Microbial Community Structure and Dynamics of Artificial, Engineered Microalgal Biofilms

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Cell & Systems Biology
University of Toronto

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Abstract

Microalgae can be grown as immobilized biofilms with a high potential for the production of biofuel and bioproducts. This thesis investigates the community structure and microbial community dynamics of engineered microalgal biofilms. Our research looked specifically at understanding how different concentrations (12% versus 0.04%) of CO$_2$ may affect the diversity of the microbial community in engineered microalgal biofilm photobioreactors originally seeded with *Scenedesmus obliquus*. DNA was extracted from biofilm samples at specific intervals over a 26-day experiment and used for Sanger and Illumina MiSeq based sequencing of the 16S rRNA and 18S rRNA genes. It was determined that the communities detected on day 4 in both the 12% and 0.04% CO$_2$ treatments was dominated by the seed species, *S. obliquus*, but by day 26, *Leptolyngbya sp.* was the dominant species. Therefore, further efforts will be needed to optimize growth conditions to favour species producing the most desirable biomass.
Acknowledgments

This thesis is dedicated to my grandfather, Joseph Albert Alexandre Paquette. I’ve been told that we are very alike and I wish I would have had the opportunity to meet him. It is my honor and privilege to dedicate this thesis to him.

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Chapter 1
Introduction

1.1 Background

In 2016, the widespread use of fossil fuels in industrial and commercial endeavors accounted for 13,276.3 million tonnes of oil equivalent (mtoe) of the global primary energy consumption i.e. coal (28%), oil (33%) and natural gas (24%) [1]. These resources are natural, but non-renewable energy sources. The heavy reliance on fossil fuels is likely to continue into the future due to advances in the technology of extracting fossil fuels, potential reserves and increased exploitation of newer unconventional reserves [2]. Such reliance is a matter of great concern given that these resources are both limited, non-renewable, and subject to political strife [2]. The overarching issue with fossil fuels is associated with greenhouse gas (GHG) emissions as well as pollutants. Combustion and consumption of fossil fuels were the dominating source of CO₂, contributing >60% of global GHG emissions in 2015 wherein over 30 billion tonnes of CO₂ were emitted from fossil fuels [3]. It is now generally accepted that this level of CO₂ production has a harmful and large-ranging impact on the global environment [4].

The need to mitigate CO₂ emissions and to find alternative energy sources has encouraged research into alternative, renewable energy sources such as wind, solar and hydro power [5]. Furthermore, research into the feasibility of bioenergy products has also occurred sporadically, since the 1970s. The best and perhaps most successful example of biofuel production is ethanol derived from the processing of farm-grown corn. Over 14.8 billion gallons of corn ethanol was produced in the United States in 2015, most of which was used as a supplement in gasoline mixtures [6]. Other biofuels such as the production of biodiesel from vegetable oils and animal fats are also on the rise. These fuels are thought to be the solution to
low emission energy sources that matched petroleum diesel fuel in the amount of energy produced per unit [2, 7, 8]. However, renewable biofuels have high costs involved in terms of growing crops, land requirements and the competing demands between land use for food and bioethanol [2, 9]. It is for these reasons that renewed interest and research has focused on microalgae as an additional renewable energy source.

Microalgae are oxygenic photosynthetic microorganisms that grow under a wide range of environmental conditions. They have very rapid growth rates compared to agricultural crops, and are estimated to produce more oil per m² of land than agricultural crops [5, 10, 11]. Moreover, using microalgae as a source of biofuel avoids the dilemma of food versus biofuel production [5, 10, 11]. In an industrial setting, microalgae are grown in closed photobioreactors, open ponds, and biofilm photobioreactors. The cultivation of algae in open pond systems was established in the 1950’s. Open pond systems are feasible due to low maintenance and investment costs and the simplicity of their architecture [2, 5]. Open ponds are typically constructed in a shape similar to a racetrack with endless loops, where the algal culture is circulated using paddle wheels (Figure 1). However, open ponds are directly exposed to environmental conditions, making it difficult to avoid contamination and control growth conditions, such as pH, light and temperature [5, 7]. Open pond systems have poor biomass productivity and a large area of land is required to build these systems [2].
Figure 5: Schematic of a typical raceway pond [11]

Closed photobioreactors typically have higher biomass productivity and biomass concentration than open ponds due to the ability to adjust most growth conditions and reduce the probability of contamination [2, 5]. While light utilization efficiency is much better in these closed systems, there are also some drawbacks. Most notably, the very high capital costs associated with construction of photobioreactors and their ongoing upkeep costs [5]. There are four common designs for closed photobioreactors: (1) tubular; (2) flat plate; (3) column; (4) large scale plastic bag photobioreactors [2]. Tubular photobioreactors have small diameter tubes constructed in different patterns, typically made of plastic or glass. Tubular systems have large illumination surface areas, are suitable for outdoor cultures, have good biomass productivities and are relatively cheap (Figure 2A) [2, 12]. The limitations of tubular photobioreactors is the relatively large requirement of land and the difficulty in controlling growth conditions along the tubes like pH, temperature and dissolved oxygen or CO₂. Flat plate photobioreactors are commonly known to have large illumination surface areas and are made of transparent materials.
for maximum utilization of solar light energy (Figure 2B) [2, 5, 12]. These flat plate systems are easy to sterilize, have high biomass productivities and low oxygen build up, unlike tubular systems. Column photobioreactors are usually cylinder in shape, compact and can be vertical or horizontal (Figure 2C). The column systems have high potential for scalability, high mass transfer, reduced photoinhibition and photo-oxidation and good mixing, with low shear stress. [2, 5, 12]. Plastic bag photobioreactors have recently been a focus in the large scale production of microalgae because of the low cost to build them (Figure 2D) [12, 13]. The plastic bags can be built with an aerator to increase cell yield and combined with a water pool to cut costs and control temperature [12, 13].

Figure 6: Schematic of a (A) Tubular photobioreactor (B) Flat plate photobioreactor (C) Column photobioreactor and (D) Large scale plastic bag photobioreactor

Algal biomass is grown planktonically in open pond systems and closed photobioreactors, both of which face the common problem of harvesting and dewatering of the
algal biomass [14, 15]. Up to 30% of the overall cost of producing biomass in these systems is attributed to the latter two processes and make the process economically impractical for all but very high-value products (like carotenoids and astaxanthin)[14]. This has led to an alternative strategy of cultivating algae in engineered microalgal biofilm photobioreactors which produces biomass on a solid surface, in a concentrated and largely dewatered state, wherein harvesting of the biomass involves scraping of the biofilm off the surface of the photobioreactor. Biomass productivity in an engineered microalgal biofilm photobioreactor can be as high or higher than planktonic algal systems [16]. Other advantages of these systems include improved CO₂ mass transfer and minimization of light limitation [17].

1.2 Biofilm Photobioreactor Designs

Engineered microalgal biofilm photobioreactors require a surface that acts as a support to the biofilm. While there are many designs of microalgal biofilm photobioreactors, they are mainly categorized as stationary or rotating, based on the movement of the biofilm support. The orientation of the biofilm support can be used to further categorize stationary biofilm systems as horizontal, vertical or flow cells. The movement of medium through the photobioreactor can be done in three ways: (1) perfused biofilm systems, which involves the cultivation medium flowing over a permeable biofilm support; (2) constantly submerged systems where the microalgal biofilm is consistently immersed in cultivation medium; (3) intermittently submerged systems that are based on rotating and rocker microalgal biofilm photobioreactors wherein the biofilm is constantly moving between the growth medium and air [17-20]. The figure below (Figure 4) depicts the microalgal biofilm system designs that were described above.
Figure 7: Schematic of the basic design concepts for microalgal biofilm photobioreactors [20].

Perfused biofilm systems are also known as twin layer systems [18, 21, 22] and consist of a liquid conducting biofilm support with a semipermeable membrane. This acts to supply nutrients and moisture to the microalgal cells on the membrane, without contaminating the liquid medium, thus allowing the medium to be recirculated [18, 21, 22]. Some advantages of perfused systems include exposing the microalgal biofilm directly to light and air, facilitating gas exchange and reducing diffraction of light through liquid [18, 21, 23]. However, with this system there is the risk of drying out the microalgal biofilm [18]. Constantly submerged systems typically have microalgae grown on an inclined solid support that is covered by a layer of cultivation medium. This type of system is usually constructed as a flow cell or channels where a pump or pump-like device moves the medium through the photobioreactor [17-19]. Intermittently submerged systems can be split into two groups; (1) systems that are derivatives of the algal turf scrubber [18, 24]; (2) systems that have a surface that moves. The algal turf
scrubber that was built by Adey et al [24] is designed for algae to attach to sloped surfaces and wastewater is pulsed over the surfaces [24]. The systems that have been designed based on the algal turf scrubber differ from the original design wherein these systems have flow cells/channels where the intermittent submersion is due to the fluctuation in the flow rate of the cultivation medium [18]. However, for systems that have a moving surface the opposite happens, i.e. the surface actually moves through the cultivation medium instead of the periodic surges of the liquid medium to the biofilm. Rotating algal biofilm (RAB) systems are one example of the moving surfaces where algae attach to the surface of a wheel that is constantly rotated. Both designs of intermittently submerged systems have the advantage of the biofilm coming in contact with fresh medium and being directly exposed to light and air [17-19, 25].

