled laboratory studies to field studies.

**Recommendations**

Successful integration of algal biofilms into wastewater treatment processes for nutrient removal requires engineering versatile systems that maximize algal productivity, subsequently enhancing nutrient uptake capacity and providing substantial amounts of biomass to justify investments required for bioproducts production. Standard operating procedures (SOPs) should be developed, validated, and tested for nutrient removal and biomass production using wastewater at bench and pilot scale levels. Accordingly, pilot scale studies should be utilized to investigate scale up issues. Based on this critical review of algal-based biofilm systems, the research focus should specifically be directed to:

1. Designing and testing biofilm bioreactors including choice of substratum in field conditions to address issues of space allocation, water requirements, light utilization, algal attachment, and construction and maintenance costs.

2. Determining the system limits by understanding the fundamental algal biofilm processes such as mass transport mechanisms as determined by its structure, heterotrophic–autotrophic interactions, and community characterization.

3. Correlating wastewater characteristics from different sources with biofilm growth, species composition, succession progression, and nutrient removal trends.

4. Determining the influence of light, temperature, and nutrients (N & P) on algal biofilm growth and development, especially in pilot scale algal biofilm systems.
5. Determining the interactions of environmental variables on nutrient uptake and biomass production.

Conclusions

There exists a large potential for the development of algal biofilm based technology for wastewater treatment. However, application of algal biofilm-based systems in wastewater treatment is still limited by the lack of information on system performance, sustainability, reliability, and techno economic and life cycle analysis from bench to field scale operations. These needs can be addressed by conducting research that involves the use or testing of algal biofilms in pilot and large scale wastewater operations in conjunction with implementing biomass to bioproducts options to utilize the generated algal biomass. The existing infrastructure of full scale wastewater treatment systems for municipal, industrial, and agricultural wastes provides a ready platform and an opportunity to evaluate large scale applications of algal-based biofilm technologies to integrate wastewater remediation with large-scale biomass production and algal-based bioproduct development.

References


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CHAPTER 3

DISSOLVED INORGANIC CARBON-ENHANCED GROWTH, NUTRIENT UPTAKE, AND LIPID ACCUMULATION IN WASTEWATER-GROWN MICROALGAL BIOFILMS

Abstract

Microalgal biofilms grown to evaluate potential nutrient removal options for wastewaters and feedstock for biofuels production were studied to determine the influence of bicarbonate amendment on their growth, nutrient uptake capacity, and lipid accumulation after nitrogen starvation. No significant differences in growth rates, nutrient removal, or lipid accumulation were observed in the algal biofilms with or without bicarbonate amendment. The biofilms possibly did not experience carbon-limited conditions because of the large reservoir of dissolved inorganic carbon in the medium. However, an increase in photosynthetic rates was observed in algal biofilms amended with bicarbonate. The influence of bicarbonate on photosynthetic and respiration rates was especially noticeable in biofilms that experienced nitrogen stress. Medium nitrogen depletion was not a suitable stimulant for lipid production in the algal biofilms and as such, focus should be directed towards optimizing growth and biomass productivities to compensate for the low lipid yields and increase nutrient uptake.

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1.0 Introduction

Cultivation of microalgae in wastewater streams has been proposed as a means of reducing competition for freshwater sources, as an inexpensive source of nutrients, and as a biological wastewater treatment alternative (Cai et al., 2013; Sturm and Lamer, 2011). Microalgae can utilize nutrients in wastewater for growth to generate considerable amounts of biomass. However, recovery of microalgae from the liquid medium is difficult and represents a substantial capital cost in suspended cultivation systems (Greenwell et al., 2010; Hoffmann, 1998), consequently there is a growing interest in attached algal growth platforms. Algal biofilm based systems such as the rotating algal biofilm reactor (RABR), algal turf scrubber (ATS™), revolving algal bioreactor (RAB), and Algaewheel® have been developed, and algal biofilm growth demonstrated in bench and pilot scale operations (Christenson and Sims, 2011; Christenson and Sims, 2012; Gross et al., 2013; Pizarro et al., 2006; see chapter 2). However, there is still limited fundamental information on algal biofilm physiological processes and growth especially in wastewater remediation.

Widespread application of algal biofilm-based systems is also limited but can be promoted through integration of wastewater treatment with the production of valuable bioproducts from the harvested algal biomass. Algal biomass composition (i.e., lipid, carbohydrate, and protein content) is influenced by the chemical composition of the medium and the environmental growth conditions (e.g., temperature, pH, and light), which subsequently determines the by-products that can be synthesized. Conventionally, microalgae grown as feedstock for biofuels require a two stage process where biomass accumulation occurs under nutrient-rich conditions followed by an environmental
challenge to induce secondary byproduct accumulation (*e.g.*, tri-acylglycerols as energy storage compounds) (Su et al., 2011). Nutrient starvation is typically employed as an environmental stress to stimulate lipid biosynthesis in microalgae cultures (Devi et al., 2012; Rodolfi et al., 2009; Sharma et al., 2012). However, stimulation of lipid production in algal biofilms as a result of nutrient starvation has not been as successful as in suspended cultures (Bernstein et al., 2014; Schnurr et al., 2013).

Furthermore, information on the use of other lipid inducing techniques such as chemical addition, pH stress, and temperature either independently evaluated or in combination with nutrient starvation is limited in algal biofilm studies. For example, addition of bicarbonate salts (HCO$_3^-$) was reported as an effective trigger for lipid production in nutrient limited suspended microalgae cultures (Gardner et al., 2012; Gardner et al., 2013; Peng et al., 2014; White et al., 2013). The bicarbonate salts not only induce lipid production, but also provide a stable and readily available source of inorganic carbon essential for photosynthesis and microalgae growth (Chi et al., 2013; Mus et al., 2013; Wensel et al., 2014). In addition, Glud et al. (1992) observed an increase in photosynthetic rates and a simultaneous reduction in respiration rates (17%) in a diatom-dominated biofilm community amended with bicarbonate.

The potential use of bicarbonate in minimizing photorespiration is especially of interest in algal biofilms because of the high O$_2$/CO$_2$ ratios due to localized supersaturated oxygen concentration from active oxygen photosynthesis (Bernstein et al., 2014; Glud et al., 1992). Photorespiration is a competing process to carboxylation, where ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO) acts as an oxygenase, thereby inhibiting carbon dioxide fixation and subsequently reducing photosynthetic
efficiency. The study presented here evaluated the effects of adding dissolved inorganic carbon in the form of 2 mM HCO$_3^-$ to synthetic wastewater medium to grow algal biofilms in order to:

(1) Enhance algal biofilm growth, nutrient uptake, and lipid accumulation during nutrient deplete culturing

(2) Increase photosynthetic rates with biofilm depth within the photic zone

2.0 Materials and Methods

2.1 Microalgal biofilm culturing and sampling

The chlorophyte isolate *Botryococcus* sp. strain WC-2B, previously described in Bernstein et al. (2014), was cultured in 8 L laboratory scale rotating algal biofilm reactors (RABRs) operated at 12 rpm and 25°C. Each reactor was comprised of two plastic cylindrical wheels (10 cm diameter) onto which 3/16 inch (diameter) untreated cotton cord was attached as the biofilm substratum. Synthetic wastewater was made to simulate typical medium strength domestic wastewater for total nitrogen (TN) and total phosphorus (TP) concentrations without a carbon source (Metcalf and Eddy, 2003). The medium consisted of 60 mg L$^{-1}$ NH$_4$Cl, 150 mg L$^{-1}$ NaNO$_3$, 16 mg L$^{-1}$ Na$_2$HPO$_4$, 15 mg L$^{-1}$ K$_2$HPO$_4$, 4 mg L$^{-1}$ KH$_2$PO$_4$, 75 mg L$^{-1}$ MgSO$_4$·7H$_2$O, 25 mg L$^{-1}$ CaCl$_2$·H$_2$O, and micronutrients (8.82 mg L$^{-1}$ ZnSO$_4$·7H$_2$O, 1.44 mg L$^{-1}$ MnCl$_2$·4H$_2$O, 0.71 mg L$^{-1}$ MoO$_3$, 1.57 mg L$^{-1}$ CuSO$_4$·5H$_2$O, 0.49 mg L$^{-1}$ Co(NO$_3$)$_2$·6H$_2$O and 4.98 mg L$^{-1}$ FeSO$_4$).

The experimental set up consisted of four laboratory RABRs under fluorescent lights with a photosynthetically active radiation (PAR) of 227±65 µmol m$^{-2}$ s$^{-1}$ on a 14:10 L/D cycle. Duplicate reactors were amended with 2 mM HCO$_3^-$ in the form of NaHCO$_3$ and another duplicate set without HCO$_3^-$ amendment was cultured for comparison. The
reactors were operated in sequenced batch mode with a 5 day hydraulic retention time (HRT) for a period of 18 days, after which nitrogen stress was induced for an additional 5 days by replacing all liquid medium with synthetic wastewater without a nitrogen source. For each cycle of hydraulic retention time, the reactors were drained, cleaned, and filled with fresh medium. Prior to the start of the experiment, the medium was inoculated with microalgae and the RABRs operated for 3 days (seeding period) to allow the microalgae to attach to the rope strands. As shown in Figure 3-1, after the seeding period, the RABRs with the exception of the substratum (rope strands) were covered with black polyethylene sheet to minimize microalgae growth in the liquid medium. Culturing and sampling was performed under non-aseptic conditions (open air).

Figure 3.1: Laboratory set up showing rotating algal biofilm reactors (RABRs) before microalgae attachment (left) and after biofilm growth (right). Duplicate RABRs were amended with bicarbonate and no bicarbonate addition, respectively
Rope samples with attached microalgae were excised for oxygen microsensor measurements, microscopy characterization, biomass dry weight measurements, and lipid analysis. Biomass cell dry weights (CDW, gcdw m⁻²) were obtained by removing the biofilm from a known length of cord into a pre-weighed aluminum weigh boat using a flat end spatula. The biomass was dried at 70°C for 18 h until the biomass weight was constant. Biomass CDWs were calculated by subtracting the dry weight of the oven dried boat with biomass and normalizing by the total cylindrical surface area for the length of cotton cord substratum excised.

2.2 Water quality monitoring

Nitrate (NO₃⁻), nitrite (NO₂⁻), and orthophosphate (PO₄³⁻) concentrations were monitored in the bulk medium and measured by ion chromatography (IC) using a Dionex IonPac AS22 carbonate eluent anion-exchange column set at a flow rate of 1.2 mL min⁻¹. IC data was analyzed by Chromeleon 7 Chromatography Data system (CDS) software. Ammonium (NH₄⁺-N) concentrations were determined according to the 2-phenylphenol method (Rhine et al., 1998) with a BioTek PowerWave XS microplate reader (Vermont, USA) at an absorbance of 660 nm. The dissolved inorganic carbon (DIC) was measured on 8 mL filtered (0.2 µm pore size filters) medium samples using a Skalar Formacs¹HT/TN TOC/TN analyzer (model CA16, Netherlands) and Skalar LAS-160 autosampler. DIC was quantified using peak area correlation against a standard curve from a bicarbonate-carbonate mixture (Sigma Aldrich). Culture pH and optical density (OD) measurements were taken using a standard laboratory Accumet pH electrode (Fisher Scientific) and Genesys 10 UV-Model 10-S spectrophotometer (Thermo Electron Corporation), respectively.
2.3 Oxygen microsensor analysis

Clark-type oxygen microelectrodes (10 μm tip diameter; OX-10 Unisense) and specialized computer controlled hardware (Unisense) were used to analyze the reactive transport of dissolved oxygen with biofilm depth under steady-state diffusive conditions corresponding to light and dark conditions. Photosynthetic rates (coupled with photorespiration) were estimated using the light/dark shift technique (Kühl et al., 1996; Revsbech and Jørgensen, 1986). The light/dark shift measurements are valid under the following assumptions: (1) initial steady state oxygen distribution is achieved before darkening, (2) oxygen consumption rates before and after dark incubation are identical, and (3) identical diffusive fluxes are maintained during the measurement time at each position. Two point calibrations were performed for the oxic conditions (medium saturated with air) and anoxic conditions (medium sparged with nitrogen gas).

2.4 Biodiesel analysis

Biodiesel precursors *i.e.* free fatty acids (FFAs), mono-acylglycerols (MAGs), di-acylglycerols (DAGs), and tri-acylglycerols (TAGs) were extracted from dried biomass by bead beating extraction and the biodiesel potential (total FAMEs) was determined by direct *in situ* transesterification according to protocols published by Lohman et al. (2013). The total FAMEs and the fatty acid compositions of these FAMEs were quantified using gas chromatography-mass spectroscopy (GC-MS; Agilent 6890N and 5973 Network MS). The FFAs, MAGs, DAGs, and TAGs were analyzed using gas chromatography flame ionization detection (GC-FID; Agilent 6890N).
2.5 Microscopy imaging and lipid profiling

Samples were qualitatively characterized for morphology and TAG accumulation through fluorescence staining and examined using confocal microscopy. Three components of these biofilms were visually analyzed: (1) chlorophyll autofluorescence, (2) neutral lipids via Bodipy 505/515 staining, and (3) biofilm substratum via Calcofluor white M2R staining (cellulose containing cotton-cord strands) (Cooper et al., 2010). Each component/stain was visualized with distinct excitation/emission spectra. Images were acquired using a Leica TCS SP5 with Leica Advanced Suite-Advanced (LAS-AF) software (version 2.5.1.6757). Excised biofilm samples were embedded in a viscous matrix (OCT, tissue-tek) on dry ice and cut into clean intact cross sections using a clean razor blade. Each cut section was stained with Bodipy 505/515 (2 uL mL⁻¹ of diH₂O) and calcofluor white M2R (20 uL mL⁻¹ of diH₂O) for 20 minutes. Bodipy and chlorophyll autofluorescence were excited by 488 nm and 633 nm lasers, and captured with emission ranges 499-547 and 647-761 nm, respectively. Calcofluor white M2R was excited by a 405 laser and captured with an emission range of 419-474 nm. Samples were rinsed once with filter-sterilized de-ionized water (diH₂O) prior to imaging. To minimize movement, each sample was partially embedded in 2% low electroendosmosis (EEO) Agarose (Fisher Scientific). Composite images were obtained with combined z-stacks up to the maximum depth at which autofluorescence by chlorophyll was detected using a 40x water immersion objective. Planar images were captured every 0.8 µm. Z-stacks were compiled into Maximum Intensity Projection (MIP) images using Imaris x64 (version 7.5.2, Bitplane Scientific Software).
3.0 Results and discussion

3.1 Microalgae growth rate and yield

Microalgae successfully attached to the cotton cord and grew as a biofilm for the entire study period (Figure 3-1). The lag phase was minimized by the 3-day seeding period. The microalgal biofilms were in exponential growth from days 3 - 10 as determined from linear the portion of the natural log transformed growth data and the stationary phase occurred after 10 days of growth (Figure 3-2). Curve fitting of the growth data also showed that the 1st order equation provided a better description of the microalgal growth between day 3-10 compared to the zero order equation with R² values of 0.946 and 0.999 for biofilm amended with bicarbonate and those without bicarbonate respectively (Figure B-1). The maximum specific growth rates measured during the exponential phase were 0.18|0.07 (mean|range) and 0.20|0.07 day⁻¹ for algal biofilms amended with bicarbonate and the unamended control, respectively. The maximum areal biomass density measured during the stationary phase was 20.95 and 25.98 g m⁻² for biofilms with bicarbonate and biofilm samples without bicarbonate, respectively. Additionally, the biofilm production rates, calculated as the total biomass accumulated per rope surface area divided by the time taken to reach stationary phase, were 1.45 and 1.79 g m⁻² day⁻¹ for biofilms with bicarbonate and biofilm without bicarbonate added, respectively.
Figure 3-2: Algal biofilm growth curves from natural log transformed data showing the exponential phase (day 3-10) and stationary phase (day 11-18). Insert: Equations and $R^2$ values describing the exponential phase of biofilms with and without bicarbonate amendment, respectively

Growth curves for the algal biofilms (attached to rope) and microalgae growth in suspension are shown in Figure 3-3A. Microalgae growth in the bulk medium was negligible over the study period indicating that covering the reactors with black plastic effectively prevented light penetration and minimized growth in suspension. There was no statistical difference observed in growth characteristics for algal biofilms amended with bicarbonate and biofilms that did not receive bicarbonate ($p$ value of 0.4517 from $t$ test). Although it was hypothesized that the addition of bicarbonate would increase the algal biofilm growth, this was not observed. With the 8L medium reservoirs, even the unamended algal biofilms were not carbon limited, such that bicarbonate addition did not
enhance growth in this reactor system. DIC measurements remained relatively constant for each 5-day retention time with slight differences observed in the medium concentrations, with the exception of the first 3 days, (Figure 3-3B).

Figure 3-3: Growth curves for attached (solid lines) and suspended (dotted lines) microalgae (A) and dissolved inorganic carbon (DIC) concentrations (B) in laboratory-
RABRs amended with bicarbonate and without bicarbonate addition. Error bars for algal biofilm areal density and DIC measurements represent the standard deviation (n=4). Error bars for suspended growth represent the range (n=2). Vertical dotted lines represent end of 5 day hydraulic retention time.

3.2 Removal of nitrogen and phosphorus from synthetic wastewater using algal biofilms

A basic requirement of wastewater treatment is the removal of nutrients (i.e., nitrogen and phosphorus) to acceptable limits prior to discharge. Microalgae based systems promote nutrient removal through plant uptake and subsequent harvesting of the nutrient-rich biomass from the effluent. In addition, microalgae increase the medium pH via photosynthesis thereby promoting volatilization of ammonia and possible precipitation of phosphate ions (Boelee et al., 2012). It should be noted that all the RABRs were covered in black polyethylene, cleaned, and had the bulk medium replaced every 5 days to minimize algal growth in the bulk medium, which also minimized the pH increase of the medium resulting from photosynthesis. Therefore, at the measured pH of 8.5 ± 0.15 for medium amended with bicarbonate and 7.97 ± 0.22 for medium without bicarbonate respectively, nutrient removal was attributed to the activity of the biofilms.

The synthetic wastewater was prepared with ammonia and nitrate salts as the only nitrogen sources. Initial concentrations of total nitrogen and phosphorus in the medium were approximately 40 mg-N L⁻¹ and 7 mg-P L⁻¹, respectively, giving a molar N:P ratio of approximately 13:1. The measured residual total nitrogen concentrations (including NO₂⁻-N) ranged from 7.95 – 19.66 and 8.20 – 19.72 mg-N L⁻¹ for RABRs with and without bicarbonate amendment, respectively. Similarly, final total phosphorus concentrations ranged from 3.39 – 3.57 and 3.35 – 3.55 mg-P L⁻¹ for RABRs with and
without bicarbonate amendment, respectively. The lowest N and P residual concentrations were obtained during the retention time cycles corresponding to the exponential growth phase of the biofilms (Figure 3-4). Therefore, as expected, nutrient removal from the wastewater was closely linked to algal biofilm growth i.e., higher removal efficiencies were obtained during the exponential growth phase of the biofilm compared to the onset of the stationary phase.

The N and P removal efficiency ranged from 27 - 74% (NO$_3^-$-N), 89 -100% (NH$_4^+$-N), and 19 - 41% (PO$_4^{3-}$-P) during the experiments, with no significant difference observed between liquid samples from reactors amended with bicarbonate and those that did not receive additional dissolved inorganic carbon. Similarly, for the entire duration residual N and P concentrations followed the same trend in cultures amended with bicarbonate and those that did not receive bicarbonate (Figure 3-4). Complete uptake of ammonium ions was observed unlike nitrate ions in this study, probably due to preferential uptake of ammonia by microalgae compared to nitrate (Eustance et al., 2013). Microalgal cultures supplied with mixed nitrate and ammonium sources may repress NO$_3^-$-N uptake due to feedback inhibition, since ammonium is an end product of assimilatory nitrate reduction (Crofcheck et al., 2012). Similar to the algal biofilm growth results, phosphate and nitrogen removal rates were not influenced by the addition of bicarbonate to the medium. Maximum nutrient removal from wastewater with algal biofilms can be attained via harvesting at the end of the exponential growth phase preferably after 8-10 days of growth using this RABR system.

The nitrite concentrations observed in solution were probably a result of incomplete nitrification of ammonia since the algal biofilms were grown in a non-aseptic
oxygenated environment (Figure 3-4). An abiotic control was used to verify that the presence of NO$_2^-$-N ions was due to biological processes (Table B-1). The chemoautotrophic bacteria involved in nitrification require a carbon source such as CO$_2$ or HCO$_3^-$, therefore the reactor with bicarbonate treatment possibly had more favorable initial conditions for the bacteria to grow, thus the higher nitrite concentrations observed (Figure 3-4). However, quasi-steady state concentrations of nitrite were eventually attained and the difference ceased to be significant later in the experiment.

Figure 3-4: Ammonium, nitrate, nitrite, and phosphate ion concentrations in medium amended with bicarbonate and without bicarbonate addition. Error bars represent range for (n=2). Vertical dotted lines represent end of 5 day hydraulic retention time.
3.3 Microalgal biofilm photosynthesis and coupled respiration

3.3.1 Oxygen microprofiles under illumination

Oxygen microprofiles were taken before and after N-deprivation was initiated, at 18 and 23 days of RABR operations. Steady state oxygen microprofiles for biofilm samples under light showed an initial increase in oxygen concentrations (compared to equilibrium with saturated air $\approx 260 \, \mu\text{M oxygen}$), which peaked at a depth of $200 \pm 25 \, \mu\text{m}$ from the biofilm surface (biofilm/air interface) for both N-replete and N-deprived biofilms (Figure 3-5A and B). Oxygen production in illuminated algal biofilms is a result of photosynthesis, and spatial gradients of light are known to affect the rate of oxygenic photosynthesis and corresponding oxygen concentrations in algal biofilms (Wieland and Kühl, 2000). Photosynthetic activity was highest in the upper layers of the biofilm and decreased with biofilm depth, possibly due to light attenuation and/or substrate diffusion limitations. Biofilms cultured under N-replete conditions had peak oxygen concentrations that were twice that of N-deprived biofilms (Figure 3-5A and B). Furthermore, under illumination there were no anoxic zones observed in N-replete biofilms, an indication that the oxic zone (oxygen penetration depth) extended into the cotton cord substratum (Figure 3-5A). In nitrogen replete systems, the steady state oxygen microprofiles showed no significant differences under either light or dark conditions for biofilms with or without bicarbonate (Figure 3-5A and C).

On the contrary, differences in steady state oxygen microprofiles were revealed between N-deprived algal biofilms with and without bicarbonate amendment (Figure 3-5B and D). For example, bicarbonate amended biofilms had higher oxygen concentrations compared to biofilms that did not receive bicarbonate. This is an
indication of either higher photosynthetic rates and/or reduced oxygen consumption rates due to respiration. Indeed, higher photosynthetic rates and lower areal respiration rates (in the light) were calculated for bicarbonate amended biofilm samples under N-stress (Table 3-1). Additionally, anoxic zones were observed in N-deprived algal biofilms and the depth of oxygen penetration for the bicarbonate amended biofilms was 1500 µm compared to 850 µm for biofilms without bicarbonate addition (Table 3-1).
3.3.2 Oxygen microprofiles in the dark

Oxygen is consumed by algal biofilms in the dark as a result of respiration. Assuming oxygen diffusivity is constant, the rate at which oxygen decreases (slope) is an indication of the consumption rate \( i.e., \) a steeper decline in oxygen concentration indicates greater consumption and a smaller depth of oxygen penetration can be assumed to occur as a result of high heterotrophic activity (Glud, 2008). Steady state oxygen concentrations for biofilms in the dark decreased with depth to anoxic conditions for both N-replete and N-deprived biofilms (Figure 3-5C and D). Biofilms under N-replete culturing showed a more gradual decline in oxygen concentration compared to N-deprived biofilms, where steeper slopes and shorter oxygen penetration depths were observed. This was an indication of greater potential for heterotrophic oxygen consumption in N-deprived biofilms compared to N-replete biofilms, an observation that is contrary to what was reported in Bernstein et al. (2014). The current study provided a longer N-starvation period of 120 h compared to 60 h in the study by Bernstein et al. (2014), which may have promoted greater heterotrophic activity in the N-deprived biofilms.
Both before and after N-deprivation, biofilm samples amended with bicarbonate had greater oxygen penetration depths under dark conditions compared to biofilms that did not receive bicarbonate. Oxic zones of 1100 ± 25 µm and 950 ± 25 µm in depth were estimated for biofilms amended with bicarbonate and without added bicarbonate under N-replete culturing. Similarly, oxygen penetration depths of 650 ± 25 µm and 300 ± 25 µm for biofilms amended with bicarbonate and without added bicarbonate during N-deprivation were observed (Table 3-1). This showed that the bicarbonate amended biofilms had lower oxygen consumption in the dark compared to the biofilms without bicarbonate amendment for both nutrient conditions.

3.3.3 Spatial rates of photosynthesis and respiration

The gross photosynthesis profiles were generated at a spatial resolution of 100 µm vertical depth using the volumetric photosynthetic rates (i.e., the rate of oxygen depletion within 3 seconds of dark incubation) determined from the light/dark shift technique. Photosynthesis occurred within a depth of 500 µm from the biofilm surface (Figure 3-5E and F). Similarly, increasing rates of areal gross photosynthesis (P_g) resulted in higher areal net biofilm photosynthesis (P_n) and photic zone photosynthesis (P_n, phot), which corresponded to deeper oxic zones (Table 3-1).

However, photosynthetic rates significantly varied with both nutrient conditions and presence/absence of bicarbonate in medium. Biofilm samples under nutrient replete culturing had higher photosynthetic rates (P_g, P_n, and P_n, phot) compared to N-deprived algal biofilms indicating a greater potential for photo-productivity when nutrient replete (Figure 3-5 and Table 3-1). Biofilms amended with bicarbonate also had higher photosynthetic rates (P_g, P_n, and P_n, phot) compared to the biofilms that did not receive
bicarbonate for both N-replete and deprived conditions (Table 3-1). The distribution of \( P_n \) and \( P_{n,\text{phot}} \) as a fraction of the gross photosynthesis in the bicarbonate amended biofilms was different from that of biofilms that did not receive bicarbonate. \( P_n \) and \( P_{n,\text{phot}} \) represented a greater proportion of gross photosynthesis under N-deprived conditions for bicarbonate amended biofilms, whereas for biofilm samples that did not receive bicarbonate the reverse was observed \( i.e., \) \( P_n \) and \( P_{n,\text{phot}} \) represented a greater proportion of gross photosynthesis under nutrient replete conditions.

Dark respiration and photorespiration are the two basic types of respiration that occur in photosynthesizing microalgae. Dark respiration is assumed to be constant and occurs both in the light and dark whereas photorespiration is mostly active in the light and a few seconds after dark incubation (Wieland and Kühl, 2000). The dark respiration term (\( R_{\text{dark}} \)) was obtained as the slope of the initial portion of the \( O_2 \) microprofiles (linear part) in the dark. The light respiration terms (\( R_{\text{light}} \) and \( R_{\text{phot}} \)) were determined as the difference between \( P_g \), and \( P_n \) and \( P_{n,\text{phot}} \), respectively. Although, there was no clear trend observed for respiration rates (\( R_{\text{light}} \) and \( R_{\text{phot}} \)) across nutrient conditions, addition of bicarbonate to the biofilms revealed some differences. For biofilms cultured under N-replete conditions, higher areal respiration rates (\( R_{\text{light}} \) and \( R_{\text{phot}} \)) were observed in bicarbonate amended biofilms compared to biofilms that did not receive bicarbonate (Table 3-1). This may have been due to the higher photosynthetic rates and subsequent increase in oxygen concentration in the biofilms amended with bicarbonate during N-replete culturing (Figure 3-5). For algal biofilms cultured under N-deprived conditions, lower \( R_{\text{light}} \) and \( R_{\text{phot}} \) were observed with added bicarbonate compared to biofilm samples.
without bicarbonate (Table 3-1). This indicated that addition of bicarbonate reduced light respiration in N-deprived biofilms possibly due to an increased DIC supply.

Dark respiration measurements were greater for N-deprived biofilms indicating a higher capacity for heterotrophic (or light independent) respiration. The influence of bicarbonate addition on $R_{\text{dark}}$ values varied with nutrient condition. For example, N-replete cultures had higher $R_{\text{dark}}$ in biofilms that did not receive bicarbonate, whereas higher $R_{\text{dark}}$ were observed in biofilms amended with bicarbonate for N-deprived cultures (Table 3-1).

Table 3-1: Measurements of photosynthetic rates, respiration rates, and relevant depth parameters for laboratory grown microalgal biofilms with and without bicarbonate amendment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bicarbonate</th>
<th>No bicarbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-replete</td>
<td>N-deprived</td>
</tr>
<tr>
<td>Gross photosynthesis, $P_g$ µmol O$_2$·cm$^{-2}$·sec$^{-1}$</td>
<td>6.27E-04</td>
<td>2.26E-04</td>
</tr>
<tr>
<td>Net areal rate of biofilm photosynthesis, $P_a$ (% $P_g$)</td>
<td>2.43E-04 (38.74%)</td>
<td>9.2E-05 (40.76%)</td>
</tr>
<tr>
<td>Net areal rate of photic zone photosynthesis $P_{a\text{phot}}$ (% $P_g$)</td>
<td>2.99E-04 (47.72%)</td>
<td>1.36E-04 (60.26%)</td>
</tr>
<tr>
<td>Areal respiration of the biofilm, $R_{\text{light}}$ (% $P_g$)</td>
<td>3.84E-04 (61.26%)</td>
<td>1.34E-04 (59.24%)</td>
</tr>
<tr>
<td>Areal respiration of the photic zone, $R_{\text{phot}}$ (% $P_g$)</td>
<td>3.28E-04 (52.28%)</td>
<td>8.97E-05 (39.74%)</td>
</tr>
<tr>
<td>Respiration in the dark, $R_{\text{dark}}$</td>
<td>0.59E-04</td>
<td>1.49E-04</td>
</tr>
<tr>
<td>Depth of photic zone, $L_{\text{phot}}$, µm</td>
<td>1000 ± 100</td>
<td>600 ± 100</td>
</tr>
<tr>
<td>Depth of oxic zone in light, µm</td>
<td>1500</td>
<td>&gt;1950</td>
</tr>
<tr>
<td>Depth of oxic zone in the dark, µm</td>
<td>1100 ± 25</td>
<td>650 ± 25</td>
</tr>
</tbody>
</table>
3.4 Biofuel precursor production

Extractable biofuel precursor molecules (FFAs, MAGs, DAGs and TAGs) and total biofuel potential (as FAMEs, i.e. extractable and non-extractable molecules) for each biofilm type, both before and after N-starvation, were measured and are presented in Table 3-2 and Figure 3-6. An increase in total extractable precursor concentrations was observed in the biofilms after the 120 h N-starvation period (Table 3-2). Stressed microalgae have been reported to accumulate TAG as a carbon and energy storage material (Mus et al., 2013). The sum of extractable precursors increased from 5.62% to 7.13 % (w/w) for biofilms amended with bicarbonate and 4.84% to 5.18% (w/w) for the biofilms that did not receive bicarbonate, respectively (Table 3-2). Although the FFA, MAG, and DAG concentrations remained relatively constant, twice as much TAGs accumulated in the biofilms after N-starvation leading to the overall increase in total biofuel precursor molecules (Table 3-2). Bicarbonate amended algal biofilms had higher weight percentage of extractable molecules.