1.3 Microalgal biofilm formation

Engineered microalgal biofilm photobioreactors can also be used for wastewater bioremediation and purification. Wastewater from municipal treatment facilities is a cheap and rich source of nutrients (e.g., nitrogen and phosphorus) that can support and enhance microalgal growth. Removal of these nutrients by microalgae prior to discharge of the wastewater into the environment is a promising means for reducing local eutrophication of freshwater bodies that adjoin the wastewater treatment plants [19, 26, 27]. In some cases, these engineered microalgal biofilm photobioreactors are designed to use bacteria within the wastewater as primary colonizers that bind to an attachment material in the photobioreactor, initiating the formation of the biofilm [18]. Formation of the microalgal biofilm can be based on the binding of microalgae to a surface due to an interaction with microorganisms that have already colonized the surface, or the interaction between the microalgal cells and the attachment surface [18, 28]. When microorganisms like bacteria attach to the surface prior to the microalgae, they begin producing a gluey substance known as extracellular polymeric substance (EPS) [15, 18]. The EPS can consist
of proteins, phospholipids, polysaccharides and nucleic acids [29]. Bacterial-derived EPS forms a matrix that acts as a surface adhesive facilitating attachment of the microalgae to the photobioreactor system [26, 30, 31] (Figure 2). The EPS formed matrix also provides stability and facilitates the interaction between bacteria and microalgae [30]. As the microalgal biofilm matures, the cells are immobilized and forms a multi-layered structure that depends on diffusion for the movement of nutrients and waste materials [18]. The microalgal biofilm will develop with different species growing in zones that are favourable to them, creating this heterotrophic structure [26, 31].

![Figure 8: Schematic of microalgal biofilm formation [20]](image)

1.4 Attachment materials for microalgal biofilm systems

Attachment material is an important factor in the design of a microalgal biofilm system. When choosing an attachment material there are three criteria to consider; (1) cost; (2) durability; (3) degree of cell attachment. Previous work by Gross et al [25] and Christenson and Sims et al
investigated attachment materials for their rotating algal biofilm photobioreactor. Gross et al explored 16 different types of materials and discovered that armid fiberglass, chamois cloth, and cotton duct had good attachment by microalgal species, with cotton being the best material for cell attachment, low cost and high durability[25]. Meanwhile, Christenson and Sims et al discovered that the texture of the cotton cord had an effect on cell attachment [16]. This led Gross et al to look at different cotton material to determine which one had the best attachment based on the texture of the cotton. The types of cotton used was rag, denim, corduroy and duct, with cotton duct having the best attachment and biomass productivity (g m$^{-2}$ day$^{-1}$) [25].

Although in another study by Gross et al, it was found that cotton deteriorates within 3 months, leading to extra costs to continuously replace the cotton and causes downtime where the photobioreactor is non-operational [32]. Lee et al [33] examined cell attachment for a mixed group of microalgal species, such as Scenedesmus, Chlorella, Pediastrum, Nitzschia, and Cosmarium, to the following materials; polycarbonate plate, polyethylene plate, nylon mesh, and stainless mesh. It was determined that stainless mesh had the best cell attachment and the polycarbonate plate was the worst, however Lee et al decided to use the nylon mesh for their experiments because it had the second best attachment and was cheaper than the stainless mesh [33]. In another study, the potential attachment of Chlorella sp. to different attachment materials, such as polystyrene foam, cardboard, polyethylene landscape fiber, loofah sponge, polyurethane foam and nylon sponge, was explored [10]. Polystyrene foam and cardboard were the two highest biomass productivities, but the cardboard surface was not very durable. However, polyurethane, loofah and nylon had cell attachment, but were difficult to harvest from since the material is porous [10]. Adhesion of Chlorella vulgaris (chlorophyceae), Nitzschia amphibia (bacillariophceae) and Chroococcus minutus (cyanobacteria) to stainless steel, titanium, copper and glass was examined, of which copper had the least attachment while
stainless steel and titanium had the most [34]. The broad range of attachment materials used implies that the material chosen has an impact on biofilm formation and biomass productivity, and is a factor that depends on the microalgal species of interest.

1.4 The effect of growth conditions on biofilm formation and photobioreactor performance

Research in algal biofilm photobioreactors (PBRs) has largely focused on the ultimate objective of optimizing biomass and biocompound productivity, in which abiotic factors, such as flow velocity/shear stress, pH, nutrient limitation, light intensity and carbon dioxide, are important to consider. However, this is a largely understudied area and a potential focus for future research.

1.4.1 Flow velocity and shear stress

The integrity of the biofilm is dependent on the rate at which liquid flows (flow velocity) as well as the movement between the liquid flow and the biofilm which causes shear stress. As the biofilm matures, it is prone to sloughing from the velocity of the liquid flow, as in the case of stationary biofilms, or the rotation speed for rotating biofilms [25]. Therefore, the flow velocity in microalgal biofilm photobioreactors can have a detrimental effect when the flow velocity is too strong which causes the microalgal cells to detach from the growth surface and hinder the biomass productivity of the system [20]. Gross et al [25] investigated biomass productivity in a rotating microalgal biofilm photobioreactor based on the rotation speed of the wheel. The speeds tested were 0.333, 0.667, 2, 4 and 6 rpm, in which speeds below 4 rpm had the lowest biomass productivity due to drying from a longer exposure time to air. A wheel speed of 6 rpm caused the biofilm to be sheared off, but at 4 rpm there was less shear stress and the biofilm was less likely
to dry out [25]. Zippel et al [35] examined the effect of flow velocity in a flow lane photobioreactor that was growing freshwater and marine microalgal biofilms. The major conclusion of the study was that a lower flow velocity at the beginning of the experiment allowed for improved cell attachment, while a higher flow velocity is better for mature biofilm growth [35]. Liu et al [36] investigated the effect of flow velocity on biomass production in an outdoor mixed microalgal biofilm turf scrubber. They investigated four levels of flow rates ranging from 2 to 8 L min⁻¹, where it was determined that a higher flow rate of 8 L min⁻¹ produced a higher biomass production (1.2–2.0 g DW m⁻² d⁻¹), while a lower flow rate of 2 L min⁻¹ produced a lower biomass production (0.8–1.7 g DW m⁻² d⁻¹) [36].

1.4.2 pH of the cultivation medium

The pH of the cultivation medium is known to affect the growth of microalgae and establishment of the biofilm. [37]. Biofilms grown at pH 3.0 and pH 10 showed a difference in biofilm thickness, in which biofilms grown under acidic conditions were 2/3 the size as that grown at pH 10 [38]. Chen et al [39] investigated the effect of pH on the growth of a *Pseudomonas fluorescens* biofilm at different pH’s, such as 5.5, 7 and 8.5. At a pH of 5.5 and 8.5, biofilm development took 3-5 days longer than biofilms grown at a neutral pH. Chen et al [39] also found that the pH can vary across the photobioreactor, such that the liquid medium around the biofilm can be different than the microalgal layers within the biofilm. The influence of the pH medium on the adhesion of *Nitzschia amphibia* (bacillariophceae) to different attachment materials, such as stainless steel, glass and copper, was examined by Sekar et al [34]. This was tested at pH 6, 7, 8 and 9, where pH 7 and above had higher attachment [34]. While there is some evidence that pH is a vital growth condition in microalgal attachment, biofilm formation and growth, further work should investigate the influence of pH on biomass productivity in microalgal biofilm photobioreactors.
1.4.3 Nutrient-limitation

There is immense interest in increasing lipid production from algae because of the ability to convert it to biodiesel through transesterification. In planktonic systems, abiotic factors such as pH, temperature, light and CO₂ concentrations are known to induce the production of triacylglycerol, however the most effective way is through nutrient starvation, particularly nitrogen and silicon. For example, in planktonic growth systems of *Nannochloropsis* sp., *Neochloris oleoabundans*, *Scenedesmus obliquus* and *Chlorella vulgaris*, nitrogen starvation leads to a 20-40% increase in lipid production compared to nitrogen-replete conditions [40-43]. Yu et al [44] examined how nitrogen and silicate limitation affect the lipid content of *Thalassiosira pseudonana*, where it was determined that the silicate-limited *Thalassiosira pseudonana* had a higher increase in lipid content (24% increase) than the nitrogen-limited [44]. These studies reveal that in planktonic growth systems nitrogen and silicon limitation can affect lipid content, but microalgal species can respond differently to the nutrient that is limited. However, a study examining the effect of nitrogen limitation on lipid content of microalgal biofilms from a biofilm photobioreactor have found the opposite outcome compared to their planktonic counterparts. Schnurr et al [45] worked with biofilms of *Scenedesmus obliquus* (*S.obliquus*), where the biofilms were starved of nitrogen to examine if lipid content would increase. Biofilm samples were grown for 20-21 days, where nitrogen starvation began at the 13-14th day [45]. The growth kinetics of the biofilms were measured continuously during the 21-day period. However, soon after the nitrogen-starvation was started, the growth kinetics could not be measured because the growth of the biofilm had stopped completely and sloughing of the biofilm occurred [45]. Therefore, nitrogen-starvation had no effect on lipid production of the *S. obliquus* biofilms. In fact, Schnurr et al [45] observed that nitrogen-starvation led to the
microalgal cells resorting to a planktonic life-style. This was a dramatic example of nutrient availability affecting the physical stability of microalgal biofilms.