The total FAME-weight percent and yield for N-replete and N-starved biofilms with or without bicarbonate amendment were similar (Figure 3-6A and C). Although, the total FAME potential ranged from 12 – 20 % (w/w) of the biomass (Figure 3-6B) the total extractable lipids were less than 10% (w/w) (Table 3-2). As previously reported by Bernstein et al. (2014), the most notable difference regarding lipid production in the RABR-grown algal biofilms was the difference in the total extractable weight percent of lipids between the N-replete and deplete conditions (Table 3-2). Depletion of nitrogen and addition of dissolved inorganic carbon in the medium were not effective in stimulating substantial lipid production in the microalgal biofilms. Qualitative analysis of
lipid profiles using images from CLSM showed the same result, the microalgal biofilms only showed a slight increase in lipids after N-starvation (Figure 3-7). Previous studies have attributed the inability of N-depletion in the growth medium to induce lipid production in algal biofilms to possible nutrient re-cycling within the biofilms and resilience of algal biofilms to environmental stress (Bernstein et al., 2014; Schnurr et al., 2013).

Table 3-2: Total and percent composition of extractable biofuel precursor weight (%) in laboratory grown microalgal biofilms with and without bicarbonate amendment

<table>
<thead>
<tr>
<th>Extractable biofuels precursor molecules, Weight % (w/w)</th>
<th>Condition</th>
<th>Nutrient Replete</th>
<th>Nutrient deplete</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aBicarbonate</td>
<td>aNo bicarbonate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aBicarbonate</td>
<td>aNo bicarbonate</td>
</tr>
<tr>
<td>C14 FFA</td>
<td>1.44±0.01</td>
<td>0.67±0.14</td>
<td>1.07±0.18</td>
</tr>
<tr>
<td>C16 FFA</td>
<td>1.11±0.26</td>
<td>1.49±0.03</td>
<td>0.86±0.58</td>
</tr>
<tr>
<td>C18 FFA</td>
<td>0.73±0.21</td>
<td>1.53±0.05</td>
<td>0.48±0.35</td>
</tr>
<tr>
<td>C16 MAG</td>
<td>0.11±0.05</td>
<td>0.18±0.01</td>
<td>0.11±0.09</td>
</tr>
<tr>
<td>C18 MAG</td>
<td>0.11±0.01</td>
<td>0.16±0.01</td>
<td>0.09±0.04</td>
</tr>
<tr>
<td>C16 DAG</td>
<td>0.09±0.03</td>
<td>0.10±0.02</td>
<td>0.09±0.05</td>
</tr>
<tr>
<td>C18 DAG</td>
<td>0.21±0.06</td>
<td>0.19±0.06</td>
<td>0.19±0.06</td>
</tr>
<tr>
<td>C16 TAG</td>
<td>0.49±0.41</td>
<td>0.17±0.08</td>
<td>0.58±0.41</td>
</tr>
<tr>
<td>C18 TAG</td>
<td>1.32±1.30</td>
<td>0.36±0.37</td>
<td>3.65±1.51</td>
</tr>
<tr>
<td>Sum of extractables</td>
<td>5.62±1.08</td>
<td>4.84±0.71</td>
<td>7.13±0.57</td>
</tr>
<tr>
<td>Weight % (w/w)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Areal concentration (gm m^-2)</td>
<td>1.03</td>
<td>1.22</td>
<td>1.30</td>
</tr>
</tbody>
</table>
Figure 3-6: Total FAMEs and free fatty acid composition of the FAMEs. A: Percent FAME per total FAME (w/w), B: percent FAME per biomass (w/w), C: areal concentration (g m$^{-2}$). Error bars represent range (n=2). ND and NR represent nitrogen deprived and replete algal biofilms, respectively.
Figure 3-7: Representative image of a nutrient deprived algal biofilm showing A: microalgae auto fluorescence (red), lipids stained with Bodipy (green) and B: same as A with rope strands stained with Calcofluor white M2R (cyan)

4.0 Conclusions

For this study, there was no significant difference in algal biofilm growth, nutrient removal, and lipid accumulation between algal biofilms amended with bicarbonate and those that did not receive bicarbonate. However, an increase in photosynthesis rates was observed in algal biofilms amended with bicarbonate. The influence of bicarbonate on photosynthetic and respiration rates was especially noticeable in biofilms that experienced nitrogen stress, as compared to biofilms in nutrient replete conditions.

Medium N-depletion may not be a suitable stimulant for lipid production in algal biofilms; rather focusing on optimizing growth, nutrient removal rates, and/or biomass productivities may be more beneficial.
References


CHAPTER 4

INTERACTION OF MICROALGAE AND BACTERIA IN SUSPENDED AND ATTACHED MICROALGAE COMMUNITIES

Abstract

The influence of algae-bacteria interactions on the productivity of algal biofilms is of interest in the development of algal biofilm technologies for wastewater treatment because wastewaters contain large populations of microbial organisms. This study first investigated the effect of algae–bacteria interactions on productivity in suspended mixed cultures and then immobilized co-cultures using microalgae species of Scenedemus obliquus and Chlorella vulgaris with Escherichia coli. Enhanced growth was observed in the suspended co-cultures compared to the controls (single species) with optical density measurements. However, the colony counts of viable E.coli concentrations showed a declining trend in the same samples. It is probable that microalgae inhibited bacteria growth but benefited from the nutrients and growth factors released by the bacteria, an indication of an antagonistic relationship. The trend for the algal biofilms was inconclusive and thus the effect of algal–bacteria interaction on productivity was not determined. Further research on algae-bacteria interactions using species native to the wastewater grown algal biofilms is recommended.

Co authors: Bethany Jensen, Ryan Putman, Charles Miller, and Ronald C. Sims
1.0 Introduction

Biofilms are communities of microorganisms, which attach to solid surfaces in an enclosed matrix of extracellular polymeric substances (EPS) (Cao et al., 2011). Natural biofilm communities comprise of several microorganisms including algae, bacteria, fungi, protozoa, and insects, which may or may not depend on each other for survival (Leadbeater and Callow, 1992). The role of algae–bacteria interactions on the function or structure of biofilm communities is not clearly defined, however, the nature of the relationship varies from synergistic to antagonistic (Unnithan et al., 2014).

Synergistic relationships between microalgae and bacteria result into direct exchange and internal re-cycling of nutrients essential for biofilm growth and survival (Subashchandrabose et al., 2011). Bacteria potentially minimize oxygen accumulation in the microenvironments around the algal cells by using the oxygen to degrade dissolved organic matter such as algal photosynthates (Jones and Cannon, 1986). The oxygen would otherwise inhibit photosynthetic activity. Initial surface colonization by the bacteria has also been reported to enhance algal biofilm formation (Roeselers et al., 2007). Microalgae in turn utilize CO₂ from bacterial degradation of organic compounds and growth factors like vitamins from bacteria metabolism, in addition to protecting bacteria against adverse environmental conditions (Espeland et al., 2001; Jones et al., 1973).

On the contrary, antagonistic relationships between microalgae and bacteria do inhibit algal biofilm growth. Microalgae release toxic metabolites (antimicrobials) and increase medium pH or oxygen concentrations that inhibit bacterial activity. Likewise,
bacteria inhibit microalgae growth by producing phycotoxins (Le Chevanton et al., 2013; Unnithan et al., 2014).

In wastewater treatment, removal of biological oxygen demand (BOD) using stabilization ponds/lagoons is already an established technology that takes advantage of the symbiotic nature of algal-bacterial interactions (Fallowfield and Garrett, 1985). The microalgae utilize the nutrients in the wastewater lagoons for growth and release oxygen via photosynthesis, which is used by bacteria as an electron acceptor in the degradation of organic matter (Oswald et al., 1953). This eliminates the need for an aeration system, which is usually energy intensive. This concept can be extended to algal biofilm based systems thereby improving system sustainability, while simultaneously meeting the wastewater treatment goals and biomass production.

This subsequent study was carried out to provide preliminary information on the influence of algal-bacterial interactions on productivity of both suspended and immobilized mixed algae-bacteria cultures. Microalgae strains *Scenedesmus obliquus* CCALA 453 and *Chlorella vulgaris* UTEX 2714, and bacteria strain *Escherichia coli* were selected because of their abundance in wastewaters (Oswald, 2003).

### 2.0 Materials and methods

#### 2.1 Preparation of antibiotic stock

Stock concentration of commonly used antibiotics chloramphenicol (34 mg/ml), ampicillin (50 mg/ml), and kanamycin (50 mg/ml) were prepared and stored in ethanol (chloramphenicol) and water (ampicillin and kanamycin) in a freezer until use. A 1:1000 dilution was recommended for any of the antibiotics used giving a working concentration of 34, 50, and 50 µg/ml of chloramphenicol, ampicillin, and kanamycin, respectively. In
order to determine microalgae sensitivity to any of the antibiotics, different antibiotic concentrations/ dosages were used in the initial screening studies. The concentrations tested included: chloramphenicol (34, 20, 10 µg /ml) and kanamycin and ampicillin (50, 25, 10 µg/ml).

2.2 Growth medium preparation

Bolds Basal medium (BBM) was prepared, stored at 4°C, and autoclaved before use. The medium composition consisted of 25 mg L⁻¹ NaCl, 25 mg L⁻¹ CaCl₂.H₂O, 250 mg L⁻¹ NaNO₃, 175 mg L⁻¹ KH₂PO₄, 75 mg L⁻¹ K₂HPO₄, 75 mg L⁻¹ MgSO₄.7H₂O, 11.42 mg L⁻¹ H₃BO₃, and micronutrients (8.82 mg L⁻¹ ZnSO₄.7H₂O, 1.44 mg L⁻¹ MnCl₂. 4H₂O, 0.71 mg L⁻¹ MoO₃, 1.57 mg L⁻¹ CuSO₄. 5H₂O, 0.49 mg L⁻¹ Co (NO₃)₂.6H₂O and 4.98 mg L⁻¹ FeSO₄). No organic carbon source was added to the media so that bacteria could only survive on dissolved organic carbon from microalgae exudates.

2.3 Microalgal strains and stock culture maintenance

Microalgae stock cultures of *C. vulgaris* and *S. obliquus* from the algae culture collection at the University of Texas at Austin (UTEX 2714) and the culture collection of autotrophic organisms at the Institute of Botany of the Academy of Science, Czech Republic (CCALA 453), respectively, were grown in Bold Basal medium in 250 ml shaker flasks on an illuminated shaker table at room temperature. Stock cultures were maintained by adding 10% inocula to fresh media every two weeks. All transfers were performed under a laminar flow hood.
2.4 Bacterial strains and stock culture maintenance

Bacterial strain *E. coli* with an antibiotic resistant gene encoded in a high copy plasmid was obtained. The stock culture was grown and maintained on LB medium with antibiotics at 37°C overnight.

2.5 Growth studies for suspended axenic microalgae cultures

Antibiotics screening experiments were carried out on suspended *S. obliquus* and *C. vulgaris* cultures in triplicate 250 ml shaker flasks containing 100 ml microalgae cultures for a period of 2 weeks. An initial optical density (OD_{750}) of 0.1 was used for all the flasks inoculated with algae. After, selecting the suitable antibiotics, microalgae growth in terms of OD_{750} was correlated to its total suspended solids (TSS) and chlorophyll a measurements. Five hundred milliliters shaker flasks (duplicate) containing 200 ml microalgae cultures + antibiotics were utilized to obtain OD correlations with TSS and chlorophyll a measurements. The microalgae cultures were also cultured for 2 weeks with a starting OD_{750} of 0.1. The correlation (equations) obtained could then be used to give an estimate of biomass or chlorophyll a concentration using OD_{750} readings. Validation of the equations was done by taking a random sample from the stock solutions, measuring its OD_{750} and using the equations to predict the TSS, and chlorophyll a concentrations. The predicted values were then compared to the actual measured values and the % error determined.

2.6 Growth studies for suspended co-cultures of algae and bacteria

Duplicate 250 ml shaker flasks containing 100 ml of mixed algae-bacteria cultures were cultured in a growth chamber under continuous light (innova® 42 incubator
shaker series, New Brunswick Scientific) and controlled temperature (25°C) at 100 RPM. An initial OD$_{600}$ of 0.05 was used for *E. coli* and 0.1 for microalgae. It was hypothesized that bacteria (in this case *E. coli*) would survive on algal photosynthates as the carbon source and the nutrients in Bolds Basal medium. The initial experiment involved growing *E.coli* in BBM (no carbon source), in BBM with 1% glucose (carbon source present), and in LB medium (ideal) to determine whether the medium had enough nutrients to sustain *E.coli* growth when a carbon source was present. Duplicate flasks were prepared and the experiment carried out for 72 h. OD$_{600}$ measurements were taken and *E.coli* growth in the BBM flasks compared to control (LB flask). Another experiment was carried out for at least 14 days, which involved the following microalgae and bacteria combinations in duplicate:

1. *E. coli* + BBM + 1% glucose (OD control)
2. Microalgae (*C. vulgaris* or *S. obliquus*) + BBM (OD control)
3. *E. coli* + BBM (bacteria count control)
4. *E. coli* + BBM + *S. obliquus*
5. *E. coli* + BBM + *C. vulgaris*

OD$_{600}$ readings were taken for all the flasks (1-5) and bacteria plate counts for flasks 3-5 at days 0, 2, 4, 6, 8, 10, 12, and 14. OD$_{600}$ readings from the mixed culture flasks represented a rough estimate of the total cells in solution i.e., both the bacteria and microalgae contribute to the final reading:

Rough estimate of total cells as $OD_{600} = OD_{600}$ due to bacteria cells + $OD_{600}$ due to microalgae cells
2.7. Algal biofilm growth using drip flow reactors (DFRs)

The experimental set up consisted of drip flow reactors (Biosurface Technologies, Bozeman MT) under fluorescent lighting (PAR= 200 µmol/m²/s) on a 14:10 L/D cycle at room temperature (Figure 4-1). This reactor system allowed for more environmental control and axenic culturing in order to assess algal-bacterial interactions. The reactors were modified for phototrophic biofilm growth by replacing the polycarbonate lids with a glass lid to increase light penetration. As a result of the modification, the reactor air vent was sacrificed, which necessitated addition of 2 mM bicarbonate (inorganic carbon source for microalgae) in the medium to compensate for absence of adequate airflow (CO₂ source). All drip flow reactor apparatus, tubing, and medium were autoclaved before start of the experiment. The medium was allowed to cool to room temperature prior to addition of filter sterilized antibiotics and bicarbonate in a fume hood. The medium was stored in 10L polypropylene carboys connected to the DFRs by Masterflex 16 and 14 Noprene™ tubing. Cole-Palmers Masterflex L/S Peristaltic pumps were used to pump the sterile medium from the carboys to the reactors at a flow rate of 0.2 ml/min.

C. vulgaris and S. obliquus were cultured independently and as artificial co-cultures with E. coli on pre-weighed glass fiber filters (47 mm diameter) or microscope glass slides (75 x 25 mm). The wells of the DFRs containing the growth substratum were inoculated with 20 ml algal cells suspension under sterile conditions. For the mixed cultures, an additional 3 ml of bacteria suspension was added to each well. The algae and bacteria inoculum used in the DFRs was obtained from the respective stock solutions. A seeding period of two days was allowed before the experiment was started. After the seeding period, continuous drop wise flow of medium into each DFR well was started via
a 23-G syringe (BD precision glide needle) for a growth period of 7-14 days. Glass flow breaks were used to prevent contamination due to back flow of media into the storage carboys.

2.8 Biomass measurements

Chlorophyll a and optical density absorbance measurements of microalgae cultures were taken using a UV Spectrophotometer (Shimadzu Corporation, Japan). Methods 10200 H and 2540D were followed in the determination of Chlorophyll a and TSS respectively (APHA 2005). Triplicate glass fiber filter papers were removed from the DFRs reactors at day 7 (for single microalgae biofilms) and day 14 (algal biofilms cocultures), dried at 70 °C to a constant weight, cooled in a desiccator and weighed on a balance. For the microscope slides, the biomass was scrapped off the glass surface into pre-weighed aluminum tins and then dried at 70 °C. The difference in weights between the initial and final weight of the filters or aluminum tins represented the biofilm weight.