1.4.4 Light Intensity

Microalgae are oxygenic photoautotrophic organisms, that need light to metabolize carbon dioxide to produce organic compounds and oxygen. This light-driven biomass growth is known as photosynthesis. The effect of photon flux density and the different directions that biofilms are illuminated from has been investigated in microalgal biofilm photobioreactor research. Schnurr et al [46] examined if illumination from different directions affected biomass and lipid productivity (fatty acid methyl esters). These photobioreactors were constructed such that one side of the biofilm adheres to a solid, transparent surface while the other side is exposed to a nutrient solution, which supports its growth. Schnurr et al used biofilm thickness (µm) and dry weight/area substrate (g/m²) to evaluate the effect of light direction on the biofilm [46]. Overall, there was no difference in biofilm thickness and dry weight/area substrate for the different directions the biofilm was illuminated with a constant light intensity of 100 total µmol/m²/s [46]. This result implies that the cells received enough light no matter what direction the light was coming from [46]. After the initial harvest of the biofilm from the glass coupon, the coupon was inserted back into the photobioreactor to see if light direction made a difference on initial cell attachment (re-growth experiment). Illumination from the nutrient side enhanced initial cell recruitment to the biofilm over the first seven days of re-growth experiments [46]. It is possible that light direction has an effect only on the initial cell attachment and not biofilm thickness or dry weight/area substrate, however further research is required to confirm this.

Although light direction was not shown to have an impact on the performance of a microalgal biofilm photobioreactor, the opposite is true for the effect of photon flux density on
biomass productivity and lipid production. Schnurr et al [47] focused on the effect of photon flux density on fatty acid content and biomass productivities. Fatty acid content was calculated by comparing the mass of fatty acid methyl esters (FAME) per dry weight of algal biomass (% w/w), while biomass productivity was measured as the dry weight of algal biomass per area per unit of time (g/m²/d). Duplicate experimental runs were conducted at the photon flux densities of 150 µmol/m²/s and 600 µmol/m²/s, however triplicate runs were carried out for photon flux densities of 50 µmol/m²/s and 300 µmol/m²/s [47]. The biofilm was sampled on the fourth day of the experiment and every 2-4 days thereafter, for a total of 26 days or until the biofilm was 3 mm thick. When the photon flux density was increased from 50 µmol/m²/s to 150 µmol/m²/s, the FAME concentration increased from 5% to 8% (w/w), but there was no further change at photon flux densities of 300 and 600 µmol/m²/s [47]. The biomass productivity in this study was found to increase from 1 g/m²/d to ~3.5 (g/m²/d) in relation to an increase in photon flux density from 50 µmol/m²/s to 300 µmol/m²/s and the productivity was very similar to the 300 µmol/m²/s at 600 µmol/m²/s [47]. Therefore, increasing photon flux density can lead to an increase in lipid production and biomass productivity, but there comes a point where increasing photon flux density will no longer improve biomass productivity and lipid production.

1.4.5 Carbon dioxide concentrations

Carbon dioxide is a crucial component in the phototrophic growth of microalgae and can possibly enhance biofilm growth and lipid production [25, 48-50]. Blanken et al [49] investigated how a change in CO₂ concentration would affect biomass productivity, using a rotating disk microalgal biofilm photobioreactor. The disks were submerged for some time in the cultivation medium that contained dissolved inorganic carbon. It was determined that a decrease in CO₂ concentration resulted in a reduction of the biomass productivity [49]. Kesaano et al [48] also used a rotating algal biofilm photobioreactor (RABR) with inorganic carbon dissolved in the
growth medium, but came to a different conclusion than Blanken et al [51]. The analysis of their results revealed that there was no noticeable increase in biomass productivity when increasing the inorganic carbon concentration in the growth medium [48]. Gross et al [25] found a similar conclusion with no significant increase in productivity at different CO2 concentrations. In this study [25] an RABR was designed to expose the biofilm to different gaseous CO2 concentrations. The effect of a change in CO2 concentration on the growth kinetics of Scenedesmus obliquus biofilms was examined by running different CO2 experiments with a constant light intensity of 100 µmol/m²/s of red light (620nm - 640nm) [50]. The different CO2 concentrations ranged from 0.04% CO2 (atmospheric CO2) to 12% (v/v) CO2 (0.04%, 1%, 2%, 6%, 8%, 10% and 12%), in which only the 1%, 6% and 10% v/v CO2 experiments were run in triplicate [50]. At a CO2 concentration of 0.04%, the biomass productivity was ~0.5 g/m²/d, however the biomass productivity was 4 times higher for the 2% CO2 experiment (~2 g/m²/d). The productivity plateaued between 2% - 12% CO2 with a biomass productivity of ~2 g/m²/d [50]. This indicated that microalgal biofilms grown at CO2 concentrations above 2% did not utilize the extra CO2. It is evident from the studies above that it is still inconclusive if a change in CO2 concentration has an actual effect on biomass productivity.

While it is clear that abiotic factors, such as flow velocity/shear stress, pH, nutrient limitation, light intensity, and carbon dioxide, have an effect on algal biofilm biomass productivity, lipid production, biofilm growth and the attachment of microalgal cells to a biofilm, there is a lack of studies investigating the effect of abiotic factors on the stability of the microbial community structure and community dynamics of artificially engineered microalgal biofilms [20, 52]. This thesis will examine biofilm samples collected from an engineered microalgal biofilm photobioreactor that was used to investigate the effect of CO2 on biomass productivity [14]. The
The purpose of this thesis will be to investigate how inorganic carbon concentrations influence the microbial community structure and dynamics of *Scenedesmus obliquus* biofilms.

### 1.5 Engineered microalgal biofilm photobioreactor

For the research described in this thesis, *Scenedesmus obliquus* biofilms were grown in a semi continuous flat plate horizontal photobioreactor with 18 replicate photobioreactors designed and built by Schnurr et al. [45, 50] (Figure 5). Development of the biofilms started with the addition of wastewater as a source of nutrients (i.e. nitrogen and phosphorus) and bacteria (i.e. primary colonizers of the biofilm). The wastewater was circulated throughout the system for 48 hours, after which 90% of the wastewater was removed. Then, CHU10-diatom medium and the seed culture, *Scenedesmus obliquus*, was added to the system. The seed culture was allowed to settle and adhere to the bioreactor surface for 24 hours before re-commencing circulation of the growth medium. The biofilm samples that were studied for this thesis were collected from two CO₂ experiments (0.04% and 12% CO₂) that were grown over a 26-day period at a constant light intensity of 100 μmol/m²/s of red light (620nm - 640nm) [50]. Temperature and pH were kept constant at 25°C and 7, respectively. Biofilm samples were collected at different time points (day 4, 10/11, 15, 19/20 and 26) where two samples were taken at each time point from two of the 18 replicate photobioreactors.
1.6 Microbial community analysis:

Microbial ecology is the study of microorganisms’ interactions with one another, with other microbes and with their environment [53]. When studying microbial communities there are two things to consider: 1) the biodiversity of microorganisms and 2) the activities of microorganisms [53]. The biodiversity of microorganisms refers to how many microbes are present in a sample and can include measures of eveness. When studying the activities of microorganisms, the goal is to investigate how these microbes function and contribute to the community [53]. The focus of this thesis is on the biodiversity of the microorganisms in the engineered microalgal biofilms since we were particularly interested in determining the species composition of the biofilm communities.

The traditional microbial ecology approach to determine the biodiversity of a community involves microscope observations combined with the application of determinative staining [54]. Cultivating microorganisms in the laboratory is another traditional approach which has always been a challenge as a majority of microorganisms are uncultivable in a laboratory environment.
Advancements in molecular techniques and technologies led to progress in studying microbial communities. One of those molecular techniques is DNA sequencing, which involves ‘reading’ the nucleotides that are present in a marker gene [54, 56]. The sequencing results are compared to a database of known sequences to identify the genera and species the sequences are most closely related to [55].

One way of using DNA sequencing for identification is to use a marker gene like small ribosomal RNA (SSU rRNA) [54, 56, 57]. Using a marker gene like SSU rRNA can be beneficial because all cells have ribosomes which are essential for survival and are their sequences are conserved through evolutionary time [54, 55, 57]. Eukaryotic microorganisms can be identified using 18S rRNA genes, while 16S rRNA genes are used to detect prokaryotic cells and eukaryotic organelles [56]. Polymerase chain reaction (PCR) is used to amplify the marker genes, after which they are sequenced using one of two methods: 1) Sanger sequencing or 2) high-throughput sequencing [54, 58]. Sanger sequencing is a “first generation” sequencing technology that involves the addition of chain-terminating dideoxynucleotides by DNA polymerase throughout an artificial DNA replication [54, 59, 60]. Further advancements in sequencing technologies led to high-throughput sequencing and is currently the most recent generation of sequencing technologies [54, 61]. High-throughput sequencing is a method that is capable of sequencing multiple DNA molecules in parallel, allowing millions of DNA molecules to be sequenced at a time, creating large sequence data sets. [62].

Nucleotide sequences can be analyzed using software packages like “mothur” and R to trim, screen, and align similar sequences. The data can then be used to determine phylogenetic distances between sequences, assign sequences to operational taxonomic units (OTU), and to describe the α and β diversity [63]. The extent to which the sequencing effort has managed to
capture the community composition can be measured using a rarefaction analysis which takes into account the number of sequences and OTUs that are obtained [64]. A steep slope on the rarefaction curve is indicative of insufficient sampling and low coverage of microbial diversity [64]. A shallow slope suggests that a majority of the OTUs within a community has been captured. Microbial community diversity is measured through α-diversity, a measure of the diversity within a specific area or sample (i.e. within a community), and β-diversity, a measure of the difference in species composition between samples or areas (i.e. between communities) [65-67]. Metrics used to measure the α-diversity includes Chao1, Shannon and Simpson index, while β-diversity metrics are either quantitative which takes into account the sequence abundance (i.e. Bray Curtis or Weighted Unifrac) or qualitative, taking into account the presence or absence of species (i.e. Unweighted Unifrac) [58, 65-68]. Phylogeny is considered for Unifrac metrics, but not for Bray Curtis [54, 65, 66, 68].