Figure 4-1: Set up of a drip flow reactor (DFR)
3.0 Results and Discussion

3.1 Screening of antibiotics

Sensitivity of microalgae species (*C. vulgaris* and *S. obliquus*) to varying concentrations of chloramphenicol, kanamycin and ampicillin was determined as shown in Figure 4-2. Antibiotics have different modes of action i.e., inhibition of bacterial cell wall synthesis (ampicillin), inhibition of translation on the 50S ribosomal subunit and prevention of peptide bond formation (chloramphenicol), and inhibition of ribosomal translocation by binding to 70S ribosomal subunits (kanamycin), therefore it was hypothesized that their effect on microalgae if any should also vary accordingly.

*C. vulgaris* was not affected by the antibiotics tested i.e., growth of the microalgae cells in the flasks with and without antibiotics was similar for all the antibiotics. However, *S. obliquus* showed sensitivity to chloramphenicol at all dosages tested, and kanamycin at the highest dosage and recommended working concentration (Figure 4-2). All chloramphenicol dosages were inhibitory to *S. obliquus*, however, the highest and recommended working concentration of 34 µg/ml was lethal (the microalgae cells were killed within 6 days). A similar observation was reported in a study by Zhang et al. (2013), which showed that exposure of chloramphenicol to *S. obliquus* significantly inhibited its growth while *C. pyrenoidosa* exhibited less sensitivity. Similarly, the highest dosage for kanamycin (50 µg/ml) initially inhibited growth of *S. obliquus* as shown by the lag in growth compared to the control, but the inhibition was eventually overcome after about 9 days of growth (Figure 4-2). Ampicillin did not affect growth of either microalgae species and as a result, ampicillin concentration 50 µg/ml (Amp 50) was chosen for further use in the subsequent studies.
3.2 Microalgae growth rates and OD correlation with TSS and Chlorophyll a for suspended cultures

As above-mentioned, all subsequent experiments had Amp 50 since it did not have any effect on the growth of the microalgae species. The specific growth rates of *C. vulgaris* and *S. obliquus* were determined during the exponential phase as 0.28 and 0.26 day\(^{-1}\) respectively (Figure 4-3). The stationary phase was reached after approximately 8 days of growth in the suspended single microalgae cultures (without *E.coli*). The exponential phase was between day 2 and day 8 as evidenced by the R\(^2\) values of 0.957 and 0.991 for *C. vulgaris* and *S. obliquus* respectively (Figure 4.3). The overall growth of the microalgae species in this study was not statistically significant as determined by a t test (p value of 0.7155).
Figure 4-3: Growth curves for suspended axenic microalgae cultures. Insert: curves showing the exponential phase of *S. obliquus* and *C. vulgaris*

The OD$_{750}$ measurements were correlated to the biomass concentration (TSS) and chlorophyll a concentration for the two species in the suspended cultures (Figure 4-4), respectively. Only the absorbance values for the undiluted samples for the OD$_{750}$ vs. TSS experiment (i.e. OD below 0.1) were considered, which corresponded to six days of growth. A fairly good correlation was obtained for *C. vulgaris* compared to *S. obliquus* cultures. Using the derived equations, the TSS and chlorophyll a values were predicted and compared to the measured values to validate the equations (Table 4-1). A more accurate prediction of the TSS was obtained for the cultures in exponential phase (less than a week of growth) as shown by the results for *C. vulgaris* than cultures in stationary phase (*S. obliquus*). The same observation applied to predicted chlorophyll a measurements.
Figure 4-4: Correlating OD with TSS (top) and chlorophyll a (bottom) for axenic microalgae cultures in suspension
Table 4-1: Validation of equations from correlating OD Vs. TSS and OD Vs. Chlorophyll a data

<table>
<thead>
<tr>
<th>Species</th>
<th>Measured OD</th>
<th>Parameter (mg/L)</th>
<th>Predicted</th>
<th>Measured</th>
<th>% error</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. obliquus</em></td>
<td>0.88</td>
<td>Chlorophyll a</td>
<td>2180</td>
<td>3177</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TSS</td>
<td>240</td>
<td>515</td>
<td>53</td>
</tr>
<tr>
<td><em>C. vulgaris</em></td>
<td>0.246</td>
<td>Chlorophyll a</td>
<td>1735</td>
<td>1416</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TSS</td>
<td>74</td>
<td>105</td>
<td>29</td>
</tr>
</tbody>
</table>

3.3 Growth studies for mixed algae-bacteria cultures in suspension

3.3.1 *Bacteria (E.coli) growth estimate in Bold's Basal Medium using plate counts*

*E.coli* growth in LB medium, BBM, and glucose + BBM was as expected (Figure 4-5). *E.coli* growth in BBM was negligible because of the lack of a carbon source; however, the bacteria did survive and maintained a stationary growth trend for 72 h without dying possibly due to residual nutrients from the stock culture. With the 1% glucose added to BBM (carbon source), an increase in *E. coli* growth concentrations was observed, which peaked at about 24 h and remained stationary for the rest of the time period (72 h). A similar trend was observed for *E. coli* growing in LB medium (control) but with higher bacteria concentrations. Since, *E. coli* did not show any drastic drop in concentrations (death) when grown in BBM, the experiment was continued on the assumption that adding microalgae would provide the required carbon source and promote bacteria growth.
Interestingly, colony counts of viable *E.coli* concentrations (CFU/ml) from the co-cultures did not show any appreciable increase within the 72 h growth period. In fact, *E. coli* growth in the BBM (no carbon source) was similar to that of the algae-bacteria co-culture flasks (Figure 4-5), an indication that the 3-day period was probably not sufficient to show a significant difference in bacteria growth for the algae-bacteria co-cultures. The experimental time was therefore extended to at least 2 weeks in order to determine if the trend would eventually change.

Figure 4-5: *E. coli* colony counts in LB and BBM for 72 h growth period
3.3.2 Total cells (microalgae-bacteria) growth estimate using OD measurements

As previously mentioned, the OD$_{600}$ measurements were influenced by the presence of both microalgae and bacteria in the medium. Figure 4-6 shows all the growth curves for both algae-bacteria co-cultures and their respectively axenic cultures. The mixed algae-bacteria cultures had higher OD$_{600}$ readings compared to the single microalgae (C. vulgaris/ S. obliquus) or bacteria (E. coli) cultures (Figure 4-6). Therefore, enhanced growth was obtained as a result of co-culturing algae with bacteria compared to single cultures of microalgae or bacteria alone (controls).

Figure 4-6: OD$_{600}$ measurements for all single and mixed culture flasks
3.3.3 Influence of microalgae or bacteria presence on the total OD measured

By adjusting for the microalgae controls (S. obliquus/C. vulgaris + BBM) or bacteria control (E. coli + BBM), an estimate of the increase in OD$_{600}$ due to the presence of bacteria or microalgae, respectively, was obtained (Figure 4-7 and 4-8). The presence of microalgae in the mixed algae–bacteria flasks contributed to the bulk of the absorbance readings recorded at the 600 nm wavelength as shown by the closely coupled curves for both the total and adjusted OD values (Figure 4-7). However, Figure 4-6 also showed that growth of axenic microalgae cultures was still lower than that of the microalgae growing with E. coli. This showed that even though microalgae did contribute to the bulk of the OD readings observed, presence of bacteria enhanced its growth.

![Figure 4-7: Absorbance due to presence of microalgae obtained after accounting for OD readings from (E. coli +BBM) control](image-url)
3.3.4 Microalgae and E.coli growth in suspended algae-bacteria co-cultures

Both *S. obliquus* and *C. vulgaris* chlorophyll a measurements increased with time in the co-culture flasks as expected. The enhanced growth was attributed to the presence of *E.coli*. A linear correction between chlorophyll a and OD$_{600}$ was obtained with an $R^2$ of 0.49 and 0.86 for *C. vulgaris* and *S. obliquus* respectively (Figure 4-9), an indication that *S. obliquus* was more directly influenced by the presence of bacteria than *C. vulgaris*. However, due to the low $R^2$ values obtained, the correlation results are not reliable and thus cannot be used to infer a meaningful relationship between chlorophyll a and OD$_{600}$. 

Figure 4-8: Absorbance due to presence of bacteria (adjusted) obtained after accounting for OD readings from (microalgae + BBM) control
The longer growth period (14 days), did allow for *E. coli* growth from the algae-bacteria co-culture flasks to be distinguished from the control flask (*E. coli* + BBM) as shown in Figure 4-10. Agar plates with and without ampicillin were used for the bacteria counts in order to account for possible influence of ampicillin degradation on the bacteria in the shaker flasks given the long growth period. Since the *E. coli* utilized in this study
had the ampicillin resistance gene encoded in a high copy plasmid, it is probable that lack of selection pressure (ampicillin degradation) could lead to the bacteria eliminating the plasmid and failing to grow on the agar plates with ampicillin. Results from the bacteria counts showed a decreasing trend in bacteria concentrations from the algae-bacteria co-culture flasks plated on all the LB agar plates regardless of presence or absence of ampicillin (Figure 4-10). However, the plates with ampicillin did have lower viable bacteria concentrations (CFU/ml) compared to those with out ampicillin as anticipated. Interestingly, bacteria counts from the control flask (E. coli + BBM) remained constant throughout the experimental period and only showed a decline after 14 days of growth. This observation implies that the presence of microalgae inhibited E. coli growth in the mixed culture flasks.

Contrary to what was observed with the OD measurements, where mixed cultures showed enhanced growth compared to the controls. The OD measurements may not have captured this trend because of lack of sensitivity in the method since both bacteria and microalgae influence the absorbance readings at 600 nm. It is probable that the bacteria started to die after about 4 days of growth in absence of an organic carbon source and presence of a growth-inhibiting factor released by the microalgae, while the microalgae continued to grow due to release of nutrients from the bacteria. This is an indication of an antagonistic relationship between the E. coli and both species of algae (S. obliquus and C. vulgaris).
Figure 4-10: *E.coli* colony counts from LB – Amp (top) and LB+AMP plates (bottom) for mixed algae-bacteria cultures and control (*E. coli* + BBM). Error bars represent standard deviation.

3.4 Algal biofilm growth studies in drip flow reactors

With the drip flow reactors, algae growth as a biofilm was demonstrated in a sterile environment. The microalgae biofilms were cultured on two commonly used substrata i.e., glass and glass microfiber papers. Single point biomass measurements at 7 days of growth for single algae species and both 7 and 14 days of growth for the mixed
algae-bacteria cultures were made. Growth of *S. obliquus* and *C. vulgaris* were similar on both glass and the glass microfiber paper, with the exception of one measurement that showed higher growth of *S. obliquus* on glass than the paper (Figure 4-11). Also, the growth of both species was not different with and without antibiotics. This was similar to what was observed in the suspended *S. obliquus* and *C. vulgaris* samples.

Figure 4-11: Single microalgae species biofilm growth on microfiber glass filter paper and microscope glass slides. Error bars represent standard deviation (n=3)

Algal biofilm growth of the co-cultured *C. vulgaris + E. coli* and *S. obliquus + E. coli* showed an increase in growth from 7 days to 14 days of growth (Figure 4-12). The reason for the increase in growth could be due to either presence of bacteria or the longer growth period. The results obtained in this study were inconclusive and so extending the control experiments to a 14-day period would help rule out the effect of time on biofilm
growth. However for the 7-day growth period, there was no difference in growth between the single algae or algae- *E. coli* co-culture biofilms.

Figure 4-12: Algal biofilm growth of mixed algae-bacteria species and single microalgae species (control) on glass microfiber filter paper. Error bars represent standard deviation (n=3).

**Conclusion**

Growth of *S. obliquus* and *C. vulgaris* was enhanced by presence of *E. coli* in the suspended algae-bacteria co-cultures, but the algae inhibited *E. coli* growth. The growth trend observed with suspended co-cultures could not be verified in the algal biofilms because of insufficient information.

**Recommendations/Future work**

Successful implementation of algal biofilm-based systems will require understanding of the system dynamics and limits in terms of the influence of different
microbial interactions and predation on the biofilm function and structure. Algal biofilms in wastewater environments comprise of a complex and diverse mixture of phototrophs and heterotrophs, whose role in the community is not well defined or known. Consequently, there is need for further research in order to better understand the underlying science and also apply to knowledge to advance engineering design of algal biofilm based systems. Based on this study, future work should include:

1. Determining the difference in growth of algae-bacteria co-cultures at a longer growth period (14 days) as compared to the single axenic cultures in order to ascertain if the enhanced algal biofilm productivity is due to time or presence of bacteria.

2. Correlate chlorophyll a measurements and bacteria concentration with biofilm areal density. This will show if the growth trends of both microalgae and bacteria observed in suspended algae-bacteria co-cultures are similar to that of the attached/biofilm algae-bacteria co-cultures.

3. Determine how the algal-bacteria interactions affect the biofilm structure through microscopy and substrate utilization especially inorganic nutrients. Microscopy will help show the arrangement of the species within the biofilm and the substrate utilized will determine which organisms are present or thriving in the biofilm.

4. The microbial consortia and microalgae community in the wastewater environment (Logan City sewage lagoons) should be identified and characterized. After which, the bacteria and microalgae species already existing in the wastewater grown microalgae biofilms (field-RABR) should be isolated and their specific interactions studied instead of relying on literature as employed in this study.
References


CHAPTER 5

SUMMARY

The challenge of algal biofilm based technologies for wastewater treatment is not whether microalgae can grow and subsequently take up nutrients from wastewater, but if the productivity or nutrient removal capacity is sufficient to meet the needs of the biomass end use and/or wastewater treatment goals. There is a need therefore to improve predictability, feasibility, sustainability, and scale up of these systems through research on the fundamental physiological processes occurring in the algal biofilm communities. This study was carried out in order to provide information on algal biofilms with regards to their photosynthetic activity and structure, their performance as a nutrient removal option and feedstock for biofuels, and algae-bacteria interactions.

The algal biofilms were characterized using microsensor-based methodology to identify photosynthetic activity with depth of the biofilm and microscopy (confocal laser scanning microscope) to visualize the biofilm structure on cotton cord substratum. Medium nutrient concentrations were monitored and analyzed using ion chromatography in order to determine the nutrient removal capacity of the biofilms. Stimulation of lipid production in the algal biofilms was attempted through nitrogen stress and bicarbonate addition and algae-bacteria interactions investigated in both suspended and immobilized cultures.

Results obtained from this study showed that algal biofilm production rates per rope surface area ranged from 1.45 - 1.79 g m\(^{-2}\) day\(^{-1}\) for the laboratory grown biofilms and 1.68 g m\(^{-2}\) day\(^{-1}\) for biofilms from the outdoor RABR. The maximum measured areal biomass density (observed during the stationary phase) ranged from 20.95 - 36.90 g m\(^{-2}\)
for laboratory grown biofilms and 65.98 g m$^{-2}$ for biofilms under field conditions. The maximum nutrient removal rates were observed during the exponential growth phase of the algal biofilms, and nutrient removal efficiencies ranged from 89-100%, 27 -74%, and 19-41% for NH$_4^+$-N, NO$_3^-$-N, and PO$_4^{3-}$-P, respectively.

Oxygen microprofiles and the corresponding spatial photosynthetic and respiration rates of the biofilms were influenced by nutrient availability, orientation to the light, culturing conditions, and species composition. Only a slight increase in lipids production was observed as a result of nitrogen stress or a combination of nitrogen stress with bicarbonate addition. In addition, the lab-RABR grown biofilms did not experience carbon limitation possible due to the large reservoir of DIC in the medium. Medium nitrogen depletion was not a suitable stimulant for lipid production in the algal biofilms. The bacteria-algae interactions positively influenced productivity in suspended mixed algal cultures but the effect on algal biofilms was inconclusive.

In conclusion, it is possible to directly measure photosynthetic parameters of algal biofilms using oxygen based microsensor techniques and visualize the different components of an intact biofilm morphology using microscopy. The RABR systems require process optimizing to increase the biofuel potential of the algal biofilms.

**RECOMMENDATIONS**

1. Focus should be directed towards optimizing growth and biomass productivities to compensate for the low lipid yields and increase nutrient uptake. Algal biofilm growth optimization in terms of growth conditions such as light and nutrients and also species composition should be investigated especially in field scale operations.
Wastewater characteristics from different sources should also be correlated with algal biofilm growth.

2. Research in other lipid enhancing techniques such as pH stress should also be considered and/or determine better ways to directly stress algal biofilms. Indirect methods for example medium nutrient depletion employed in this study did not effectively stimulate lipid production in the algal biofilms probably because attached microalgal cultures may be more resilient to stress compared to suspended cultures.

3. The effect of light penetration on the photosynthetic activity with depth of biofilm and optimization of the light flux in the biofilms should be determined. Light is an important component for algal growth and yet very little information is available on spatial light utilization in algal biofilm communities.
APPENDICES
APPENDIX A

DIRECT MEASUREMENT AND CHARACTERIZATION OF ACTIVE PHOTOSYNTHESIS ZONES INSIDE WASTEWATER REMEDIATING AND BIOFUEL PRODUCING MICROALGAL BIOFILMS

Abstract

Microalgal biofilm based technologies are of keen interest due to their high biomass concentrations and ability to utilize light and CO₂. While photoautotrophic biofilms have long been used for wastewater remediation, biofuel production represents a relatively new and under-represented focus area. However, the direct measurement and characterization of fundamental parameters required for industrial control are challenging due to biofilm heterogeneity. This study evaluated oxygenic photosynthesis and respiration on two distinct microalgal biofilms cultured using a novel rotating algal biofilm reactor operated at field- and laboratory-scales. Clear differences in oxygenic photosynthesis and respiration were observed based on different culturing conditions, microalgal composition, light intensity and nitrogen availability. The cultures were also evaluated as potential biofuel synthesis strategies. Nitrogen depletion was not found to have the same effect on lipid accumulation compared to traditional planktonic microalgal studies. Physiological characterizations of these microalgal biofilms identify fundamental parameters needed to understand and control process optimization.

1.0 Introduction

Photoautotrophic microorganisms are used as biotechnology platforms for many applications including biofuel production, wastewater remediation, carbon sequestration, and agriculture (Christenson and Sims, 2011; Ordog et al., 2004; Pokoo-Aikins et al., 2010). Of these, microalgal biofuel production has been identified as especially promising due to its potential for sustainable supplementation or replacement of fossil fuels (Chisti, 2007; Hu et al., 2008). Traditionally microalgae biotechnologies have focused on suspended, planktonic, culturing methodologies designed to facilitate photo-production; the capture and conversion of energy from photons into chemical energy stored in extractable biomolecules (e.g., lipids). This study focuses on characterization of oxygenic photosynthesis and respiration in photo-biofilm reactors, an alternative and often under-represented growth scenario with benefits over planktonic cultures such as high cell density; which inherently facilitates harvesting and reduces water requirements.

Biofilms are matrix-enclosed microbial cells attached to biological or non-biological surfaces (Hall-Stoodley et al., 2004). Photoautotrophic biofilms, composed of microalgae and/or cyanobacteria, are ubiquitous to nearly all photic aquatic environments. An important attribute of biofilms is that they both create and are functionally controlled by gradients in substrates, products and energy sources (Stewart and Franklin, 2008). Spatial gradients in light have been shown to directly control rates of oxygenic photosynthesis and corresponding oxygen concentrations inside biofilms (Kuhl et al., 1996). Oxygen gradients in biofilms are directly influenced by diffusion rates and can result in localized supersaturated concentrations (with respect to air oxygen concentrations) during active oxygenic photosynthesis. The resulting high oxygen
concentrations can inhibit CO2 fixation by competing as a substrate for ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) (Falkowski and Raven, 1997; Glud et al., 1992; Kliphuis et al., 2011). This competition subsequently limits photo-production of carbon storage compounds. Thus, the characterization of spatial gradients in oxygenic photosynthesis and respiration activities is a key consideration for microalgal biofilm-based technologies.

This study employed a recently developed rotating algal biofilm reactor (RABR) that was designed, built and tested at both the laboratory scale (lab-RABR) and pilot field scale (field-RABR) (Christenson and Sims, 2012) (Figure A-1). The advantage of the RABR is the ability to simultaneously facilitate algal growth and dewatering while achieving high biomass concentration. Biofilm reactors can also reduce the water and energy requirements for biomass and photo-production compared to traditional suspended culturing strategies (Ozkan et al., 2012). The RABR and other algal-based biofilm technologies have been investigated for their potential to concurrently remediate wastewater and produce biofuel precursor molecules (Boelee et al., 2012; Farooq et al., 2013). The RABR can facilitate efficient biomass harvesting via the reported spool harvesting technique (Christenson and Sims, 2012). However, optimal biomass harvesting practices need to be determined in the context of biofilm specific physiology, such as optimal biomass areal density and biofilm thickness as it relates to active photo-production and photosynthesis zones.

The current study focuses on spatial physiological characterization of microalgal biofilms cultured through the RABR method. The specific aims of this study were to:
1) Characterize and compare two different RABR biofilms (wastewater remediating and potentially biodiesel producing) in the context of active photosynthesis zones by directly measuring spatial gradients in oxygen and photosynthesis microprofiles, as well as, determining rates of photosynthesis and respiration.

2) Characterize and compare the biofuel potential and (neutral lipid) precursor biomolecule composition in these biofilms. In addition to specific aim 2, nitrate starvation was investigated as a potential strategy for inducing lipid accumulation in the lab-scale RABR biofilms.

2.0 Materials and Methods

2.1 Laboratory strains, culturing conditions, and biomass sampling

The Chlorophyte isolate Botryococcus sp. strain WC-2B (hereinafter referred to as WC-2B) was cultured with 8 L lab-RABRs operated in batch mode. WC-2B was isolated from an alkaline stream in Yellowstone National Park (USA), confirmed unialgal using SSU 18S rDNA and revealed 99% alignment with Botryococcus sedeticus UTEX 2629, which has previously been described (Senousy et al., 2004). Reactors were operated in triplicate and grown at 25°C in Bold’s basal medium buffered with 25 mM 2-[N-cyclohexylamino]-ethane-sulfonic acid (CHES, pKa 9.3) and rotated at 15.3 RPM. All RABR experiments were loaded with untreated cotton cord as the biofilm-substratum (0.64 cm diameter) (Christenson and Sims, 2012). The lab-RABRs consisted of cords coiled onto plastic cylindrical-spool (10 cm diameter) submerged approximately 5 cm in the liquid medium. The lab-RABRs were cultured under custom light emitting diode (LED) banks (Box Elder Innovations, LLC and T&L Design, Box Elder UT) programmed with LabVIEW (National Instruments Corp.) to simulate a diurnal cycle
with photosynthetically active radiation (PAR) values ranging from 0 to 900 µmol photons·m⁻²·sec⁻¹ on a 14:10 L/D diel cycle following Eq. (1).

\[ I = \cos \left( \frac{\pi}{t_L} (t - t_M) \right)^2 \]  
Eq. 1

Where \( I \) is the light intensity, \( t_L \) is the total light time in minutes, \( t \) is the current time, and \( t_M \) is the midpoint time corresponding to the maximum light intensity. Nitrate concentrations in the medium were monitored using NitraVer 5 pillow packets (HACH). Concentrated medium (10X) and supplemental diH₂O (de-ionized) were added, as needed, to maintain nutrient replete conditions and offset evaporation. Culturing and sampling were performed under non-aseptic conditions (i.e., open-air). Nitrate depletion was induced (after 28 days of replete culturing) by removing all liquid medium from the reactors followed by immediate replacement with Bold’s basal medium without nitrate. Analysis of the biofilms after nitrate depletion was performed 60 h post depletion.

Biomass cell dry weights (CDW, g_{CDW}·cm⁻²) were obtained throughout culturing by excising a known length rope substratum and attached biofilm, followed by biofilm removal into preweighed aluminum weigh boats. The biomass was dried at 70°C for 18 h until the biomass weight was constant. Biomass CDWs were calculated by subtracting the dry weight of the preweighed aluminum boat from the oven dried boat with biomass and using an assumed cylindrical surface area of the known length of rope substratum.

2.2 Outdoor culturing conditions

Field scale biofilms were cultured outdoors (August 10th–October 17th 2012, Logan, UT, USA) with a pilot scale RABR (field-RABR) unit constructed in accordance with previously described methods (Christenson and Sims, 2012). Briefly, biofilms were
grown on cotton cord (identical to lab-RABR experiments) coiled onto aluminum wheels (193 cm in diameter), which rotated (1.25 RPM) partially submerged in approximately 14,000 L tanks (approximately 10,700 L liquid volume). An important difference from the lab-RABR was that the cord-substratum of the field-RABR was exposed to light and nutrients from top and bottom (discussed further below). The field-RABR was placed in a continuous flow channel of wastewater (18.9 °C and pH 7.4) fed at approximately 1.25 L min⁻¹, which was drawn from the final pond of the outdoor wastewater lagoon facility (Logan, UT, USA).

2.3 Oxygen microsensor analysis

Microsensor measurements were performed using Clark-type oxygen micro-electrodes with outside tip diameters of 25 µm, response time < 5 s and < 5% stirring sensitivity (Unisense, A/S) (Revsbech, 1989). Amplification and sensor positioning were controlled with a microsensor multi-meter coupled with an ADC216 USB converter and a motor controlled micromanipulator. Data collection was aided by software packages, SensorTrace Prover. 3.0.1 and Sloper ver. 3.0.3 (Unisense, A/S). Two point calibrations were performed in air-saturated diH₂O ([O₂] ≈ 260 µM) and in a 1 M NaOH, 0.1 M ascorbic acid solution (anoxic standard). Calibrations were repeatedly checked in the anoxic standard and in air-saturated diH₂O throughout the experiments. Microsensor measurements were performed between 21-25 °C under both dark and light conditions (PAR = 700 µmol photons·m⁻²·sec⁻¹). Spatial O₂ measurements were performed in one dimension (depth-wise) from the biofilm-air interface down towards the cotton cord substratum in 25-100 µm steps. The effective diffusion coefficient (Dₑ) for O₂ in the algal
biofilms was estimated to be $1.2 \cdot 10^{-5}$ cm$^2$·sec$^{-1}$, by assuming it to be 50% of the aqueous value corresponding to fresh water at 25 ºC (Stewart, 1998).

The oxygen micro-profile and light:dark shift techniques used here have been previously described in detail (Bernstein et al., 2013; Glud et al., 1992; Kuhl et al., 1996; Lassen et al., 1998). Briefly, Fick’s law was used to calculate the total oxygen flux exported from the surface of the biofilm (net areal rate of biofilm photosynthesis or Pn) and from the photic zone inside the biofilm (net areal rate of photosynthesis of the photic zone or Pn,phot). Additionally, the light:dark shift measurements were used to estimate gross photosynthesis profiles and areal rates (Pg) which represent the total amount of oxygenic photosynthesis under the assumptions that:

(i) There is an initial steady-state O$_2$ distribution prior to darkening,
(ii) The O$_2$ consumption rate is identical between the light and dark time periods,
(iii) The O$_2$ diffusion coefficient remains constant during the measurement time at each position

Detailed calculations for oxygen transport, photosynthesis, photosynthesis-coupled respiration and dark-respiration processes are included in the supplementary data (Appendix B).

2.4 Lipid analysis

At the time of oxygen microsensor analysis, bulk biomass was harvested from the RABRs and washed four times by centrifugation (5000x g) and re-suspended in diH$_2$O to remove medium salts. After washing, the biomass was centrifuged and the pelleted biomass was frozen for lyophilization and lipid analysis. Analysis of free fatty acids, mono-, di-, and tri-acyl glycerols (FFA, MAG, DAG, and TAG, respectively) was
performed in accordance to the bead beating extraction method coupled with gas chromatography–flame ionization detection (GC FID) reported by Lohman et al. (2013). Additionally, biofuel potential, defined as total fatty acid methyl esters (FAME) produced directly from the biomass (Eustance et al., 2013; Gardner et al., 2013), along with FAME profiles were determined by a previously described method of direct in situ biomass transesterification using gas chromatography–mass spectroscopy (GC–MS) (Lohman et al., 2013).

2.5 Microscopy and Imaging

Biofilm structure and lipid accumulation were examined using confocal microscopy. Three components of these biofilms were observed: (i) chlorophyll autofluorescence, (ii) neutral lipids via Bodipy 505/515 staining, and (iii) biofilm substratum via Calcofluor white M2R staining (cellulose containing cotton-cord strands). Each component/stain was observed with distinct excitation/emission spectra. Images were acquired using a Leica TCS SP5 with Leica Advanced Suite-Advanced (LAS-AF) software (version 2.5.1.6757). Clean cross-sections of intact RABR biofilms samples were embedded in O.C.T. Compound (Tissue-Tek) on dry ice and cut using a clean razor blade. To stain, each cut sample was immersed in 5 mL of filter sterilized diH$_2$O, and stained with Bodipy 505/515 (5 mM) (final concentration of 10 µM) for approximately 20 minutes. Bodipy and chlorophyll were excited using 488 nm and 633 nm lasers, and fluorescence signal was captured with emission ranges 499-547 and 647-761 nm, respectively. To observe the biofilm substratum (cellulose containing cotton-cord strands), samples were stained for an additional 20 minutes with 10 µL Calcofluor white M2R (Invitrogen) per mL of diH$_2$O. Calcofluor white M2R was excited by a 405 laser
and captured with an emission range of 419-474 nm. Samples were rinsed once with filter-sterilized diH₂O prior to imaging. To minimize movement, each sample was partially embedded in 2% Agarose (Fisher). Composite images were obtained with combined z-stacks up to the maximum depth at which autofluorescence by chlorophyll was detected (~200 µm) using a 63x water immersion objective. Planar images were captured every 0.6 µm. Image planes (z-stacks) were compiled into Maximum Intensity Projection (MIP) images using Imaris x64 (version 7.5.2, Bitplane Scientific Software).

3.0 Results and Discussion

3.1 Biofilm Cultivation

Biofilms were cultured on cotton cord substratum during field and laboratory-scale RABR experiments (Figure A1). Samples from the lab-RABR were analyzed based on nitrate replete or deplete conditions. Samples from the field-RABR were separated according to growth orientation on the substratum. The field-RABR ‘top’ and ‘bottom’ samples correspond to biofilms formed on the outer and inner section of the rotating wheel, respectively. The field-RABR top biofilms were cultured in an orientation directly exposed to ambient sunlight (average daily maximum PAR = 1715 µmol photons·m⁻²·sec⁻¹) compared to the more shaded bottom biofilms (average daily maximum PAR = 231 µmol photons·m⁻²·sec⁻¹). Hence, there were four chosen biofilm sample-types analyzed and compared in this study: (i) lab-RABR biofilm that is nitrate replete, (ii) lab-RABR biofilm that is nitrate deplete (60 h deplete culturing), (iii) field-RABR biofilm cultured on the top (outer wheel biofilm), and (iv) field RABR biofilm cultured on the bottom (inner wheel biofilm). It is important to emphasize that the laboratory and field-RABR systems are not identical, represent two different process objectives and are
intended to be compared independently of each other. However, a future goal for the RABR technology is to better integrate the wastewater remediating and biofuel producing processes; hence a minimal number of comparisons based on general biofilm physiology are made between the two systems.