This thesis will examine the collected biofilm samples using Sanger and Illumina MiSeq (high-throughput) sequencing, and the sequencing results will be used to determine the community diversity (α-diversity) and differences in species composition (β-diversity) of biofilms inoculated with the algae Scenedesmus obliquus.

1.7 Hypothesis

The productivity of microalgal biofilm photobioreactors (biomass growth, lipid production, etc.) is directly related to growth conditions, such as light and carbon dioxide. However, it is not known if these growth conditions affect the microalgal biofilm structure and community dynamics, which could impact biomass growth and lipid production. I hypothesize that species composition in the nonsterile, engineered microalgal biofilm photobioreactor would remain stable throughout the cultivation, with the seed culture being the dominant species. In
addition, I hypothesize that an abiotic factor like CO₂ concentration will have no effect on the community dynamics of the biofilm.

1.8 Objective

The aim of this research was to determine the composition and community dynamics of engineered microalgal biofilms derived from the biomass productivity studies of Schnurr et al [50]. Here, I ask the question, do biofilms seeded with *Scenedesmus obliquus* and grown in a controlled photobioreactor maintain a stable composition over time? In addition, the research will assess how CO₂ concentration affects the community dynamics of the biofilm. The outcomes of this research can help determine the conditions that favour biofilm community stability. This knowledge can be used to optimize an artificial engineered microalga biofilm photobioreactor that will produce a biofilm with the most desirable biomass for oil production.
1.9 Approach

The diagram below illustrates the approach to investigate the effect of CO$_2$ on the microbial community structure and community dynamics of artificial, engineered microalgal biofilms.

![Flow chart](image)

Figure 10: A flow chart describing the approach taken to complete the objective
Chapter 2
Contrasting the community dynamics of microalgal biofilm communities grown at different CO$_2$ concentrations

2.1 Introduction

The growth of microalgal biomass in engineered raceway ponds or photobioreactors are promising technologies for sustainable biofuel and biochemical production, for carbon capture from industrial flue gas, and for the remediation of waste water, among other uses [5, 11, 69]. Over the last decade a variety of pond and reactor designs have been tested for optimized microalgal growth either as planktonic cultures or as biofilms [2, 18, 19, 70, 71]. Understandably, many studies have focused on the effects of abiotic factors on biomass and lipid productivity etc. As a result, a wealth of data is now available to inform choices on bioreactor design in order to obtain a desired product or outcome [2, 18, 19, 70, 71].

It is likely that industrial scale, microalgal bioprocessing will frequently demand nonsterile operation in order to be economical. For example, the use of wastewater as a source of growth-promoting nutrients has the advantage of being freely, or nearly freely available, and allows for additional remediation of wastewater while stimulating the growth of valuable algal biomass. But, wastewater inherently contains diverse, but unknown, microbial community on its own. Thus, a critical understanding of the impact of microbial community composition and dynamics in an engineered bioreactor setting is vital for optimized microalgal productivity. Nonsterile operation will also require developing knowledge of conditions that will selectively promote the growth of desirable, eukaryotic algal biomass within the context of a microbial consortium. Though these types of community data are emerging for planktonic microalgal systems[27, 72-78], the microbiomes of engineered algal biofilms have only infrequently been characterized[52, 79-81].
Our recent studies have examined the effects of nutrient availability and stress [45], light direction and intensity [46, 47], CO₂ concentration [50] and light-CO₂ interaction [50] on algal biofilm growth, biomass and lipid productivities in flat-bed photobioreactors. Algal biofilms were initiated on pre-conditioned glass plates that had been treated with municipal wastewater as an initial source of nutrients, extracellular polymeric substances (EPS) and biofilm-forming bacteria. Plates were then heavily inoculated with a microalgal seed culture in defined growth medium and biomass productivity followed over a 26-day time course. During these experiments we also collected samples for microbiome analysis in order to relate productivity to species composition. We anticipated that species composition of the nonsterile, engineered microalgal biofilms would remain stable throughout the cultivation, with the seed culture being the dominant species. However, pilot-level Sanger sequencing indicated a much more dynamic microbial community than hypothesized. Here we use high-throughput 16S and 18S rRNA gene sequencing to report on the effects of CO₂ concentration on the composition and community dynamics of a microalgal biofilm grown in an engineered photo-bioreactor.

2.2 Methods

2.2.1 Experimental Setup and Sampling

We analyzed the community structure of algal biofilm samples derived from a previous study conducted by Schnurr et al. [50]. In that study, a semi-continuous flat-plate horizontal algal biofilm photobioreactor was used to grow biofilms for up to 26 days at low CO₂ concentrations approximating atmospheric levels (0.04% v/v), and at high CO₂ levels (12% v/v) mimicking the concentrations of flue gases emitted from some industrial stacks. At the beginning of the experiment, the bioreactor was ‘preconditioned’ for 48 h with unchlorinated wastewater obtained from Ashbridge’s Bay Wastewater Treatment Facility, Toronto, ON. After preconditioning, the
waste water was replaced with a synthetic growth medium and was seeded with a unialgal culture of *Scenedesmus obliquus* (strain CPCC 5, Canadian Phycological Culture Centre, University of Waterloo, ON, Canada) [45, 50]. For all experimental treatments, 50 µl – 100 µl samples of the algal biofilm were collected on days 4, 10/11, 19 and 26 as described in by Schnurr et al. [50].

### 2.2.2 DNA extraction

Nucleic acids were extracted from the biofilm samples following the procedure of Short et al. (2011). There were 18 biofilm samples, in which 8 samples came from the 12% CO₂ treatment and 10 were from the 0.04% CO₂ treatment. Briefly, washed and sterilized glass beads (212–300 mm and 425–600 mm sizes) and 550 µl of buffer AP1 and 5 µl of RNase A (QIAGEN, Louisville, USA) were added to 2 mL screw-cap tubes with biofilm samples, and cells were homogenized twice for 3 min using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, USA); the tubes were allowed to cool for 3 min between these disruption cycles. Nucleic acids were then extracted using a DNeasy Plant Mini Kit (QIAGEN, Louisville, USA) according to the manufacturer’s specifications. The DNA concentration in each extracted sample was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and all DNA samples were stored at -20°C immediately following extraction.

### 2.2.3 PCR, Ligation, Transformation, Cloning and Sanger sequencing

Sample DNAs were PCR-amplified using primers targeting 18S rDNA (Euk 1A – Euk 516r) [82], 16S rDNA (357f – 907r) [83], and psbA [84], in which the DNA came from 8 biofilm samples, 4 samples each from the 0.04% and 12% CO₂ treatments. Fifty microliter PCRs for 18S rDNA and 16S rDNA genes included 5 µl template DNA, 25 µl of GoTaq® G2 Green Master Mix (Promega, USA), 0.2 µM each of the forward and reverse primer and 17 µl of H₂O. For the
psbA primers, all volumes were the same except 0.4 µM of each primer was added and the volume of H₂O was 14 µl. Negative controls were included for every reaction by substituting 5 µl of H₂O for template DNA. Thermal cycling conditions for both the 18S and 16S rDNA amplifications were: initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 20 min in an C1000 Thermal Cycler (Bio-Rad Laboratories). For psbA, thermal cycling began with a denaturation step at 95°C for 2 min and was followed by 35 cycles of 95°C for 30 s, 52°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 20 min. Ten microliters of each PCR were electrophoresed in 1.5% agarose gel, stained in a 0.5 µg ml⁻¹ ethidium bromide solution and were visualized using a Gel Doc XR (Bio-Rad Laboratories)[85].

PCR products were purified using an EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, Ontario) following the manufacturer’s recommendations. Amplicon concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scienti-fic, Waltham, MA, USA). Ligation and transformation reactions were performed as previously described, and bacterial colonies were checked by PCR using T7 and SP6 promoter primers (Integrated DNA Technologies, Coralville, USA) to verify that insert fragments were appropriately sized [85]. Following electrophoresis, samples with the appropriate band size were purified before being sent for Sanger sequencing at The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children, Toronto, Ontario, Canada.

2.2.4 Sequence, Phylogenetic, Rarefaction and unweighted UniFrac analysis – Sanger Sequencing

Sequences were edited (i.e., vector and primer sequences were trimmed off) and analyzed using the software MEGA6 and Bioedit [86, 87]. Individual sequences were assigned to OTUs
using a sequence identity matrix created in BioEdit with a 97% sequence identity criteria [87].

Reference sequences for 18S and 16S OTU’s were identified using BLASTn, while PSI-BLAST was used to identify translated psbA sequences [88, 89]. OTU’s and reference sequences obtained from NCBI were aligned using MUSCLE [90] and neighbour-Joining phylogenies were created with bootstrap support values based on 550 replicates using MEGA6. MEGA6 and Adobe Illustrator CS (Adobe Systems, San Jose, CA, USA) were used for tree viewing and drawing [86]. In order to describe and compare the microbial communities grown at 12% and 0.04% CO₂, a software program called Mothur [63] was used to analyze the sequence data. A rarefaction analysis was conducted to evaluate if the sequencing effort captured all the diversity in the community. Finally, a qualitative measure known as unweighted Unifrac, which is a phylogeny based analysis, was used to determine the dissimilarity between communities (p<0.05) [68].