The maximum specific growth rates, measured during exponential phase, were 0.09 and 0.17 day\(^{-1}\) for the laboratory and field cultured biofilms, respectively. The maximum measured biomass areal density (observed during stationary phase) were 36.90 and 65.98 g\textsubscript{CDW} m\(^{-2}\) for the lab- and field-RABRs, respectively. The final biomass areal density decreased by 0.01 g\textsubscript{CDW} cm\(^{-2}\) 60 h post nitrate depletion in the lab-RABR biofilms, potentially indicating minor biomass sloughing or degradation. The measured biofilm thickness (distance from substratum to biofilm surface at late stationary phase) was approximately 1 mm for each lab-RABR biofilm (nitrate replete or deplete) and approximately 2 mm for each field-RABR biofilm (top and bottom).

Figure A-1: Representative photographs for the field-RABR (A) and lab-RABR (B) culturing systems designed for algal biofilm culturing (insert shows cross-sectioned excised cotton cord substratum with biofilm growth). Note the ‘top’ and ‘bottom’ biofilm orientation corresponding to the inner and outer sections of the field-RABR, respectively.
3.2 Field-RABR for wastewater remediation

3.2.1 Biofilm heterogeneity

Direct, spatially resolved measurements of steady-state oxygen profiles revealed differences between the biofilms formed on the top and bottom of the field-RABR wastewater remediating system. The illuminated portions of both biofilms near the surfaces became supersaturated with O₂, reaching concentrations over 600 µM, which was approximately 3X, the measured O₂ concentration of the bulk wastewater (Figure A2. A and B). Both biofilms were oxic to depths of approximately 1800 µm beneath the biofilm surface while illuminated (Table A-1). Steady-state oxygen profiles were also obtained after 15 min of dark conditioning (Figure A-2. C and B) and the corresponding oxic-zone depths were 700 and 450 µm in the top and bottom biofilms, respectively. This is evidence for higher oxygen consumption potential in the less-illuminated (bottom) biofilm on the inside of the spools (discussed in more detail below).

Oxygen gradients measured in the steady-state microprofiles show that these wastewater remediating biofilms maintain spatially varied microenvironments which may promote niche environments capable of supporting different microbial physiologies. A significant portion of both biofilms (top and bottom) remained anoxic during constant illumination at 700 µmol photons m⁻² s⁻¹ (approximately 10%) and in the dark (approximately 50%). However, it is possible that these biofilms become fully oxic at or near peak solar irradiance during field cultivation. In the field, these systems are also subject to temporal gradients in solar irradiance, temperature and nutrient flux. It is important to note that the measurements reported in this study are specific for
standardized and constant incident irradiance and only represent comparative physiological potentials for these biofilms.

The field-RABR was inoculated with the native wastewater microbial community and was composed of a complex mixture of environmental biofilm-forming microorganisms including phototrophs and heterotrophs. Initial 454 pyrosequence analyses indicated a high level of diversity in the field-RABR biofilms, where cyanobacteria (predominately *Oscillatoria* sp. and *Leptolyngbya* sp.) and bacterial heterotrophs accounted for significant fractions of the microbial population. However, further molecular work is required to elucidate the microbial community differences between the two biofilms with respect to their orientation of growth. It is important to re-emphasize that the ‘top’ and ‘bottom’ biofilms were formed simultaneously on different sides of the same cotton cord substratum and analyzed with microsensors ex situ under identical conditions. Other than growth orientation, these biofilms were cultured identically and were only spatially separated by the diameter of the cotton cord substratum (0.64 cm).
Figure A-2: Field-RABR: Dissolved oxygen microprofiles measured in the light extending from the surface of the biofilms (x = 0) on the (A) outer wheel surface (‘top’) and (B) inner wheel surface (‘bottom’); dissolved oxygen profiles measured in the dark for biofilms grown on the (C) outer wheel surface (‘top’) and (D) inner wheel surface (‘bottom’); and photosynthesis profiles extending from the surface for biofilms grown on the (E) outer wheel surface (‘top’) and (F) inner wheel surface (‘bottom’). Note that the biofilm surface position (depth = 0 µm) is approximated by the position at which oxygen
responses were measurable (subject to ±25 µm error or ±100 µm error for the photosynthesis profiles where each data point is a representative gross volumetric photosynthesis rate from 2-3 replicates) and individual data points represent the mean values from 3-4 replicate measurements in both light and dark conditions. Error bars represent plus or minus one standard deviation. Dotted lines indicate the photic-zone termination depth, estimated from the light: dark method. Note the scale change on the x-axis.

Table A-1: Measurements of areal photosynthesis rates, areal respiration rates and relevant depth scales for the laboratory- and field-RABR cultured biofilms

<table>
<thead>
<tr>
<th>Areal rates (µmol O₂·cm⁻²·sec⁻¹)</th>
<th>Field RABR Top Biofilm</th>
<th>Field RABR Bottom Biofilm</th>
<th>Laboratory RABR Nitrate Replete</th>
<th>Laboratory RABR Nitrate Deplete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthesis, P₉</td>
<td>a11.84·10⁻⁴</td>
<td>a5.23·10⁻⁴</td>
<td>a7.51·10⁻⁴</td>
<td>a5.70·10⁻⁴</td>
</tr>
<tr>
<td>Net areal rate of biofilm photosynthesis, P₉ (%P₉)</td>
<td>3.01·10⁻⁴ (25.4%)</td>
<td>3.55·10⁻⁴ (67.9%)</td>
<td>2.31·10⁻⁴ (30.8%)</td>
<td>2.41·10⁻⁴ (42.3%)</td>
</tr>
<tr>
<td>Net areal rate of photic zone photosynthesis, P₉,phot (%P₉)</td>
<td>3.64·10⁻⁴ (30.7%)</td>
<td>3.96·10⁻⁴ (75.7%)</td>
<td>3.10·10⁻⁴ (41.3%)</td>
<td>2.91·10⁻⁴ (51.1%)</td>
</tr>
<tr>
<td>Areal respiration of the biofilm, R₉,light (%P₉)</td>
<td>8.83·10⁻⁴ (74.6%)</td>
<td>1.68·10⁻⁴ (32.1%)</td>
<td>5.20·10⁻⁴ (69.2%)</td>
<td>3.29·10⁻⁴ (57.7%)</td>
</tr>
<tr>
<td>Areal respiration of the photic zone, R₉,phot (%P₉)</td>
<td>8.20·10⁻⁴ (69.3%)</td>
<td>1.27·10⁻⁴ (24.3%)</td>
<td>4.41·10⁻⁴ (58.7%)</td>
<td>2.79·10⁻⁴ (48.9%)</td>
</tr>
<tr>
<td>Respiration in the dark, R₉,dark</td>
<td>0.54·10⁻⁴</td>
<td>1.11·10⁻⁴</td>
<td>0.65·10⁻⁴</td>
<td>0.74·10⁻⁴</td>
</tr>
<tr>
<td>Depth of photic zone, L₉,phot (µm)</td>
<td>b1100 ± 200</td>
<td>b900 ± 200</td>
<td>b675 ± 25</td>
<td>b650 ± 25</td>
</tr>
<tr>
<td>Depth of oxic zone in light (µm)</td>
<td>b1750 ± 25</td>
<td>b1800 ± 25</td>
<td>&gt; 2675</td>
<td>&gt; 2675</td>
</tr>
<tr>
<td>Depth of oxic zone in dark (µm)</td>
<td>b700 ± 25</td>
<td>b450 ± 25</td>
<td>b850 ± 25</td>
<td>b1150 ± 25</td>
</tr>
</tbody>
</table>

a Mean of 2-3 independent measurements plus or minus a range of 25% from the mean, b Plus or minus measurement step-size, n = 2-3
3.2.2 Oxygenic photosynthesis

Direct measurements of oxygenic photosynthesis rates quantified fundamental physiological differences in the field-RABR biofilms based only on orientation of biofilm formation (Figure A-2E and F). The measured areal rate of gross photosynthesis ($P_g$) in the top biofilm was approximately 2X greater than the bottom, signifying a much higher potential for photo-productivity (proportional to $P_g$) (Table A-1). This result was attributed to the availability of solar irradiance (PAR) during biofilm growth/formation, which differed between 1715 and 231 $\mu$mol photons m$^{-2}$ s$^{-1}$ for the top and bottom biofilms, respectively.

The active zone of photosynthesis is defined here as the position in the biofilm where the volumetric gross photosynthesis rate [$P_{g(z)}$] is greater than zero and its depth is assumed to be equal to the biofilm photic zone ($L_{phot}$). The $L_{phot}$ value was only slightly higher in the top biofilm (Table A-1) indicating that the penetration depths of actinic light are comparable when illuminated at the same incident irradiance. The minor differences observed in $L_{phot}$ values may translate into minor differences in biofilm material properties which may influence the oxygen effective diffusion coefficient, as $L_{phot}$ is resolved based on photogenic oxygen changes; however, these variances are expected to be very small based on previously reported measurements (Stewart, 1998) and were not considered here in detail. A key observation for this system is that the top oriented biofilms are capable of producing oxygen at greater than twice the rate per photon attenuated than the neighboring bottom biofilm while the respective zones of active photosynthesis are nearly identical under standardized incident irradiance. This observation qualitatively indicates that the areal quantum yields are greater for the
biofilms formed under a higher incident solar irradiance. Rigorous quantification of spatially defined quantum yields and photosynthetic efficiencies are beyond the scope of this study although the present results are consistent with established photo-physiological observations (Falkowski and Raven, 1997).

Net areal photosynthesis rates were equated to the diffusive flux of oxygen transported from the biofilm surface (P_n) or the photic zone (P_n,phot), and both measurements were greater in the bottom formed biofilms compared to the top oriented samples (Table A1). This difference is more pronounced and meaningful when interpreted as a percentage of P_g, which is a proxy for the total photosynthetically, derived oxygen. Net photosynthesis rates for the entire biofilm (P_n) represent 67.9% of P_g in the bottom biofilm as compared to only 25.4% in the top. These percentage differences are even greater when evaluated for P_n,phot, which includes consideration of oxygen transported to the anoxic portions in the biofilm. These results confirm that net oxygen production rates alone are not representative of the oxygenic photosynthesis potential for these samples and that the bottom biofilms have the capacity to provide a greater flux of oxygen to bulk wastewater environment.

The P_n values measured for this study are only representative of steady-state reaction and diffusion processes. However, the rotating mechanism employed by the RABR alternates the biofilms between different light and fluid regimes in a periodic fashion corresponding to the submerged-liquid and ambient air surroundings. Diffusive oxygen flux was measured inside the biofilms and the steady-state oxygen profiles obtained on biofilms exposed to ambient air did not provide enough resolution to identify or determine the thicknesses of the diffusive boundary layer (DBL) at the surface of the
biofilms. However, DBLs almost certainly were present and are not ruled out as important regulating factors in the oxygen transport processes, especially while being exposed to the liquid medium during rotation. It has been previously established that DBL thickness is a function of the velocity differential between the biofilm and bulk fluid (Jorgensen and Marais, 1990; Kuhl et al., 1996). This is an important consideration for RABR operation since the rotational speed can be optimized to reduce the effects of mass transfer limitations external to the biofilm. This highlights a future area of characterization for the field-RABR biofilms that has the potential to enhance photosynthetic biofilm productivity by minimizing mass transfer limitations.

3.2.3 Areal respiration rates

The difference between gross and net areal photosynthesis rates provided direct measurements of photosynthesis-coupled respiration and revealed physiological distinctions between the two field-RABR biofilms. Areal photosynthesis coupled respiration rates were measured during illumination for the entire biofilm ($R_{\text{light}}$) and within just the photic zone ($R_{\text{phos}}$). Both measurements were more than 5X higher in the top biofilms compared to the bottom (Table A-1). Respiration rates accounted for greater percentages of $P_g$ than the corresponding $P_n$ values in the top biofilms. The opposite was true for the bottom biofilms. In contrast to the photosynthesis-coupled respiration rates, areal respiration rates in the dark ($R_{\text{dark}}$) were approximately 2X greater for the bottom biofilms compared to the top biofilms (Table A-1). Respiration rates corresponded directly to higher localized oxygen concentrations. This observation indicates that respiration in these biofilm consortia increases with oxygen concentration and production rate, which are both functions of actinic light availability. This provides evidence of
photorespiration processes acting in concert with heterotrophic oxygen consumption. The bottom biofilms appear to have a higher capacity for light-independent heterotrophic respiration compared to the top biofilms, which is evidenced by the higher $R_{\text{dark}}$ values.

Photosynthesis-coupled respiration is defined here to include any respiration occurring in the active zone of photosynthesis and can be advantageous to overall photosynthesis by lowering the localized $O_2/CO_2$ ratio inside the biofilm and resulting in higher selectivity for $CO_2$ fixation at the RuBisCO complex (Falkowski and Raven, 1997; Glud et al., 1992; Kliphuis et al., 2011). Oxygen removal via heterotrophic or non-oxygenic community member activity is hypothesized to be a beneficial attribute to these wastewater remediating biofilm ecosystems. Hence, the encouragement and control of localized respiration processes, independent of photorespiration, is identified here as a potentially important design feature for RABR operation and other photosynthetic biofilm reactor technologies and should be considered for future optimization of photosynthesis.

The top oriented field-RABR biofilm samples showed the highest rates of gross-oxygenic photosynthesis and respiration (both $R_{\text{light}}$ and $R_{\text{phot}}$). These two processes are tightly coupled inside biofilms and cannot be considered independent from each other. In fact, it has been shown previously that photosynthesis and respiration increase concurrently with increasing irradiance in tightly controlled laboratory cultured algal biofilms (Jensen and Revsbech, 1989). The differences in the photosynthesis and respiration capacities for these biofilms might be the result of the differences in exposure to solar irradiance during the culturing process. The top oriented biofilms were formed with a 7.4-fold higher incident irradiance (PAR) compared to the bottom of the cotton
cord substratum. This is a practical result since it is well established that different growth environments with respect to solar irradiance availability have been shown to promote different expression levels of components comprising the light harvesting complexes, non photosynthetic accessory pigments (e.g., carotenoids) and respiration components (e.g., terminal oxidases) in photosynthetic systems (Falkowski and Raven, 1997).

3.3 Nitrogen depletion in lab-RABR samples

3.3.1 Biofilm heterogeneity

The lab-RABR biofilms, formed from the known lipid accumulating strain WC-2B, established oxygen gradients under both illuminated and dark conditions. The microprofiles revealed only subtle differences between biofilms subjected to nitrate replete and deplete conditions. Similar to the field-RABR biofilms, the illuminated surface associated positions from both replete and deplete biofilm samples became supersaturated with O$_2$, reaching approximately 3X the measured O$_2$ concentration of the medium (Figure A-3A and B). During illumination, the oxic zone extended to depths greater than 2675 µm below the biofilm surface (approximately 1675 µm into the substratum) where the flux of oxygen became very low. The WC-2B biofilms showed oxygen transport, driven by consumption, in portions of the substratum indicating that some biofilm was formed within the cotton cord pore volume. This was also observed by confocal scanning laser microscopy (Figure A-4). These lab-RABR biofilms showed a higher degree of spatial heterogeneity with respect to replicate oxygen profiles compared to the field-RABR biofilms (evident by the larger standard deviations in Figure A-3 as compared to Figure A-2). This increased variance between measurements taken below Lphot positions could result from biofilm spatial heterogeneity specific for cells attached
within the cotton material. Steady-state oxygen profiles were also obtained after 15 min of dark conditioning (Figure A-3C and D). The oxic zones in the absence of light ranged from 850 to 1150 µm for the nitrate replete and deplete biofilms, respectively; indicating that the nitrogen starved biofilms had a lower potential for heterotrophic oxygen consumption (discussed in more detail below).

3.3.2 Oxygenic photosynthesis and respiration

Direct measurements of oxygenic photosynthesis and respiration rates indicated physiological differences in the RABR grown WC-2B biofilms cultured under nitrate replete and deplete conditions (Figure A-3E and F). Again, photosynthesis rates were measured as both net and gross production of photo-chemically derived oxygen at the biofilm scale. The WC-2B biofilms exhibited higher $P_g$ values (approximately 30%) during nitrate replete conditions indicating a greater potential for photo-productivity when not starved for nitrogen (Table A-1). The active zones of photosynthesis, evaluated as the portion of the biofilm between the surface and $L_{phot}$, were practically indistinguishable (within 25 µm) between the two nitrate availability conditions. This measurement supports the observation that actinic light was fully attenuated by the same depth and that the oxygenic photosynthesis reaction volumes were near identical under both conditions.