2.2.5 Preliminary Illumina MiSeq-based sequencing of the 16S rRNA genes

The extracted DNA samples used in Sanger sequencing were sequenced again using a high throughput sequencing platform. A total of 8 extracted DNA samples, 4 from each CO₂ treatment (12% and 0.04%) were sequenced by the Centre for the Analysis of Genome Evolution and Function (CAGEF, University of Toronto, ON, Canada), for a preliminary analysis of the biofilm communities. Only the 16S rRNA gene was sequenced for these samples using the V4 hypervariable region of this gene. The DNA samples were barcoded, from which amplicon libraries were created and ran on Illumina MiSeq using 150bp paired-end (x2) V2 chemistry. UNOISE pipeline was used by CAGEF, for the sequence analysis, where the sequences were assembled, trimmed, evaluated for errors, aligned and operation taxonomic units (OTUs) were
determined. Results of the sequence analysis was used to run an unweighted UniFrac assessment (p<0.05).

2.2.6 16S and 18S Amplicon Sequencing and Library Preparation

Extracted DNA samples were PCR amplified, and paired-end sequencing was conducted following the general protocol described by Sharp et al. [52]. 16S and 18S rRNA gene fragments were amplified using the 16S 'universal pyrotag' PCR primers 926wF (5’-AAACTYAAAKGAATTGRCGG3’) and 1392R (5’-ACGGGCGGTGTGTRC3’) [91] and the 18S primers TAReuk454FWD (5’-CCAGCASCYGCGTATTCC -3’) and TAReukREV3 (5’-ACTTTCGTTCTTGATYRA -3’) [92]. The universal pyrotag primer set targeted the V6-V8 variable region of the 16S rRNA gene from Archea and Bacteria [91], whereas the 18S primer set was designed to target the conserved regions adjoining the 5’ and 3’ region of the V4 rRNA loop [92]. Triplicate PCRs were performed independently for each DNA sample using the 16S and 18 primers in accordance with Sharp et al. [52], but thermal cycling conditions were modified for the 18S primers. Reaction conditions for 18S rRNA gene amplifications were as follows: initial denaturation at 95 °C for 3 min, followed by 10 cycles of 30 s at 95 °C, 45 s at 53 °C and 60 s at 72 °C, and another 15 cycles of 30 s at 95 °C, 45 s at 48 °C and 60 s at 72 °C, ending with a 5 min elongation step at 72 °C. All PCR reactions were purified using AMPure XP magnetic beads (Beckman Coulter, Indianapolis, IN), and a second round of PCR was conducted to attach dual indices and Illumina sequencing adapters (Illumina, San Diego, CA) to the amplicons [52]. Second round PCR reactions were purified again using the AMPure XP magnetic beads (Backman Coulter, Indianapolis, IN) and DNA concentrations were determined using a Qubit Fluorometer with the Quant-iT dsDNA HA Assay Kit (Life Technologies, Burlington, ON) [52]. A Bioanalyzer 2100 (Agilent, Santa Clara, CA) was used to check amplicon library sizing, and library concentrations were normalized before being pooled and
sequenced on a MiSeq Personal Sequencer using the 2 × 300 bp MiSeq Reagent Kit v3 (Illumina) [52].

2.2.7 Data Analysis

Analysis of the amplicon sequences was completed using the online amplicon data analysis pipeline MetaAmp (http://ebg.ucalgary.ca/metaamp), following Sharp et al. [52]. First, paired-end raw sequences were merged and those that did not align, had overlapping regions smaller than 100 bp, or that contained more than 8 mismatches in the overlapping region were discarded. The remaining paired-end reads were checked for the presence or absence of the forward and reverse primers, which were then trimmed off [52]; sequences that did not have the primers or had more than 1 mismatch in the primer sequences were discarded. Quality filtering, read truncation, unique sample identification, and sample pooling were performed described in Sharp et al. [52]. Following a check for chimeras, sequences were pooled into operational taxonomic units (OTUs) using a 97% sequence similarity cut-off. The OTU data was then used to compute rarefaction and rank abundance curves, and estimate alpha and beta diversity [52]. Sample to sample comparisons (i.e., divergence) were based on; 1) hierarchical clustering of samples, 2) nonmetric multidimensional scaling (NMDS), and 3) principal coordinate analysis (PCoA) [52]. Finally, NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify representatives of each OTU.
2.3 Results and Discussion

Section 2.3.0: Overview

Schnurr et al [50] explored how different CO₂ concentrations affected biomass productivity in a semi-continuous algal biofilm photobioreactor that was treated with municipal wastewater as a source of biofilm-forming bacteria and extracellular polymeric substances (EPS). Following the wastewater treatment, the photobioreactor was inoculated with an algal seed culture consisting of the chlorophyte alga *Scenedesmus obliquus* in artificial growth medium. This experiment revealed that biomass productivity increased when CO₂ concentrations were raised from atmospheric levels (0.04%) to 2%, and that productivity plateaued at CO₂ concentrations above 2% [50]. However, it was unclear if the seed species, *S. obliquus*, was responsible for the observed increase in biomass productivity.

To investigate the composition of the biofilm, we extracted DNA from the samples collected by Schnurr et al [50]. The sample used for our analysis were collected over a period of 26 days (day 4, 10/11, 15, 19/20 and 26), and from two CO₂ treatments (0.04% and 12%). Biofilm microbiomes were characterized using Sanger sequencing of PCR amplicons, generated using primers that targeted psbA genes from photosynthetic species, 18S rRNA genes from eukaryotes, and 16S rRNA genes from prokaryotes and eukaryotic organelles. These sequencing efforts revealed a more complex community than expected and was reinforced by a rarefaction analysis (Appendix Figure 14 & 15), where the rarefaction curves for Sanger sequencing of the 16S rRNA, 18S rRNA and psbA genes were linear and this suggested that more sampling was required to capture the complexity of the biofilm community. Therefore, we also used Illumina MiSeq-based sequencing of 16S rRNA and 18S rRNA genes to analyze biofilm community
composition more deeply. The Illumina MiSeq-based analysis was conducted on biofilm samples that were collected from two replicate incubations in the photobioreactor. As different environmental conditions may have led to variability between these replicates, we report this data as a range of relative abundances, except in cases where replicates were in agreement, and therefore no range is reported (sections 3.2 and 3.3). Although the biofilms that grew were consistently dark green, suggesting the predominance of the seed species, our sequence analysis revealed complex community dynamics in biofilms that were not predominantly composed of the seed culture algal species.

Section 2.3.1: Community composition based on 18S rRNA, 16S rRNA and psbA gene amplification using Sanger Sequencing

To examine community composition in biofilms cultivated at 0.04% and 12% CO2 growth conditions, we first identified oxygenic photosynthetic organisms from their psbA gene sequences. Sequences related to the seed species, *S. obliquus*, comprised 15% of all psbA sequences after 4 days of growth in 12% CO2. By day 10, no *S. obliquus* sequences were detected, but when the experiment was terminated on day 26, sequences related to *S. obliquus* reached 23% (Figure 6A). For the cyanobacteria, *Leptolyngbya sp.*, we observed an increase in abundance over time in 12% CO2, from not detected at day 4, to 13% at day 10, to day 26 by which point *Leptolyngbya sp.* dominated the culture at 73%. In contrast, for biofilms grown in 0.04% CO2, 38% of the psbA sequences were identified as *S. obliquus* on day 4, but this declined to 23% by the end of the experiment on day 26. Sequences related to *Leptolyngbya sp.* comprised 62% of psbA sequences by day 4, and had the highest relative abundance (73%) by the end of the experiment on day 26 (Figure 7A). Thus, the oxygenic photoautotrophic communities, as inferred from psbA sequences, were unexpectedly dominated by the cyanobacteria *Leptolyngbya* sp., and not the seed species *S. obliquus*, under both 0.04% and 12% CO2 growth conditions.
Figure 11: The effect of CO₂ concentration on the composition and community dynamics of engineered microalgal biofilms. OTUs were identified by Sanger sequencing of PCR amplicons using primer sets for *psbA* (A), 18S rDNA (B) and 16S rDNA (C). Biofilms, initially seeded with *S. obliquus*, were grown over a 26 day (D) time course at 0.04 % or 12 % (v/v) CO₂ and sampled periodically.
We next characterized the eukaryotic microbial community in both CO2 treatments using 18S rRNA gene amplification. The 12% and 0.04% CO2 treatments followed a similar trend where sequences identified as *S. obliquus* dominated the amplicon library on day 4, decreased by day 10 and again dominated the amplicon library on the final day (day 26). Interestingly, we observed the opposite trend for sequences related to grazers, i.e., a phylum with species that can feed on fungi, bacteria, other protozoa and algae. Sequences classified as Rhizaria represented the largest proportion of sequences on day 10, but were below our level of detection at the beginning (day 4) and at the end of the experiments (day 26) (Figure 7B). Thus, a possible reason for the decrease in the 18S rRNA sequences related to the seed culture alga could be due to grazing, which has been observed in open raceway ponds and closed photobioreactors and can be due to contaminating organisms introduced with wastewater [93-95]. In general, the decrease of the seed species could be a result of the grazers affecting the growth of the seed culture or bacteria, as well as competing for nutrients or space on the glass slide in the replicate photobioreactors. The results from the final day of each treatment showed that despite the possible effects of grazing, and/or competition from another species like *Leptolyngbya* sp., *S. obliquus* still dominated the 18S rRNA amplicon libraries.

We used 16S rRNA gene sequencing to identify bacteria involved in biofilm formation and to determine if the bacterial community composition was affected by differing CO2 concentrations. Analysis of the community composition on day 4 of the 12% CO2 treatment, revealed that a substantial portion of the 16S rRNA gene sequences were classified as belonging to *S. obliquus* (30%), which were presumably amplified from chloroplast DNA. We detected no sequences related to *S. obliquus* throughout the rest of the experiment. In addition, sequences identified as *Leptolyngbya* sp. increased from day 10 (9%) to day 26 (55%) (Figure 7C). As expected, over the 26 days, we consistently identified sequences related to the Proteobacteria.
classes, α-proteobacteria, β-proteobacteria and Y-proteobacteria [80, 81, 96, 97](Figure 7C). Similarly, for biofilms grown in 0.04% CO2, 22% of the 16S rDNA sequences were identified as *S. obliquus* on day 4, and this declined to 3% by day 26 (Figure 7C), while sequences that were related to *Leptolyngbya sp.* increased over time and dominated the amplicon library (44%) on day 26 (Figure 7C). Once again, sequences classified as α-proteobacteria, β-proteobacteria and Y-proteobacteria were found throughout the experiment (Figure 7C).

In summary, analysis of 16S rRNA sequences revealed that under both treatments (12% and 0.04% CO2), the proportion of *S. obliquus* declined dramatically from day 4 to 26, while the proportion of sequences related to *Leptolyngbya sp.* increased. This suggests that *Leptolyngbya sp.* outcompeted the *S. obliquus*. Assuming the source of the cyanobacteria and heterotrophic bacteria was the wastewater used to condition the biofilm substrates, it is possible that after the wastewater was removed from the photobioreactor very few colonies of *Leptolyngbya* remained, making it difficult to detect in the sequence analysis of day 4. We expected that *S. obliquus* would be easier to detect at the beginning of the experiment because it was used to inoculate the photobioreactor. Thus, we expected that on day 4 the eukaryotic alga would greatly outnumber any other phototrophs such as *Leptolyngbya sp.* As the experiment progressed, *Leptolyngbya sp.* may have reproduced faster than *S. obliquus* and hindered its growth. Throughout the entire experiment, and in both CO2 treatments, there was a readily detected community of Proteobacteria (α-proteobacteria, β-proteobacteria and Y-proteobacteria) which are known to initiate biofilm formation in both naturally occurring and engineered algal biofilms[80, 81, 96, 97]. There is a mutualistic relationship between bacteria and algae wherein the bacteria promote the growth of the seed algal species by producing CO2 as well as eliminating pathogens. Meanwhile, the algae use the CO2 to produce O2, which the bacteria use. [98-101].
PCR amplification, cloning, and Sanger sequencing of *psbA*, 18S rDNA and 16S rDNA libraries efficiently captured the dominant members of the microalgal biofilm community. However, based on rarefaction analysis, Appendix Figures 14 & 15, it was evident that the Sanger-based sequencing efforts just scratched the surface with respect to the complexity and diversity of these microbial communities. Additionally, a quantitative comparison of the biofilm communities using unweighted UniFrac (uwUniFrac) resulted in all comparisons of the Sanger-based sequencing having a p>0.05 (Appendix Table 3). Hence, it became clear from the rarefaction analysis and uwUniFrac community comparison, that a deeper sequencing effort was needed, so we changed our experimental approach from Sanger sequencing of PCR clone libraries to high-throughput Illumina MiSeq-based sequencing.

**Section 2.3.2: Analysis of the Eukaryotic Community using Illumina MiSeq-based sequencing on 18S rRNA genes**

Illumina MiSeq – based sequencing of 18S rRNA gene fragments was conducted following the approach described in Sharp et al [52] with the same DNA extracts used for the work described in section 3.1. Rarefaction curves and Chao1 richness estimates indicated that while the sequencing efforts did not saturate the richness, a significant portion of the eukaryotic community was captured (Appendix Figure 12).
Figure 12: The effect of CO₂ concentration on the community dynamics of the eukaryotic population of engineered microalgal biofilms. OTUs were identified by Illumina sequencing of PCR amplicons using an 18S rDNA primer set. The figure shows relative abundance and taxonomic assignments for the most abundant OTUs (>1%). Biofilms, initially seeded with *S. obliquus*, were grown over a 26 day (D) time course at 12 % (A) or 0.04 % (v/v) CO₂ (B) and sampled periodically. Samples (s1 & s2) from two separate photobioreactors operating in parallel were taken for each of the measurement days (D). The number in parenthesis refers to the number of OTUs represented by each subdivision of taxa.
On day 4, the analysis of the biofilm grown at 0.04% CO$_2$ revealed that three algal species from the phylum Chlorophyta dominated the amplicon library: *S. obliquus* (51-60%), *C. vulgaris* (3-27%) and *C. reinhardtii* (6%) (Figure 8A&B). By day 11, we detected a substantial increase in Ciliophora, Rhizaria, Rotifera and Stramenopiles, phyla that include species that can act as grazers, which comprised 37-41% of the 18S rRNA gene fragments. The proportion of sequences classified as Fungi and *S. obliquus* were 26% and 16-22%, respectively (Figure 8B). By day 15, the percentage of sequences related to *S. obliquus* remained unchanged, but the sequences associated with grazers further increased to 45-48% (Figure 8B). By day 20, sequences related to grazers decreased to 26-34%, and remained at a similar level on the final day of treatment (day 26). Finally, we observed an increase in sequences related to *S. obliquus* (32-34%) on day 20, which then decreased to 6-12% on the final day (Figure 8B).

In the 12% CO$_2$ treatment, 59-70% of the amplicon library was related to *S. obliquus*, 2-25% identified as *C. vulgaris* and 4-12% were classified as grazers on day 4. By day 10, there had been a switch in the dominant algal species from *S. obliquus* (18%) to *C. vulgaris* (42%) and the portion of sequences related to grazers remained similar to day 4 (Figure 8A). However, by day 19 sequences related to *C. vulgaris* (25%) and *S. obliquus* (4%) decreased, while sequences identified as grazers increased (45-53%) (Figure 8A). On day 26, 3% of the sequences were classified as *C. vulgaris* and 5% as *S. obliquus*, however the percentage of sequences related to grazers remained relatively the same (42-46%). The 18S rRNA gene sequencing results from both CO$_2$ treatments revealed a diverse community in the grown algal biofilms, which followed a similar trend, i.e. a decrease in the proportion of sequences related to the *S. obliquus* and an increase in relative abundance of grazers over time.
Data from our two sequencing approaches (Sanger Sequencing and Illumina MiSeq) revealed similar trends in species abundance over time. For example, both methods revealed that *S. obliquus* dominated the biofilm community on day 4 at both CO$_2$ treatment levels. The shift in community structure on day 10/11 from *S. obliquus* to grazers was also evident from both sequencing methods. However, different outcomes were observed on the final day (day 26) of both treatments, where *S. obliquus* was the dominant species from the Sanger sequencing results, while grazers dominated the amplicon library from the Illumina MiSeq-based sequencing.

Overall, both sequencing methods indicate that *S. obliquus* dominated the amplicon library at early time points, and the proportion of sequences associated with grazers increased over time. It was speculated that the wastewater initially added to the system introduced species that are known as grazers, which seem to pose a negative effect on the growth of *S. obliquus*. The effect of the grazers brings to light potential pitfalls in using wastewater in a photobioreactor i.e. wastewater can be used as a source of nutrients and primary colonizers, but has the possibility of introducing contaminating microorganisms that can have an unexpected effect on the algal species of interest.
In order to measure the species diversity in a given community, we used the Shannon and Simpson index as a diversity metric (Figure 9A and B). The Shannon and Simpson index value was the lowest on day 4 for both treatments, which is supported by a large portion of *S. obliquus*–related sequences in the amplicon library. After day 4 there was a consistent increase in the Shannon and Simpson indices until day 26, where the biofilm community had the highest diversity. To quantitatively compare the biofilm species composition between 0.04% and 12% CO₂ treatments, we generated a PCoA plot based on a matrix of the Bray Curtis community
similarities (Figure 9C). This analysis revealed that on day 4 the biofilm communities at 0.04% and 12% CO₂ were similar to each other (Figure 9C). This suggests that even at different CO₂ concentrations, the initial biofilm community started off with the same type and number of species. From the PCoA plot it was evident that the community composition on day 11, day 15 and day 20 of the 12% CO₂ treatment clustered together and day 10 and day 19 of the 0.04% CO₂ treatment were in separate clusters (Figure 9C). This difference in community similarity shows evidence of an unstable community with respect to species composition. At the end of the experiment (day 26), the algal biofilm communities in both treatments were very similar to each other in the number and type of species (Figures 9C). The overall results of the Illumina MiSeq-based sequencing of the 18S rRNA gene amplification suggest that biofilms grown at 0.04% and 12% CO₂, with a constant light intensity of 100 μmol/m²/s, did not favour the seed culture (S. obliquus).