Differences in the net areal rates of photosynthesis (both $P_n$ and $P_{n,phot}$) between the two nitrate availability conditions were not as pronounced. However, both $P_n$ and $P_{n,phot}$ represented a greater percentage of $P_g$ under nitrate deplete conditions. This observation is attributed to lower areal rates of photosynthetically-coupled respiration during nitrate starvation. Again, the $R_{light}$ and $R_{phot}$ values were measured as the
difference between $P_g$ and respective net areal photosynthesis rates. Nitrate replete conditions promoted approximately 20% increase in photosynthesis coupled respiration rates. The $R_{dark}$ measurements were greater during nitrate starvation indicating a higher capacity for heterotrophic (or light independent) respiration. However, the maximum areal respiration rates were observed during illumination and corresponded with increased $P_g$. This was consistent with the observations made on the field-RABR biofilms.

Although as a whole, there were only small differences observed in rates of photosynthesis and respiration between the nitrogen replete and deplete lab-RABRs, the data suggest two important findings: First, nitrate depletion in the medium might not have a strong effect on the general physiology of the biofilm because only a small fraction of the biofilm (outer surface) is actively performing photochemical production even under nitrogen replete conditions; secondly, the biofilms remained photosynthetically active under non-growth conditions highlighting the importance of maintenance energy for cell viability and the potential for nitrogen (re-) cycling. These are important observations, within the setting of algal lipid production, since nitrogen stress is a common strategy for triggering triacylglycerol accumulation in planktonic microalgal cultures (Converti et al., 2009; Mus et al., 2013; Stephenson et al., 2010).

The first specific aim of this study was to characterize and compare the two different RABR biofilms (lab- and field-scale) in the context of active photosynthesis and spatial gradients in steady state oxygen and photosynthesis. Of the physiological parameters measured for this specific aim, photosynthesis-coupled respiration is of special interest and should be considered a potent design parameter for controlling local
O₂/CO₂ ratios to promote carbon fixation and subsequent photo-productivity. One potential strategy for maximizing gross photosynthesis while minimizing localized oxygen concentration would be to promote heterotrophic activity via mixed culturing techniques. Evidence for this lies in the observation that the field-RABR top-oriented biofilm community, as compared to the WC-2B lab-RABR biofilms, displayed a higher potential for electron acquisition from the environment (proportional to Pₑ) while channeling much greater percentages of photosynthetically derived oxygen into respiration processes. A combination of the wastewater remediating and biofuel production processes, may be better achieved via mixed species inoculation or ‘seeding’ with known lipid accumulating photoautotrophic community members combined with compatible heterotrophic oxygen scavengers. Consortial cooperation in microbial biofilm technology has previously been demonstrated in a number of different cell factory systems (Bernstein and Carlson, 2012).
Figure A-3: Lab-RABR: dissolved oxygen profiles measured in the light extending from the surface for biofilms grown in (A) nitrate replete and (B) nitrate deplete conditions; dissolved oxygen profiles measured in the dark for biofilms grown in (C) nitrate replete and (D) nitrate deplete conditions; and photosynthesis profiles extending from the surface for biofilms grown in (E) nitrate replete and (F) nitrate deplete. Note that the biofilm surface position (depth = 0 µm) is approximated by the position at which oxygen responses were measurable (subject to ±25 µm error or ±100 µm error for the photosynthesis profiles where each data point is a representative gross volumetric
photosynthesis rate from 2 to 3 replicates) and individual data points represent the mean values from 3 to 4 replicate profiles in both light and dark conditions. Error bars represent plus or minus one standard deviation. Dotted lines indicate the photic-zone termination depth, estimated from the light:dark shift method. Note the scale change on the x-axis

Figure A-4: Cross-sectional image of a lab-RABR biofilm with nitrate depleted WC-2B cell material attached to the cotton cord substratum. The nitrate depletion time for this sample was greater than the 60 hours imposed for the quantitative GC-MS and GC-FID analyses. This sample was stained with Bodipy 505/515, Calcofluor white and DAPI where: red (chlorophyll autofluorescence), green-intracellular (neutral lipids), blue-green (cotton cord fibers). DAPI (20 µg/mL) was found to enhance samples stained with Calcofluor white without staining intracellular nucleic acid. The scale bar represents 40 µm
3.4 Biofuel precursor production

Extractable lipid fractions were recovered from all biofilm samples and analyzed by gas chromatography for assessment of biofuels properties (Table A-2). In addition, direct transesterification was performed on the lyophilized biomass to identify fatty acids and to determine total biofuel potential (extractable and non-extractable) for each biofilm-type (Table A-3). Modest increases of extractable precursor concentrations were measured in the nitrate deplete biofilms, as compared to the nitrate replete conditions. This observation was also qualitatively confirmed in microscopy images (Figure A-5A and B) where Bodipy 505/515 was used to visualize the neutral lipid precursors. The total FAME-weight%, representative of the total biofuel potential of the biofilm, was also modestly higher for the lab-RABR biofilms that were deplete of nitrate.

Figure A-5: Confocal Microscopy images of intact RABR biofilms. Red (chlorophyll autofluorescence), green (neutral lipids), and light blue (cotton-cord strands -evident in panel C only). The top panels indicate lipid accumulation in the lab-RABR (A) before
and (B) after nitrate stress exhibiting higher neutral lipid content. Lower panels represent field-RABR biofilm samples (C) ‘top’ and (D) ‘bottom’. Scale bars represent 30 µm.

The most notable differences regarding lipid production in the lab-RABR biofilms were the differences in the total extractable weight% of lipids (sum of the FFA, MAG, DAG, and TAGs) between the nitrate replete and deplete conditions, 4.3 ± 0.4% and 7.3 ± 0.7% (w/w), respectively (Table A-2). The largest differences were observed in the DAG and TAG weight% and the respective areal concentrations. Although the WC-2B biofilms exhibited reasonable biofuel potentials; the lab-RABR production-system is not considered optimized for biofuel production. Only 7.3% (w/w) of extractable precursors accumulated, which is significantly less than planktonic cultures of WC-2B that can accumulate up to 13.9% (w/w) of extractable precursors (7.7% (w/w) of which is TAG) under high pH and nitrate deplete conditions (Gardner, unpublished data). This evinces that medium nitrate depletion alone may not be an effective condition for inducing TAG accumulation in microalgal biofilms, likely due to heterogeneous distributions of nutrients like nitrate caused by mass transfer limitations as well as differences in pH stress and differences in inorganic carbon availability throughout the biofilms. It should be noted that comparisons of these preliminary biofilm oil-production systems to well mixed planktonic systems does not account for culturing times, biomass production rates or differences associated with required operating costs (e.g., energy required for mixing, biomass harvesting or water input requirements).

Biofilms cultured on the field-RABR had the lowest weight percentage in both extractable precursor molecules and potential FAMEs, 2.9 ± 1.1% and 5.1 ± 1.0% (w/w), respectively (Tables A2 and 3). This observation coincides with the relatively high
respiration rates measured in the samples (discussed earlier). The field-RABR biofilm samples are clearly not optimized for biodiesel production (i.e., total FAMEs) under the current culturing conditions. This could be, in part, due to colonization of a non-lipid accumulating microbial community native to the wastewater (Figure A-5C and D).

However, the field-RABR exhibited higher biomass productivity (P = Dgcdw/Dtime) and total biomass areal density compared to the lab-RABR. Hence, the amount of FAME recoverable from the field-RABR biofilms per unit area was similar to the amount recoverable from the nitrate deplete WC-2B biofilms from the lab-RABRs (Table A-3).

Table A-2: Mean extractable biofuel precursor weight % and areal concentrations for the laboratory- and field-RABR cultured biofilms (n=3 with one standard deviation error, or n=2 with range reported as error)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Free Fatty Acid</th>
<th>Monoaclglycerol</th>
<th>Diacylglycerol</th>
<th>Triacylglycerol</th>
<th>Total Extractable</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR-Replete</td>
<td>2.24 ± 0.39</td>
<td>1.17 ± 0.21</td>
<td>0.76 ± 0.35</td>
<td>0.11 ± 0.09</td>
<td>4.27 ± 0.37</td>
</tr>
<tr>
<td>LR-Deplete</td>
<td>2.91 ± 0.46</td>
<td>1.48 ± 0.21</td>
<td>1.98 ± 0.32</td>
<td>0.94 ± 0.31</td>
<td>7.32 ± 0.70</td>
</tr>
<tr>
<td>FR²</td>
<td>1.49</td>
<td>0.50</td>
<td>0.91</td>
<td>0.48</td>
<td>0.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Free Fatty Acid</th>
<th>Monoaclglycerol</th>
<th>Diacylglycerol</th>
<th>Triacylglycerol</th>
<th>Total Extractable</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR-Replete</td>
<td>0.78 ± 0.19</td>
<td>0.41 ± 0.10</td>
<td>0.26 ± 0.10</td>
<td>0.04 ± 0.03</td>
<td>1.48 ± 0.24</td>
</tr>
<tr>
<td>LR-Deplete</td>
<td>0.97 ± 0.22</td>
<td>0.50 ± 0.10</td>
<td>0.66 ± 0.14</td>
<td>0.31 ± 0.08</td>
<td>2.44 ± 0.38</td>
</tr>
<tr>
<td>FR²</td>
<td>0.98</td>
<td>0.33</td>
<td>0.60</td>
<td>0.31</td>
<td>0.22</td>
</tr>
</tbody>
</table>

LR: laboratory scale RABR, FR: Field scale RABR, a Mean and range (|) for n=2
Table A-3: Mean FAME %, weight %, and areal concentration from the laboratory- and field-RABR cultured biofilms. Biomass was directly transesterified to determine total biofuel potential from all fatty acid precursor molecules (extractable and non-extractable) (n=3 with one standard deviation error, or n=2 with range reported as error)

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>LR-Replete</th>
<th>LR-Deplete</th>
<th>FR</th>
<th>LR-Replete</th>
<th>LR-Deplete</th>
<th>FR</th>
<th>LR-Replete</th>
<th>LR-Deplete</th>
<th>FR</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>N/D</td>
<td>0.24 ± 0.06</td>
<td>0.54</td>
<td>0.04</td>
<td>N/D</td>
<td>0.03</td>
<td>0.01</td>
<td>N/D</td>
<td>0.01</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.39 ± 0.01</td>
<td>0.39 ± 0.06</td>
<td>1.65</td>
<td>0.69</td>
<td>0.03</td>
<td>0.003</td>
<td>0.04</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.40 ± 0.02</td>
<td>0.43 ± 0.09</td>
<td>0.60</td>
<td>0.04</td>
<td>0.03</td>
<td>0.003</td>
<td>0.05</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>C16:1-3</td>
<td>19.85 ± 1.34</td>
<td>19.50 ± 0.41</td>
<td>22.97</td>
<td>2.64</td>
<td>1.67</td>
<td>0.02</td>
<td>2.60</td>
<td>0.03</td>
<td>1.17</td>
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<tr>
<td>C16:0</td>
<td>16.68 ± 1.61</td>
<td>18.68 ± 1.14</td>
<td>15.58</td>
<td>0.93</td>
<td>1.41</td>
<td>0.23</td>
<td>1.96</td>
<td>0.06</td>
<td>0.79</td>
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<tr>
<td>C18:1-3</td>
<td>50.15 ± 2.60</td>
<td>50.42 ± 1.76</td>
<td>42.28</td>
<td>2.02</td>
<td>4.24</td>
<td>0.47</td>
<td>5.30</td>
<td>0.34</td>
<td>2.15</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.88 ± 0.08</td>
<td>0.95 ± 0.07</td>
<td>2.90</td>
<td>3.21</td>
<td>0.07</td>
<td>0.01</td>
<td>0.10</td>
<td>0.01</td>
<td>0.14</td>
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<tr>
<td>C20:4-5</td>
<td>3.35 ± 1.12</td>
<td>2.51 ± 0.65</td>
<td>0.28</td>
<td>0.10</td>
<td>0.26</td>
<td>0.06</td>
<td>0.29</td>
<td>0.10</td>
<td>0.10</td>
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<tr>
<td>C20:1-3</td>
<td>0.71 ± 0.13</td>
<td>0.64 ± 0.19</td>
<td>N/D</td>
<td>N/D</td>
<td>0.06</td>
<td>0.01</td>
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<td>N/D</td>
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<tr>
<td>C20:0</td>
<td>N/D</td>
<td>N/D</td>
<td>0.18</td>
<td>0.37</td>
<td>N/D</td>
<td>N/D</td>
<td>0.01</td>
<td>0.02</td>
<td>N/D</td>
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<tr>
<td>C22:0</td>
<td>0.48 ± 0.08</td>
<td>0.51 ± 0.13</td>
<td>0.63</td>
<td>0.07</td>
<td>0.04</td>
<td>0.01</td>
<td>0.05</td>
<td>0.01</td>
<td>0.03</td>
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<tr>
<td>C24:0</td>
<td>0.28 ± 0.04</td>
<td>0.29 ± 0.01</td>
<td>0.63</td>
<td>0.03</td>
<td>0.02</td>
<td>0.002</td>
<td>0.03</td>
<td>0.000</td>
<td>0.03</td>
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<tr>
<td>C26:0</td>
<td>0.37 ± 0.03</td>
<td>0.42 ± 0.04</td>
<td>0.56</td>
<td>0.07</td>
<td>0.03</td>
<td>0.003</td>
<td>0.04</td>
<td>0.004</td>
<td>0.03</td>
</tr>
<tr>
<td>C28:0</td>
<td>N/D</td>
<td>0.35 ± 0.31</td>
<td>0.90</td>
<td>0.41</td>
<td>N/D</td>
<td>N/D</td>
<td>0.04</td>
<td>0.03</td>
<td>N/D</td>
</tr>
<tr>
<td>Other</td>
<td>6.46 ± 2.45</td>
<td>4.68 ± 1.30</td>
<td>4.90</td>
<td>0.05</td>
<td>0.54</td>
<td>0.18</td>
<td>0.49</td>
<td>0.14</td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>8.45 ± 0.61</td>
<td>10.51 ± 0.33</td>
<td>5.07</td>
<td>0.99</td>
<td>2.92</td>
<td>0.42</td>
</tr>
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</table>

LR: laboratory scale RABR, FR: Field scale RABR, a Mean and range () for n=2, N/D: not detected

The second specific aim of this study was to characterize and compare the biofuels potential and (neutral lipid) precursor biomolecule composition in these biofilms. Although the current RABR systems are not considered optimized, lipid accumulation in algal biofilms is possible and reasonable if the microbial composition is constrained to known lipid producers such as the WC-2B isolate used here in the lab-RABR system. Future optimization is needed including the investigation of other industrially relevant algal strains such as Botryococcus braunii or Chlorella vulgaris. The field-RABR design is a more practical and industrially scalable system compared to the lab-RABR. However, the current system is not considered viable for biodiesel production.
since it only accumulated $2.9 \pm 1.1\%$ and $5.1 \pm 1.0\%$ (w/w) precursor molecules and FAMEs, respectively. Future optimization and experimentation of the field-scale system will require methodologies for enhanced control of the microbial community composition to select for better lipid accumulation. It should be noted that although biodiesel production from fatty acid containing precursor compounds is low in the field system, it is a potentially viable technique for biomass production from wastewater. Biofuel production from the field- RABR has previously been reported through the conversion of algal biomass into acetone, butanol, and ethanol by Clostridium saccharoperbutylacetonicum (Ellis et al., 2012).

As part of the objective from specific aim 2, nitrate starvation was investigated as a potential strategy for inducing lipid accumulation in the lab-scale RABR cultures. Although a modest increase in extractable precursors was observed, nitrogen stress as implemented here with 60 h cultivation in the absence of nitrogen was not identified to be as effective in “triggering” lipid accumulation in biofilms as previously reported for suspended cultures (Gardner et al., 2011; Mus et al., 2013; Gardner, unpublished data). This biofilm specific result is consistent with another recently reported study which focused on nutrient starvation (including nitrate) in cultures composed of the fresh water green alga Scenedesmus obliquus and the marine diatom Nitzschia palea (Schnurr et al., 2013). That study tested biofilm growth and lipid accumulation in algae cultured under relatively low shear in flat plate biofilm reactors and reported no significant changes in lipid concentration (% dry weight) between nitrate replete and deplete conditions. This is in minor contrast to the results from the current study, which observed an approximate 2–3% w/w increase after nitrate depletion. Additionally, the Schnurr et al. (2013) study
reported significant and near complete biomass sloughing post nitrate depletion which was not observed to the same extent in the lab-RABR biofilms during the 60 h of nitrate starvation. This could be due to the different substratum materials (i.e., glass-plate compared to cotton cord) or localized shear-stress at the biofilm surfaces. The combined results between the current and previously reported study (Schnurr et al., 2013) indicate that inducing lipid accumulation via nutrient starvation in biofilms may be possible but future research is needed to identify strategies for inducing lipid accumulation in algal biofilms, such as nitrogen or pH stress, or chemical addition (Gardner et al., 2011, 2012; Guckert and Cooksey, 1990; Mus et al., 2013; Valenzuela et al., 2012).

**Conclusion**

This manuscript explores critical photosynthetic parameters related to the production of biofuel precursor molecules by algal biofilms cultured on rotating algal biofilm reactors (RABR). The lab-RABR systems exhibited moderate biofuel capabilities yet require process optimization. The wastewater remediating field- RABR systems exhibited higher rates of photosynthesis and respiration depending on the position with respect to sunlight, but are not currently a viable biodiesel production platform. This study developed a methodological foundation and establishes a benchmark for directly measuring photosynthetic parameters fundamental to understanding and ultimately controlling the physiology of algal biofilms and to designing efficient, photosynthetic platforms for biofilm-based product generation.
References


Supplementary data

For: Dissolved inorganic carbon enhanced growth, nutrient uptake and lipid accumulation in wastewater grown microalgal biofilms.

Microalgae growth analysis: calculations and supplementary methodology

Definition of terms

X: biomass (g)
t: time (d)
dx: increase in biomass (g)
dt: time interval (d)
µ: specific growth rate (d\(^{-1}\))

In an exponentially growing microalgae culture,

\[
\frac{dx}{dt} = \mu X \quad \text{………………Eq. S.1}
\]

Re-arranging equation (A.1)

\[
\frac{dx}{X} = \mu dt \quad \text{By integration from the initial biomass } X_o \text{ to } X
\]

\[
\ln X = \mu t + \ln X_o \quad \text{………………Eq. S.2}
\]

Comparing equation S.2 to the general equation of a straight-line \(y = mx + b\), \(\mu\) the specific growth rate is the slope of this line. For the microalgae utilized in this study, specific growth rates were obtained using equations from Fig. S2.

Supplementary Table captions

Table B-1. Nutrient concentrations in medium with bicarbonate and without bicarbonate amendment under abiotic conditions for a 5-day hydraulic retention time and N-deprived conditions
Supplementary Figure Legends

Figure B-1. Growth curve of algal biofilms with and without bicarbonate amendment curve fitted with zero and first order kinetic equations

Table B-1.

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<th></th>
<th>Abiotic conditions</th>
<th>N-deprived</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>( \text{NO}_3^- \text{N} ) mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>1.94</td>
<td>1.73</td>
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<tr>
<td>No bicarbonate</td>
<td>1.82</td>
<td>1.87</td>
</tr>
<tr>
<td>( \text{NO}_2^- \text{N} ) mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>No bicarbonate</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>( \text{PO}_4^{3-} \text{P} ) mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.21</td>
<td>0.14</td>
</tr>
<tr>
<td>No bicarbonate</td>
<td>0.20</td>
<td>0.21</td>
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</table>
For: Direct measurement and characterization of active photosynthesis zones inside wastewater remediating and biofuels producing microalgal biofilms.

**Oxygen Microsensor Analysis: Calculations Supplementary Methodology**

**Oxygen Flux and Net Areal Rates of Photosynthesis.** The one-dimensional diffusive flux of oxygen ($J$) inside the biofilm matrix was calculated with respect to the biofilm depth ($z$-axis) using Fick’s law (Eq. S1).

$$J = -D_e \frac{dC_{O_2}}{dz}$$  \hspace{1cm} \text{Eq. S1}

A previously described analysis can be used to equate the diffusive flux of oxygen from various biofilm specific positions to the net areal rates of oxygenic photosynthesis or respiration (Glud et al., 1992; Kuhl et al., 1996). For example, under illuminated conditions and active photosynthesis, the flux of oxygen being expelled from the surface of the biofilm was equated to the net areal rate of photosynthesis of the biofilm ($P_n$, Eq. S2). In this study, $P_n$ was calculated at the surface positions of the biofilm; however, it may also be calculated in the diffusive boundary layer (DBL) at the biofilm fluid interface if there is sufficient resolution of the DBL from the microprofile data. Note that the diffusive flux through the DBL would be calculated with the aqueous diffusion coefficient. Additionally, if the photic zone termination position ($L_{phot}$) is known, the flux of oxygen moving through that positional plane can be used in conjunction with $P_n$ to calculate the net areal rate of photosynthesis of the photic zone ($P_{n,phot}$, Eq. S3).

$$P_n = J_{z=0}^{biofilm} = J_{z=0}^{DBL}$$  \hspace{1cm} \text{Eq. S2}

$$P_{n,phot} = |P_n| + |J_{z=L_{phot}}|$$  \hspace{1cm} \text{Eq. S3}
The areal rate of biofilm respiration in the dark ($R_{dark}$) was calculated from the steady-state oxygen profiles in the dark in an identical fashion as $P_n$ was calculated under illuminated conditions.

**Gross Photosynthesis, Photosynthesis-coupled Respiration and Photic-zone Identification.** The total amount of oxygenic photosynthesis or gross photosynthesis ($P_g$) was calculated by the previously described light:dark shift method (Glud et al., 1992; Kuhl et al., 1996). During these measurements the tip of the oxygen microsensor was placed at some position ($z$) inside the illuminated biofilm. Time dependent oxygen concentration measurements were obtained through a period spanning the illuminated steady-state and initial darkening (see supplementary Figure.B-2). The time dependent and steady-state oxygen mass balances are described by Equations S4 and S5, respectively.

\[
\frac{dc_{o_2}}{dt} = D_e \frac{d^2c_{o_2}}{dz^2} + P_g(z) - R_{light}(z) \quad \text{Eq. S4}
\]

\[
P_g(z) = R_{light}(z) - D_e \frac{d^2c_{o_2}}{dz^2} \quad \text{Eq. S5}
\]

The light:dark shift measurements are based on non-steady-state measurements directly following the darkening procedure when the photosynthesis term is equal to zero (Eq. S5). The initial-slope of time dependent oxygen response to darkening can be equated to the $P_g(z)$ by summing equations S4 and S5 (resulting in Eq. S6). The initial-slopes were measured between 0 and 3 seconds for this study representing $P_g(z)$ (Eq. S7).

\[
\frac{dc_{o_2}}{dt} = -R_{light}(z) + D_e \frac{d^2c_{o_2}}{dz^2} \quad \text{Eq. S6}
\]
\[
\frac{dC_{\text{O}_2}}{dt} = -P_g(z) \quad \text{Eq. S7}
\]

Steady-state photosynthesis profiles were generated by taking \( P_g(z) \) measurements through the depth positions-\( z \) in the biofilm. The active-photonic zone was defined here as the depth of the biofilm from the surface to \( L_{\text{phot}} \). The position \( L_{\text{phot}} \) was assumed to be equal to the position-\( z \) where \( P_g(z)=0 \). This assumption is validated in the current study by the observation that \( L_{\text{phot}} \) occurs within the oxic zones of the biofilms and is not dictated by a strong heterotrophic respiration component. The gross areal rate of photosynthesis \( (P_g) \) of the biofilm was calculated by integrating the function \( P_g(z) \) from \( z=0 \) to \( z=L_{\text{phot}} \). Integration was performed by the Simpson’s five point quadrature rule.

The areal rates of biofilm and photic zone respiration in the light were calculated by subtracting the respective net areal rates of photosynthesis \( (P_n \text{ and } P_n,\text{phot}) \) from \( P_g \).

**References Cited**


Supplementary figures

**Figure B-2.** A representative light:dark measurement showing the transition from steady-state localized oxygen concentration (proportional to % air saturation) to time dependent oxygen depletion initiated by darkening. The slope of the initial oxygen decrease over time (red line) can be equated to $-P_g(z)$. 
To Whom It May Concern:

I, Rob Gardner, as a co-author of “Direct measurement and characterization of active photosynthesis zones inside wastewater remediating and biofuels producing microalgal biofilms” and “Dissolved inorganic carbon enhanced growth, nutrient uptake and lipid accumulation in wastewater grown microalgal biofilms” give Maureen Kesaano permission to reprint these manuscripts in their entirety in her dissertation.

Sincerely,

Robert D Gardner
Assistant Professor
University of Minnesota

To Whom It May Concern:

I, Ellen Lauchnor as a co-author of “Dissolved inorganic carbon enhanced growth, nutrient uptake, and lipid accumulation in wastewater grown microalgal biofilms” give Maureen Kesaano permission to reprint this manuscript in its entirety in her dissertation.

Sincerely,

Ellen Lauchnor

To Whom It May Concern:

I, Karen Moll, as a co-author of “Direct measurement and characterization of active photosynthesis zones inside wastewater remediating and biofuels producing microalgal biofilms” and “Dissolved inorganic carbon enhanced growth, nutrient uptake and lipid accumulation in wastewater grown microalgal biofilms” give Maureen Kesaano permission to reprint these manuscripts in their entirety in her dissertation.

Sincerely,

Karen Moll
To Whom It May Concern:

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Dipl.-Ing. Robin Gerlach (Ph.D.); Professor, Chemical and Biological Engineering; Center for Biofilm Engineering, Thermal Biology Institute, Energy Research Institute; Phone: 406-994-1840; E-mail: robin.g@coe.montana.edu

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CURRICULUM VITAE

Maureen Kesaano

Email: maureen.kesaano@aggiemail.usu.edu

EDUCATION

Ph.D. Biological Engineering, Utah State University (GPA: 3.87/4.00) May 2015
Dissertation: Characterization and performance of algal biofilms for wastewater treatment and industrial applications
Advisor: Dr. Ronald C. Sims

M.S. Civil and Environmental Engineering, Utah State University (GPA: 3.81/4.00) Dec 2010
Thesis: Sustainable management of duckweed biomass grown for nutrient control in municipal wastewaters
Advisor: Dr. Ryan R. Dupont

B.S. Civil engineering, Makerere University, (GPA 4.03/5.00) Jul 2008
Senior year project: Evaluation of the aptness of pH and free chlorine levels for bathing water quality in Kampala’s public swimming pools
Advisor: Dr. Robinah Kulabako

RESEARCH EXPERIENCE

Graduate Research Assistant May 2011 – May 2015
Sustainable Waste-to-Bioproducts Engineering Center (SWBEC), Utah State University Logan, UT
• Characterized algal biofilms via spatial photosynthetic activity and microscopy
• Evaluated lipid accumulation due to nutrient stress and bicarbonate addition for biofuels production
• Examined nutrient removal capacity of algal biofilms, growth rates and algal-bacterial interactions

Center for Biofilm Engineering (CBE), Montana State University Bozeman, MT
• Acted as a liaison between the algal research groups at Utah state university and Montana state university

Graduate Research Assistant Jan 2009 – Dec 2010
Utah Water Research Laboratory (UWRL), Utah State University Logan, UT
• Evaluated duckweed biomass management options via anaerobic digestion for methane production, fermentation for ethanol production, and animal feed options
Undergraduate Researcher  
Makerere University  
Kampala, Uganda  
Monitored bathing water quality by analyzing spatial and temporal variations of pH, free chlorine, turbidity, apparent colour, and bacterial levels in swimming pools

REVIEWER ACTIVITIES

• Journal of Algal Research
• Canadian Journal of Microbiology
• Biotechnological Advances

TEACHING EXPERIENCE

Teaching Assistant  
Department of Environmental Engineering, Utah State University  
Logan, UT  
Jan 2011 – May 2011  
• Coordinated field trips, led laboratory sessions on physical and chemical processes in water treatment, and assisted graduate students with laboratory reports
• Contributed to the course by designing a laboratory procedure for anaerobic digestion

EMPLOYMENT EXPERIENCE

Intern  
Directorate of Water Development (DWD), Ministry of Water and Environment  
Kampala, Uganda  
Jun 2007 – Aug 2007  
• Kept site records through daily progress reports, inspected general construction works on the stabilization ponds and pipes laid for the sewerage network, and engaged in community sensitization activities on clean water and sanitation

Students’ Internet kiosk administrator  
Directorate of ICT support (DICTS), Makerere University  
Kampala, Uganda  
Sep 2004 – Jun 2007  
• Monitored and supervised the general operation of the computer laboratory. Assisted students with computer and/or Internet related problems.
• Prepared daily reports on student usage of the computer facilities

VOLUNTEER EXPERIENCE

National Society of Black Engineers (NSBE) – Utah state university chapter  
Jan 2010 – Present  
• Participated in awareness campaigns, fundraising activities, and book drives for the club
• Mentored 3rd grade students at a summer engineering education experience for kids’ camp (SEEK 2012 - Oakland, CA)
• Presented and won the NSBE design Olympiad competition in collaboration with students from RWTH Aachen University, Germany (Pittsburgh, PA).  
Project title: Solar powered water pumping system for rural under developed African communities, Case Study: Mbeere Community- Kenya

Engineers without Borders-Utah state university and Salt Lake City chapter
Dec 2008 – Dec 2010
• Helped to construct houses, install solar panels, and water tanks for elderly women living with dementia at Indian reservations (Navajo Nation)
• Acted as a translator during the EWB-USU assessment trip to Uganda and assisted in the EWB-Uganda team’s fundraising activities

Student assistant at Battelle Chlorinated conference, Monterey, CA May 2010
• Provided relevant information to the conference participants and reported concerns/issues raised to the organizers

SKILLS
Trained on microscopy (Confocal Laser scanning microscope), oxygen microsensor based techniques, gas chromatography mass spectroscopy (GC-MS), and ion chromatography (IC), water quality monitoring and assessment, water and wastewater treatment, and biomass waste management.

PUBLICATIONS

PRESENTATIONS AND POSTERS
• Presenter: Institute of Biological Engineering (IBE), National conference, St. Louis MO Mar 5-7 2015
• Poster presenter: Algal Biomass, Bioproducts and Biofuels (ABBB), National conference, Santa Fe NM Jun 15-18 2014
• Poster presenter: Institute for Biological Engineering (IBE), Western Regional conference, Logan UT (2011, 2012)
• Poster presenter: Intermountain graduate research symposium (IGRS), Logan UT, 2011
• Co- presenter: Spring Runoff conference, Logan UT, April 2010
• Presenter: Water Environment Association of Utah (WEAU), Midyear conference Nov 2010
• Poster presenter: Water Environment Association of Utah (WEAU) conference, West Valley City UT, Nov 2009

AWARDS
• Biological engineering outstanding citizenship award 2015
• Research assistantship (2009-2010; 2013-2015)
• Presidential fellowship for 2011- 2012 academic year
• 2nd place poster presentation at IBE-regional conference 2011
• First place student presentation at Spring Runoff conference 2010
• Awarded best undergraduate student project presentation 2008
• Full government sponsorship for undergraduate program (2004 -2008)

CERTIFICATIONS

• Engineer in Training (EIT)