Section 2.3.3: Analysis of the Prokaryotic Community using Illumina MiSeq-based sequencing of the 16S rRNA genes

We discovered a possible competitor (Leptolyngbya sp.) to the growth of S. obliquus from the analysis of the biofilm community using 16S rRNA gene amplification. We also observed that a large number of sequences were related to α-proteobacteria, β-proteobacteria and γ-proteobacteria throughout the 0.04% and 12% CO₂ treatments. The Illumina MiSeq-based sequencing of the 16S rRNA gene fragments, captured chloroplast genes from several algal species (C. vulgaris, C. reinhardti and S. obliquus) (Figure 10A and B). Rarefaction curves and Chao1 richness estimates indicated that a significant portion of the prokaryotic microbial community was assessed (Appendix Figure 13).
Analysis of the biofilm community on day 4 of the 12% CO$_2$ treatment revealed that $C. \textit{vulgaris}$ (5-21%) and $S. \textit{obliquus}$ (38-42%), made up a majority of the amplicon library and the remaining was composed of Bacteroidetes (2-7%) and classes from the phylum Proteobacteria ($\alpha$-proteobacteria, $\beta$-proteobacteria, $\gamma$-proteobacteria and $\delta$-proteobacteria) which represented 32-48% (Figure 10A and B). We noticed a shift in the community on day 10 with an increase in $C. \textit{vulgaris}$ (34%) and a decrease in $S. \textit{obliquus}$ (4%), at the same time a new species appeared related to a Cyanobacteria called $\textit{Leptolyngbya}$ sp. (7-12%). By day 19, the proportion of sequences classified as $C. \textit{vulgaris}$ and $S. \textit{obliquus}$ dropped to 9% and 0.8%, respectively, while the $\textit{Leptolyngbya}$ sp. stayed constant. (Figure 10A). On the final day, day 26, the community was dominated by $\textit{Leptolyngbya}$ sp., comprising 55% of the amplicon library and $C. \textit{vulgaris}$ and $S. \textit{obliquus}$ made up less than 2% (Figure 10A).
Figure 14: The effect of CO2 concentration on the community dynamics of the prokaryotic population of engineered microalgal biofilms. OTUs were identified by Illumina sequencing of PCR amplicons using a 16S rDNA primer set. The figure shows relative abundance and taxonomic assignments for the most abundant OTUs (>1%). Biofilms, initially seeded with *S. obliquus*, were grown over a 26 day (D) time course at 12 % (A) or 0.04 % (v/v) CO2 (B) and sampled periodically. Samples (s1 & s2) from two separate photobioreactors operating in parallel were taken for each of the measurement days (D). The number in parenthesis refers to the number of OTUs represented by each subdivision of taxa.
The amplicon library on day 4 of the 0.04% CO2 treatment, resembled that of day 4 under the 12% CO2 treatment. On day 11, the sequences that were classified as C. vulgaris and S. obliquus decreased to 1% and 16%, respectively, whereas the percentage related to Proteobacteria (41-49%) and Bacteroidetes (24-30%), increased (Figure 10B). By day 15, the portion of sequences related to Leptolyngbya sp. increased considerably (43-49%) compared to 5% on day 11, whereas sequences classified as Proteobacteria (20%), Bacteroidetes (17%), C. vulgaris (0.5%) and S. obliquus (2%), decreased (Figure 10B). A substantial portion of the amplicon library on day 20 was identified as Leptolyngbya sp. (68-73%), there were 8-11% of sequences classified as Proteobacteria, while the proportions of C. vulgaris and S. obliquus did not change (Figure 10B). We observed that by the end of the 0.04% CO2 treatment (day 26), 63% of the sequences were related to Leptolyngbya sp. and 6% to Proteobacteria, but C. vulgaris and S. obliquus were undetectable (Figure 10B).

In summary, the analysis of the biofilm communities using Illumina MiSeq-based sequencing of the 16S rRNA gene fragments revealed that by the end (day 26) of the 12% and 0.04% CO2 treatments, sequences classified as Leptolyngbya sp. dominated the amplicon library and S. obliquus was merely detected. This falsified our hypothesis that S. obliquus would dominate the amplicon library by day 26. Throughout both CO2 treatments we constantly detected Proteobacteria and Bacteroidetes. These species are commonly found in the growth of microalgae in suspension and biofilms [73, 81, 102, 103], but further work is needed to investigate their role in these systems. In general, both of our sequencing methods (Sanger sequencing versus Illumina MiSeq), captured a similar algal biofilm community through the 26-day experiment, as seen from Figures 7C and 11. When comparing Illumina MiSeq-based sequencing of 16S rRNA to 18S rRNA gene fragments, we found that the communities of both CO2 treatments had a decrease in S. obliquus over time. Thus, if we assume that the pre-
treatment with wastewater to the photobioreactor introduces *Leptolyngbya* sp. and grazers, it is probable that the decrease in *S. obliquus* is due to the effect of grazers and the growth of *Leptolyngbya* sp..

**Figure 15:** Alpha diversity metrics for the prokaryotic microbial community described in Figure 4. A Shannon and Inverse Simpson analysis of the community composition of biofilms grown at 12% (A) and 0.04% (B) CO$_2$. Principal coordinate analysis (PCoA) plot (C) with Bray-Curtis dissimilarity of the prokaryotic microbial community (p<0.05).

The Shannon and Simpson diversity metrics for both treatments had the highest index values at Day 10/11 which correlates with the large diversity of species that were found on those days (Figure 11A and B). The diversity index values remained the same for the biofilm community of the 12% CO$_2$ treatment on day 19, but decreased slightly on day 26 (Figure 11A). In the case of the 0.04% CO$_2$ experiment, after day 11 the Shannon and Simpson index values
decreased on day 15 and was at its lowest on day 26 (Figure 11B). This decrease in the index values for day 26 of both treatments connects with the majority of sequences being classified as *Leptolyngbya sp.* on this day (Figure 10, 11A and 11B). A Bray Curtis similarity metric was used to determine how similar the communities were to each other on each day of the treatments (Figure 11C). We observed that the biofilm community on day 4 of both experiments were similar as they grouped together on the PCoA plot (Figure 11C). From the PCoA plot day 10 and day 19 of the 12% CO₂ experiment clustered together, which corresponds with the above result of the Shannon and Simpson index values being the same. We discovered that the community composition on day 11 of the 0.04% CO₂ treatment seemed to have a community that was dissimilar to the other days. This may be due to sequences related to Proteobacteria and Bacteroidetes dominating the amplicon library on day 11. A distinct cluster was observed for days 15, 20 and 26 of the 0.04% CO₂ treatment and day 26 of the 12% CO₂ treatment (Figure 11C). This cluster is most likely due to sequences identified as *Leptolyngbya sp.* dominating the amplicon library on these days.

### 2.4 Conclusion

Results of this study indicate that Sanger sequencing did not capture the complex community dynamics that are occurring in the artificial engineered microalgal biofilm photobioreactors. In contrast, we were able to conduct a thorough analysis of the biofilm species composition using Illumina MiSeq sequencing. We determined that at the beginning of the 0.04% and 12% CO₂ treatments the majority of sequences were related to the seed culture, *S. obliquus*, but by the end of the treatments the growth of the seed culture was not promoted and most sequences were classified as a cyanobacteria, *Leptolyngbya sp.*. The results also suggest that the presence of grazers had an effect on the community structure and dynamics, which dominated the species composition on day 10/11. This study shows that sequences related to Proteobacteria classes, α-
proteobacteria, β-proteobacteria and Y-proteobacteria, persist throughout the entirety of both experiments, suggesting that these Proteobacteria are important to the biofilm structure. Considering the above, the results point to the increase in biomass productivity observed by Schnurr et al [50] actually being an increase in other species (Leptolyngbya sp. and grazers) and not the seed culture. Therefore, further efforts are needed to optimize growth conditions to favour species producing the most desirable biomass.
Chapter 3
Conclusion and Future Directions

3.0 Conclusion & Future Directions

Considering the results of this thesis, there is a clear message that artificial engineered microalgal biofilms have complex communities which were not captured by Sanger sequencing. This was supported by a rarefaction analysis where the lines representing Sanger sequencing did not converge (Appendix Figures 14 and 15). The opposite was observed for the lines denoting Illumina MiSeq-based sequencing which were closer to reaching a horizontal asymptote (Appendix Figures 14 & 15). One possible reason Sanger-based sequencing was unable to capture the diversity was a result of sequence depth where an average of 40 sequences were obtained per biofilm sample (Appendix Table 1). However, the average number of sequences acquired per sample was 9,030 for Illumina MiSeq-based sequencing (Appendix Table 2). The lack of sequences from Sanger sequencing appears to have an effect on the qualitative measure of the dissimilarity between biofilm communities, demonstrated through unweighted UniFrac which showed that there was a difference in the structure of biofilm communities, but the results were not significant (p>0.05) (Appendix table 3). Meanwhile, the same analysis with the Illumina MiSeq-based sequencing, conducted through Centre for the Analysis of Genome Evolution and Function (CAGEF), showed that the biofilm communities were significantly different (p<0.05), (Appendix Table 3). The advantage to using Illumina MiSeq over Sanger is that MiSeq allows for millions of fragments to be sequenced in a single run versus sanger sequencing which only produces one forward and reverse read. However, the tradeoff is that methods like MiSeq are not as accurate as Sanger sequencing, which is 99.99% accurate, and produce a maximum read length of 300 base pairs, unlike Sanger sequencing which can produce reads of 400-900 base pairs.
Furthermore, depending on the primers that were used for the Sanger-based sequencing there was a difference in the community structure that was identified. This is exemplified by the sequencing results of the psbA and 16S rRNA genes on the last day of both treatments, where a small portion of sequences were related to the seed culture, while the opposite was indicated for the sequencing of the 18S rRNA genes (Figure 7A, B & C). Additionally, the sequence analysis with the psbA primer set detected large number of sequences related *Leptolyngbya* on day 4 compared to the results of the 16S rRNA primers where *Leptolyngbya* was not detected until day 10 for both CO2 treatments (Figure 7A & C). The differences between primer sets for the Sanger-based sequencing is possibly due to the lack of sequence depth and primer bias. Results of Illumina MiSeq-based sequencing of the 16S rRNA and 18S rRNA genes resembled each other, for example, both amplicon libraries on day 4 of both treatments were dominated by the seed culture and followed the same trend with a decline in the proportion of sequences related to the seed culture overtime (Figures 8 & 10). In general, both Illumina MiSeq and Sanger based sequencing of the 16S rRNA and 18S rRNA genes were able to detect the increase in the proportion of sequences classified as grazers and *Leptolyngbya* throughout both treatments.

In spite of the wealth of data on the performance of microalgal biofilm photobioreactors, there is a lack of effort in characterizing the communities of these engineered microalgal biofilms. The primary goal of this thesis was to determine how CO2 effects the microbial community dynamics of engineered microalgal biofilms. This thesis revealed that an increase in biomass productivity, due to a change in one parameter, such as CO2, [50] is not necessarily correlated with an increase in the species of interest. It is suggested that the first step when designing an engineered microalgal biofilm photobioreactor should be determining the conditions that will favour the growth of the microalgal species of interest and allow for a reproducible community composition. Furthermore, all corresponding studies that look at the
microbial community dynamics of engineered microalgal biofilms, should use a high throughput sequencing method i.e. Illumina MiSeq-based sequencing of the 16S rRNA and 18S rRNA genes. This type of sequencing method allows for a more in depth analysis of the species that are a part of the microbial community.

Future experiments should look at the effects of light intensity on community dynamics of engineered microalgal biofilms. Other studies have looked at how different wavelengths of light (white, red and blue) affect the composition of algal biofilms in a photobioreactor and have found that depending on the wavelength the species composition shifts and favours one species over others [52]. This same study was able to find the wavelength that favoured their species of interest [52].

Temperature is another aspect that could be further investigated since it has been illustrated that laboratory biofilms grown at higher temperatures have faster biofilm colonization and bacterial growth [104]. Meanwhile, algal strains grown in suspension have also been known to have optimum growth temperatures. For example, *Scenedesmus sp.* grows well in a range of 20°C–40°C, with an optimum growth temperature of 30°C [105-107]. Therefore, investigating the biofilm composition at different temperatures may help determine a temperature that will favour an algal species, like *S. obliquus*, over a cyanobacteria like *Leptolyngbya sp.*

Additional research could focus on identifying the initial microbial colonizers involved in the algal biofilm formation. If the initial colonizers are identified, then these colonizers could be cultivated and added directly to the photobioreactor to form the initial layer of the biofilm instead of using wastewater as the source for the colonizers. Without the use of wastewater this would reduce the chance of another species out competing the seed culture. It might also be worthwhile to characterize the community in the wastewater for downstream comparison to the community
of the microalgal biofilm. Alternatively, further research efforts should focus on using aeroterrestrial algae which are highly plastic, thus being able to withstand constantly changing environments and are known to form biofilms in nature [108].

The final potential experiment that could be investigated is the effect of growing the engineered microalgal biofilm for longer than 26 days to form a stable community that might favour the seed culture. In these experiments, it was determined that 12% and 0.04% CO₂ had similar community compositions at day 4 and day 26, however, the latter was not dominated by the seed culture. On the contrary, the communities had completely different species composition at the other time points (days 10 and 19) when comparing 12% and 0.04% CO₂. It would be interesting to see if over time the seed culture would be able to reestablish itself as the dominant species in the community.
References


Figure 16: Rarefaction (A, B) analysis of the DNA sequencing effort for data from Figure 7. Also, Chao 1 (C, D) analysis of OTU diversity versus the observed OTU diversity of the eukaryotic community. Biofilms, initially seeded with S. obliquus, were grown over a 26 day (D) time course at 12 % (A, D) or 0.04 % (v/v) CO₂ (B, C). Samples (s1 & s2) from two separate photobioreactors operating in parallel were taken for each of the measurement days (D).
Figure 17: Rarefaction (A, B) analysis of the DNA sequencing effort for data from Figure 9. Also, Chao 1 (C, D) analysis of OTU diversity versus the observed OTU diversity of the eukaryotic community. Biofilms, initially seeded with S. obliquus, were grown over a 26 day (D) time course at 12 % (A, C) or 0.04 % (v/v) CO₂ (B, D). Samples (s1 & s2) from two separate photobioreactors operating in parallel were taken for each of the measurement days (D).
Table 4: Total sequences in clone library, singleton OTUs and total number of OTUs obtained from Sanger-based sequencing of the *psbA*, 16S rRNA and 18S rRNA genes.

<table>
<thead>
<tr>
<th></th>
<th>psbA 12% CO₂ Experiment</th>
<th>18S 12% CO₂ Experiment</th>
<th>16S 12% CO₂ Experiment</th>
<th>psbA 0.04% CO₂ Experiment</th>
<th>18S 0.04% CO₂ Experiment</th>
<th>16S 0.04% CO₂ Experiment</th>
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<td><strong>Day 4</strong></td>
<td>22</td>
<td>24</td>
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<td>22</td>
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<td>87</td>
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<td>5</td>
<td>4</td>
<td>3</td>
<td></td>
<td>14</td>
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<tr>
<td><strong>Day 19</strong></td>
<td>4</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td><strong>Day 26</strong></td>
<td>43</td>
<td>41</td>
<td>47</td>
<td>43</td>
<td>174</td>
<td>45</td>
</tr>
<tr>
<td><strong>Total OTUs</strong></td>
<td>21</td>
<td>26</td>
<td>12</td>
<td>8</td>
<td></td>
<td>2</td>
</tr>
</tbody>
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Table 5: Number of reads and OTUs obtained from Illumina MiSeq-based sequencing of 16S rRNA and 18S rRNA genes.

<table>
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<tr>
<th>18S 12% CO₂</th>
<th>Day 4, s1</th>
<th>Day 4, s2</th>
<th>Day 10, s1</th>
<th>Day 10, s2</th>
<th>Day 19, s1</th>
<th>Day 19, s2</th>
<th>Day 26, s1</th>
<th>Day 26, s2</th>
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<td>No. of OTUs</td>
<td>62</td>
<td>67</td>
<td>106</td>
<td>87</td>
<td>108</td>
<td>112</td>
<td>119</td>
<td>108</td>
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<td>18S 0.04% CO₂</td>
<td>Day 4, s1</td>
<td>Day 4, s2</td>
<td>Day 11, s1</td>
<td>Day 11, s2</td>
<td>Day 15, s1</td>
<td>Day 15, s2</td>
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<td>72</td>
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<tr>
<td>16S 0.04% CO₂</td>
<td>Day 4, s1</td>
<td>Day 4, s2</td>
<td>Day 11, s1</td>
<td>Day 11, s2</td>
<td>Day 15, s1</td>
<td>Day 15, s2</td>
<td>Day 20, s1</td>
<td>Day 20, s2</td>
</tr>
<tr>
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<td>1877</td>
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<td>4306</td>
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<td>5859</td>
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<tr>
<td>No. of OTUs</td>
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<td>67</td>
<td>77</td>
<td>151</td>
<td>160</td>
<td>133</td>
<td>120</td>
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Table 6: Qualitative comparison of the biofilm communities from Sanger and Illumina MiSeq based sequencing using the β-diversity metric, unweighted UniFrac (p<0.05). Illumina MiSeq sequencing results obtained through the Centre for the Analysis of Genome Evolution and Function (CAGEF) was used for this analysis.

<table>
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<th>unweighted UniFrac</th>
<th>uwscore</th>
<th>p&lt;0.05</th>
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<td>0.813</td>
</tr>
<tr>
<td>16S Sanger 12% versus 0.04%</td>
<td>0.556</td>
<td>0.652</td>
</tr>
<tr>
<td>psbA Sanger 12% versus 0.04%</td>
<td>0.353</td>
<td>0.967</td>
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<td>16S MiSeq 12% versus 0.04%</td>
<td>0.454</td>
<td>&lt;0.05*</td>
</tr>
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</table>
Figure 18: Rarefaction (A, B) analysis of the DNA sequencing effort for data from Figure 7C & 10. Biofilms, initially seeded with *S. obliquus*, were grown over a 26 day (D) time course at 12 % (B) or 0.04 % (v/v) CO₂ (A). Samples (s1 & s2) from two separate photobioreactors operating in parallel were taken for each of the measurement days (D).
Figure 19: Rarefaction (A, B) analysis of the DNA sequencing effort for data from Figure 7B & 8. Biofilms, initially seeded with *S. obliquus*, were grown over a 26 day (D) time course at 12 % (B) or 0.04 % (v/v) CO₂ (A). Samples (s1 & s2) from two separate photobioreactors operating in parallel were taken for each of the measurement days (D).